Vascular Pharmacology of Migraine and Preeclampsia

Problems of the Fair Sex

Saurabh Gupta

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Vascular Pharmacology of Migraine and Preeclampsia: Problems of the Fair Sex

Vasculaire Farmacologie van Migraine and Preeclampsie: Problemen van het Vrouwelijk Geslacht

Thesis

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by

Saurabh Gupta

born in Palampur, India

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Promotor:	Prof.dr. P.R. Saxena
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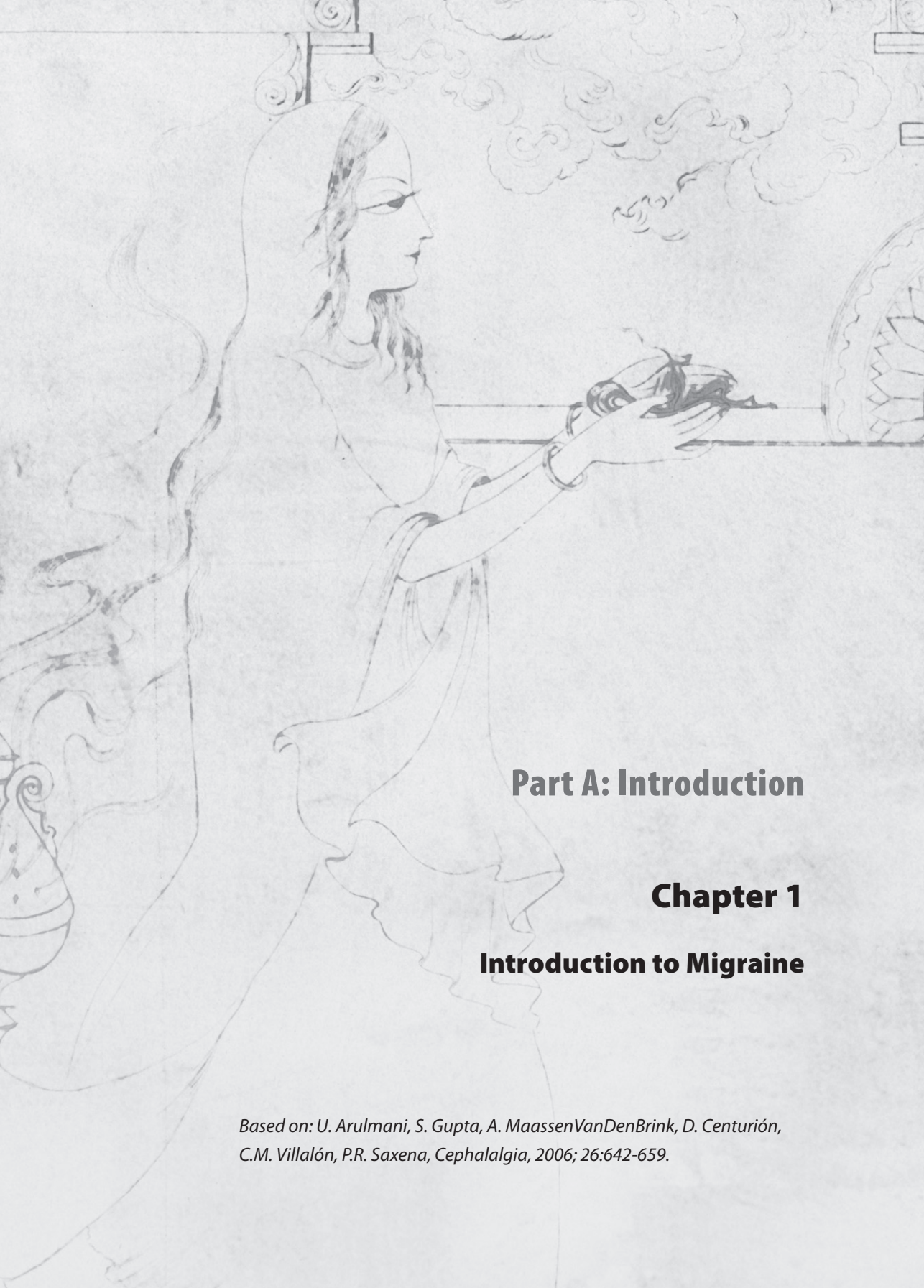
For My Parents
&
My Country

CONTENTS

What my mind elaborates has no value untill nature which I study has told me whether I am right or wrong (Dr. Christian de Duve)



Part A: Introduction

Chapter 1

Introduction to Migraine

Based on: U. Arulmani, S. Gupta, A. MaassenVanDenBrink, D. Centurión, C.M. Villalón, P.R. Saxena, Cephalalgia, 2006; 26:642-659.

ABSTRACT

Although the understanding of migraine pathophysiology is incomplete, it is now well accepted that this neurovascular syndrome is mainly due to a cranial vasodilatation with activation of the trigeminal system. Several experimental migraine models, based on vascular and neuronal involvement, have been developed. Obviously, the migraine models do not entail all facets of this clinically heterogeneous disorder, but their contribution at several levels (molecular, *in vitro*, *and in vivo*) has been crucial in the development of novel antimigraine drugs and in the understanding of migraine pathophysiology. One important vascular *in vivo* model, based on an assumption that migraine headache involves cranial vasodilatation, determines porcine arteriovenous anastomotic blood flow. Other models utilize electrical stimulation of the trigeminal ganglion/nerve to study the neurogenic dural inflammation, while the superior sagittal sinus stimulation model takes into account the transmission of trigeminal nociceptive input in the brainstem. More recently, the introduction of integrated models, namely electrical stimulation of the trigeminal ganglion or systemic administration of capsaicin, allows studying the activation of the trigeminal system and its effect on the cranial vasculature. Studies using *in vitro* models have contributed enormously during the preclinical stage to characterise the receptors in cranial blood vessels and to study the effects of several putative antimigraine agents. The aforementioned migraine models have advantages as well as some limitations. The present review is devoted to discussing various migraine models and their relevance to antimigraine therapy.

1.1 INTRODUCTION

Migraine is a complex, disabling, multifactorial, typically episodic neurovascular disorder of unknown aetiology; this condition affects a significant proportion of the adult population and it is a familial syndrome with a genetic component (1-3). Migraine attacks are characterised by an intense, throbbing and pulsatile headache, which is often unilateral in onset and is accompanied by anorexia, nausea, vomiting, photophobia and/or phonophobia (3). Furthermore, one third of the migraine sufferers experience aura, a focal neurological symptom including scintillating scotoma, muscle weakness and sensory disturbances (2, 3).

The pathophysiology of migraine involves the activation of the trigeminovascular system which, in turn, results in headache produced due to changes in cranial blood vessels (2, 4). These pain-producing cranial blood vessels are innervated by trigeminal nerves that release calcitonin gene-related peptide (CGRP), which produces a potent vasodilatation (5). This dilatation activates trigeminal nerves, which transmit vascular nociception to higher centers in the central nervous system (5, 6). On the basis of these observations, various experimental models for migraine have been developed (7, 8); these will be considered in this review in detail.

1.2 PATHOPHYSIOLOGY OF MIGRAINE

The knowledge of the underlying pathophysiology of migraine has enhanced over the years and it is now well accepted that migraine is principally a disorder of the brain (Figure 1.1) (2). Based on clinical symptoms, the pathophysiology of migraine can be divided in to three phases: (i) the trigger phase characterised by neuronal hyperexcitability, (ii) the aura phase possibly involving cortical spreading depression and, finally, (iii) the headache phase due to cranial vasodilatation precipitated by activation and sensitization of the trigeminal system at the peripheral and central levels (4, 9-11). Exploring each phase of migraine reveals unique mechanisms and divulges novel therapeutic targets.

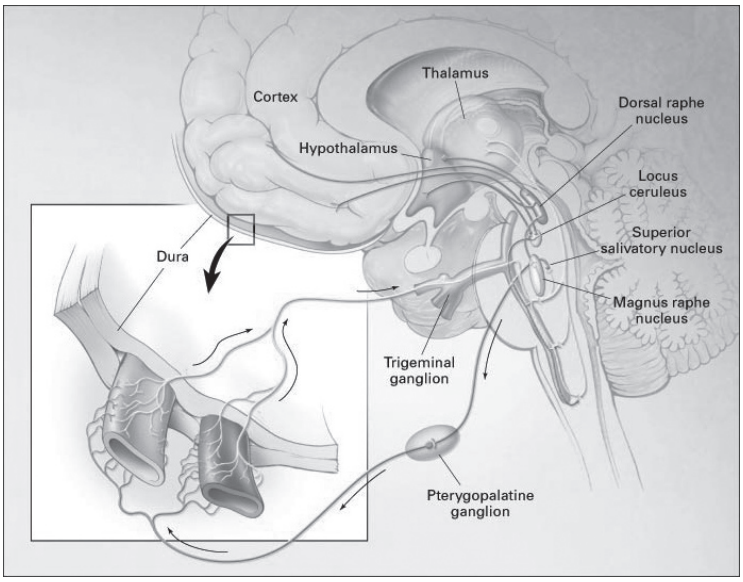


Figure 1.1. Pathophysiology of migraine
Sensory fibres innervating the cranial vessels arise from trigeminal ganglion neurons that contain neuropeptides. Trigeminovascular inputs from dural meningeal vessels pass through the trigeminal ganglion and synapse on second order neurons. These neurons project to the quintothalamic tract and synapse with neurons in the thalamus. There is also a reflex connection between neurons in the pons in the superior salivatory nucleus, which results in a cranial parasympathetic outflow that is in part mediated through the pterygopalatine (sphenopalatine) ganglion. From Goadsby et al. (2) with permission.

1.2.1 Trigger phase

Several lines of evidence, including studies using positron emission tomography, indicate that brainstem structures, rather than cortical structures, are activated during a migraine attack (2, 9). This activation remained unaffected after the resolution of the headache by sumatriptan treatment, suggesting that brainstem activation is a fundamental part of migraine (2, 12). In addition, certain genetic abnormalities may be responsible for altering the response threshold to migraine-specific triggers in the brain, e.g. mutations of the P/Q-type calcium channel gene that plays an important role in familial hemiplegic migraine (1, 2). The subsequent events following the trigger phase leading to the symptoms observed during the aura and headache phases can be explained on the basis of neurovascular involvement (6, 11).

1.2.2 Aura Phase

Up to 15-30% of migraine sufferers experience aura symptoms that last for 5 to 60 min before the onset of headache and may involve cortical spreading depression (13, 14). Cortical spreading depression is a wave of transient intense spike activity that spreads along the cortex slowly at rates between 2 and 6 mm min^{-1}, possibly leading to a long-lasting neuronal inhibition (2, 14). With the advent of non-invasive methods, including magnetic resonance imaging and magnetoencephalography (15), it has been shown that a wave of cortical spreading depression is followed by a decrease in regional cerebral blood flow (2, 14). Most clinicians believe that the migraine aura is due to a neuronal dysfunction, probably resulting from cortical spreading depression (15, 16), rather than ischemia.

1.2.3 Headache Phase

Although elegant functional neuroimaging procedures have refined the underlying concept of migraine, the mechanism by which the aura transduces into headache still remains elusive (13). Clinical and experimental considerations suggest that the pathogenesis of the migraine headache is intimately linked to the trigeminal innervations, which when activated possibly following cortical spreading depression, causes dilatation of cranial blood vessels, including arteriovenous anastomotic shunts (11, 17, 18). The involvement of shunt vessels in migraine has once again attracted attention because patients with right to left cardiac shunts have been reported to have a high incidence of migraine headaches that are substantially reduced after shunt repair (see 19, 20, 21). Moreover, it is well known that patients with cranial arteriovenous malformations have a high incidence of migraine, which is reduced after correction of such malformations (22-25). Thus, migraine pain is due to activation of the nociceptors in intracranial structures, in concert with a reduction in the function of endogenous pain-control pathways (2). This nociceptive information from the cranial blood vessels is conveyed to central neurons in the trigeminal sensory nucleus that, in turn, relay the pain signals to higher centres, where headache is perceived (4, 13). In addition, trigeminal pathways may get sensitized (10, 26, 27) as well as release CGRP, thus reinforcing vasodilatation relaying the nociceptive impulses to the central nervous system (11).

1.3 EXPERIMENTAL MODELS FOR MIGRAINE

In general terms, experimental models are a part of strategy that is employed to develop new and better therapeutic agents for a particular ailment (Figure 1.2). The experimental models are developed based on pathophysiological features of the disease and the pharmacological action of the existing drugs. The new compounds found active in the models are subjected to clinical evaluation. If the compound is devoid of clinical efficacy and pharmacokinetic reasons are excluded, the model must be modified or discarded. On the other hand, when the compound is found clinically effective, it not only benefits patients, but further evaluation of its pharmacology leads to modification of experimental models and, in some cases, to entirely new models that may yield new compounds ultimately becoming novel therapeutic agents.

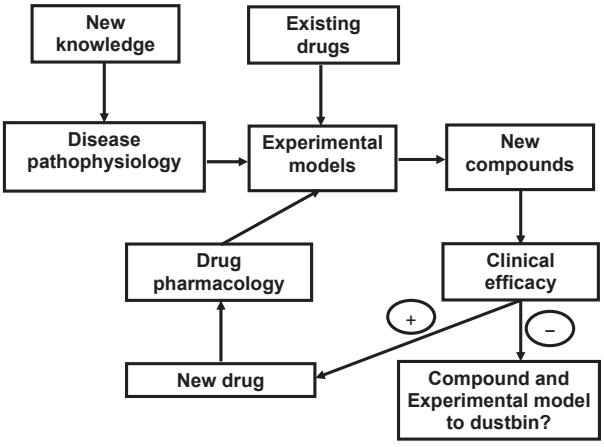


Figure 1.2. Experimental models based on disease pathophysiology are critical for the drug development process. New compounds found effective in these models undergo clinical evaluation. If found effective, the pharmacological properties of the new drugs help in evolving new experimental models, which provide the basis for improved new drugs.

The experimental models for migraine developed in the last two decades (11, 18) investigate principles such as: (i) constriction of dilated extracranial blood vessels, including carotid arteriovenous anastomoses (e.g. carotid vasculature or isolated blood vessels; vascular involvement), (ii) inhibition of the trigeminal system (e.g. blockade of plasma protein extravasation and/or central trigeminal inhibition, neurogenic involvement) (2), and (iii) a combination of the two (e.g. inhibition of neurogenic vasodilatation).

1.3.1 Migraine models based on vascular involvement

1.3.1.1 In vivo animal models

Constriction of carotid arteriovenous anastomoses in anaesthetized pigs

Although a complete understanding of migraine pathogenesis remains elusive, there seems to be little doubt that dilatation of cranial blood vessels, including carotid arteriovenous anastomoses, is involved in the headache phase of migraine (11, 18). In addition to headache, migraine patients also experience facial paleness, a reduction in facial temperature, an increase in temporal artery pulsations and swelling of the frontal vein on the side of the headache (28, 29). Thus, Heyck (17) measured the oxygen saturation difference between the arterial and external jugular venous blood samples (A-V SO_2 difference) during and after the headache phase of migraine and compared it with the healthy control groups. As shown in Figure 1.3 (left panel), he observed that the A-V SO_2 difference was abnormally small during the headache phase of migraine, likely due to dilatation of the carotid arteriovenous anastomoses, and that this decrease was normalised after spontaneous or drug-induced (ergotamine) alleviation of the headache (17).

Arteriovenous anastomoses are precapillary communications between the arteries and veins (Figure 1.3, right panel); they are predominantly located in the head skin, ears, nasal mucosa, eyes and dura mater in several species, including humans and pigs (30). In conscious animals, the arteriovenous anastomoses are constricted under a strong influence of the sympathetic neuronal tone, thereby shunting only a small (<3%) fraction of the total carotid blood flow (31, 32). In contrast, under pentobarbital anaesthesia, approximately 80% of the total carotid blood flow in the pig is shunted via arteriovenous anastomoses into the jugular venous circulation (32). Consequently, opening of the carotid arteriovenous anastomoses during migraine shunts a large quantity of oxygenated blood directly into the veins thereby resulting in facial pallor, a lowering of skin temperature and an increase in vascular pulsations (30). This increase in vascular pulsations stimulates the so-called 'stretch receptors' present in the wall of

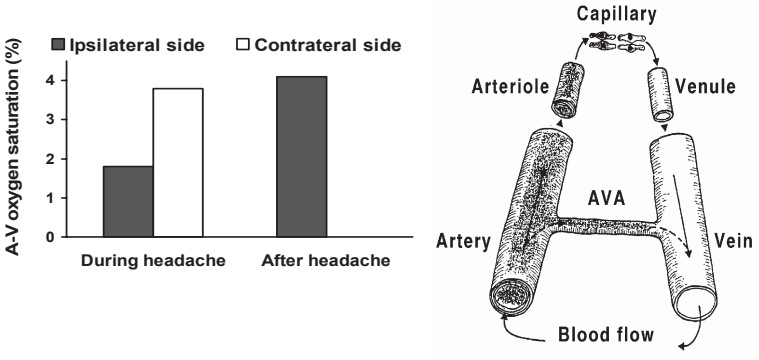


Figure 1.3. Arterio-jugular vein oxygen saturation difference on the ipsilateral side is lower than on the contralateral side during migraine headache (left panel, data from Heyck, 17), probably due to opening up of arteriovenous anastomoses (AVA), which, being direct communications between arteries and vein, shunt oxygen-rich blood to the venous side (right panel).

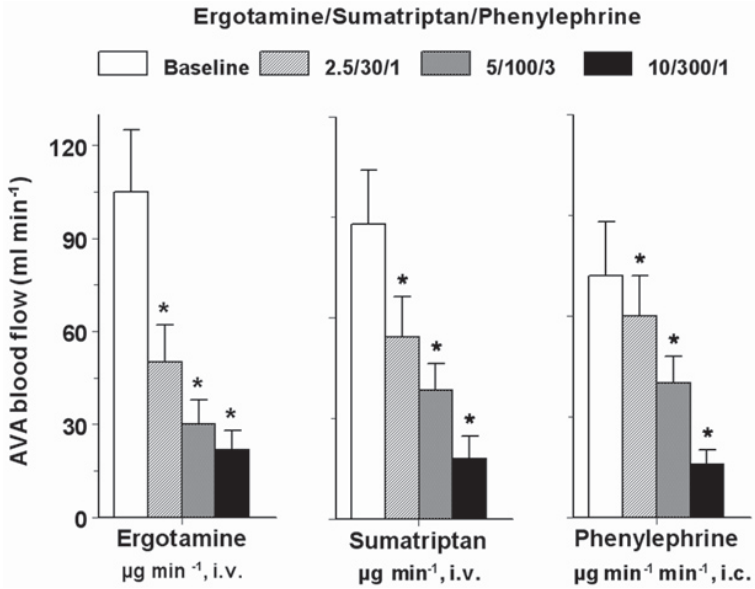


Figure 1.4. Arteriovenous anastomotic (AVA) blood flow measured at baseline and after infusions of ergotamine (2.5, 5 and 10 µg kg^{-1}, i.v.) sumatriptan (30, 100 and 300 µg kg^{-1}, i.v.) and phenylephrine (1, 3 and 10 µg kg^{-1} min^{-1}, intracarotid artery infusion). *, $P < 0.05$ vs. baseline values. Data, presented as mean ± s.e.m., are from: ergotamine (33), sumatriptan (34) and phenylephrine (35)

blood vessels, with ensuing activation of perivascular trigeminal nerves containing peptides (e.g. CGRP; 11, 18). The trigeminal cranial nerve conveys nociceptive information to central trigeminal nuclei that, in turn, relay the pain signals to higher centers where headache is perceived (5).

In accordance with the above findings, it is reasonable to assume that counteracting carotid arteriovenous anastomotic vasodilatation may abort migraine (11, 18). Therefore, an animal experimental model in anaesthetized pigs was developed; radioactive microspheres were used to measure the carotid arteriovenous anastomotic blood flow and the effects of antimigraine drugs on this parameter (30). Over the years, this model has proven predictive of antimigraine activity in the clinic (11, 18) and, in fact, two of the early molecules in the sumatriptan development programme were evaluated and found active in this model (P.R. Saxena, personal observations, 1981). The major advantage of the porcine model is that one can simultaneously study different vascular beds in order to evaluate the cranioselectivity of antimigraine drugs (18). Based on this notion, all acutely active antimigraine agents, including ergot alkaloids, triptans as well as α-adrenoceptor agonists potently constrict the porcine carotid bed and the corresponding arteriovenous anastomoses (18, 33-37); Figure 1.4 shows that ergotamine, sumatriptan and phenylephrine dose-dependently decrease arteriovenous anastomotic blood flow. These results, together with Heyck's (17) observations of decreased A-V SO_2 difference during migraine headache, shed light on the mechanisms of action of acutely-acting antimigraine drugs. Hence, the porcine carotid (arteriovenous anastomotic) circulation is an experimental model highly predictive of antimigraine activity, but this model will only pick up potential antimigraine drugs acting via vascular mechanisms.

Constriction of the canine external carotid bed

All acutely effective antimigraine drugs available thus far also produce selective constriction of cephalic blood vessels in the dog, including the external carotid artery, its temporal branches and arteriovenous anastomoses (11, 18). In this context, although serotonin (5-HT) (38), sumatriptan (38, 39), ergotamine and dihydroergotamine (40, 41) elicit a selective external carotid vasoconstriction in vagosympathecto-

mised dogs, different mechanisms are involved. Thus, 5-HT and sumatriptan produced a dose-dependent vasoconstriction amenable to blockade by the 5-$HT_{1B/1D}$ receptor antagonist GR127935 (42), suggesting that 5-$HT_{1B/1D}$ receptors are involved, but the profile of antagonism is different. The vasoconstrictor responses to both 5-HT and sumatriptan are completely blocked by GR127935, but in case of 5-HT a dose-dependent vasodilator component is unmasked (43).

A further pharmacological analysis of the 5-HT-induced vasodilator responses in animals pre-treated with GR127935 revealed that this effect is mediated by the 5-HT_7 receptor (44). Moreover, with the use of selective 5-HT_{1B} (SB224289) and 5-HT_{1D} (BRL15572) receptor antagonists, it was shown that the external carotid vasoconstrictor response is mediated by the 5-HT_{1B} (not 5-HT_{1D}) receptor (45).

The ergot alkaloids ergotamine and dihydroergotamine (46, 47) produce selective and long-lasting vasoconstriction in the canine external carotid bed (48), but the pharmacological profile of this effect is complex (49). Using GR127935, yohimbine and prazosin as antagonists, we showed that it is mainly mediated by 5-$HT_{1B/1D}$ receptors and α_2-adrenoceptors (41); the latter is consistent with subsequent findings showing that both α_1- and α_2-adrenoceptors mediate the canine external carotid vasoconstriction to adrenaline and noradrenaline (50). Since α_2-adrenoceptors include at least three receptor subtypes (α_{2A}, α_{2B} and α_{2C}) (51), we investigated the pharmacological profile of the above responses to ergotamine and dihydroergotamine. Thus, using selective antagonists, such as SB224289 (5-HT_{1B}), BRL15572 (5-HT_{1D}), rauwolscine (α_2-adrenoceptors), BRL44408 (α_{2A}), imiloxan (α_{2B}) and MK912 (α_{2C}), it was revealed that the canine external carotid vasoconstriction to dihydroergotamine (52) and ergotamine (53) is mediated by 5-HT_{1B} receptors and $\alpha_{2A/2C}$-adrenoceptors. Therefore, the canine external carotid bed is an experimental model highly useful for the screening of new compounds with potential antimigraine activity via a vasoconstrictor mechanism, also involving subtype-selective α-adrenoceptor agonists (54).

1.3.1.2 In vivo human models (i.v. infusion of vasodilator agents)

Several studies have reported that there is an increase in plasma CGRP concentrations associated with cranial vasodilatation during a migraine attack (see 18, 55, 56). Thus, the human *in vivo* models for migraine are based on the intensity and duration of the headache induced by infusions of headache-inducing substances: (i) nitroglycerin, (ii) histamine, (iii) prostaglandin E_1, (iv) m-chlorophenylpiperenzine, or (v) CGRP (57). These substances are infused in both migraineurs and non-migraineurs (healthy controls) and the responses are evaluated on the basis of a verbal scoring method (pain intensity) established by the international headache society (IHS).

Amongst the substances that induce headache, nitroglycerin is most useful because it affects vascular tone, inflammation, central desensitisation and pain transmission (58). The major advantages of using nitroglycerin are: (i) good tolerance, (ii) short half-life, (iii) blood-brain barrier penetration, (iv) ease of administration, and (v) relatively few side effects. Nitroglycerin produces an immediate, short-lasting and pulsatile headache in healthy volunteers. In contrast, in patients with a history of migraine, a mild to moderate headache is initially observed; nevertheless, a genuine migraine-like headache is observed 5-6 hours after nitroglycerin infusion (57). The mechanism of action of nitroglycerin to produce headache involves the release of nitric oxide, a potent vasodilator that plays a role in nociception. The importance of nitric oxide as a potential initiator of the migraine attack opens a new avenue for the treatment of migraine and other vascular headaches (57). Migraineurs seem to be supersensitive to nitric oxide donors (57) and the vascular effects of CGRP released from trigeminal nerves, at least in a rat migraine model, are partly mediated by the endothelial release of nitric oxide (59). Indeed, i.v. infusions of h-αCGRP in migraineurs can produce a migraine-like headache (58); hence, the trigeminal release of CGRP is a reliable marker for migraine (56). Similar to nitroglycerin, i.v. infusions of histamine in migraineurs produced both an immediate and a delayed headache, which was blocked by the histamine H_1 receptor antagonist mepyramine (60). Moreover, the phosphodiesterase-5 inhibitor dipyridamole also produced a mild to severe headache in healthy volunteers (61).

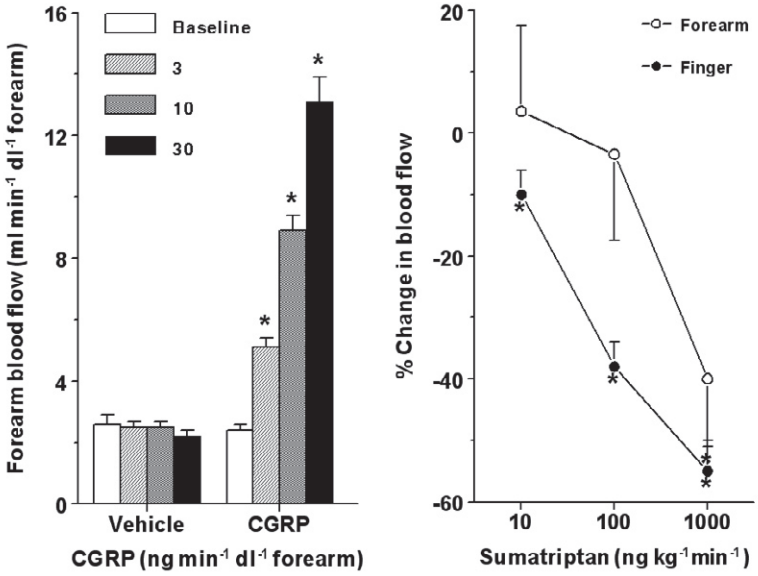


Figure 1.5. Left panel: Changes in human forearm and finger (right panel only) blood flow to infusions of CGRP and sumatriptan, as assessed by venous occlusion plethysmography. *, P<0.05 vs. respective baseline values. Data, presented as mean±s.e.m., are from De Hoon et al. (62; left panel) and Van Es et al. (63; right panel).

Another model in humans determines the effects of vasodilator substances on the forearm blood vessels (62). Infusions of CGRP in the brachial artery result in a marked vasodilatation of the forearm vasculature, as assessed by venous occlusion plethysmography (Figure 1.5, left panel) and this vasodilatation is, at least in part, dependent on the local release of nitric oxide (62). In fact, the vascular beds of the forearm and fingers can be used to study the mechanisms of the antimigraine drugs in the peripheral vasculature (63, 64); in this respect, sumatriptan (Figure 1.5, right panel) and some second generation triptans constrict brachial blood vessels, suggesting that vasoconstriction is an important mechanism behind the therapeutic efficacy of triptans (63, 64). Van Es et al. (63) demonstrated that sumatriptan also reduces arteriovenous anastomotic blood flow in human forearm.

Despite the availability of volunteers (healthy as well as migraineurs) and the numerous advantages implicit in human models of migraine, such benefits are limited by practical and ethical issues. Therefore, human isolated blood vessels are increasingly being employed as experimental models.

1.3.1.3 In vitro human models

These models offer several advantages: (i) drug-receptor interactions at equilibrium, (ii) the possibility to carry out a detailed pharmacological analysis, mounting multiple segments of blood vessel in parallel, (iii) no influence by pharmacokinetic factors, and (iv) exclusion of central and autonomic mechanisms as well as the effects produced by circulating hormones, distending pressure, etc. Notwithstanding, the benefits provided by the *in vitro* models are only complementary to the information obtained with *in vivo* models. The *in vitro* models that stand out in characterizing potential antimigraine drugs are isolated cranial (meningeal, temporal, basilar) and coronary arteries; the results obtained from these blood vessels allows one to assess cranial selectivity.

Isolated cranial blood vessels

As previously pointed out, the therapeutic efficacy of acutely acting antimigraine drugs is most probably mainly mediated by constriction of dilated cranial arteries (6, 11, 18). Therefore, this model considers the

use of three cranial arteries, namely the middle meningeal, the basilar and the temporal (65-68). The human middle meningeal artery is heavily innervated with afferent sensory fibres containing substance P, neurokinin A and CGRP and originating from the trigeminal ganglion (69). Cumulative concentration response curves are used to determine the vasoconstrictor potency and efficacy of the prospective antimigraine agent. The rank order of potency of 5-HT receptor agonists in this preparation positively correlates with affinity measurements in cell lines expressing the 5-HT_{1B} receptor (65). Molecular studies have detected mRNA encoding predominantly 5-HT_{1B} receptors, but also other 5-HT receptors like 5-HT_{1P}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4 and 5-HT_7 (70, 71). In line with the above findings, it has been demonstrated that during a migraine attack blood flow velocity in the middle cerebral artery significantly decreases, and this is normalized after administration of sumatriptan (72). Indeed, sumatriptan also constricts cranial vessels *in vitro* (66, 67, 73). Therefore, studying human isolated cranial blood vessels gives reliable information regarding the behaviour of these vessels in migraine headache and the effects of novel therapeutic agents.

CGRP has also been widely studied by using *in vitro* human models. In the development of CGRP receptor antagonists, like BIBN4096BS, as a viable option for acute antimigraine therapy, not only studies in human arteries (74, 75) but also in other species (76-78) help in understanding the mechanism of action of CGRP antagonists. Denudation of the arteries provides information whether the receptors are present on the endothelium or in the vascular smooth muscle of arteries. The *in vitro* models also give us insight into downstream signalling, as CGRP activates adenylyl cyclase to increase cyclic adenosine monophosphate (cAMP), hence causing vasodilatation. This increase in cAMP induced by CGRP is attenuated by the CGRP receptor antagonist BIBN4096BS (75, 80). Further, to investigate the role of endogenous neuropeptides in perivascular nerve endings, artery segments can be stimulated chemically (81) or electrically to release CGRP and other neuropeptides.

Isolated coronary blood vessels

Human isolated coronary arteries are useful in analyzing a major side-effect potential of antimigraine drugs, namely, the chest symptoms (chest pressure, tightness and pain). Admittedly, the chest symptoms may not be in all cases due to coronary vasoconstriction, but they occur in up to 15% of patients treated with sumatriptan (82, 83). The right epicardial coronary artery is the most commonly used segment for the *in vitro* studies.

It is now well known that triptans constrict coronary arteries, but the effect on cranial vessels, where, contrary to peripheral arteries, the 5-HT_{1B} rather than 5-HT_2 receptor dominates, is more marked (see 46, 83). Eletriptan (Figure 1.6; 68, 84), sumatriptan (68, 84), rizatriptan (70), frovatriptan (85), almotriptan (86) as well as donitriptan (87) have been demonstrated to be several fold more potent in contracting the human middle meningeal artery than the coronary artery. More importantly, the magnitude of contraction elicited by triptans in cranial vessels is much more than that in the coronary arteries. Therefore, at therapeutic plasma concentrations, triptans contract cranial vessels much more than the coronary artery (Figure 1.6, 68, 84-87). The reason for this is not clear, but it may be related to the higher density of 5-HT_{1B} receptors in the cranial compared to coronary arteries (70).

Other studies have analyzed the effects of CGRP receptor ligands in the coronary arteries (75, 80, 88). Accordingly, BIBN4096BS, the only CGRP receptor antagonist evaluated in clinical trials for acute antimigraine therapy, does not constrict coronary arteries (75); thus, BIBN4096BS seems to posses a clear advantage over the triptans. However, it is yet to be ascertained whether the antagonism of coronary artery dilatation by BIBN4096BS has consequences during pathophysiological conditions, where endogenous CGRP may be important, for example, cardiac preconditioning (89).

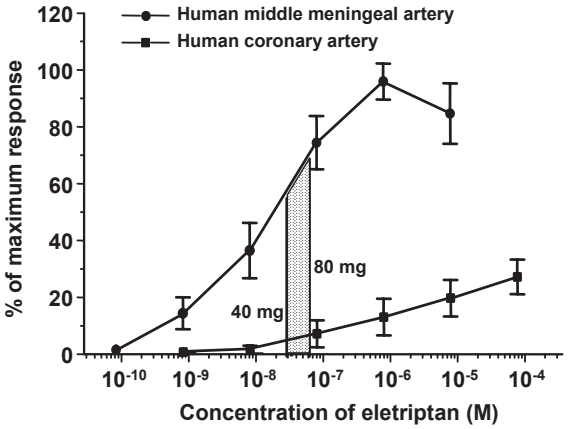


Figure 1.6. Concentration response curves of eletriptan on human middle meningeal and coronary arteries. Superimposed on these curves are the free C_{max} (protein-unbound fraction of the maximum plasma concentration) of eletriptan observed in human subjects after oral ingestion of a single 40-mg or 80-mg tablet. It may be noted that the drug elicits a substantial contraction of the middle meningeal artery, while there is only a minimal effect on the coronary artery (79).

1.3.2 Migraine models based on neurogenic involvement

The basic perception behind the development of neurogenic models for migraine is that migraine pain is due to a sterile neurogenic inflammation within the meninges followed by activation of trigeminal nerve terminals (90) to release neuropeptides (substance P, neurokinin A and CGRP) responsible for some features of migraine (2). Thus, the efficacy of acutely-acting antimigraine drugs is believed to be due to a presynaptic action on sensory nerves, thereby inhibiting neuropeptide release (2). Moreover, mechanisms, which do not seem to be mediated solely by the 5-$HT_{1B/1D}$ receptor have also been implicated in migraine relief (91). These include inhibition of the trigeminovascular system peripherally and/or centrally (2, 27).

1.3.2.1 Inhibition of plasma protein extravasation after trigeminal stimulation

The concept of plasma protein extravasation in migraine pathogenesis is based on the observation that plasma protein extravasation is produced by antidromic stimulation of trigeminal ganglia and sensory nerves in rats and guinea-pigs (92). Since clinically effective antimigraine agents, such as the ergots, triptans, opioids and valproate, inhibited this sterile neurogenic inflammation, suggesting that inhibition of plasma protein extravasation could be predictive of antimigraine therapeutic activity (92). Indeed, sumatriptan was shown to inhibit plasma protein extravasation and this effect was attenuated by the 5-$HT_{1B/1D}$ receptor antagonist GR127935 in both rats and guinea-pigs, showing the involvement of 5-$HT_{1B/1D}$ receptor subtypes (90). However, in mice this effect proved to be mediated by the 5-HT_{1B} receptor, whereas in guinea-pigs and rats, it was a 5-HT_{1D} receptor-mediated response (90, 93, 94). Although triptans have high affinity for both 5-HT_{1B} and 5-HT_{1D} receptor subtypes, they may also act on other subtypes (90). In this respect, plasma protein extravasation was also inhibited by 5-carboxamidotryptamine and CP122,288 in 5-HT_{1B} receptor knockout mice and this effect was not prevented by GR127935 in guinea-pigs (93, 95). Likewise, CP122,288 (which displays high affinity for 5-ht_{1F} receptors) showed a much higher potency than sumatriptan in rats and this did not correlate with its affinity for 5-HT_{1B} or 5-HT_{1D} receptors. (93, 95); this suggests that at least part of the CP122,288 action may be via the 5-ht_{1F} receptor. Moreover, a number of 5-HT_1 receptor agonists that inhibit plasma protein extravasation in guinea-pig dura mater display a rank order of potency that correlated with their affinity towards the 5-ht_{1F} rather than the 5-HT_{1B} or 5-HT_{1D} receptor subtype (90, 96). Accordingly, a selective 5-ht_{1F} receptor agonist, LY344864, was developed (96, 97). This compound inhibited plasma protein extravasation

following trigeminal ganglion stimulation in rats and was found modestly effective in acute migraine treatment (98). However, it remains unclear whether the two properties are related. LY344864 also inhibited the activation of brainstem neurons in response to the stimulation of dura mater as well as the c-fos expression in trigeminal nucleus caudalis (90); this suggests that the primary mechanism of LY344864 is central (i.e. interruption of the ascending pain pathways) rather than peripheral (inhibition of plasma protein extravasation) (90). Interestingly, the selective 5-HT_{1D} receptor agonist PNU-142633F, which blocked plasma protein extravasation in guinea-pigs (99), was ineffective in migraine treatment (100). Other studies have shown that plasma protein extravasation can be inhibited by the CGRP receptor antagonist $CGRP_{8-37}$ (101, 102).

Plasma protein extravasation models do not always predict antimigraine efficacy (2), as clearly evidenced by the failure of several compounds in clinical trials, including: (i) the NK_1 receptor antagonist, lanepitant (103), (ii) specific plasma protein extravasation inhibitors, such as CP122,288 and 4991W93 (104), (iii) the $ET_{A/B}$ receptor antagonist bosentan (105), and (iv) the neurosteroid ganaxolone (106). In addition, the clinical antimigraine predictability of plasma protein extravasation assays became questionable following an elegant clinical study in migraine showing no increases in retinal or choroid permeability (107); this contrasts with the increase in retinal or choroid permeability following trigeminal ganglion stimulation in rats (90).

1.3.2.2 Central trigeminal neuronal inhibition

The importance of the brainstem in the migraine pathogenesis is emphasised by its activation during migraine attacks, where blood flow increases in the cerebral hemispheres (cingulate, auditory and visual cortex) as well as brainstem (108). Sumatriptan relieved the headache and reversed the increase in cerebral blood flow, but not in the brainstem, indicating that persistent brainstem activation is due to other factors, including increased activity of the endogenous antinociceptive system. Moreover, the brainstem activation may be inherent of the migraine process itself, and continuous activation of the brainstem (despite symptom resolution by sumatriptan) may account for the headache recurrence (108).

Based on this finding, animal migraine models have been developed to study c-fos activation of the trigeminal nucleus caudalis; interestingly, this effect was not altered by sumatriptan (109-111). However, the second generation triptans, such as zolmitriptan (112), naratriptan (113) and eletriptan (113, 114) as well as dihydroergotamine (115) inhibited the action potentials generated in the trigeminal nucleus caudalis after superior sagittal sinus stimulation in cats and dural stimulation in rats (116). This discrepancy could be due to poor central penetration by sumatriptan (84) as compared to second-generation triptans with central inhibitory effects (2). Consequently, it has been argued that the blood-brain barrier may be disrupted during migraine (117); indeed, under experimental disruption of the blood-brain barrier by hyperosmolar mannitol, sumatriptan produced central inhibitory effects (118). However, there is little or no evidence for a disrupted blood-brain barrier based on computerised tomography or MRI findings in migraine patients (119, 120).

Several lines of pharmacological evidence indicate that potent antimigraine agents act on the second order trigeminal neurons to reduce cell activity, suggesting that trigeminocervical complex neurons in the caudal brainstem could be a possible target for antimigraine activity (27, 108). It is likely that this central inhibitory effect is mediated by 5-$HT_{1B/1D}$ receptors since the central inhibitory effect of eletriptan in cats is amenable to blockade by GR127935. In addition, the involvement of 5-HT_{1D} rather than 5-HT_{1B} receptors is crucial for this effect (113, 114). Moreover, CGRP mediates sensory nerve transmission between the first and second order afferent inputs from the cranial blood vessels, and centrally penetrating CGRP receptor antagonists may attenuate these sensory nerve transmissions. Recently, adenosine A_1 receptors were localised in human trigeminal ganglia, suggesting a potential usage of adenosine A_1 receptor agonists to inhibit trigeminal nociception (13).

A strong link between migraine and glutamatergic system is suggested on the basis that migraine pain-relay centres contain glutamate-positive neurons and, glutamate is implicated in cortical spreading depression, trigeminovascular activation, and central sensitization (121). Thus, glutamate receptor-subtype antagonists may be useful in migraine; indeed, some compounds are effective in preclinical models of migraine and probably in the clinic (122, 123). Moreover, the selective NMDA receptor antagonists memantine and MK-801, dose-dependently and significantly reduced *c-fos* expression induced by capsaicin (124).

The selective blockade of NK_1 receptors decreased *c-fos* response in the trigeminal nucleus caudalis after electrical (125) or chemical (126) stimulation of the trigeminovascular system. Another major molecular marker extensively studied in relation to migraine is the nuclear factor-κB (NF-κB) (127). This factor plays a pivotal role in iNOS induction and controls the transcription of the acute phase proteins (128), which, under basal conditions, sequesters NF-κB within the cytoplasm. In a rat migraine model, infusion of the nitric oxide donor glyceryl trinitrate produced activation of the NF-κB in the dura mater, which was suppressed by the anti-inflammatory parthenolide (127). This model based on probing the neuronal effects of nitric oxide has provided interesting insights into the neuroanatomic circuits and neuropharmacological mechanisms involved in the initiation and repetition of migraine attacks (7). This is in accordance with the efficacy of anti-inflammatory agents such as parthenolide and aspirin to reduce the frequency and intensity of migraine attacks (129, 130). These molecular markers in combination with *in vitro* and *in vivo* techniques might provide crucial insight into the migraine pathophysiology.

1.3.2.3 Central pain sensitization

Burstein and co-workers, who reported that application of an 'inflammatory soup' containing histamine, 5-HT, bradykinin an prostaglandin E_2 on rat dura activated trigeminal neurons and enhanced their sensitivity to mechanical stimuli, proposed that this chemosensitivity and sensitization is characteristic of some types of headaches and may contribute to throbbing of migraine pain (131). Early sumatriptan intervention (i.e. together with application of inflammatory soup) effectively blocked the development of all aspects of central sensitization (expansion of dural receptive fields, reduction of neuronal response threshold, spontaneous firing rate, increased neuronal response magnitude to skin brushing, and reduced response threshold to skin heating), but late sumatriptan intervention (2 hours after inflammatory soup) only counteracted the first two aspects of central sensitization (132). When both peripheral (trigeminal ganglion) and central (medullary dorsal horn) trigeminovascular neuronal potentials were simultaneously recorded in rats, sumatriptan prevented the induction of sensitization and normalized the heightened intracranial mechanical sensitivity following sensitization in central but not in peripheral neurons; however, the drug failed to attenuate the increased spontaneous activity established during sensitization in both neurons (27). These authors concluded that sumatriptan inhibits neither peripheral nor central trigeminovascular neurons directly, but it exerts its action via presynaptic $5\text{-}HT_{1B/1D}$ receptors in the dorsal horn to block synaptic transmission between axon terminals of the peripheral trigeminovascular neurons and cell bodies of their central counterparts, thus suggesting that the analgesic action of triptans manifests in the absence, but not in the presence of central sensitization (27).

In agreement with their studies in rats, Burstein and co-workers reported that over 75% of migraine patients develop cutaneous allodynia during migraine within the referred pain areas, initially on the ipsilateral side of the head and later on the contralateral side and on ipsilateral forearm and they hypothesized that the cutaneous allodynia can be used to predict the effectiveness of triptans (10, 133). Indeed, only 15% of patients with, compared to 93% without cutaneous allodynia were rendered pain-free by sumatriptan within 2 hours of treatment and similar responses were observed in the two groups whether sumatriptan was administered early or late in migraine attack (132). Interestingly, sumatriptan effectively terminated the throbbing of migraine pain (peripheral sensitisation) in the vast majority of patients, even when pain relief was incomplete or allodynia was not suppressed (132).

The value of this model, as indeed of other neurogenic models, is still unclear. So far, only sumatriptan has been studied and one is uncertain if this triptan indeed passes the blood-brain barrier effectively (see 6). However, the stock of this model will increase if a new compound, selected on the basis of this model and devoid of vasoconstrictor properties, is found clinically effective.

1.3.2.4 Trigeminal primary afferents in vitro

Slices of rat trigeminal nucleus caudalis were used to study the release of neurotransmitters, namely CGRP (134). Similarly, slices of rat neocortex were used to study cortical spreading depression induced by exposure to artificial cerebrospinal fluid with elevated potassium chloride (135). In line with this model, ifenprodil, an NR2B receptor subunit-selective NMDA receptor antagonist, suppressed the spreading depression induced in slices of mouse entorhinal cortex (136).

1.3.3 Integrated migraine models

The pathogenesis of migraine appears to be an integrated process involving the trigeminovascular system (11, 18). Accordingly, innovative models of migraine have been developed in which the trigeminal ganglia/nerves are stimulated and the neuroinflammatory processes, such as vasodilatation, trigeminal nociception and trigeminal CGRP release are determined (11, 18). CGRP not only dilates cranial blood vessels, but also transmits vascular nociception (5). The obvious advantage of these models is that they allow studying both presynaptic and postsynaptic actions of any potential antimigraine compound.

1.3.3.1 Electrical stimulation of the trigeminal ganglia/nerves and superior sagittal sinus

Electrical stimulation of the trigeminal nerve in humans evokes the release of CGRP in cranial venous blood (2). Moreover, during the headache phase of migraine, the plasma concentration of CGRP, but not of substance P, increases in the jugular venous blood (2). Therefore, CGRP released from trigeminal sensory nerves innervating cranial blood vessels produces vasodilatation thereby causing headache (90, 137, 138). On this basis, several animal models have been developed to demonstrate: (i) cranial vasodilatation associated to the trigeminal release of CGRP, and (ii) the inhibitory effects of antimigraine drugs on this parameter. Triptans attenuate cranial vasodilatation induced by trigeminal stimulation as well as CGRP release in rats. However, carotid vasodilatation in guinea-pigs following trigeminal ganglion stimulation is mediated by vasoactive intestinal peptide, which was not amenable to blockade by antagonists at CGRP or tachykinin receptors (139, 140). Therefore, another model was developed in which trigeminal sensory Aδ-fibres that only release CGRP are electrically stimulated and the dural blood vessel diameter is measured by an intravital microscope through a closed cranial window (Figure 1.7) (90, 137, 138). Electrical stimulation of this cranial window as well as intravenous infusion of substance P and αCGRP in rats increase dural blood vessel diameter (90, 94). Interestingly, the NK_1 receptor antagonist, RP 67580, clearly antagonised substance P-induced vasodilatation, but not the neurogenic vasodilatation (90).

In contrast, the CGRP receptor antagonist, $CGRP_{8-37}$, abolished the vasodilatation induced by both αCGRP and neurogenic stimulation (138, 141) (Figure 1.8, upper left panel), demonstrating that the neurogenic vasodilatation is mediated by endogenous CGRP released from trigeminal sensory nerves. This observation is consistent with clinical data showing that the levels of CGRP, but not those of substance P, are elevated during the headache phase of migraine (2). A recent study on this model showed that BIBN4096BS produced a significant inhibition of both CGRP- and electrically-induced increases in the diameter of meningeal arteries, but not of pial and cerebral arteries (142). Significantly, sumatriptan attenuated dural vasodilatation following trigeminal stimulation (Figure 1.8, right panels), but not the response to CGRP (Figure 1.8, lower left panel; 137), probably via presynaptic inhibition of CGRP release. The use of selective antagonists suggest that the above inhibitory effect of sumatriptan is mediated via prejunctional $5\text{-}HT_{1B}$ receptors in rats and $5\text{-}HT_{1D}$ receptors in guinea-pigs, cats and humans (90).

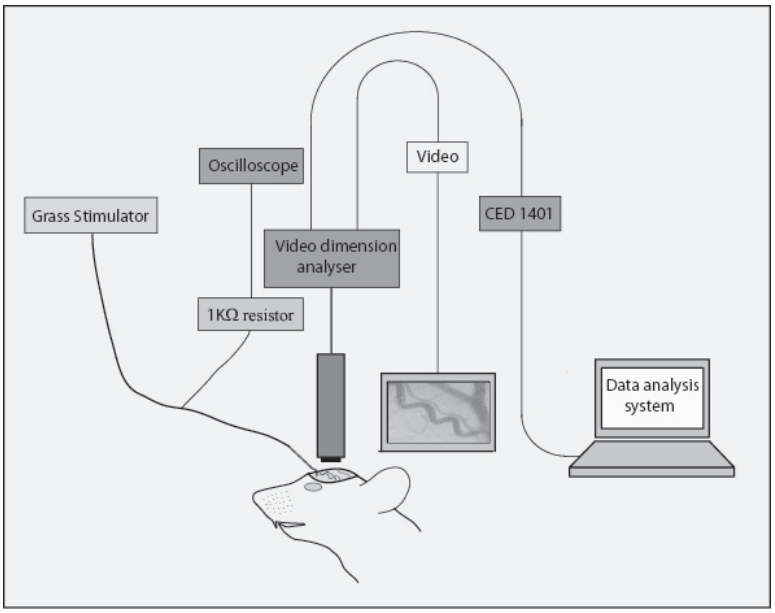


Figure 1.7. Overview of the intravital set-up for neurogenic dural vasodilatation
The magnified image of the dural blood vessel is displayed on a television monitor. The diameter of the blood vessel is measured by the video dimension analyser, the signal from which passes through the video and then onto the monitor, so that the visual display can be recorded. There is a second output from the video dimension analyser that passes the diameter signal through the CED 1401, which enables analysis of the data online, and then finally into the computer with the Spike 4v2 software which displays the data online for analysis. The figure also shows how the cranial window is given electrical stimulation from the Grass stimulator, and the current, representing the contact between the electrode and skull surface, was displayed on an oscilloscope after passing through a 1kΩ resistor.

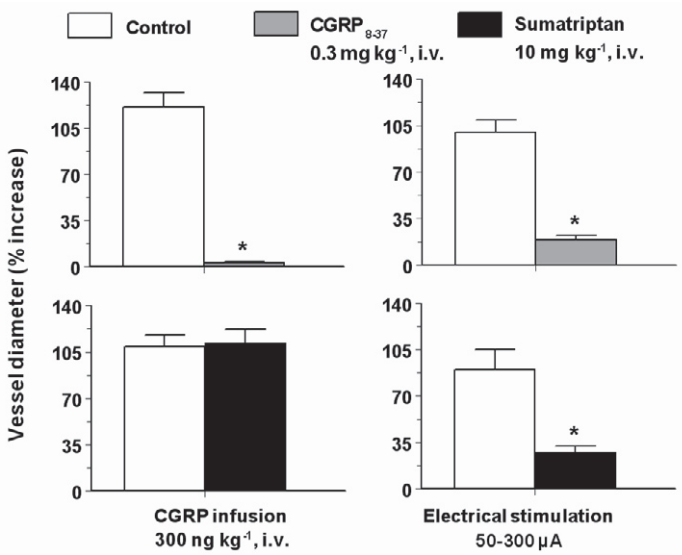


Figure 1.8. Increase in the dural vessel diameter (%) produced by CGRP (left panels) and electrical stimulation of the surface of a cranial window (right panels) in the anaesthetised rat before (control) and after $CGRP_{8-37}$ or sumatriptan.*, $P < 0.05$ compared with the control values. Data, presented as mean±s.e.m., are from Williamson et al (137, 138).

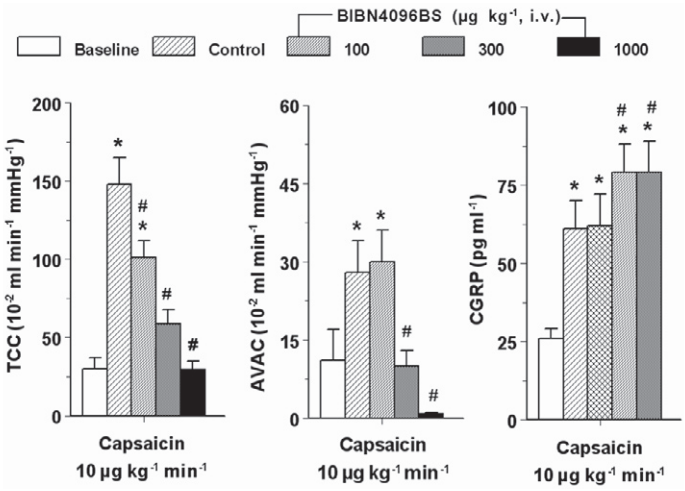


Figure 1.9. Total carotid conductance (TCC), arteriovenous anastomotic conductance (AVAC) and jugular venous plasma CGRP concentrations measured at baseline and following infusions of capsaicin (10 µg kg^{-1} min^{-1}, intracarotid artery infusion) given in anaesthetised pigs before (control) and after i.v. administrations of BIBN4096BS (100, 300 and 1000 µg kg^{-1}). *, P < 0.05 vs. baseline values; $^{\#}$, P<0.05 vs. response after the corresponding volume of vehicle (data not shown). All data, presented as mean±s.e.m., are from Kapoor et al. (144).

1.3.3.2 Chemical stimulation of sensory nerve fibres with capsaicin

Stimulation of sensory nerve fibres results in the release of neuropeptides in both the central and peripheral nervous system (143). In the former, these neuropeptides transmit nociceptive signals to the spinal cord and brain, whilst in the latter they promote neurogenic inflammation, such as plasma protein extravasation, neuropeptide release and vasodilatation. This model allows us to study the inhibitory interactions between antimigraine agents and peripheral trigeminal fibres.

Several studies have shown that sensory nerves innervating the cerebral vasculature contain substance P and CGRP (5); however, capsaicin-induced relaxation of guinea-pig isolated basilar artery is mainly mediated by CGRP (145, 146). In this context, in an established porcine migraine model, capsaicin was infused into the carotid artery and the carotid haemodynamic responses as well as trigeminal CGRP release were investigated using BIBN4096BS (144). The results clearly show that capsaicin-induced carotid and arteriovenous anastomotic vasodilatation are mainly mediated by CGRP (Figure 1.9). Admittedly, as reported earlier (147), vasodilator responses to capsaicin tend to wear off following subsequent infusions of capsaicin, suggestive of tachyphylaxis, which was rather limited, possibly due to a neuronal reuptake of released CGRP into capsaicin-sensitive perivascular nerves (148).

1.4 FUTURE DIRECTIONS AND PERSPECTIVES

Overall, migraine models have provided significant insights into pathophysiological mechanisms. Indeed, a new era in migraine research has dawned with the revelation of several genetic mutations linked to this neurovascular disorder. Mutations in the P/Q-type calcium channel CACNA1A, Na^+/K^+ pump ATP1A2 or neuronal voltage-gated $Na_v1.1$ sodium channel SCN1A genes (149-153) can result in familial hemiplegic migraine. A knock-in mouse carrying the human pure FHM-1 R192Q mutation gene has reduced threshold and increased velocity of cortical spreading depression (154). Although the role of these genes is yet to be clarified, incorporating knowledge of the heritability of migraine in existing migraine models may shed further light on the pathogenesis of migraine. Also, a major part of migraine research should be fo-

cussed on preventive medications, which are believed to affect abnormal neuronal changes in the brain (155). In this respect, putative prophylactic antimigraine drugs, such as botulinum toxin type A (156) and flunarizine (157), may decrease CGRP release from trigeminal neurons and reduce spontaneous synaptic currents in rat neocortex, respectively. These findings suggest that migraine models can be used to study the mechanisms involved in migraine prophylaxis. However, the challenge of finding novel and more effective therapeutic strategies that prevent or control migraine attacks still remains open.

The continuing evolution of appropriate migraine models is instrumental in understanding the complex pathophysiology of this syndrome and development of more selective antimigraine drugs. Evidently, this requires an integrative approach that involves the use of several models (rather than only one) so that the advantages add up to the progress and the limitations are minimized. In this respect, the introduction of transgenic animal models and pharmacogenomics will further advance our understanding at genetic and molecular levels. This will ultimately result in new therapeutic options for patients whose migraine attacks presently remain inadequately controlled.

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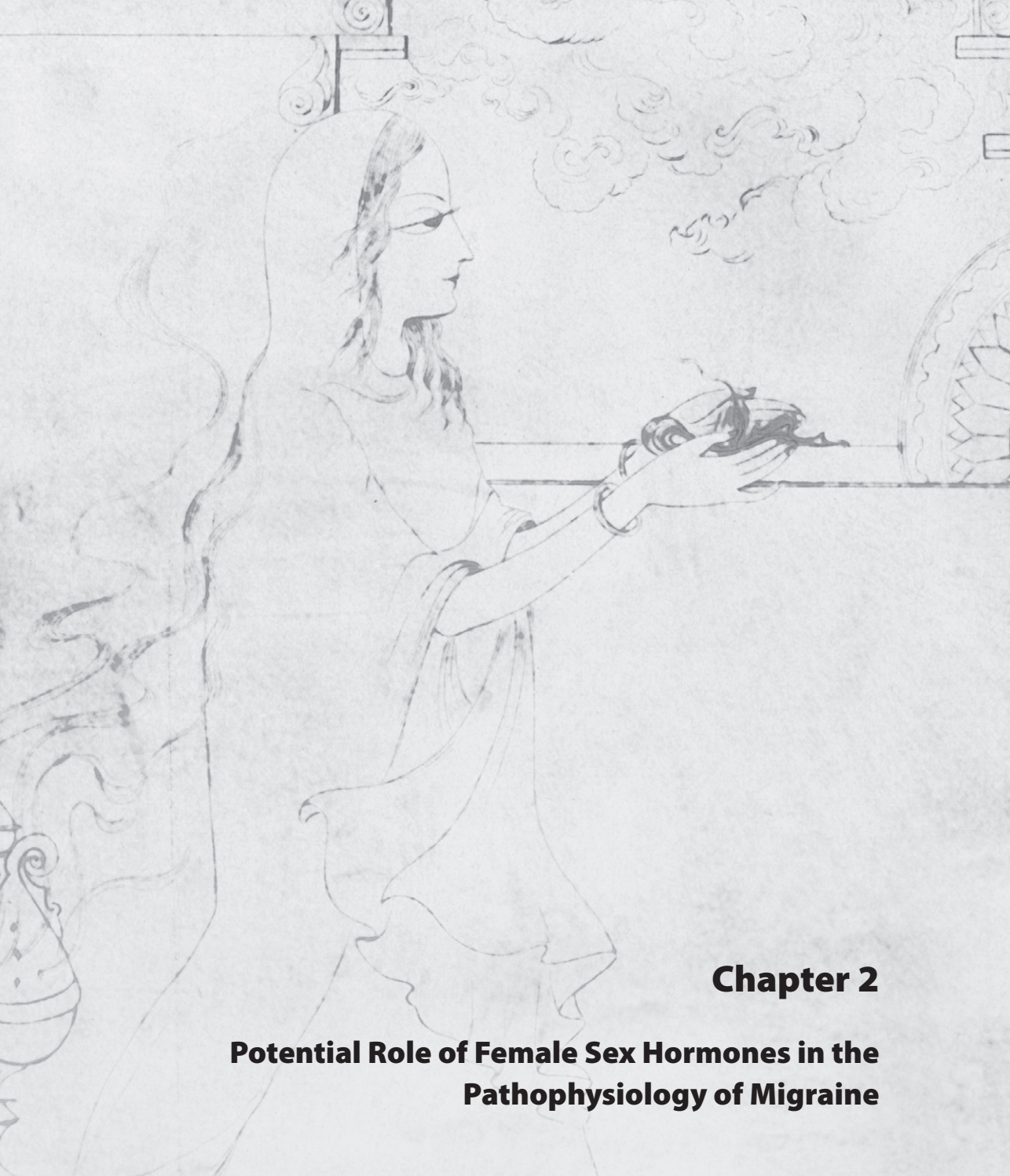

Chapter 2

Potential Role of Female Sex Hormones in the Pathophysiology of Migraine

Based on: S. Gupta, S. Mehrotra, C.M. Villalón, M. Perusquía, P.R. Saxena, A. MaassenVanDenBrink, Pharmacology & Therapeutics (Accepted with revisions)

ABSTRACT

Clinical evidence indicates that female sex steroids may contribute to the high prevalence of migraine in women, as well as changes in the frequency or severity of migraine that are in tandem with various reproductive milestones in women's life. While estrogen and progesterone do not seem to be involved in the pathogenesis of migraine *per se*, they may modulate several mediators and/or receptor systems *via* both genomic and non-genomic mechanisms. These actions may be perpetuated at the central nervous system, as well as at the peripheral (neuro)vascular level. For example, estrogen enhances neuronal excitability by elevating Ca^{2+} and decreasing Mg^{2+} concentrations; this may act in concurrence with other mechanisms triggering migraine. Further, estrogen is reported to enhance the synthesis and release of nitric oxide and neuropeptides, such as calcitonin gene-related peptide; this in turn reinforces vasodilatation and activates trigeminal sensory afferents with a subsequent stimulation of pain centres. In addition, female sex steroids may increase the function of receptors mediating vasodilatation, while responses of receptors inducing vasoconstriction are attenuated. The serotonergic, adrenergic and GABAergic systems are also modulated by sex steroids, albeit to a varying degree and with potentially contrasting effects on migraine outcome. Taken together, female sex steroids seem to be involved in an array of components implicated in migraine pathogenesis. Future studies will further delineate the extent and the clinical relevance of each of these mechanisms, and will thus expand the knowledge on the femininity of migraine.

2.1 INTRODUCTION

Migraine is a common neurovascular syndrome, which is typified by intense, unilateral, throbbing and pulsatile headache attacks, lasting for 4-72 h and accompanied by anorexia, nausea, vomiting, photophobia and/or phonophobia (1, 2). In about 15% of patients, an aura may precede the migraine headache within about one hour (*migraine with aura*). Migraine prevalence peaks between ages of 35-45 years (3), a period, which is most productive in life. Thus, not only does migraine reduce quality of life, as evident from the fact that the WHO ranks it among the world's most disabling illnesses (4), but it also results in a considerable economic loss to the society in terms of lost workdays (5). Although in the past one and a half decade constructive progress has been made in understanding migraine pathogenesis, the exact cause of this complex disorder has not yet been unravelled.

Since the prevalence of migraine is 2-3 fold higher in women than in men (3, 6) (Figure 2.1), and reproductive milestones such as menarche, pregnancy and menopause are associated with changes in migraine frequency and/or severity, female sex hormones have been implied to play a role in the migraine

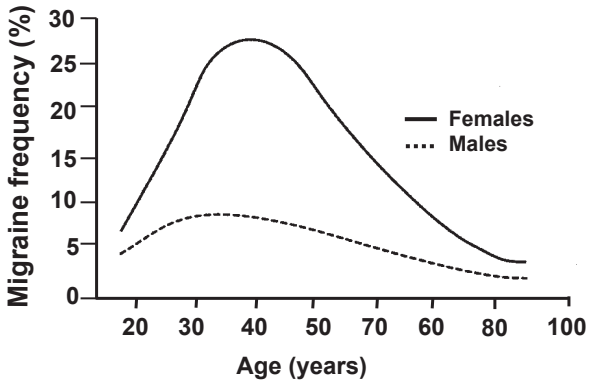

Figure 2.1. Adjusted age-specific prevalence of migraine by sex. Adapted from Lipton et al. (3).

pathophysiology. This review elaborates the changes in migraine prevalence during females' life, as well as the potential mechanisms *via* which female sex steroids may play a role in the pathophysiology of migraine.

2.2 PREVALENCE OF MIGRAINE

Migraine afflicts up to 15-20% of the general population (3, 6). The estimates of the prevalence of migraine across various studies shows large variations, as it may be influenced by age, gender, race, geography and socio-economic status (7, 8). Different stages in a woman's life are highlighted by changes in female hormone levels, especially estrogen and progesterone. As illustrated in the following sections, there is a concurrent change in the prevalence and severity of migraine during these different stages.

2.2.1 Before puberty

Paediatric migraine is one of the most common causes of headache in children, with a prevalence increasing from 3% at an age of 5 years to 14% at 15 years, with an overall prevalence of 11% (9). Although children with migraine often remain suffering from this disease as an adult, about one quarter of children suffering from migraine have been reported to be migraine-free before the age of 25 years (10). In very young children ($\leq$7 yrs) the prevalence of migraine is higher in boys than in girls (11), while with increasing age the incidence in females starts rising.

2.2.2 After puberty

Starting at puberty, the migraine prevalence in women increases and considerably exceeds that in men. At its peak (between 35 and 45 years), the prevalence in women is almost three times higher than in men (12). As many as 60% of female migraineurs report attacks during perimenstrual periods, i.e. two days before to three days after menstrual bleeding (13, 14). To address this phenomenon, the revised International Classification of Headache Disorders includes "candidate criteria" for two entities: *menstrually-related migraine* and *pure menstrual* migraine (Headache Classification Committee of the International Headache Society, 15). *Pure menstrual migraine,* which is not frequent (7-8%), is defined as migraine without aura that occurs exclusively on Day 1±2 of menstruation in at least 2 out of 3 menstrual cycles, while no migraine occurs at other times of the cycle. If such additional attacks, with or without aura, occur at other times of the cycle, it is defined as *menstrually-related migraine*. More than 50% women with migraine

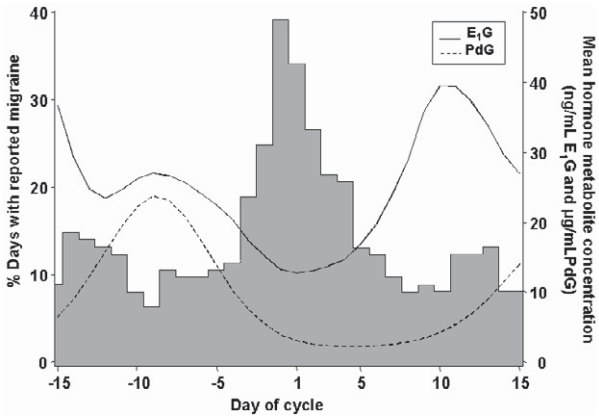

Figure 2.2. Migraine incidence and urinary estrone-3-glucuronide (E1G) and pregenandiol-3-glucuronide (PdG) levels recorded on each day of the menstrual cycle in 120 cycles from 38 women. Adapted from Massiou and MacGregor (280).

report an association between migraine and menstruation (16, 17). During menstruation, there is a rapid change in hormone levels and during the perimenstrual period there is an abrupt fall in female sex hormone levels towards the end of the luteal phase. MacGregor et al (18) reported a negative association between migraine and urinary estrogen levels across the menstrual cycle (Figure 2.2). The relative risk of a severe migraine increases by 3.4 fold on Days 1 to 3 of menstruation, as compared with other times of the month (14). In addition, headache severity was positively correlated with premenstrual syndrome severity on Days -3 to +6 of the menstrual cycle in a post-hoc study (19). Since studies suggested that the fall in female hormone levels is the trigger for migraine attacks, the use of estrogen supplements as preventive strategy has been assessed. In an elegant study, Somerville (20) showed that in 6 women suffering from menstrually-related migraine attacks could be postponed by a single injection of estradiol, and similarly Magos et al. (21) observed an improvement of more than 80% in women with menstrual migraine treated with subcutaneous estradiol implant. However, in a later study, Somerville (22) did not observe improvement of menstrual migraine with estradiol implant, although this negative result may have been related to the variable plasma concentrations of estradiol observed in this study. Interestingly, several days of high estrogen levels are thought to be a prerequisite before estrogen withdrawal can precipitate migraine attack (20).

2.2.3 After hormonal contraceptives

Hormonal contraceptives are generally composed of an estrogen analogue, usually ethinyl estradiol, and a progestin analogue. Some decades ago, the risk for migraine attacks in women previously not suffering from migraine was reported to be about 10 times higher in those starting with an oral contraceptive than in those without these hormones (23); the use of oral contraceptives in patients with pre-existing migraine increased the frequency of attacks (24). However, since the dose of ethinylestradiol in oral contraceptives has been reduced from $\geq$50 µg to 35 µg or even less, the influence of oral contraceptives on migraine seems to have been diminished (25), although in a recent study migraine was still more likely among women using oral contraceptives containing estrogen (26). Further, it is common that patients report either an improvement or worsening of their migraine attacks after starting oral contraceptives (27, 28), but a continued use of oral contraceptives tends to improve headaches that occur at the start of oral contraceptives (29). Migraineurs with aura are more likely to experience worsening of their attacks after starting with oral contraceptives, as compared to patients without aura (30, 31). This is further substantiated by the observation that visual, sensory and motor aura develop for the first time after start of oral contraceptives (32).

2.2.4 During pregnancy

During pregnancy, plasma levels of 17β-estradiol, estriol and progesterone increase to a level 10 to 100 times higher than in the nonpregnant state. The majority of women suffering from migraine without aura and menstrual migraine experience improvement during pregnancy (33). In patients suffering from migraine without aura, Sances et al. (34) reported improvement in 47% during the first, 83% during the second and 87% during the third trimester, while complete remission was attained in 11%, 53%, and 79% of patients, respectively. It is worth mentioning that women suffering from menstrually-related migraine experience maximum improvement in their migraine attacks (35, 36), probably due to constant levels of hormones. However, in some women attacks remain unchanged, worsen, or even appear for the first time during pregnancy, especially the attacks with aura (31, 37). Postpartum, the incidence of migraine may again increase (33). Breast feeding seems to protect from migraine recurrence postpartum (34); indeed headache during the first 3 months postpartum in breastfeeding mothers was reported to be similarly low as in the second trimester of pregnancy (38). The protective effect of breast feeding may be attributed to increased levels of oxytocin and vasopressin, which have anti-nociceptive properties (39, 40) or alternatively to low levels of the estrogen and progesterone in this period.

2.2.5 During and after menopause

Migraine worsens in many cases just before or during the menopause, while it generally improves afterwards. Changing hormone levels in the perimenopausal years seem to be responsible for the increase in migraine prevalence during this period (22, 41). Further, low levels of estrogen and high levels of follicle-stimulating hormone may be associated with lower migraine prevalence (42). Since migraine decreases in two third of women with spontaneous menopause and in only one third of women following surgical menopause, aging ovaries are thought to produce factors that may improve headache (43). Hormone replacement therapy (HRT) has a variable effect on migraine prevalence; in this respect, HRT has been reported to improve migraine in 45% of women, worsen in 46%, have no effect in the rest of women (44). Additionally, migraine attacks with aura may, similar to the situation in pregnancy, develop for the first time after the start of HRT (45). HRT can be adjusted by reducing the dose, employing different estrogens or using less fluctuating hormone regimens (46).

Further, knowledge about the role of female sex hormones in migraine may be obtained from some special conditions in which hormonal interventions are required. For example, in a retrospective study, male to female transsexuals have been reported to have a similar migraine prevalence as genetic females (47), suggesting that the prevalence of migraine may have been increased by the administration of female sex hormones to these subjects. Obviously, a prospective study, including female-to-male transsexuals as a control group for excluding a stress-related mechanism as cause for the high prevalence of migraine in transsexuals, would be necessary to confirm this finding. Further, in women undergoing *in vitro* fertilization, where an analogue of gonadotropin-releasing hormone was administered to downregulate estrogen levels, the low levels of 17β-estradiol correlated with increased headache prevalence (48). Interestingly, migraineurs have recently been reported to have a higher frequency of endometriosis and menorrhagia, possibly related to haematological, immunological, or genetic factors (49).

These epidemiological observations underline that there is a hormonal component in migraine pathophysiology, although the exact neurobiological mechanism has not yet been deciphered. In some conditions, such as the onset of puberty or menopause, when plasma levels of 17β-estradiol change (50), there is a concurrent change in migraine prevalence (3); however, in menstrual migraine, it is the rapid decline of female hormones, in literature mostly referred to as 'withdrawal', that is thought be the trigger for migraine (50). The fact that the correlation between plasma levels of female sex hormones and the prevalence of migraine is not simply positive or negative makes this correlation both complex and intriguing to investigate.

2.2.6 Genetics of migraine

The genes involved in the pathophysiology of migraine are difficult to search as migraine is an episodic disorder with a high prevalence, displaying both a clinical and genetic heterogeneity. However, three 'migraine genes' have now been identified in families with familial hemiplegic migraine (FHM), a rare from of migraine with an autosomal dominant inheritance pattern. These genes, CACNA1A (51), ATP1A2 (52) and SCN1A (53), are all involved in ion transport and encode subunits of $Ca_v2.1$ (α_{1A}) voltage-dependent P/Q type calcium channels, Na^+/K^+ pumps, or neuronal voltage-gated $Na_v1.1$ sodium channels, respectively. Possibly, these genes could also be involved in more common forms of migraine (54). It is not exactly known how these mutations could trigger migraine attacks, but it is believed that alternations in intracellular Ca^{2+} concentrations and abnormal K^+ levels, leading to increased neuroexcitability, play a critical role. Most intriguingly, as will be discussed in further detail later in this review, female sex steroids may also enhance neuronal excitability, and thus female sex steroids might enhance the effects of the ion channel or pump mutations mentioned above. Indeed, the prevalence of FHM is 2.5 times higher in women than in men (55).

The genetic predisposition for migraine is similar in men and women (56, 57), but the 3-fold higher incidence in females at peak prevalence, and the increased risk of migraine in first degree relatives of

only male probands (58, 59) suggests the possibility of an X-linked dominant gene. In accordance, Nyholt et al. (60, 61) have reported that a locus on the X chromosome (Xq24-28) may be related to migraine susceptibility in some families, although it is unlikely that this locus completely accounts for the sex difference in migraine prevalence, even when one disregards the influence on migraine prevalence due to changes in hormone levels during life.

Some genetic studies have tried to decipher the tentative relation between female sex hormone receptors and migraine. Individuals with a polymorphism at G594 (exon 8) of the estrogen receptor 1 (ESR1) gene are twice likely to suffer from migraine than those with the wild type gene (62). Similarly, in a Spanish population, women carrying the C352 genotype were over 3 times more likely to suffer from migraine than those carrying the G325G genotype (63). Further, individuals carrying the PROGINS inserts of the progesterone receptor gene polymorphism are twice as likely to suffer from migraine than control subjects (64). However, it is worth mentioning that polymorphism in the case of estrogen receptor increases the expression of the receptor (62), whereas in the case of progesterone it negatively impacts its expression. Further, it is interesting that the PROGINS allele of the progesterone receptor acts synergistically with the 594A allele of ESR1 to increase the risk of migraine by 3.2 times (64). Genetic studies investigating the CAG repeat of the androgen receptor revealed no correlation with migraine (64). Similarly, the T/C *Pvu* II single nucleotide polymorphism in intron 1 of the ESR1 and the C325G single nucleotide polymorphism in exon 4 are not associated with migraine (65). Taken together, these studies further underscore the relevance of hormonal factors in migraine pathogenesis, although genetic variation in the receptors for these hormones is unlikely to be a major cause for the higher prevalence of migraine in women.

2.3 PATHOPHYSIOLOGY AND TREATMENT OF MIGRAINE

Although the pathophysiology of migraine is largely unknown, migraine attacks most probably start in the brain, possibly within the brain stem (66, 67). Migraine is best understood as a neurovascular disorder; neuronal events are likely to result in the dilatation of blood vessels, which in turn induces pain and further nerve activation (1, 68). The basic mechanisms believed to be involved in migraine pathophysiology are: (i) neuronal hyperexcitability during the interictal phase (69, 70), (ii) cortical spreading depression during the aura phase, and (iii) activation and sensitization of the trigeminal system at the peripheral and central levels that results in cranial vasodilatation. This trigeminal stimulation may involve the release of neuropeptides, in particular, calcitonin gene-related peptide (CGRP) (71, 72) and nitric oxide (NO) (73), thus reinforcing vasodilatation and perivascular nerve activity (74). While the early events during a migraine attack are unlikely to account for the headache, because the brain is largely pain-insensitive, trigeminal activation and vasodilatation are most probably responsible for the headache phase (68, 75).

The pharmacologic treatment of migraine can be divided into drugs that are taken daily in order to reduce the frequency or severity of the attacks (prophylactic drugs) or those that are taken to abort attacks (acutely-acting drugs). Further, a distinction can be made between non-specific treatments (e.g., paracetamol and NSAIDS) (76) and specific treatments, such as ergot alkaloids (α-adrenoceptors and 5-HT receptor agonists) (77, 78) and the triptans (5-$HT_{1B/1D}$ receptor agonists) (68, 74). The mechanism of action of prophylactic drugs in migraine is largely unknown, although it may depend, especially for the antiepileptics, on reduction of cortical excitability (79). In contracts, it is well known that the acutely-acting specific anti-migraine drugs may act *via* three different modes of action: (i) constriction of dilated cranial arteries, (ii) inhibition of trigeminal neuropeptide release, and (iii) a central action. Since vasoconstrictor properties of anti-migraine drugs may potentially lead to coronary vasospasms after their use, an attempt is currently being made to develop anti-migraine drugs that are devoid of vasoconstrictor properties. One such approach is the development of the CGRP receptor antagonist olcegepant (BIBN4096)

(80), which was recently demonstrated to be effective in the treatment of migraine (81). Alternatively, in view of the involvement of NO in the pathophysiology of migraine, nitric oxide synthase (NOS) inhibitors have been successfully investigated as anti-migraine treatment (82). Blockade of CGRP receptors or inhibition of NOS does not induce vasoconstriction *per se*, and the use of olcegepant (81, 83, 84) or the NOS inhibitor L-NMMA (85, 86) in humans did not induce any significant cardiovascular side effect. However, these studies were performed in a limited number of subjects, mostly healthy volunteers and the drugs were administered acutely. Especially in view of the possible prophylactic use of such drugs, it is important to study their cardiovascular side-effect potential, as they may inhibit the physiological protective properties of CGRP and NO (87, 88).

In the following sections, we will focus on the (potential) action of sex steroids on several mechanisms and receptor systems that are involved in the pathophysiology of migraine (ion balance, transcription factors) and/or that are pharmacological targets for current (α-adrenoceptors, 5-HT receptors) or future (CGRP, NO) anti-migraine drugs.

2.4 SEX STEROIDS AND THEIR CENTRAL AND VASCULAR MECHANISM OF ACTION

It is widely documented that sex steroid hormones regulate several biological functions *via* two main mechanisms, which are triggered by genomic (transcription dependent) and/or non-genomic (non-transcription dependent) stimuli. Ovarian steroids are capable of crossing the blood brain barrier by passive diffusion because of their lipophilic nature and low molecular weight (89). Thus, the blood serum levels of the steroids are mirrored within the brain, and sex steroids induce a wide array of central effects. In addition, a number of enzymes involved in steroid production has been demonstrated in the human brain (90), suggesting that such hormones may also be produced within the central nervous system.

2.4.1 Central effects of sex steroids

The non-genomic effects of steroids in different excitable tissues have been extensively studied over the past decades. Initially, the non-genomic modulation by steroids in the central nervous system resulted in numerous observations of steroid-induced changes in neuronal activity to induce anaesthetic (91-93), sedative/hypnotic (94) and anticonvulsant effects (95); these are rapid effects, not mediated by intracellular receptor occupancy (96, 97). Although the receptors and mechanisms involved in their non-genomic action are not yet completely elucidated, at least three different mechanisms have been identified *via* which the steroids may non-genomically modulate the central nervous system (for review, see 98): (i) modulation of the coupling of a receptor to its first messenger system, such as uncoupling of μ-opioid and γ-aminobutyric acid B ($GABA_B$) receptors from their effector systems, which would result in increased neuroexcitability (99) (ii) altered conductance of ion channels by allosteric modulation, such as increase in the opening time of neuroinhibitory $GABA_A$ receptors (100-102), which, in contrast, would decrease neuroexcitability and (iii) enhanced neuronal excitability after acute exposure (103). Most interestingly, the increase in neuronal excitability under normal, physiological conditions changes into a reduction in neuronal excitability after arterial occlusion (103), underlining the importance of an integrated neurovascular approach when studying the potential role of female sex steroids in the pathophysiology of migraine.

As mentioned above, the brain stem (66, 67), possibly especially the periaqueductal grey (104, 105), seems to be involved in the generation of migraine attacks. The facts that estrogen receptors are abundantly expressed in this brain area (106), and that the periaqueductal grey is an important antinociceptive center in the brain, make it tempting to suggest that abnormal activity of the brain stem, inducing migraine attacks, may be modulated via estrogen receptors located in this brain area.

The genomic mechanisms involved in the central action of sex steroids encompass two different receptors for estrogen (ERα and ERβ) (107), as well as two progesterone receptors (PR-A and PR-B) (108). In contrast to the non-genomic effects, the central function of these nuclear receptors has not yet been well characterized, but they may alter receptor expression and may be implicated in the release and synthesis of various neurotransmitters and hormones. For example, CGRP (a facilitator in pain transmission) (109, 110), galanin (a modulator of gonadotropin releasing hormone) (111), neuropeptide Y (a regulator of inflammation and central nociception) (112), as well as the neurotransmitters glutamate (113) and serotonin (114) have been reported to be genomically modulated by sex steroids. The genomic effects of estrogen may be mediated via extracellular signal-regulated kinase (115), by increased phosphorylation of cAMP response-element binding protein (CREB) (116), or by modulating the enzymes involved in the synthesis and metabolism of neurohormones and/or -peptides (114). Progesterone receptors are often co-localized with estrogen receptors, and the presence of estrogen may be required for expression of progesterone receptors in some brain areas. Remarkably, progesterone can act in a synergistic, antagonistic or neutral manner compared to the effects of estrogen (117-122).

2.4.2 Vascular effects of sex steroids

As previously described for their central actions, steroid hormones are capable of inducing non-genomic effects to regulate vasomotion, which occur at times too quickly to be explained by an altered gene expression (123). In this respect, female sex steroids have been reported to possess a protective cardiovascular role and one of their most important responses is inhibition of vascular tone (124). A large body of evidence has shown a marked vasorelaxation effect elicited by estrogens in a variety of vascular beds from different species (124-128), including humans (129-131). Likewise, progesterone (129, 132-135), as well as some natural (132) and synthetic (136, 137) progestins also induce a relaxant effect on the vasculature.

On the other hand, it has been reported that 17β-estradiol induces vasorelaxation by both genomic and non-genomic mechanisms, *via*: (i) production of vasodilator agents, such as NO, cGMP, cAMP, adenosine and prostacyclin, (ii) alteration of receptor expression of these mediators, and (iii) alterations in ion channel activity (124).

Although endothelial factors might be involved (138), steroid-induced vasorelaxation is preserved in blood vessels without endothelium or pre-treated with NOS inhibitors (130, 132, 133, 139-141). The complexity of mechanisms involved in steroid-induced vasorelaxation which, in turn, leads to divergent results could be attributed to a myriad of factors, including species differences, the vascular bed under study and the experimental conditions, e.g. the presence of intact endothelium, specific agonists, blood vessels obtained from steroid-treated animals, etc.

Collaterally, as mentioned above for their central actions, several studies have provided evidence for a modulatory role of steroids on ion channel function. In this respect, it is suggested that two main mechanisms might help explain the vasorelaxant response elicited by male and female sex steroids, namely: i) the control of $[Ca^{2+}]$ homeostasis, including modulation of Ca^{2+} entry *via* inactivation of voltage-gated channels (133, 134, 142-144) and nonvoltage-gated pathways (132, 133, 140, 145) or ii) activation of K^+ channels (125, 127, 146), particularly on the large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}).

The analysis of these findings leads to the conclusion that the relaxant action of steroids on vascular tone is through multiple cellular mechanisms; however, the actual mode of action in this process remains to be elucidated. Furthermore, it is interesting to note that sex steroids differ in their ability and potency to exert non-genomic activity on vascular tone (132, 136, 147). Therefore, the different structural conformation of each steroid could be important for inducing vasorelaxation *via* activating different modes of action.

2.5 EFFECT OF FEMALE HORMONES ON VARIOUS MECHANISMS AND VASOACTIVE AGENTS INVOLVED IN MIGRAINE

As mentioned above, there is ample clinical evidence for a relationship between the occurrence of migraine attacks and changes in plasma levels of female sex hormones. Below, the effects of the sex hormones on different mediators that are most likely involved in the pathophysiology of migraine or that are a target of (future) anti-migraine drugs will be discussed.

2.5.1 CGRP and its receptors

CGRP is a neurotransmitter that is widely distributed in the peripheral and central nervous systems, as well as in the cardiovascular system (148). The wide distribution of CGRP-containing nerve fibers and CGRP receptors in the body suggests that this peptide plays an important role in the modulation of physiological functions. Indeed, CGRP has a number of functions in the cardiovascular system, among which potent vasodilatation (149) is probably the most vital effect of this peptide. In the central nervous system, CGRP modulates the motor, sensory and pain pathways (149) and may contribute to the maintenance of spontaneous activity in spinal trigeminal nucleus in rats (150). Additionally, CGRP antagonists have been demonstrated to inhibit trigeminocervical activity evoked by superior sagittal sinus (151). Thus, the central actions of CGRP appear to be a part of the pathophysiology of migraine (148).

CGRP is stored in perivascular nerve terminals surrounding most blood vessels at the junction of the adventitia and the media, passing into the muscle layer. Thus, this peptide is well capable of modulating vascular tone, including that of cranial blood vessels (152). Because of the extensive presence of CGRP-containing neurons in the trigeminovascular system, the release of CGRP is thought to initiate cranial blood vessel dilatation, thus playing a key role in the pathophysiology of migraine (153). CGRP levels in jugular venous plasma have been reported to increase during migraine and they are normalized after treatment with triptans (154), although this could not be confirmed recently (155). Nevertheless, the newly developed CGRP receptor antagonist olcegepant (80, 156) has been shown to be effective in the treatment of migraine (81), thus providing convincing evidence for the involvement of CGRP in migraine.

The homeostasis of CGRP in the central nervous system is strongly influenced by sex steroids. Indeed, both 17β-estradiol and progesterone may increase CGRP synthesis in dorsal root ganglion, the main site of CGRP synthesis in rats (157, 158). The role of CGRP in pain transmission (109, 110) may be modified by 17β-estradiol, as was demonstrated by selective increase in sensory nociceptor vasodilator innervation of arterioles (159). In accordance, using intravital microscopy on a closed cranial window in ovariectomized rats, we observed that maximum relaxations of dural arteries in response to neurogenic periarterial stimulation were significantly increased in 17β-estradiol-treated rats compared to placebo-treated rats (Figure 2.3; 160), which may be attributed to increased release of CGRP from sensory nerve endings (161). In this context, it is important to note that human extracerebral intracranial arteries are innervated by sensory nociceptor nerves, and these pain sensors are of key importance in the headache phase of migraine (162).

Sex steroids modulate CGRP levels, as exemplified by the fact that ovariectomy decreases CGRP plasma levels in rats, whilst subsequent treatment with 17β-estradiol, progesterone or their combination normalizes these levels (163), while the levels are even further increased during pregnancy (164). In humans, there is a higher plasma level of CGRP in females as compared to their male counterparts (165). Similar as reported in rats, plasma CGRP levels are elevated during pregnancy (166), as well as in postmenopausal women undergoing HRT (167, 168). Not only the levels of CGRP are modulated by female sex steroids, but the mRNA levels of CGRP receptors, as well as CGRP binding sites are increased during pregnancy in rats (169). Dexamethasone also increases the expression of RAMP-1 and CL receptor in vascular smooth muscle cells (170) and the hypotensive responses to exogenously administered CGRP are enhanced in

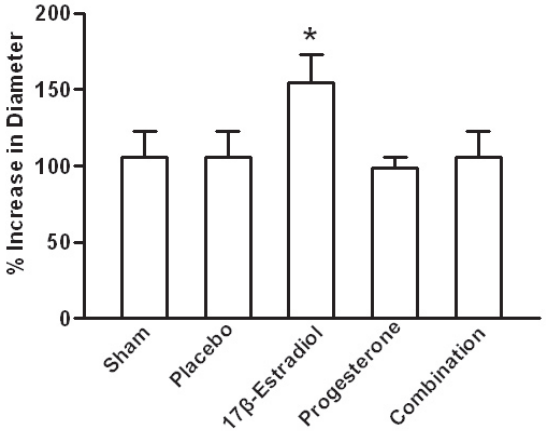

Figure 2.3. Effect of periarterial nerve stimulation (125 μA) on middle meningeal artery diameter in sham-operated or ovariectomized rats receiving placebo or hormone treatments (n=6-7). *, Significantly higher (P<0.05) than the corresponding value in the placebo group. Values are mean±SEM (160).

pregnant rats and ovariectomized rats treated with 17β-estradiol as compared to ovariectomized controls (171). In our laboratory (172), as well as in other studies (163), an increased potency of CGRP in mesenteric and caudal arteries of pregnant rats or ovariectomized rats treated with 17β-estradiol was reported as compared to those treated with placebo, although this mechanism was not observed in basilar arteries. Interestingly, progesterone may attenuate the enhance responses to CGRP by 17β-estradiol (172). Indeed, the two steroids may affect the CGRP receptor expression in opposite ways (173).

Taken collectively, these findings indicate that female sex steroids affect CGRP and its receptors in the central and peripheral nervous systems, as well as in blood vessels. Most findings point to a positive correlation between plasma levels of CGRP and 17β-estradiol, suggesting a higher prevalence of migraine in subjects exposed to relatively high levels 17β-estradiol. However, more research on the potentially opposing effects of 17β-estradiol and progesterone, as well as on the effects of estrogen withdrawal and CGRP are needed before a definite conclusion can be drawn on the relationship between female sex steroids and CGRP.

2.5.2 Noradrenaline and α-adrenergic receptors

The endogenous catecholamines, adrenaline and noradrenaline, which are released upon activation of the sympathetic nervous system, play an essential role in the regulation of a host of physiological responses *via* the activation of α- and β-adrenoceptors (174). Adrenaline is the primary hormone secreted by the adrenal medulla, while noradrenaline is a major neurotransmitter released from sympathetic neurons. Since both participate in the maintenance of vascular tone mainly *via* α-adrenoceptors, these have been implicated to have a role in migraine pathophysiology (175). α-Adrenoceptors can be divided into two major types, namely, the α_1- and α_2-adrenoceptors, and each of these types can be further subdivided into three subtypes, based on structural, transductional and operational (pharmacological) criteria (176). Within this context, the high affinity of ergotamine and dihydroergotamine at α-adrenoceptors has been proposed to be one of the main reasons for their anti-migraine action (77, 78). Indeed, activation of $\alpha_{2A/2C}$-adrenoceptor subtypes is partly involved in the canine external carotid (extracranial) vasoconstriction induced by ergotamine (177) and dihydroergotamine (178).

In the central nervous system, estradiol and progesterone may enhance hypothalamic noradrenaline release, leading to increased excitability of the ventromedial hypothalamus (179). In addition, female mice have been reported to have lower pain thresholds on a tail flick test and to be less sensitive to the analgesia induced by the α_2-adrenoceptor agonist clonidine compared with their male counterparts (180).

The authors suggest that G protein-coupled inwardly rectifying potassium channels are responsible for these differences. Furthermore, estradiol has been reported to downregulate cortical α_{2A}-adrenoceptor by as much as 50% in ovariectomized rats (181), as well as uncouple α_{2A}-adrenoceptors from G-proteins (182). In addition, estrogen may attenuate α_2-adrenoceptor-mediated anti-nociception in the spinal cord (183). Thus, it is likely that estrogen modulates the anti-nociceptive effects by compromising the expression and/or the function of α_{2A}-adrenoceptors, which are G-protein (G_i/G_o)-coupled receptors responsible for producing anti-nociception.

Female rats have higher plasma adrenaline levels than male rats, which decrease after ovariectomy (184). In contrast, both 17β-estradiol (185) and progesterone (186) are known to decrease sympathetic tone. The latter seems to be in line with a decreased sympathetic tone in migraineurs (175).

In addition to their neuronal effects, female sex hormones may affect (i.e., increase or decrease, depending on vessel system under study), vasoconstriction in response to α-adrenergic receptor agonists. For example, forearm blood flow responses to noradrenaline are higher in men than in women (187). Consistent with this finding, Gisclard et al. (188), as well as our own group (189), have reported that 17β-estradiol administration after ovariectomy depresses contractions mediated by α_2-adrenergic receptors in the rabbit femoral and rat carotid artery, respectively. Thus, an increased α_2-adrenoceptor function may, at least partly, be responsible for the increased blood pressure in females after menopause (190). In contrast, 17β-estradiol replacement in ovariectomized rats has been reported to enhance vasoconstriction induced by smooth muscle α_2-adrenergic receptor activation in rat mesenteric artery, although this effect was obscured due to an overriding influence of endothelial dilator substances, primarily NO (191). Further, both our group (189) and others (192) have reported that vascular sensitivity to noradrenaline in isolated arteries from the rat is regulated in opposite directions by female sex steroids, with the sensitivity being higher after treatment with 17β-estradiol than with progesterone.

Taken together, it appears that female sex hormones may centrally affect the noradrenergic system at the receptor level, as well as at the level of noradrenaline release in different brain regions. Both the increased cortical excitability and the lower pain threshold mediated by α-adrenergic receptors and female sex steroids may predispose women for migraine. Besides these central effects, female sex steroids in addition display an array of (sometimes contradictory) effects on blood vessels, which may be relevant in migraine pathogenesis.

2.5.3 5-HT and its receptors

In addition to being a central and peripheral neurotransmitter, serotonin (5-hydroxytryptamine; 5-HT) is capable of exerting complex cardiovascular effects, including hypotension or hypertension, vasodilatation or vasoconstriction and/or bradycardia or tachycardia; the eventual response primarily depends on the nature of the 5-HT receptors involved (193, 194). The conjunction of structural, transductional and operational criteria has led to classify 5-HT receptors into 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_7, recombinant (5-ht_5 and 5-ht_6) and 'orphan' receptors (194, 195).

5-HT may be involved in the pathogenesis of migraine at different levels. Firstly, the brain of migraine patients seems to display an increased 5-HT synthesis compared to that of control subjects (196), which could lead to cortical hyperexcitability. Indirect evidence for the involvement of 5-HT in migraine pathogenesis is given by the fact that the 'triptans', which are all agonists at $5\text{-HT}_{1B/1D}$ (and in most cases 5-HT_{1F}) receptors, are effective in the acute treatment of migraine (68, 74). Although their definite mode of action is still under debate, several mechanisms have been proposed, including: (i) inhibitory actions in the central nervous system, particularly in the *trigeminal nucleus caudalis*; (ii) prejunctional trigeminovascular inhibition at the level of cranial extracerebral arteries, with a corresponding inhibition of CGRP release; and/or (iii) a direct vasoconstrictor action on cranial extracerebral arteries (1, 68, 74).

Recent neuropharmacological evidence from a primate model of surgical menopause suggests a positive link between estrogen and 5-HT synthesis (114), which is most likely mediated by the ERβ subtype

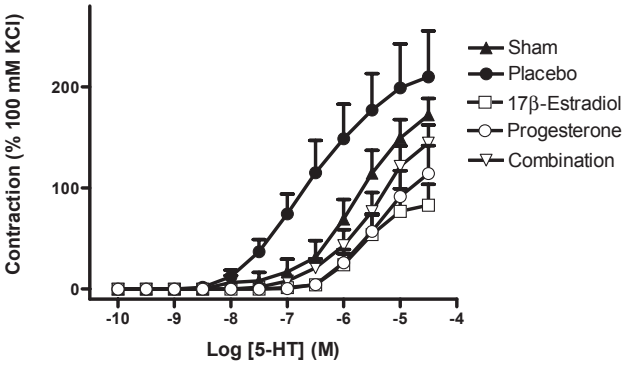

Figure 2.4. Effect of increasing concentrations of 5-HT in carotid artery obtained from sham-operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each). Values are mean±SEM (189).

(197). Such a possible link between estrogen and serotonergic signalling may be relevant in migraine, as illustrated by a small clinical study in women with status migrainosus occurring within 48 h of the discontinuation oral contraceptive (198). In this study, the neuroendocrine response to the direct central 5-HT agonist meta-chlorophenylpiperazine (m-CPP) was decreased in patients with status migrainosus, while this was restored after transdermal estradiol supplementation (198). It remains, however, to be established whether these observed differences are causal in the development of migraine, or represent an epiphenomenon that is rather a consequence of the migraine attacks. The firing rate of serotonergic neurons in dorsal raphé nucleus is higher in male than in female rats, although, apparently in contrast, it increases during pregnancy (199).

The contractile responses to 5-HT in porcine isolated coronary artery decrease after acute exposure to physiologically relevant concentrations of 17β-estradiol (123). Since classical ER antagonists, as well as *de novo* protein synthesis inhibitors did not inhibit this rapid effect, the authors concluded that this was mediated *via* a non-genomic pathway (123). Further, the chronic exposure to 17β-estradiol in the rat isolated aorta (200), as well as the rabbit isolated coronary artery and thoracic aorta (201) decreases contractions to 5-HT. We observed an attenuation of 5-HT-induced contraction by 17β-estradiol in the rat isolated carotid artery and showed that this was also the case with progesterone as well as a combination of 17β-estradiol and progesterone (Figure 2.4; 189). These actions of female sex steroids seem to be, at least partly, mediated *via* a direct effect on vascular smooth muscle cells (202) and are thus not (completely) mediated by the endothelium.

In conclusion, similar as described above for CGRP and the α-adrenergic system, female sex steroids may modulate synthesis of and/or responses to 5-HT on both central and vascular levels. Whereas the vascular effects of 17β-estradiol and progesterone are opposite in the case of α-adrenoceptor-mediated contraction (see above), these hormones seem to display the same (inhibitory) effects on contractile responses to 5-HT.

2.5.4 Ion balance

The concentration of an array of ions, both intracellular as well as extracellular, is of key importance for neurotransmission and neuronal excitability (203). As mentioned in the previous sections on Genetics and Pathophysiology of migraine, neuronal excitability is likely to play a crucial role in migraine pathophysiology, especially in the early phases of a migraine attack (204). In addition, ion concentrations modulate vasoconstriction and vasorelaxation.

Decreased magnesium concentrations are associated with cortical spreading depression in animals (205), and indeed there is evidence for low Mg^{2+} levels in the brain during (206) and between (207)

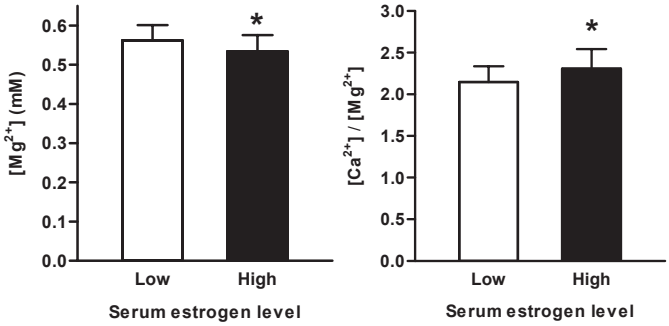

Figure 2.5. Effect of low and high concentration of estrogen in rat plasma concentration of Mg^{2+} and Ca^{2+}/Mg^{2+} concentration. *, Significantly different ($P<0.05$) from the corresponding low level of estrogen. Values are mean±SD Adapted from Muneyvirci-Delale et al. (220).

migraine attacks. Magnesium ions may also influence the vascular tone (208, 209) and Mg^{2+} concentrations are decreased in blood, serum, red blood cells, mononuclear cells, as well as in salivary secretions of migraineurs, both interictally and during attacks (210). Recent evidence shows that in up to 50% of migraine patients there is lowered ionized level of magnesium and infusion of magnesium provided sustained relief from headache in these patients (211, 212). Oral magnesium has been demonstrated to be effective prophylactic in menstrual migraine (213).

Interestingly, calcium seems to be involved in the pathogenesis of migraine in a number of ways. As mentioned above: (i) mutations in the P/Q-type calcium channel are related to familial hemiplegic migraine (51) and possibly to migraines with and without aura (54); and (ii) L-type calcium channels modulate the release of CGRP in neurons innervating trigeminal vasculature (214). In keeping with these findings, Ca^{2+} channel blockers have shown a number of effects, for example: (i) the calcium channel blocker verapamil potentiates anti-nociception in rats (215); and (ii) verapamil and flunarizine seem to be effective in migraine treatment (216, 217). Nevertheless, their putative mechanism of action is not understood, and many other calcium antagonists are ineffective in migraine (218, 219).

Significantly, serum levels of Mg^{2+} and Ca^{2+} are affected by sex steroids (Figure 2.5) and vary throughout the menstrual cycle in women (220). Interestingly, in a study in cerebral vascular smooth muscle cells the levels of both estrogen and progesterone were negatively correlated to cytosolic Mg^{2+} concentrations, whereas testosterone had no such effect (221). Furthermore, the levels of Ca^{2+} are reported to rise in parallel with estrogen levels in women (222); indeed 17β-estradiol modulates, either genomically or non-genomically, both the extracellular and intracellular Ca^{2+} concentrations (223). Both Ca^{2+} and K^+ channels are directly coupled to estrogen receptors, via pertussis toxin-sensitive G proteins. These G proteins are activated by steroids acting as a first messenger (224, 225). Transient exposure to estrogen results in a decreased potency of μ-opioid receptor agonists to hyperpolarise propiomelanocortin (POMC) neurons (226). This action is mediated by activation of the G-protein-gated, inwardly-rectifying K^+ channel subfamily known as GIRK1-4 (Kir3.1-3.4) (225, 227, 228). Further, estrogen affects the rapid action on α_1-adrenergic receptor-mediated inhibition of the small-conductance, Ca^{2+}-activated K^+ channels in GABAergic neurons (229). Although, clearly, female sex steroids modulate ion levels both in the brain and in plasma, it remains to be established to what extent this modulation of various ion channels by female sex steroids is indeed relevant for the pathophysiology of migraine. This could be feasible *via* changes in several physiological actions, including amongst others: (i) neuronal excitability; (ii) neuropeptide (particularly CGRP) release; and/or (iii) postjunctional vascular actions.

2.5.5 Nitric oxide

Nitric oxide (NO) displays a pronounced vasodilator effect and plays an important role in the physiological regulation of local blood flow and blood pressure (230, 231). It is synthesized from L-arginine and the reaction is catalysed by nitric oxide synthase (NOS) in the endothelial, lungs, as well as neuronal cells (230). Although NO can hyperpolarize vascular smooth muscle cells, activation of the endothelium can induce hyperpolarisation and vasodilatation by other means (231). Amongst other functions, NO is involved in: (i) the neurogenic control of microcirculation through autonomic efferent nerves; (ii) the vasodilatation and inflammation associated with activation of sensory nerves; and (iii) the pathophysiology of several cardiovascular and other diseases (230, 231).

Significantly, NO is able to differentially influence afferent fibers in the superficial laminae of rat spinal trigeminal nucleus caudalis, and estradiol modulates the basal expression of these transmitters and blocks the nitroglycerin effect (232). In addition, estrogen may directly affect the vascular system by stimulation of NO release. The ERα increases NOS activity in endothelial cells (233) by direct activation of the protein phosphatidylinositol 3-OH kinase in a non-nuclear and perhaps membrane-associated compartment location (234). Remarkably, the NO and platelet L-arginine pathway has been reported to exhibit a heightened activation in women with a history of menstrual migraine, especially during the luteal phase, compared to women with non-menstrual migraine and women without migraine (235). In rats, estrogen is reported to decrease myogenic tone through a NO-dependent mechanism in rat cerebral arteries, as is clear from the smaller decrease in vessel diameter in response to increasing transmural pressure in females and ovariectomized females treated with estrogen compared to the responses in males and ovariectomized females without hormone replacement (236).

The interaction of sex hormones with cytosolic/nuclear receptors triggers long-term genomic effects that could stimulate endothelial cell growth while inhibiting smooth muscle proliferation (237). Activation of plasmalemmal sex hormone receptors may trigger acute non-genomic responses that could stimulate endothelium-dependent mechanisms of vascular relaxation such as the nitric oxide-cGMP, prostacyclin-cAMP, and hyperpolarization pathways (237). Thus, the effect of NO modulating the vasoconstrictor effect of vessels is non-genomic in nature. Vascular function in postmenopausal women may be improved through increasing the serum NO level after a 3-month oral intake of estradiol valerate, while estradiol valerate plus medroxyprogesterone acetate may attenuate the beneficial effects (238), suggesting a contrasting role of estrogen and progesterone towards the vascular tone. In summary, these findings suggest a positive interaction between sex steroids and NO, which may thus augment the susceptibility to migraine.

2.5.6 Transcription factors

Nuclear factor-κB (NF-κB) has emerged as a critical and ubiquitous transcription factor underlying multiple cellular functions, e.g. induction of the inducible isoform of NOS (iNOS) and transcription of acute phase proteins (239, 240). Recently, Reuter *et al*. (241) demonstrated that nitroglycerin infusion, which triggers migraine attacks (242), increases iNOS expression and activates NF-κB within rodent dura mater several hours after its administration. Thus, it is believed that this transcriptional factor has a role in the pathophysiology of migraine, and provides a novel target for the development of anti-migraine drugs although the role of neurogenic inflammation in the pathophysiology of migraine should be considered with caution, since plasma protein extravasation, one of the components of neurogenic inflammation, does not seem to be involved in the pathophysiology of migraine (243).

The transcription factor p65/relA, a member of the NF-κB family, plays a major role in inflammation and drives the expression of pro-inflammatory mediators (244). Cell culture studies have shown that 17β-estradiol downregulates the expression of inflammatory genes, such as those coding for iNOS or matrix metalloprotease 9 (245), enzymes directly involved in the progression of the inflammatory response (246), and inhibits the biochemical and morphological activation of macrophages (247). Thus, evidence

accumulated so far is uniformly concordant in identifying estradiol as a protective agent against the induction of inflammatory responses. The gene and protein expression of NF-κB are constitutive but ovarian hormones can decrease the nuclear location of NF-κB in dorsal raphé neurons and, thereby, decrease the ability of NF-κB to drive gene expression in response to cytokines (248). Estrogen withdrawal has been reported to increase NF-κB DNA binding activity (249). Further, 17β-estradiol modulates expression of Nrf-2 (250), another transcriptional factor that might be involved in the pathophysiology of migraine, based on its increased expression in a mouse oligemia model (251), i.e. blood flow reduction without acute tissue damage that could occur in migraine.

In addition, a group of immediate early genes, including c-fos are expressed at higher levels in migraine patients (252). C-fos has been widely used in migraine and pain research to trace the neuronal activity of pain-responsive neurons. As estrogen modulates expression of c-fos, depending upon the brain region understudy (253), c-fos may represent one of the mechanisms *via* which sex steroids modulate central aspects of migraine

2.5.7 Miscellaneous

Although the GABAergic system does not seem to be primarily implicated in migraine aetiology, hyperexcitabilty of the brain is thought to be an important factor in its pathogenesis (204). Hence, the potential role of GABA, a predominant neuroinhibitory transmitter, should not be underestimated. GABA is predominantly found in the brain, and accounts for 30% of neurotransmission at all synapses (254). GABA acts mainly *via* two main receptors, namely: (i) the $GABA_A$ receptor, a ligand-gated postjunctional receptor (255); and (ii) the $GABA_B$ receptor, a G-protein coupled receptor that is expressed both pre- and post-synaptically (256).

Activation of $GABA_A$ and $GABA_B$ receptors induces neuroinhibition, which is significantly influenced by female sex steroids. Indeed, the opening time of the $GABA_A$ receptor is increased by allopregnenolone, a progesterone metabolite that does not act on progesterone receptors, thus leading to hyperpolarisation *via* an increased influx of Cl^- ions (102). In contrast, estrogen uncouples the $GABA_B$ receptor from the GIRK potassium channels in rat hypothalamus, thus decreasing the neuroinhibitory effects of $GABA_B$ receptors (226, 257, 258).

Shughrue and Merchenthaler (259) have reported that estrogen increases the expression of glutamic acid decarboxylase, the rate-limiting step in GABA synthesis, as well as GABA release. More recently, it has been shown that estrogen and/or oxytocin can rapidly increase the formation of functional GABA synapses in the adult rat supraoptic nucleus (260). In parallel to their central action, sex steroids may affect vascular GABA receptors, which appear to be localized only in certain blood vessels, such as the cerebral arteries (261-264). Nevertheless, it is noteworthy that GABA has been shown to be one of the less potent endogenous cerebrovasodilators (265, 266). Considering the above lines of evidence, it is clear that sex steroids have contrasting effects on the GABAergic system homeostasis. Depending on the experimental conditions and the steroids under study, this balance may be tilted in either direction, and might thus have a relevant contribution to the excitation threshold of the neurons implicated in the pathogenesis of migraine.

Glutamate may be involved in the cortical hyperexcitability in migraine (267). The effects of glutamate on brain excitability are mediated via the ionotropic NMDA, AMPA and kainate receptors, which are being studied as potential anti-migraine targets (268). Interestingly, estradiol and progesterone have divergent effects on the ionotropic glutamate receptors, which depend on the location in the brain. For example, estrogen increases NMDA receptor-mediated excitatory responses in the hippocampus (269, 270), but not in other brain regions (269). In contrast, progesterone is likely to decrease NMDA receptors in frontal cortex (269). The action of kainate receptors in hippocampal neurons is potentiated by estradiol (271). Significantly, glutamate may, *via* AMPA and kainate receptors, contribute to the peripheral release of

vasoactive neuropeptides such as CGRP (272), which, as described above, is modulated by female sex steroids.

The opioid system is also strongly influenced by sex-steroids. There are three major subtypes of opioid receptors, μ, κ and δ, which are further subdivided into subtypes. All these receptors are G-protein coupled, and analgesia is one of the major effects. Enkephalins and β-endorphin are endogenous ligands having high a affinity for μ and δ receptors, whereas dynorphins have a higher affinity for κ receptors. Gender differences in morphine-induced antinociception have been reported in a number of species, including rats (273), monkeys (274) as well as humans (275), with males generally showing greater antinociceptive effects than females. In pregnancy, spinal cord levels of enkephalin and dynorphins are increased, and high levels of oestrogen and progesterone are required to produce this analgesia (276). The mRNA expression of the opioid precursor gene pro-opiomelanocortin is increased after treatment with estrogen and progesterone as compared to that in ovariectomized sheep (277). Morphine increases the expression of c-Fos, one of the molecular marker of migraine, to a higher degree in males than in females, but these sexually dimorphic effects of morphine were independent of sex steroids (278). Thus, the effects of female sex steroid on the opioid system do not all point to same direction, and depend on the type of opioid receptor, the specific brain region and the type and or duration of hormone treatment.

2.6 IMPLICATIONS, FUTURE DIRECTION AND CONCLUSION

Both basic research and clinical studies suggest an intricate relation between female sex steroids and the occurrence and/or severity of migraine. At the onset of puberty, when plasma levels of estrogen increase, there is a concurrent increase in migraine frequency, while after menopause, when plasma levels of estrogen decrease, there is a parallel decrease in migraine frequency. Intriguingly, the declining levels of estrogen during the menstrual cycle do cause an increase in migraine attacks, typically migraine without aura. Finally, in pregnancy there is generally an improvement of migraine without aura, whereas migraine with aura may increase, and some women experience attacks for with aura for the first time. As elaborated earlier and summarized in Figure 2.6, there is substantial evidence that estrogen enhances neuronal excitability as well as vasodilatation, which both lead to an increased propensity for migraine.

Obviously, there are certain methodical - and some inherent - limitations in the extrapolation of results from basic research to the clinical situation. Firstly, as the pathophysiology of migraine is not completely deciphered yet, experimental models employed in migraine research are based on only a few symptoms observed in the clinical situation (e.g., focus on only neuronal or only vascular aspects), and thus do not completely mimic all aspects of a migraine attack. Thus, the parameters obtained with these studies do not necessarily apply to a real migraine attack, as there is still no unequivocal neuronal, humoral or biochemical marker for migraine.

Further, apart from obvious variables such as the species used, there are several experimental parameters that should considered, such as the route of administration and pharmacokinetic characteristics of the steroids in various studies, their dose, the duration and frequency of administration. As the effects of sex steroids may be mediated by classical genomic and/or non-genomic/rapid mechanisms, a lot of work is required in identifying non-genomic pathways in both the central nervous system and the cranial circulation. Further, while interpreting experimental results, it is important to take into account whether the sex hormones were given alone or in combination. Essentially, in most experimental models, stable levels of sex hormones are used, while clinical evidence indicates that not only do absolute levels of female sex steroids seem to be of relevance, but rather their (rate of) change. Therefore, future experimental studies should carefully take these considerations into account, and investigations focussing on the change in hormone levels, especially the decrease in 17β-estradiol after exposure to a high concentration, may reveal more insight into the pathophysiology of menstrual migraine.

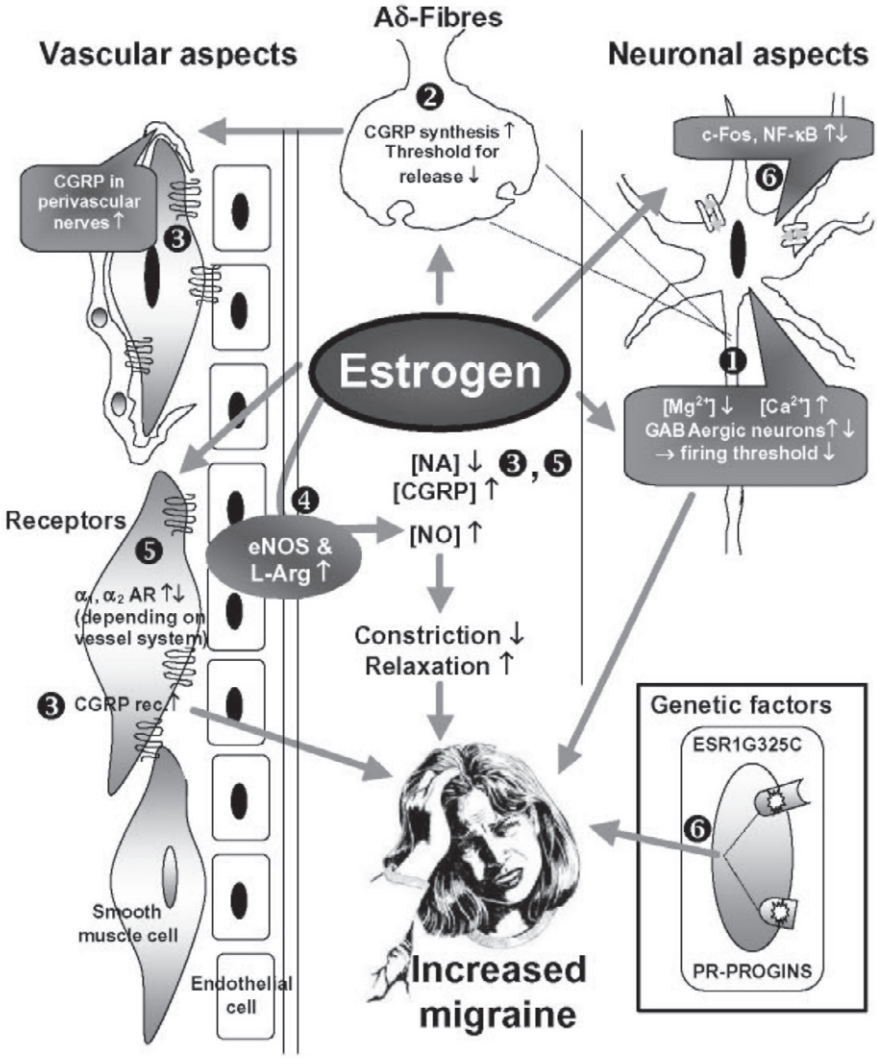

Figure 2.6. Schematic representation of main mechanisms by which estrogen may increase the incidence of migraine: ❶ Estrogen enhances neuronal excitability by decreasing levels of Mg^{2+} and increasing Ca^{2+} concentrations; ❷ Estrogen up-regulates the synthesis of calcitonin gene-related peptide (CGRP) in and its subsequent release from Aδ-fibres; ❸ On the vascular level, this increased CGRP release enhances vasodilatation, which acts in tandem with increased expression of CGRP receptors and an inhibition of the sympathetic nervous system; ❹ Further, estrogen enhances the production of nitric oxide (NO) *via* eNOS (endothelial nitric oxide synthase) from L-Arg (L-arginine) by the endothelium; ❺ The GABAergic and noradrenergic (α_1- and α_2-AR (adrenoceptors), as well as plasma concentrations of NA (noradrenaline)) systems may display both enhanced and attenuated effects in response to estrogen; and ❻ In addition, estrogen modulates the molecular markers c-fos and NF-κB, while mutations in both estrogen receptors (ESR1G325C) and progesterone receptors (PR-PROGINS) increase the risk for migraine.

Disregarding the limitations of basic research, we will inevitably need a multidisciplinary approach encompassing basic and clinical observations to understand the influence of female sex steroids on migraine. Such an integrated approach will be relevant to understand why migraine without aura seems to be predominantly triggered by a decline in estrogen levels, while migraine with aura seems to be prompted by high levels of estrogen. While migraine with and without aura are often considered as

two entities of the same disease, the differential effect of female sex steroids on these two forms of migraine suggests that, at least in some aspects, they represent separate entities. Another interesting dimension to this ever increasing enigma is the so-called mismatch between the slow genomic and rapid non-genomic actions of sex steroids (279). As the changes during menstrual cycle are drastic and rapid, the balance between excitatory and inhibitory stimuli might be disturbed, and could thus be attributed to increased susceptibility to migraine in females during this period.

Taken together, there is overwhelming clinical and experimental evidence for a relationship between migraine and levels of female sex hormones, which may influence the pathophysiology at a central, peripheral prejunctional and/or vascular levels. In-depth delineation of this relationship is an intriguing and challenging subject for future research.

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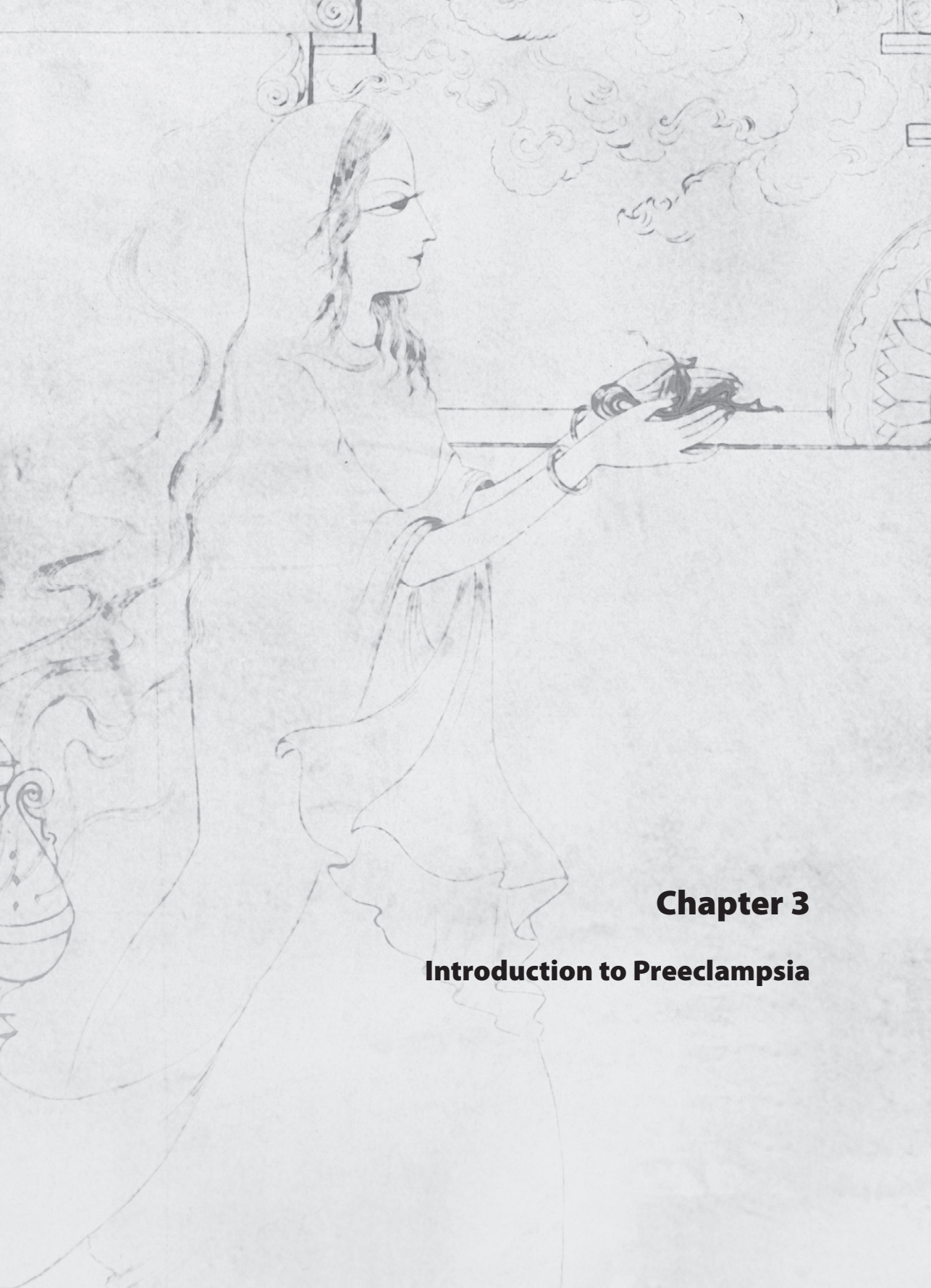

Chapter 3

Introduction to Preeclampsia

3.1 INTRODUCTION

Preeclampsia is a multisystem disorder with an incidence in pregnancy varying between 2% and 7% (1) and is still the leading cause of maternal mortality during pregnancy in The Netherlands (2). The disorder is characterised by its occurrence after the 20th week of gestation, elevated maternal blood pressure and proteinuria. In most women, the onset is near term or intrapartum and the disease is usually mild with a negligible increased risk of adverse pregnancy outcome. However, in severe cases, preeclampsia can lead to serious maternal complications, such as HELLP-syndrome (haemolysis, elevated liver enzymes, low platelet counts), pulmonary edema, acute renal failure, liver failure or haemorrhage, abruptio placentae and eclampsia. Preeclampsia is also associated with increased perinatal morbidity and mortality. Depending on the severity of the disease, intrauterine growth restriction, reduced amniotic fluid and abnormal oxygenation can occur, leading to increased occurrence of iatrogenic preterm delivery, small-for-gestational-age neonates and prenatal or perinatal death (3).

3.2 PATHOGENESIS

The cause(s) of preeclampsia still remain unknown, but most hypotheses focus on vascular dysfunction and maternal-foetal (paternal) immune maladaptation (3). The presence of the placenta or the maternal response to placentation is considered to be a key factor in the disorder. Cytotrophoblastic invasion of the spiral arteries is incomplete in preeclampsia, resulting in narrow and undilated myometrial segments (4), which may compromise uterine blood flow during pregnancy. Recently, the anti-angiogenic protein sFlt-1 (soluble fms-like tyrosine kinase 1) has been shown to be elevated in preeclampsia as compared to normal pregnancies (4). This protein is thought to be involved in antagonising vascular endothelial growth factor and placental growth factor, which may cause the endothelial dysfunction, which is present in preeclampsia (4, 5). In the future, this mechanism may become a possibility for an alternative and more causal strategy for pharmacological management of preeclampsia. Based on the present knowledge the hypothesis involved in migraine pathogenesis is summarised in Figure 3.1 (1).

An important factor implicated in the pathogenesis of preeclampsia is the close tissue contact between maternal and foetal cells, resulting from the trophoblast cell invasion into the deciduas (3). Couple-specific immune maladaption is speculated to be involved in the superficial placentation, causing increased apoptosis of cytotrophoblasts. This may trigger a systemic inflammatory response in the mother, resulting in endothelial activation and inflammation and thrombocyte activation. The latter can result in a lower production of the vasodilator prostacyclin and increased release of the vasoconstrictors thromboxane A_2 and 5-hydroxytryptamine (5-HT; serotonin). The usual physiologic adaptations in response to increased fluid volume that are observed in normal pregnancies are attenuated in preeclampsia, resulting in increased vascular resistance (6). This higher vascular resistance of the maternal vasculature in preeclampsia may be attributed to increased plasma concentrations of contractile agents (7-10), but could alternatively be attributed to an increased sensitivity of the arteries to vasoconstrictor agents like angiotensin II (11, 12), 5-HT (10) and noradrenaline (13). This combined with a decreased response to vasodilating peptides such as calcitonin gene-related peptide (14) and acetylcholine (15). Vascular hyperreactivity in preeclamptic women has been demonstrated by, for example, an increased vasoconstrictive response to the cold pressor test, which is mediated by α-adrenergic receptors (16). Obviously, increased plasma levels of a vasoconstrictor, in combination with an increased sensitivity of the respective receptors, may synergistically lead to increased vascular resistance and hence hypertension. One of the main problems of preeclampsia is that the clinical symptoms become manifest long after the compromised placentation and vascular dysfunction have developed. Management at the time of diagnosis is limited to controlling maternal symptoms, and assuring foetal well-being. Theoretically,

pharmacological interventions at the time of early placentation and before the occurrence of extensive vascular dysfunction would be needed to prevent the development of preeclampsia or to reduce the severity of the disease. However, early identification of women at risk for developing preeclampsia remains an unresolved challenge in obstetric practice, hampering the possibility of selective use of prophylactic drugs for women at high-risk.

Acetylsalicylic acid is the drug most widely used to prevent preeclampsia (17), based on its positive effect on the imbalance in the thromboxane A_2 to prostacyclin ratio, found in preeclampsia. Based on the hypothesis that antioxidants may be effective in decreasing oxidative stress and improving vascular endothelial function, 283 women at high risk were supplemented in a placebo-controlled study with vitamin C (1000 mg) and vitamin E (400 IE) from the 16th-22th week of gestation (18). The occurrence of preeclampsia was reduced from 17% to 8% in the treated group ($p<0.02$), but no reduction on the

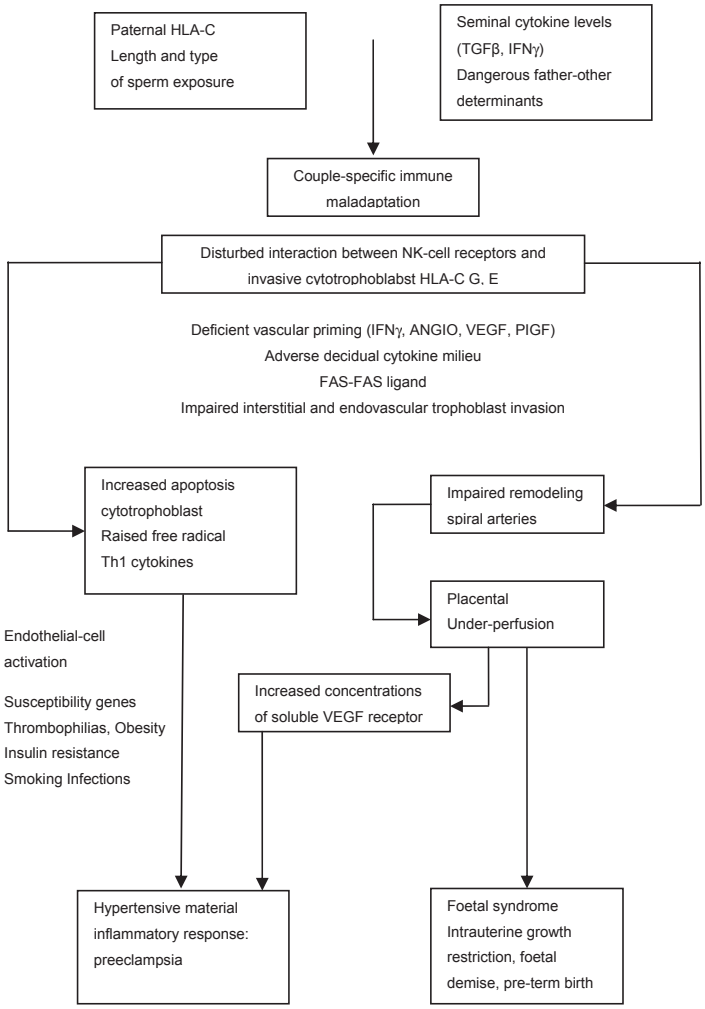

Figure 3.1. Hypothetical cause and pathogenesis of preeclampsia TGF=transforming growth factor. IFN=interferon. VEGF=vascular endothelial growth factor. PlGF=placental growth factor. ANGIO=angiopoietin II. Reprinted from Sibai, B et al. (1) with permission from Elsevier.

occurrence of severe preeclampsia or improvement in perinatal outcome could be established. Antihypertensive drugs (α-adrenoceptor, calcium channel blockers, β-adrenoceptor blockers or hydralazine) have been used orally in women with chronic hypertension with a beneficial effect on the occurrence of severe hypertension, but use of these drugs did not decrease the risk of preeclampsia according to a Cochrane review, referencing 40 clinical trials with 3797 women (19). In summary, there is no efficient prophylactic treatment available to prevent preeclampsia.

3.3 PREVENTION

One of the main problems of preeclampsia is that the clinical symptoms become manifest long after the compromised placentation and vascular dysfunction have developed. Management at the time of diagnosis is limited to controlling maternal symptoms, and assuring foetal well-being. Theoretically, pharmacological interventions at the time of early placentation and before the occurrence of extensive vascular dysfunction would be needed to prevent the development of preeclampsia or to reduce the severity of the disease. However, early identification of women at risk for developing preeclampsia remains an unresolved challenge in obstetric practice, hampering the possibility of selective use of prophylactic drugs for women at high-risk.

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3.4 MANAGEMENT OF PREECLAMPSIA WITH ANTIHYPERTENSIVE DRUGS

Delivery is the only cure for preeclampsia. In preeclampsia occurring in near-term or term patients, delivery is, therefore, recommended to minimize the risk of maternal complications. The use of antihypertensive drugs may be necessary to stabilise maternal blood pressure before delivery and to gain sufficient time to administer corticosteroids in patients with a gestational age below 34 weeks to enhance foetal lung maturity (20).

In severe preeclampsia occurring before 32 weeks of gestation, expectant management by postponing delivery using antihypertensive drugs in patients with a stable maternal and foetal condition, can be considered to improve neonatal outcome (21, 22). A recent review (23) showed that a mean pregnancy prolongation in early preeclampsia using antihypertensive treatment of 10-14 days can be established, which is considered clinically important for an improvement in neonatal outcome. Some obstetricians, however, prefer early delivery after stabilizing the maternal condition (interventionist care) to prevent the development of serious maternal complications. A meta-analysis on available studies on interventionist versus expectant care (24) stated that due to lack of randomised trials of sufficient size, no conclusions can be drawn. Recent trials show that short-term morbidity for the baby may be reduced by a policy of

expectant care (25, 26). However, improvement in perinatal outcome should never be achieved at the expense of maternal safety. This implies that only in tertiary care settings with experienced personnel, expectant management using adequate monitoring of both mother and foetus, should be considered in severe early-onset preeclamptic patients. The challenge in using antihypertensive drugs in preeclampsia is to reduce blood pressure to assure maternal safety, while at the same time not compromising uteroplacental perfusion.

There is a general consensus (27) to start antihypertensive treatment in pregnant women with sustained values of systolic blood pressures of 170 mmHg or above and diastolic blood pressures of 110 mmHg or above. The aim of such treatment is to lower the risk of harmful effects, especially stroke and other haemorrhagic complications, for the mother. Whether to target at a fixed value of systolic and diastolic blood pressure or to aim for a reduction relative to the initial maternal blood pressure remains controversial. In patients with an initial high blood pressure, the latter may lower the risk of acute foetal distress, caused by diminished uteroplacental perfusion following aggressive antihypertensive treatment, but in patients with borderline blood pressure, a relative reduction might result in over-treatment.

The ideal antihypertensive drug for treatment of severe hypertension in pregnancy should be potent, rapidly acting, controllable and without detrimental maternal or foetal side effects. Unfortunately, only limited antihypertensive drugs are studied and even less are licensed for use in preeclamptic patients. As a result, newer drugs in other medical areas have long surpassed most drugs currently used in preeclampsia. Administering antihypertensive drugs in preeclampsia is only treating the symptoms of the disease. As long as the foetus and placenta are present, the disease will not be cured and may even exacerbate during treatment. To maintain adequate blood pressure control, increased dosages, a switch from oral to parenteral administration or combination treatment are often necessary, but no consensus on choice of first-, second- or even third-line treatment is available. Considering the fact that current antihypertensive drugs differ greatly in their action, potency and side-effects, it is surprising that (inter)national recommendations on which drug to prefer, are still lacking. In the next part, the different drugs are discussed with their respective properties.

3.4.1 Methyldopa (Aldomet)

Methyldopa is an oral drug with an excellent safety record for use in pregnant women (28-30). The drug exerts its antihypertensive effect from the action of its metabolite α methylnoradrenaline on central inhibitory α-adrenoreceptors. Common dosages of methyldopa are 500-1,000 mg orally taken 3-4 times daily, with a maximum of 4 g/day. In mild preeclampsia and in the management of chronic hypertension in pregnant women, methyldopa is regarded as drug of choice, but its limited efficacy and delayed onset of action (4-6 h) preclude its use in acute, severe preeclampsia. Side effects include decreased heart rate and sedation.

3.4.2 Hydralazine (Apresolin) or dihydralazine (Nepresol)

Dihydralazine and hydralazine differ slightly in their molecular structure, but both act in a similar way as vasodilatating agents on arterial smooth muscle, causing a reduction in total peripheral vascular resistance and reflex tachycardia. The antihypertensive effects occur 10-20 min after intravenous administration, following metabolism in the vessel wall. The duration of action is 3-8 h (31). The drugs are very effective in lowering maternal blood pressure, but maternal side effects (headache, nausea, epigastric pain and fluid retention) are common and mimic symptoms of deteriorating preeclampsia. Careful monitoring of central venous pressure and adequate volume expansion is necessary in patients receiving (di)hydralazine, to prevent an excessive hypotensive effect, causing foetal distress (32).

3.4.3 Labetalol

Labetalol is a selective α-adrenoceptor blocking and non-selective β-adrenoceptor blocking agent, lowering high blood pressure while, at the same time, exerting a heart-rate-reducing effect because of the drug's β-adrenoceptor-blocking effect. The drug is used in dosages of 10 to 30 mg/h intravenously or 50 to 200 mg orally three times daily. After intravenous administration, the antihypertensive effect occurs immediately with duration of action of 4 to 6 h, whereas, after oral administration the effect is delayed for 1-4 h. The drug is tolerated fairly well, with flushing, nausea and vomiting as main side effects. Due to the β-adrenoceptor-blocking effect, its use is contra-indicated in patients with congestive heart failure, asthma or bradycardia (31). As in severe preeclampsia, high intravenous dosages of labetalol are sometimes necessary to obtain adequate maternal blood pressure in preeclamptic patients. However, these high maternal dosages of labetalol have been associated with the occurrence of severe neonatal bradycardia, hypotension and hypoglycaemia (33-35). These neonatal side effects are the major limitation for its use in severe preeclampsia.

3.4.4 Ketanserin

A drug with a different action is ketanserin. Ketanserin is a selective 5-HT_{2A}-receptor-blocking agent with minor α_1-adrenoceptor-blocking properties. Its use in preeclampsia is based upon the assumption that serotonin is involved in the vasoconstrictive processes, leading to hypertension in preeclampsia. Elevated levels of serotonin in plasma will stimulate mainly 5-HT_2 receptors in platelets causing platelet aggregation and serotonin release, 5-HT_1 and 5-HT_2 receptors in vascular smooth muscle, causing vasoconstriction. The observation that levels of platelet-derived free serotonin are found to be significantly higher in preeclamptic patients than in normal pregnancies (9) supports this theory.

Ketanserin acts as an antagonist on the 5-HT_{2A} receptor, counteracting the serotonin-depending vasoconstriction and platelet aggregation. The latter may be clinically advantageous, especially in pregnancies complicated with HELLP syndrome (36). The pharmacokinetic characteristics of the drug are not ideal for drug treatment in preeclampsia because of its long elimination half-life of 10-18.5 h, which precludes accurate titration and may lead to accumulation. Side effects of ketanserin are mainly dizziness, dryness of the mouth, nasal congestion and tiredness. A potentially serious adverse effect of ketanserin is prolongation of the QT_c interval (37) and treatment should be accompanied by ECG control.

Conflicting data exist on the efficacy of ketanserin. Bolte et al found ketanserin to achieve adequate blood pressure control (38) and with lesser maternal side effects than dihydralazine (39). Also, significantly more patients treated with ketanserin needed antihypertensive co-medication to maintain adequate blood pressure control. A Cochrane review (40) on three available studies of ketanserin versus dihydralazine found that use of ketanserin was associated with persistent high blood pressure as compared to dihydralazine. These data make the role of ketanserin in the treatment of preeclampsia ambiguous.

3.4.5 Calcium channel blocking agents

Calcium channel blocking agents inhibit the influx of calcium via voltage dependent, slow L-type calcium channels, causing peripheral vasodilatation. The safety of the use of calcium-channel blocking agents of the dihydropyridine type in pregnant women has long been debated. In animal studies (41, 42), the use of nifedipine and nicardipine has been associated with foetal acidemic responses, caused by a decrease of uteroplacental perfusion. In women with pregnancy-induced hypertension however (43), nifedipine was not associated with a negative effect on uteroplacental blood flow, and the same conclusion was drawn after reviewing the extensive experience with nifedipine as a tocolytic drug in recent years (44). Probably, the adverse foetal effects found in animals are linked to the substantially elevated dosages as compared to dosages used as tocolytical or antihypertensive drug in pregnant women.

The successful use of nifedipine in preeclampsia has been described in several studies (45, 46) and, as cited above, a recent review showed a favourable outcome of nifedipine as compared to parenteral

hydralazine (47). The advantages of the use of nifedipine are its ease of administration and its low costs. Side effects of nifedipine are limited and are mainly flushing, nausea and vomiting. The tocolytical effects of nifedipine might be disadvantageous in preeclamptic patients, delaying induction of labor or increasing the risk of postpartum haemorrhage (48). Calcium channel-blocking agents have been associated with the occurrence of pulmonary oedema, possibly caused or enhanced by the cardiovascular action of the dihydropyridine derivates (49). However, irrespective of antihypertensive treatment, pulmonary oedema is known to occur as a complication in preeclampsia, usually after iatrogenic fluid overload in combination with the administration of steroids for foetal lung maturation (31). Nicardipine is a calcium channel-blocking agent, with some potential advantages over nifedipine in preeclampsia (50). It acts more selectively on the vessels and causes less negative inotropic effects and reflex tachycardia. Another advantage is that the drug is available both for intravenous and oral administration. The drug has a short half-life of 2-5 min which increases after prolonged infusion to 1-2 h (51). It is metabolised extensively in the liver to inactive metabolites. Maternal headache and increase in heart rate are the most common side effects.

3.4.6 Other antihypertensive drugs

For treatment of an acute hypertensive emergency, hydralazine is most widely used. If hydralazine is, in rare cases, not effective, or, if the delayed onset of action of 10-20 min poses a risk to the mother, sodium nitroprusside can be used in acute situations. Sodium nitroprusside is very potent and acts almost immediately by direct dilatation of arterioles and veins. Its short duration of action (3-5 min) allows accurate titration of the blood pressure (31). Due to the possible risk of accumulation of cyanide in foetus and mother, prolonged use is not recommended (28).

Several other classes of drugs are used to treat hypertension in non-pregnant patients, but are (relatively) contra-indicated during pregnancy. The use of angiotensin-converting enzyme inhibitors during pregnancy has been associated with foetal and neonatal renal failure, oligohydramnios, intrauterine growth retardation and increased foetal mortality (28). The use of diuretics is contra-indicated in preeclampsia because plasma volume is already decreased and further volume depletion could affect the foetus adversely (6, 31). The β-adrenoceptor blocking agents metoprolol and atenolol are used orally as add-on treatment in severe preeclampsia. However, long-term use of β-adrenoceptor blocking agents has been associated with an increase in small-for-gestational-age infants, especially with atenolol (52). Neonatal bradycardia, hypoglycemia and respiratory depression have also been reported as side effects, probably due to β-adrenoceptor blocking effects.

3.5 FOETAL AND NEONATAL EFFECTS OF MATERNAL ANTIHYPERTENSIVE DRUG TREATMENT

Maternal drug use may exert unwanted effects on the foetus by a direct pharmacological action of the drug after passing the placenta or by an indirect action, caused by compromising the uteroplacental perfusion. The latter can occur with all antihypertensive drugs through an overshoot of lowering of maternal blood pressure resulting in foetal distress due to marginal placental function. Especially, the use of drugs which cause vasodilatation but which do not affect placental vascular resistance, such as (di)hydralazine, can cause a reduction in placental flow, leading to foetal distress. Indeed, a recent review (47) showed that hydralazine was associated with more adverse effects on foetal heart rate than other antihypertensive drugs. Drugs, which are effective at the level of the placental vasculature, such as calcium-channel blocking agents, should, theoretically, be able to maintain adequate placental perfusion. Many drugs cross the placenta by simple diffusion, depending on the physicochemical characteristics of the drug and placental factors such as surface area and thickness of the membrane. As the use of antihypertensive

drugs for management of preeclampsia is limited to the late second and third trimesters, teratogenic effects are not relevant. Information on the effect of the high dose of combination treatment for a prolonged period on the placental transfer and possible adverse effects on foetus and neonate is scarce. This is even more relevant as many neonates born from mothers with severe early-onset preeclampsia are premature and are probably more susceptible to adverse drugs effects. For most drugs reviewed earlier, the information is available either from animal studies or from human data regarding placental transfer. Almost all antihypertensive drugs used in preeclampsia pass the placenta freely, but information regarding a number of drugs, including ketanserin, is lacking.

Neonatal morbidity or mortality is usually reported in studies describing the use of antihypertensive drugs in severe preeclampsia but are hard to interpret due to the fact that inherent to the severity of the maternal illness, most children are born prematurely and growth-retarded, with a corresponding high morbidity and mortality in the first postnatal months. To date, only high maternal doses of labetalol have been associated in case reports with direct neonatal side effects (bradycardia, hypoglycemia) (34, 35). Further, as discussed above, the usage of antihypertensive drugs should be closely monitored, with emphasis on the side effects of drugs to both mother and foetus. In this context, we tried to explore the effect of preeclampsia on functional responses of 5-HT and CGRP receptors, in maternal and foetal arteries. We also studied the effect of ketanserin treatment on the foetal 5-HT receptors in the preeclamptic patients.

The primary cause of preeclampsia is still unknown and the research in this arena indicates that there will not be a single factor, but interplay between a number of factors, including increased vascular resistance. Immunological maladaptations, which are thought to be the result of putative misalliance of foetal trophoblasts with maternal tissue, as well as endothelial cell activation or dysfunction, seem to be crucial factors. Therefore, research to divulge the factors such as various receptor system involved in the pathogenesis of preeclampsia is vital.

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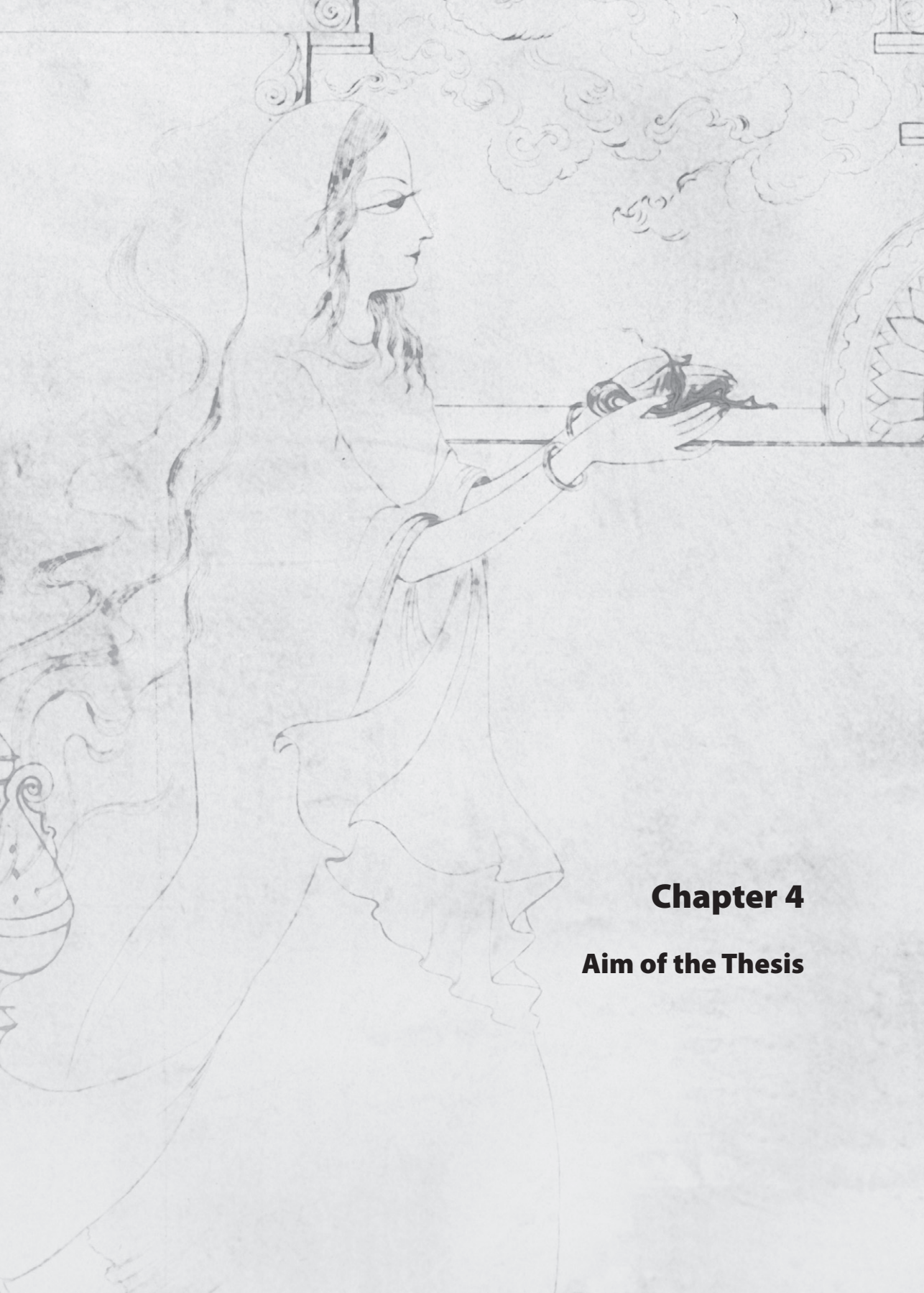


Chapter 4

Aim of the Thesis

AIMS OF THIS THESIS

1. The role of CGRP in the pathogenesis of migraine is well established. Therefore we characterised CGRP receptors in human coronary **(Chapter 5)** and meningeal **(Chapter 6)** arteries, using conventional CGRP ligands as well as a novel CGRP antagonist, BIBN4096BS.

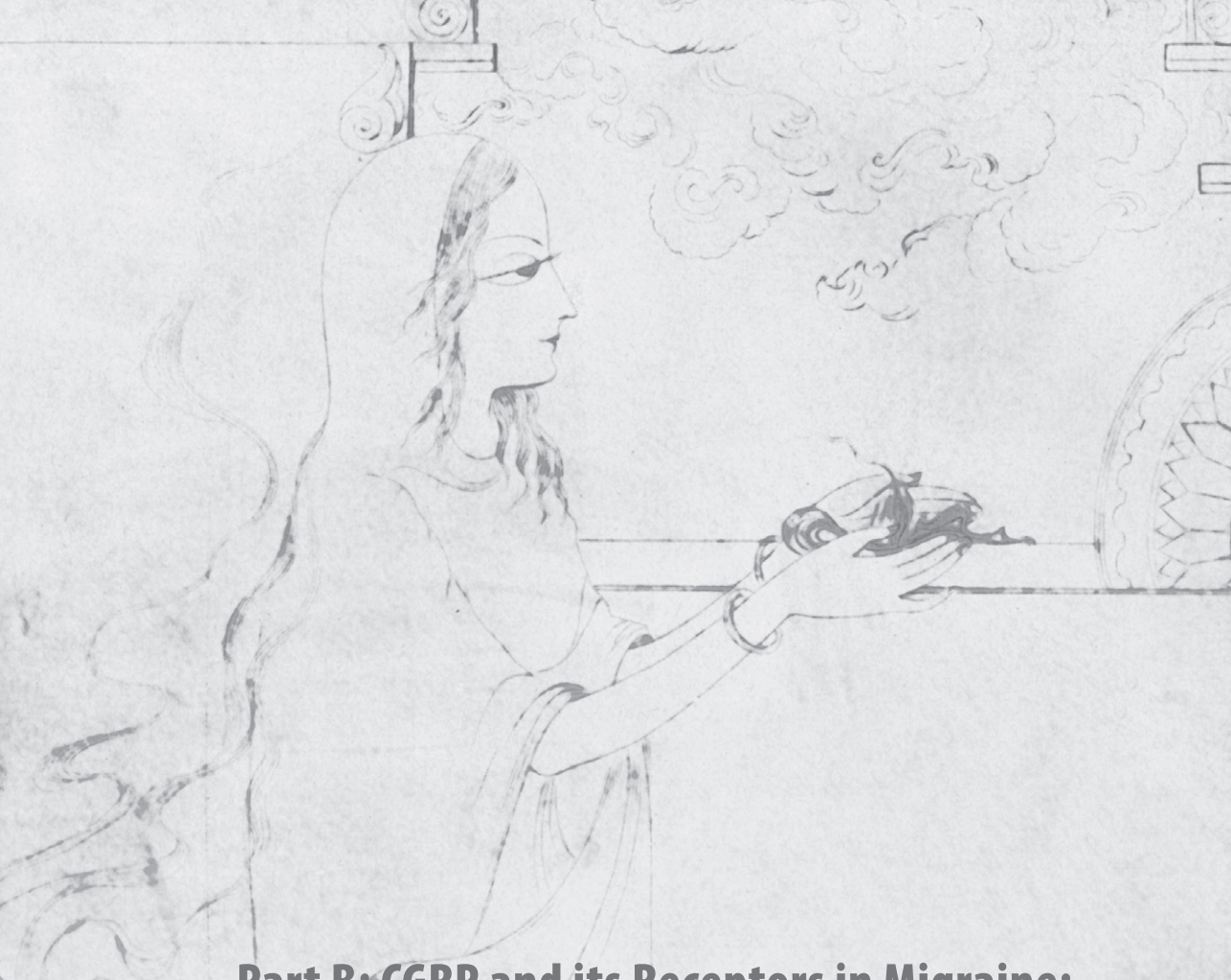
2. Further, we wanted to develop an *in vitro* model in human and porcine arteries for endogenous CGRP release in view of its role in the pathogenesis of migraine. We used capsaicin **(Chapter 7)**, a vanillod receptor agonist, which is reported to release CGRP in *in vivo* experimental models.

3. Due to the higher preponderance of migraine in females compared to males, we investigated the role of female sex steroids using *in vivo* and *in vitro* models of migraine. We investigated the role of sex steroids on vasodilatation caused by endogenous and exogenous CGRP in rats, using intravital microscopy on closed cranial window **(Chapter 8)**. We also studied the functional responses to CGRP, ACh, 5-HT **(Chapter 9)** and α-adrenoceptors ligands **(Chapter 10)** in different arteries *in vitro* in rats treated with different hormone interventions.

4. Mutations have been discovered in genes encoding for various ion channels, and these are thought to play a role in the pathogenesis of migraine. Some of these mutations now have been incorporated in mice. Thus, we developed a model of closed cranial window of intravital microscopy in mice (**Chapter 11),** which will enable us to study trigeminovascular mechanisms in these transgenic mice.

5. The model of external carotid vasodilatation in vagosympathectomized dogs is a predictive model on vascular aspects of migraine. The role of capsaicin has not been investigated in this model therefore we investigated the receptors involved in capsaicin-induced responses **(Chapter 12)**.

6. 5-HT and CGRP are implicated in the pathogenesis of preeclampsia. Therefore, we investigated 5-HT receptors in the umbilical cord and subcutaneous fat arteries obtained from preeclamptic and normotensive pregnant subjects **(Chapter 13)**. The 5-HT_{2A} receptor antagonist, ketanserin, is used for the treatment of preeclampsia; we studied the effect of ketanserin treatment on functionality of 5-HT receptors in umbilical cord artery **(Chapter 14)**. In maternal subcutaneous fat arteries obtained from normotensive and preeclamptic women, we also investigated CGRP-induced relaxations **(Chapter 15)**.



Part B: CGRP and its Receptors in Migraine: *In vitro* Approach

Chapter 5

Characterisation of CGRP Receptors in Human and Porcine Isolated Coronary Arteries: Evidence for CGRP Receptor Heterogeneity

Based on: S. Gupta, S. Mehrotra, CM. Villalón, IM. Garrelds, R. de Vries, J.P. van Kats, H. S. Sharma, P. R. Saxena & A. MaassenVanDenBrink, European Journal of Pharmacology, 2006, 530:107–116.

ABSTRACT

This study sets out to characterise calcitonin gene-related peptide (CGRP) receptors in human and porcine isolated proximal and distal coronary arteries using BIBN4096BS. Human (h)-αCGRP induced relaxations that were blocked by BIBN4096BS in all arteries studied. In contrast to the other vessels, the Schild plot slope in the human distal coronary artery segments (0.68±0.07) was significantly less than unity and BIBN4096BS potently blocked these responses (pK_b (10 nM): 9.29±0.34, n=5). In the same preparation, h-αCGRP$_{8-37}$ behaved as a weak antagonist of h-αCGRPinduced relaxations (pK_b (3 μM): 6.28±0.17, n=4), with also a Schild plot slope smaller than unity. The linear agonists, [ethylamide-Cys^{2,7}]h-αCGRP ([Cys(Et)^{2,7}]-h-αCGRP) and [acetimidomethyl-Cys^{2,7}]h-αCGRP ([Cys(Acm)^{2,7}]-h-αCGRP), had a high potency (pEC50: 8.21±0.25 and 7.25±0.14, respectively), suggesting the presence of $CGRP_2$ receptors, while the potent blockade by BIBN4096BS (pK_b (10 nM): 10.13±0.29 and 9.95±0.11, respectively) points to the presence of $CGRP_1$ receptors. Using RT-PCR, mRNAs encoding for the essential components for functional $CGRP_1$ receptors were demonstrated in both human proximal and distal coronary artery. Further, h-αCGRP (100 nM) increased cAMP levels, and this was attenuated by BIBN4096BS (1 μM). The above results demonstrate the presence of $CGRP_1$ receptors in all coronary artery segments investigated, but the human distal coronary artery segments seem to have an additional population of CGRP receptors not complying with the currently classified $CGRP_1$ or $CGRP_2$ receptors.

5.1 INTRODUCTION

The human calcitonin gene-related peptide (h-αCGRP), a 37-amino acid peptide, is one of the most potent endogenous vasodilators known. It exists in two forms, h-αCGRP and h-βCGRP, which differ from each other by three amino acids. In humans, these forms have almost similar biological actions (1, 2), but are encoded by separate genes. Although CGRP was first described in 1982 (3) the classification of CGRP receptors has been painstakingly slow because of the lack of selective and potent ligands.

Presently, CGRP receptors are functionally classified into $CGRP_1$ and $CGRP_2$ types. The $CGRP_1$ receptor has been cloned and consists of at least three main entities, namely, the calcitonin receptor like receptor (CLR), receptor activity modifying protein-1 (RAMP-1) and receptor component protein (RCP) (4, 5); in contrast, the $CGRP_2$ receptor, described in some animal tissues (1, 6), has not yet been reported in humans or deciphered molecularly. Therefore, the classification and characterisation of CGRP receptors is primarily based on different functional pharmacological responses. In this respect, the C-terminal fragment h-αCGRP$_{8-37}$ has a higher antagonist potency at the prototypic $CGRP_1$ receptor described in guinea-pig atrium (pK_b: 7-8) than at the $CGRP_2$ receptor described in rat vas deferens (pK_b: 5.5-6.5) (1, 7). However, the range of antagonist affinities reported for h-αCGRP$_{8-37}$ within and between different species (5) is too wide to be explained by the existence of just two receptors. Further, the $CGRP_2$ receptor seems to be more sensitive to the linear agonists [ethylamide-Cys^{2,7}]-h-αCGRP ([Cys(Et)^{2,7}]-h-αCGRP) and [acetimidomethyl-Cys^{2,7}]-h-αCGRP ([Cys(Acm)^{2,7}]-h-αCGRP) (pEC_{50}: ≈7) (6, 8) than the $CGRP_1$ receptor (9, 10). It should be noted that the selectivity of these linear agonists is ambiguous; in porcine large coronary artery [Cys(Acm)^{2,7}]-h-αCGRP acts like a partial agonist (11), while [Cys(Et)^{2,7}]-h-αCGRP is known to activate the $CGRP_1$ receptor in cell lines (12). However, these linear agonists still are important for the study of CGRP receptors in the absence of more selective CGRP receptor agonists and antagonists.

Interestingly, the responses to CGRP receptor agonists vary depending on the location and the size of blood vessels (13, 14). Moreover, the relaxation induced by CGRP is endothelium-dependent in the rat aorta (15), while it is endothelium-independent in human and porcine arteries (15, 16). Therefore, the extrapolation of affinities from one species to another to classify CGRP receptors should be done with caution.

BIBN4096BS, a lys-tyr dipeptide derivative, (1-piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl] carbonyl] pentyl] amino]-1-[(3,5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4-

(1,4-dihydro-2-oxo-3(2H)-quinazolinyl), displays a high antagonist potency and selectivity for human CGRP receptors (17, 18). Moreover, this antagonist is reported to have a 10-fold higher affinity for the $CGRP_1$ receptor (19) as compared to the $CGRP_2$ receptor. These properties of BIBN4096BS provide the possibility for an in-depth characterisation of CGRP receptors. Although the antagonist potency of BIBN4096BS in the human coronary artery has been studied earlier (20), a detailed investigation of CGRP receptors in human coronary arteries has not yet been performed. The fact that BIBN4096BS is effective in the acute treatment of migraine (21) underlines the need for a detailed investigation of CGRP receptors in human blood vessels. Therefore, we used BIBN4096BS and other available conventional CGRP receptor ligands to characterise CGRP receptors in human and porcine isolated coronary arteries. A part of this study has been published as an abstract (22).

5.2 MATERIALS AND METHODS

5.2.1 Functional studies

Human hearts were obtained from 'heart-beating' donors (22 male, 28 female; 48±2 years, range 3-66 years) who died due to non-cardiac causes. The hearts were provided by the Heart Valve Bank, Rotterdam after donor mediation by Bio Implant Services Foundation/Euro Transplant Foundation (Leiden, The Netherlands). Porcine hearts (pigs of either sex; 6-12 months of age) were obtained from a local slaughterhouse. In both cases, proximal (internal diameter: 2-3 mm) and distal (internal diameter: 250-600 µm) portions of the right coronary artery were dissected, placed in oxygenated Krebs bicarbonate solution (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1 mM; pH 7.4) and stored overnight at 4˚C. Vessel segments containing distinct, macroscopically visible atherosclerotic lesions were not used.

Proximal coronary artery segments (3-4 mm length) were suspended with the help of stainless-steel hooks in 15-ml organ baths with 15 mN pretension (optimal tension shown in earlier experiments). Distal artery segments (12 mm length) were placed in Mulvany myographs between two parallel titanium wires with a tension normalised to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development (23). The organ baths and myograph chambers, containing Krebs bicarbonate solution (for composition, see above) at 37˚C, were continuously bubbled with 95% O_2 and 5% CO_2. Since spontaneous contractions frequently occurred in human distal coronary arteries, these experiments were performed in the presence of the cyclo-oxygenase inhibitor indomethacin (0.1 µM). Unless mentioned otherwise, no attempt was made to remove the endothelium.

After an initial equilibration period of 30 min, all segments were challenged twice with 30 mM KCl at 30-min intervals to verify reproducibility of the responses. The integrity of the endothelium was assessed by observing relaxations to substance P (1-10 nM) after precontraction with prostaglandin $F_{2\alpha}$ (1 µM, proximal coronary arteries) or U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$, 10-100 nM, distal coronary arteries). In 4 human and 5 porcine distal coronary artery segments, the endothelium was denuded using a human hair. After 30 min, 100 mM KCl was added to determine the reference contractile response in each segment. Then, a stable contraction plateau of around 60% of the maximal contraction was obtained with KCl (30 mM) and, subsequently, the CGRP receptor agonists (i.e. h-αCGRP, h-βCGRP, [$Cys(Acm)^{2,7}$]-h-αCGRP and [$Cys(Et)^{2,7}$]-h-αCGRP; range: 0.1 nM-3 µM) were added in a cumulative manner in 0.5 log steps every 5 min or when the maximum effect of a given concentration was reached. In human proximal coronary artery, it was difficult to obtain a stable precontraction with 30 mM KCl. In fact, other compounds, such as U46619, showed even more unstable precontraction in pilot experiments, and the responses to concentrations of CGRP <30 nM were small and difficult to discriminate from the spontaneous decline in the baseline tension. Therefore, we chose to start the concentration-response curves to h-αCGRP at a higher concentration, so that quantification was less

sensitive to artefacts. Furthermore, in three human proximal vessels, but none of the distal vessels, the control segment did not respond to h-αCGRP and, consequently, all segments from these non-responding vessels were excluded from the study. Only one concentration-response curve was constructed in the arterial segments, either in the absence or presence of the antagonists (BIBN4096BS, 0.1 nM10 μM or h-αCGRP$_{8-37}$, 0.1-10 μM) that were incubated for a period of 30 min, unless mentioned otherwise.

5.2.2 Isolation of total RNA and Reverse transcriptase-polymerase chain reaction (RT-PCR) studies

After isolation from the heart, the segments of proximal and distal human coronary arteries were snap frozen in liquid nitrogen and stored at 80˚C until use. The tissues were transferred to guanidium thiocyanate buffer, homogenised (Ultra-Turrax homogeniser, model T8; Janke & Kunkel Gmbh, Staufen, Germany) and the total RNA was extracted (24, 25). The RNA concentration was measured by UV absorbance at a wavelength of 260 nm using a Gene Quant RNA/DNA calculator (Pharmacia-LKB, Uppsala, Sweden) and the quality of RNA was assessed by formaldehyde agarose gel electrophoresis and a DNA/protein ratio (OD_{260}/OD_{280}) of >1.8. Total RNA was denatured at 65°C and the first strand of cDNA was synthesised in a reaction volume of 20 μl by adding the following reagents: reverse transcription buffer (25 mM Tris HCl; pH 8.3, 50 mM KCl; 5 mM $MgCl_2$, 2 mM dithiothreitol), 1 mM deoxy nucleotide triphosphate (dNTPs), ribonuclease inhibitor (1 U/μl), random hexamer (150 ng/μg total RNA) and finally moloney-murine leukaemia virus-Reverse Transcriptase (MMLV-RT) (Pro-omega, Benelux b.v., Leiden, The Netherlands). A control reaction with all of the above ingredients, except MMLVRT, was prepared. The reactions were carried out for 90 min at 42°C, extended for another 10 min at 75°C and then cooled at 4°C. The cDNA thus synthesised was stored at -20°C until used as a PCR template.

The quality of cDNA was verified by PCR amplification of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) using specific oligonucleotide primers (see Results section). For PCR amplification, a 20 μl reaction mixture containing the following components was prepared: 1.25 mM of each dNTP, 3 mM $MgCl_2$, PCR buffer (1xPCR buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl) Ampli Taq Gold™ enzyme (0.5 U), 0.5 μM of each of the forward and reverse primers and 2 μl of the cDNA template. After a brief centrifugation the enzyme was first activated for 5 min at 94°C in a PCR thermocycler (model PTC-100™, MJ Research Inc., Watertown, MA, U.S.A.). PCR was carried out as: 45 sec at 94°C, 45 sec at 55°C and 90 sec at 72°C with a total of 40 cycles. Finally, the reaction was extended for an additional 10 min. For PCR amplification of RAMP-3 cDNA template obtained from the proximal coronary artery, we reduced the concentration of $MgCl_2$ from 3 mM to 2 mM and performed 35 instead of 40 cycles to eliminate an unspecific band, while the remaining part of the conditions remained unchanged. An aliquot of PCR reaction product was analysed on 2% agarose gel, visualised under UV light and digitally photographed. The different CGRP receptor components were semi-quantitatively assessed using 1D-analysis software (Kodak Digital Science 1D Image Analysis, Version 1.6, Scientific Imaging System, Rochester, NY, U.S.A.).

5.2.3 cAMP measurements

Human proximal and distal, as well as porcine distal coronary artery segments were incubated in a medium containing isobutylmethylxanthine (IBMX, 0.5 mM) for 30 min in the absence or presence of BIBN4096BS (1 μM). The arterial segments were exposed to KCl (30 mM), challenged with h-αCGRP (100 nM) or forskolin (10 μM) for 5 min and then snap frozen. Forskolin, which increases intracellular cAMP concentrations by activating adenylyl cyclase, was used to assess the specificity of BIBN4096BS for CGRP-mediated increases in cAMP concentrations. The samples were stored at -80°C until cAMP assay using the ELISA kit and manual (R&D Systems Europe Ltd., Abingdom, U.K.).

5.2.4 Data presentation and statistical analysis

The relaxant responses elicited by agonists are expressed as percentage relaxation of the tone induced by 30 mM KCl. All data are presented as means±S.E.M and *n* represents the number of blood vessels

obtained from different donors. The concentration-response curves for all agonists were analysed using nonlinear regression analysis and the potency of agonists was expressed as pEC_{50} using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The blocking potency of the antagonists was estimated by calculating EC_{50} ratios and plotting a Schild-plot (26) using linear regression to get the slope value. Only the EC_{50} ratios >2 were used for Schild plot analysis and for calculation of respective pK_b values. In certain cases, the slope value was significantly different from one, thus prohibiting us to calculate pA_2 values. Therefore, to enable a uniform comparison between groups, "apparent pK_b" values were calculated for the antagonists at each given concentration, constraining the slope to unity. Correlation analyses were carried out using Pearson's coefficient of correlation using SPSS 11.01 (SPSS Inc., Chicago, Illinois, U.S.A.). Statistical significance was determined by unpaired Student *t*-test, with differences considered significant at $P \leq 0.05$. The ethical committee of Erasmus MC, Rotterdam, approved the study.

5.2.5 Compounds

The compounds used in the present study (obtained from the sources indicated) were: h-αCGRP, h-βCGRP, [Cys $(Acm)^{2,7}$]-h-αCGRP, [Cys $(Et)^{2,7}$]-h-αCGRP and h-$αCGRP_{8-37}$ (Polypeptide, Wolfenbüttel Germany); BIBN4096BS (gift from Dr. Henri Doods, Boehringer Ingelheim Pharma K.G., Biberach, Germany); U46619, isobutylmethylxanthine (IBMX), forskolin and substance P (Sigma Chemicals Co., Steinheim, Germany); and KCl (Merck, Darmstad, Germany). All compounds were dissolved in distilled water except for forskolin, which was dissolved in dimelthylsulfoxide and BIBN4096BS, which was dissolved in 4% HCl (1 N) to obtain a 0.01 M stock solution; the latter was then diluted with distilled water and. All peptides and antagonists were stored in aliquots at 80°C.

5.3 RESULTS

5.3.1 Functional studies in human coronary arteries

The contraction induced by 100 mM KCl was significantly higher in the proximal (56±3 mN) than in the distal (8±1 mN) coronary arteries. In contrast, the endothelium-dependent relaxant response to substance P (10 nM) was less in the proximal than in the distal coronary segments (26±13 and 76±4%, respectively).

h-αCGRP induced a concentration-dependent relaxation in precontracted human coronary arteries (Figure 5.1). The maximal response to h-αCGRP was significantly less in the proximal (E_{max}: 43±7% of contraction to 30 mM KCl) than in the distal (E_{max}: 92±4%) segments. In distal segments where no attempt was made to remove the endothelium, we assessed the correlation between the pEC_{50} and E_{max} of h-αCGRP and the relaxation to substance P, as determined in each experiment. These correlations were not significant (Pearson's r=0.066 and 0.088, respectively; P=0.76 and 0.69, respectively, *n*=23-27), indicating that the endothelium is not involved in relaxations to h-αCGRP. Accordingly, in distal coronary segments, we also performed an additional series of experiments where the endothelium was denuded (relaxation to substance P: 1.0±1.4% and 64±22% of the precontraction to U46619 in endothelium-denuded and -intact segments, respectively). In these segments, both the E_{max} (96±2.3% and 96±2.1%, respectively) and pEC_{50} (9.23±0.44 and 9.18±0.86, respectively, *n*=4) of h-αCGRP were similar. BIBN4096BS did not change the basal tone or the contraction to KCl (30 mM) in either the proximal or distal human coronary artery. In proximal segments, the antagonism seemed to be non-competitive (Figure 5.1a) as demonstrated by a significant suppression of the E_{max} of h-αCGRP (43±7%), even at low concentrations of BIBN4096BS (E_{max}: 24±5% and 18±8% and in the presence of 0.1 nM and 1 nM of BIBN4096BS, respectively). However, in view of the large variability inherent to this preparation, as described in methods section, these data should be interpreted with caution.

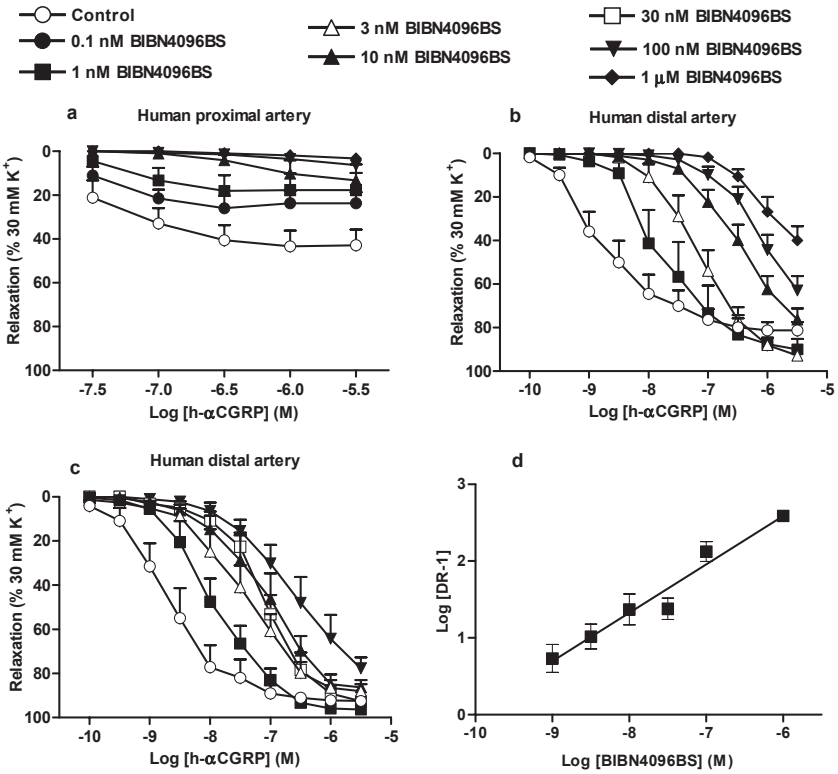


Figure 5.1. Effect of increasing concentrations of BIBN4096BS on the relaxant responses to h-αCGRP in proximal (panel a) and distal (panels b and c) segments of the human right coronary artery (*n*=6-11) and average Schild plot slope (panel d) obtained from panel c. BIBN4096BS (0.1 nM-1 µM) was incubated for 0.5 h (panels a, c and d) or 2 h (panel b).

In distal segments BIBN4096BS concentration-dependently antagonised the responses to h-αCGRP, but the Schild plot slope was significantly less than one (0.68±0.07; Table 5.1). Further experiments were carried out in the human distal coronary artery segments to investigate the anomalous nature of the slope. To exclude that this finding was due to the inability of BIBN4096BS to reach equilibrium conditions after 30 min, we performed experiments in distal coronary arteries with BIBN4096BS using a longer incubation period (2 h; Figure 5.1b). In addition, we conducted experiments using a wider range of concentrations of BIBN4096BS (Figure 5.1c) to obtain an estimate of the Schild slope based on more data points than in our initial experiments (Figure 5.1d). In both cases, the slope remained significantly less than one (0.65±0.09 and 0.64±0.05, respectively), but the longer incubation period did increase the potency of BIBN4096BS (see apparent pK_b values in Table 5.1).

h-αCGRP$_{8-37}$ also antagonised the responses to h-αCGRP in the human distal coronary artery (Figure 5.2), but with a low potency (pK_b at 3 µM: 6.28±0.17 n=4), and again the Schild plot slope was significantly less than one (0.36±0.05, n=4).

Both linear agonists induced concentration-dependent relaxations and BIBN4096BS, similarly as with h-αCGRP, antagonised the responses to [Cys(Acm)^{2,7}]-h-αCGRP in a seemingly competitive manner with a slope significantly less than one (0.49±0.13; Figure 5.3). Although the antagonism of BIBN4096BS appeared competitive in nature, it should be kept in mind that in view of the low potency of [Cys(Acm)^{2,7}]-h-αCGRP the values had to be extrapolated for calculation of the pharmacological parameters. In contrast, the slope obtained with [Cys(Et)^{2,7}]-h-αCGRP (1.13±0.25) was not different from unity. h-βCGRP relaxed the distal coronary artery with a potency not different from that of h-αCGRP (Figure 5.3; Table 5.1), and this

Table 5.1. Effect of BIBN4096BS on relaxations to CGRP receptor agonists in human distal coronary artery segments. BIBN4096BS (0.1 nM-1 μM) was incubated for 0.5 h with concentrations increasing in one-log steps, except in the case of the human coronary artery and h-αCGRP where two additional groups (+more concentrations or # 2h incubation) were used.

Agonists	E_{max}	pEC_{50}	Slope	Apparent pK_b BIBN4096BS				
				0.1 nM	1 nM	10 nM	0.1 μM	1 μM
h-αCGRP	92±4 (10)	8.05±0.23 (10)	a0.68±0.07 (5)			9.29±0.34 (5)	8.66±0.25 (10)	8.41±0.26 (10)
h-αCGRP+	90±3 (6)	8.27±0.43 (6)	a0.64±0.05 (6)		9.56±0.2(5)	9.33±0.25 (6)	9.13±0.17 (4)	
h-αCGRP#	82±4 (11)	8.60±0.22 (11)	a0.65±0.09 (9)		9.72±0.18 (4)	10.12±0.18 (9)	9.76±0.16 (10)	9.29±0.20 (6)
[Cys(Acm)2,7]	93±2 (8)	7.25±0.14 (8)	a0.49±0.13 (5)	11.03±0.42 (4)	10.24±0.26 (7)	9.95±0.11 (9)		
[Cys(Et)2,7]	90±3 (9)	8.21±0.25 (9)	1.13±0.25 (6)	§10.67±0.37 (3)	10.29±0.18 (8)	10.13±0.29 (9)	10.26±0.36 (3)	
h-βCGRP	86±4 (8)	8.80±0.21 (8)	a0.70±0.08 (8)		9.88±0.40 (2)	9.51±0.20 (8)	9.19±0.30 (8)	8.86±0.22 (8)

E_{max} was expressed as % of the response to 30 mM KCl. All data are means±S.E.M (n). [a], slopes of the Schild regression significantly different ($P \leq 0.05$) from unity. [Cys(Acm)^{2,7}], [Cys(Acm)^{2,7}]h-αCGRP; [Cys(Et)^{2,7}], [Cys(Et)^{2,7}]h-αCGRP. [§], pK_b at 0.3 nM BIBN4096BS.

Table 5.2. Detection of molecular components relevant for CGRP receptors

Components	NCBI Genebank Accession No.	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplified sequence	Size (bp)
h-CLR	AY389506	TCAAGAGCCTAAGTTGCCAAA	AATCAGCACAAATTCAATGCC	497-1057	560
h-RAMP-1	NM005855	CTGCCAGGAGGCTAACTACG	GACCACGATGAAGGGGTAGA	78-376	298
h-RAMP-2	BC040107	GGGGGACGGTGAAGAACTAT	GTTGGCAAAGTGGATCTGGT	164-391	227
h-RAMP-3	BC05385	AAGGTCTTCGCAGACATGAT	GCAGTTGGAGAAGAACTGCC	123-312	189
h-RCP	U51134	AACTGATCTGAAAGAGCAGCG	TCTTCTTCTGCTCAGCCTCTG	121-465	344
h-RDC-1	AF030297	ACGTGGTGGTCTTCCTTGTC	AAGGCCTTCATCAGCTCGTA	770-990	220
GAPDH	BC023632	TGACTTCAACAGCACCC	TACATGACAAGGTGCGGCTC	906-1254	348

Each set of forward and reverse primers was designed from the nucleotide sequences reported in the NCBI Genebank. CLR, Calcitonin receptor like receptor; RAMP, receptor activity modifying protein; RCP, receptor component protein; RDC-1, the orphan receptor (originally cloned from canine thyroid cDNA); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

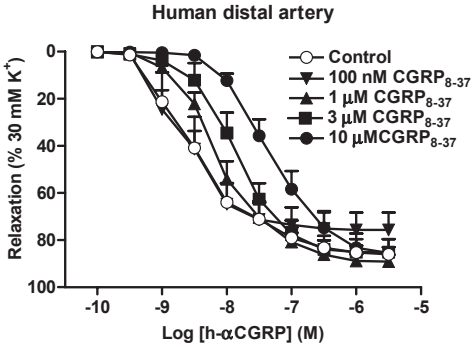


Figure 5.2. Effect of increasing concentrations of h $\alpha CGRP_{8-37}$ on the relaxant responses to h-αCGRP in distal segments of the human right coronary artery (*n*=4-12). h-$\alpha CGRP_{8-37}$ (100 nM-10 μM) was incubated for 0.5 h.

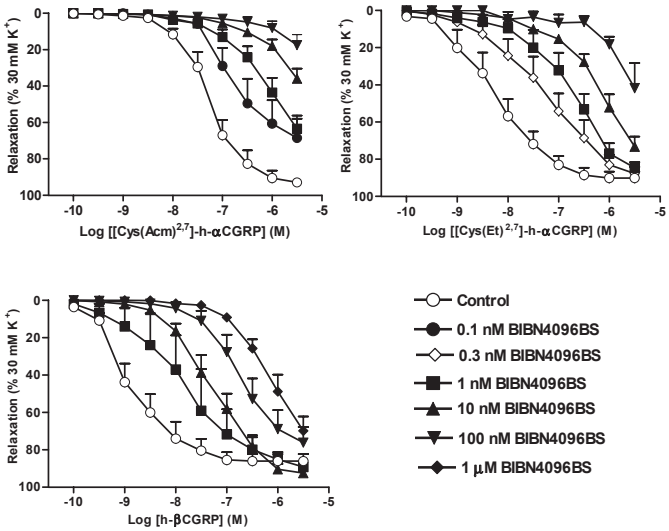


Figure 5.3. Effect of increasing concentrations of BIBN4096BS on the relaxant responses to [Cys $(Acm)^{2,7}$]h-αCGRP, [Cys $(Et)^{2,7}$]h-αCGRP and h-βCGRP in distal segments of the human right coronary artery (*n*=5-9). BIBN4096BS (0.1 nM-1 μM) was incubated for 0.5 h.

response was potently antagonised by BIBN4096BS, with a Schild plot slope (0.70±0.08) significantly less than one

5.3.2 Porcine coronary arteries

The contractile response to 100 mM KCl in proximal coronary arteries was 69±6 mN as compared to 14±4 mN in the distal segments. h-αCGRP induced concentration-dependent relaxations in both proximal and distal coronary artery segments (Figure 5.4). Endothelium denudation in the porcine distal coronary segments resulted in significantly decreased relaxations to substance P (7±4% and 78±7% of the precontraction to U46619 for endothelium-denuded and -intact artery segments, respectively). In contrast, the relaxations to h-αCGRP in endothelium-denuded segments (E_{max}: 97±2%; pEC_{50}: 8.80±0.17) were not different from those in control segments (E_{max}: 95±2%; pEC_{50}: 8.77±0.16, *n*=5). Similar to the human coronary artery, the E_{max} of h-αCGRP was significantly less in porcine proximal segments (72±3%) than in distal segments (95±2%). Moreover, h-αCGRP-induced relaxations were blocked with equal po-

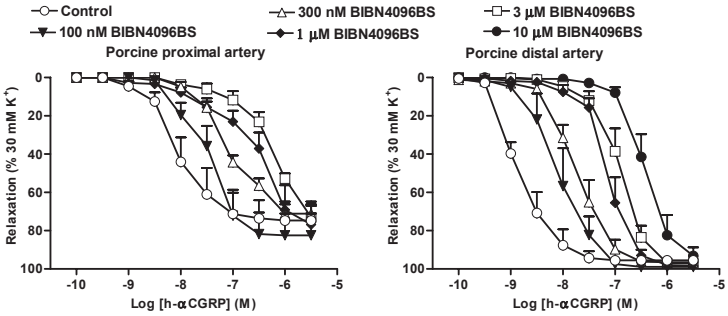


Figure 5.4. Effect of increasing concentrations of BIBN4096BS on the relaxant responses to h-αCGRP in proximal and distal segments of the porcine right coronary artery (*n*=68). BIBN4096BS (0.1 nM-1 µM) was incubated for 0.5 h.

tency by BIBN4096BS (1 µM) in the proximal and distal segments (pK_b: 7.44±0.10 and 7.63±0.14, respectively), while the Schild plot slope obtained in both cases were not different from unity (0.80±0.18 and 0.87±0.10, respectively).

5.3.3 RT-PCR studies in human coronary arteries

Using human specific forward and reverse primers, designed on the basis of nucleotide sequences of several components of the CGRP receptor family reported in the NCBI Genebank (Table 5.2), PCR products corresponding in size to CLR, RAMP-1, RAMP-2, RAMP-3, RDC and RCP were consistently amplified in both proximal and distal human coronary arteries (Figure 5.5). Semi-quantitative analysis revealed no difference in the expression levels of these CGRP receptor components, except for a two-fold higher expression of RAMP-2 in the distal as compared to the proximal segments.

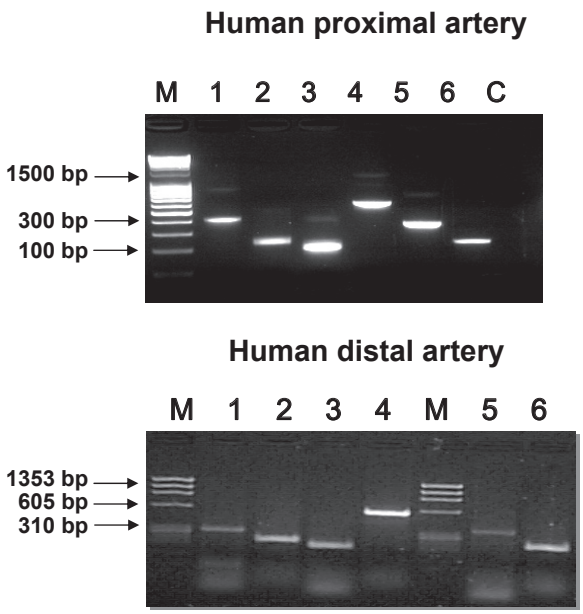


Figure 5.5. Agarose gel electrophoresis of PCR amplified products derived from cDNA obtained from proximal (upper panel) and distal (lower panel) segments of the human right coronary artery. The different lanes marked on top of the panels denote: φx174 DNA/Hae III marker (M), RAMP-1 (298 bp; 1), RAMP-2 (227 bp; 2), RAMP-3 (189 bp; 3), CLR (560 bp; 4), RCP (344 bp; 5) and RDC (220 bp; 6). The size of 3 marker bands is indicated in the left margins.

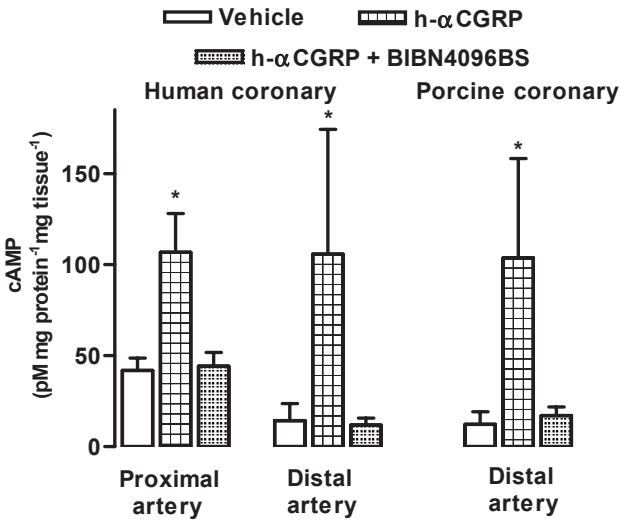


Figure 5.6. Effect of BIBN4096BS (1 μM) on the increases in cAMP concentration by h-αCGRP (100 nM) in the human (proximal and distal segments) and porcine (distal segments) right coronary artery (*n*=5-17). *, Significantly (P<0.05) different from vehicle-treated segments.

5.3.4 cAMP measurements in human and porcine coronary arteries

h-αCGRP (100 nM) increased cAMP concentrations significantly in the human proximal and distal artery segments as compared to the segments incubated with vehicle (42±7 versus 107±21 and 14±9 versus 106±69 pM mg protein-1 mg tissue-1, respectively). This increase was significantly blocked by BIBN4096BS in both the human artery segments (Figure 5.6). Similarly in porcine distal coronary arteries also there was significant increase in cAMP levels in response to h-αCGRP and this effect was antagonised by BIBN4096BS (Figure 5.6). BIBN4096BS did not significantly inhibit the forskolin-induced increase in the cAMP levels, either in human (389±134 and 292±110 pM mg protein-1 mg tissue-1 in the absence or presence of antagonist, respectively, P=0.47, n=5) or porcine (200±40 and 141±72 pM mg protein-1 mg tissue-1, respectively, P=0.61, n=4) distal coronary arteries.

5.4 DISCUSSION

5.4.1 Functional interaction between CGRP receptor agonists and antagonists

In both human and porcine proximal (internal diameter: 2-3 mm) as well as distal (internal diameter: 250-600 μm) segments of the right coronary artery, h-αCGRP elicited a concentration-dependent relaxation that was antagonised by BIBN4096BS, which proved more potent in the humans than in the pig. Moreover, our results demonstrate that these relaxations were endothelium-independent in both human and porcine distal coronary artery. Our findings, showing that the responses to h-αCGRP in all four segment types are mediated by the $CGRP_1$ receptor, are consistent with those reported recently in the human (20, 27) and porcine (28) left anterior descending coronary artery, where the CGRPinduced relaxation was also antagonised by BIBN4096BS with similar potencies.

As reported earlier (14), we also found that the efficacy of CGRP inversely related to the vessel diameter, E_{max} values in both human and porcine arteries were lower in the proximal (43±7% and 72±3%, respectively) than in the distal (92±4% and 95±2%, respectively) coronary segments. The antagonism in the case of human proximal arteries seems to be non-competitive, as demonstrated by the suppression of the E_{max}. Although these data should be interpreted with caution, it is worth noting that a similar

non-competitive behaviour of BIBN4096BS has been reported in human subcutaneous arteries at similar concentrations (0.1 nM and 1 nM) (29).

The Schild plot slope in the human distal coronary artery (0.68±0.07) was significantly lower than unity and, therefore, additional experiments were undertaken with human distal coronary arteries. To rule out that the Schild plot slope was less than unity because BIBN4096BS had not yet reached the equilibrium conditions in the distal segments, we employed a longer incubation period (2 h) with the antagonist. Notwithstanding, the slope (0.65±0.09) remained significantly less than one, although the potency of the antagonist increased with longer incubation period. Within this framework, if there were a non-equilibrium state between an antagonist and its receptor, the Schild plot slope would tend to be greater than unity (30) instead of less than one, as was the case in our experiments. The fact that a longer incubation period increased the potency of BIBN4096BS (apparent pK_b at 10 nM: 10.12±0.18) is in line with previous observations on slow on and off kinetics of BIBN4096BS (31). Similarly, as with the experiments employing a longer incubation period for BIBN5096BS, the slope of the Schild plot obtained with more concentrations of BIBN4096BS remained less than one (0.64±0.05).

In subsequent studies, we used the conventional antagonist h-αCGRP$_{8-37}$ as a tool to distinguish between CGRP1 (pK_b: 7-8) and CGRP2 (pK_b: 5.5-6.5) receptors (1, 7, 32). The fact that h-αCGRP$_{8-37}$ antagonised h-αCGRP with a low potency (pK_b: 6.28±0.17) supports the possible involvement of $CGRP_2$ receptors (33). Again, the Schild plot slope was less than unity, demonstrating that the smaller Schild slope values should most likely be attributed to a heterogeneous receptor population in the human distal coronary artery and is not due to a BIBN4096BS-specific phenomenon. Further, our finding showing that the relatively selective $CGRP_2$ receptor agonists, [Cys(Acm)^{2,7}]-h-αCGRP and [Cys(Et)^{2,7}]-h-αCGRP (1, 7, 33), also induced concentration-dependent relaxations indicate the presence of putative $CGRP_2$ receptors. However, in similar experiments performed in left anterior descending coronary artery segments (1-2 mm external diameter) obtained from patients with cardiac myopathy, Hasbak *et al*. (27) observed only a very weak relaxant effect of [Cys (Acm)^{2,7}]-h-αCGRP (1 μM) amounting to less than 5% of the effect we observed. This difference may be due to the fact that we used donors without cardiac pathology and, moreover, used smaller size segments (0.25-0.6 mm internal diameter) of the right coronary artery, which may contain different subtypes of CGRP-sensitive receptors. The selectivity of the linear agonists is not unequivocal; Waugh et al. (11), who observed that with increasing concentrations of KCl (8-15 mM) used as precontraction, the relaxation induced by [Cys(Acm)^{2,7}]h-αCGRP in pig coronary arteries decreased from 90% to almost zero, demonstrating that [Cys(Acm)^{2,7}]h-αCGRP may act as a partial agonist. However, in our preparation this was not the case, since even with a precontraction induced by 30 mM KCl the E_{max} of [Cys(Acm)^{2,7}]h-αCGRP was as high as 93±2%. Further, the other linear agonist [Cys(Et)^{2,7}]h-αCGRP activated $CGRP_1$-like receptor in SK-N-MC and Col-29 cells, while the same agonist acted on $CGRP_2$-like receptors in rat vas deferens with a pEC_{50} ≈8 (19). In the present study, [Cys(Et)^{2,7}]h-αCGRP also relaxed arteries with a similar potency (pEC_{50}: 8.21±0.25). According to Kenakin (30), different slopes obtained with Schild plots for different agonists with the same antagonist indicate a heterogeneous receptor population. Indeed, in contrast to our experiments with h-αCGRP and [Cys(Acm)^{2,7}]h-αCGRP, the Schild slopes obtained with BIBN4096BS antagonising [Cys(Et)^{2,7}]h-αCGRP was equal to unity, suggesting that these agonists do not stimulate the same receptor population or activate same receptor but with different potencies. Further, the observation that the apparent pK_b values decrease with increasing concentrations of antagonist in case the slope was less than one again points to a heterogeneous CGRP receptor population (Table 5.1).

Intriguingly, the fact that the apparent pK_b of BIBN4096BS was around 10 for both linear agonists points to the presence of $CGRP_1$ receptors as BIBN4096BS is supposed to be at least 10 times less potent for $CGRP_2$ receptors (19). Hence, it is reasonable to assume that the receptors in the human distal coronary artery do not completely comply with the pharmacological profile of the presently accepted CGRP receptor classification (33); notwithstanding, it would be prudent to consider several lines of arguments

within this framework. Firstly, Sheykhzade et al. (29) have recently suggested a non-competitive nature of antagonism of BIBN4096BS at concentrations higher than 10 pM against h-αCGRP-induced relaxations in human subcutaneous arteries. Although, as mentioned above, this might be the case in our results with the proximal coronary artery, it does not apply to the distal coronary artery, where even at high concentrations of BIBN4096BS the E_{max} to h-αCGRP or other agonist was not suppressed showing antagonism is competitive in nature. Secondly, the interaction between BIBN4096BS and the adrenomedullin receptor might be responsible for the slope of the Schild plot being less than one. However, the affinity of BIBN4096BS for adrenomedullin receptors is too low (IC_{50}: 10.3 μM) for such an interaction (17). Thirdly, metabolism of h-αCGRP or CGRP receptor ligands by tissue peptidases can affect the data obtained. Nevertheless, inhibition of these peptidases has shown that the contribution of metabolism in determining the affinity of CGRP ligands is negligible (19). Fourthly, the linear agonists might not be selective enough to form the basis for the classification of CGRP receptors. In view of our findings, we also have our reservation towards the purported selectivity of linear agonist towards $CGRP_2$ receptors, as both agonists under similar experimental conditions yielded significantly different Schild plot slopes with BIBN4096BS, suggesting that these agonists do not activate the same receptor subtypes. BIBN4096BS is suppose to be $CGRP_1$ selective receptor but it showed similar or even higher antagonist potency with linear agonist in human distal coronary artery as compared with h-αCGRP. Finally, the slope being different from one cannot be attributed only to BIBN4096BS as in case of porcine proximal and distal coronary arteries the slope was not significantly different from one, and on the other hand h-$αCGRP_{8-37}$ with h-αCGRP also produced a slope less than one, thus underlining a heterogeneous receptor population in the human distal coronary artery.

5.4.2 Molecular components of the CGRP receptor family

Our RT-PCR results showed the expression of various CGRP receptor components like CLR, RCP, RDC-1, RAMP-1, RAMP-2 and RAMP-3 in both proximal and distal human coronary arteries; these molecular studies confirmed the presence of all the essential components (CLR + RAMP-1 + RCP) required for functional $CGRP_1$ receptors in the above coronary segments. In this context, CLR is a G-protein coupled receptor and the presence of RAMP-1 ensures intracellular trafficking and maturation of the receptor (34). The third entity, RCP, is required for the formation of a high-affinity G-protein-coupled receptor, there by ensuring the signal transduction of CLR (35). Unfortunately, the molecular counterpart for the $CGRP_2$ receptor is yet to be determined.

5.4.3 Transduction mechanisms of the CGRP receptors

Both in human and porcine coronary arteries cAMP levels increased after a challenge with h-αCGRP. The fact that this increase in cAMP was abolished after incubation with BIBN4096BS clearly demonstrates that the relaxations to h-αCGRP in the above blood vessels are, at least partly, mediated via this second messenger system.

In conclusion, the relaxation to h-αCGRP in the human distal coronary arteries is mediated by $CGRP_1$ receptors as demonstrated by the high apparent pK_b values (≈9-10) obtained with BIBN4096BS. In addition, another CGRP receptor subtype, possibly also acting via an increase in cAMP, seems to be present in the human distal coronary artery. Although the nature of this receptor is not entirely clear, it shows some functional characteristics of $CGRP_2$ receptors, such as the low antagonist potency of h-$αCGRP_{8-37}$ and the high potency of the linear agonists, whereas the high apparent pK_b values of BIBN4096BS points to the presence of $CGRP_1$ receptors. A prerequisite to further characterise these receptors would be the advent of more selective CGRP receptor agonists and antagonists along with the molecular characterisation of the putative $CGRP_2$ receptor.

ACKNOWLEDGEMENTS

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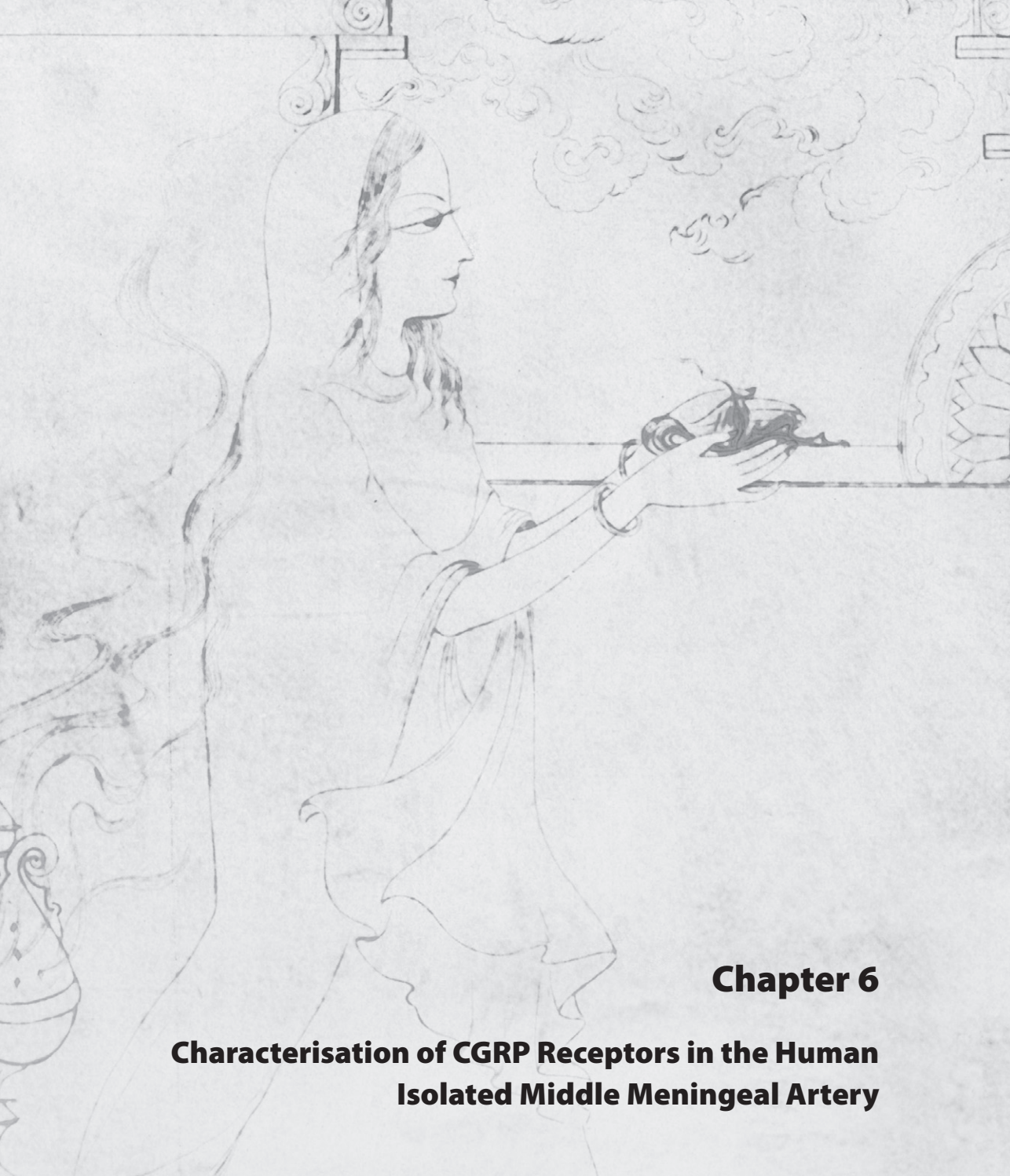

Chapter 6

Characterisation of CGRP Receptors in the Human Isolated Middle Meningeal Artery

Based on: S. Gupta, S. Mehrotra, C.J.J. Avezaat, C.M. Villalón, P.R. Saxena & A. MaassenVanDenBrink, Life Sciences 2006; 79:265-71.

ABSTRACT

Although the understanding of migraine pathophysiology is still incomplete, there seems to be little doubt that dilatation of cranial blood vessels, including meningeal arteries, is involved in the headache phase of migraine. Since calcitonin gene-related peptide (CGRP) has been implicated in this vasodilatation, the present study set out to compare the relaxant effects of the endogenous ligand h-αCGRP, and [ethylamide-Cys^{2,7}]h-αCGRP ([Cys(Et)^{2,7}]h-αCGRP), a $CGRP_2$ receptor agonist, on human isolated middle meningeal artery segments, precontracted with KCl. Classical Schild plot analysis was used to characterise the receptor population in this arery using BIBN4096BS and h-α$CGRP_{8-37}$ as antagonists. h-αCGRP relaxed arterial segments more potently than [Cys(Et)^{2,7}]h-αCGRP (pEC_{50}: 8.51±0.16 and 7.48±0.24, respectively), while the maximal responses to these agonists were not significantly different. BIBN4096BS equipotently blocked the relaxations induced by both agonists with a pA_2 of ~10 and with a Schild plot slope not significantly different from unity. h-α$CGRP_{8-37}$ also antagonised the response to h-αCGRP with a pA_2 of 6.46±0.16 and a Schild plot slope not different from unity. Furthermore, the results obtained from RT-PCR studies confirmed the presence of all the essential components required for a functional $CGRP_1$ receptor in these arteries. Considering the high antagonist potency of BIBN4096BS, coupled to the lower agonist potency of [Cys (Et)^{2,7}]h-αCGRP, it is reasonable to suggest a predominant role of $CGRP_1$ receptors in the human middle meningeal artery. This view is reinforced by Schild plot analysis, which revealed a slope of unity in all experiments, giving further evidence for a homogeneous CGRP receptor population in this vascular preparation.

6.1 INTRODUCTION

Migraine is a neurovascular syndrome that is characterized by vasodilatation of meningeal blood vessels; this vasodilatation is associated with activation of perivascular trigeminal sensory nerves and release of calcitonin gene-related peptide (CGRP) (1, 2). Indeed, plasma concentrations of CGRP, but not of other neuropeptides, are elevated during migraine attacks (3) and these levels are normalised by triptans in parallel with alleviation of headache (4). Further, the antimigraine action of triptans has been attributed to vasoconstriction of cranial arteries, including the middle meningeal artery (5, 6).

On the basis of pharmacological criteria, it is known that CGRP may act mainly on $CGRP_1$ and $CGRP_2$ receptors (7), with h-α$CGRP_{8-37}$ being a 10-fold more potent antagonist at $CGRP_1$ receptors than at $CGRP_2$ receptors (8). While $CGRP_1$ receptors are widely distributed, $CGRP_2$ receptors have only been described in rat *vas deferens*, and are more sensitive to the linear agonists [ethylamide-Cys^{2,7}]h-αCGRP ([Cys(Et)^{2,7}]h-αCGRP) and [acetimidomethyl-Cys^{2,7}]h-αCGRP ([Cys(Acm)^{2,7}]h-αCGRP) (9). Using these pharmacological tools, CGRP receptors have been characterised in human cerebral (10) and middle meningeal (11) arteries.

BIBN4096BS: (1piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl] carbonyl]pentyl] amino]-1-[(3, 5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4- (1,4-dihydro -2-oxo-3(2H)-quinazolinyl)-, [R-(R*,S*)]-).has been developed as a very potent and selective human CGRP receptor antagonist (12, 13) which: (i) displays a 10-fold higher affinity for $CGRP_1$ than for $CGRP_2$ receptors (14); and (ii) is effective in the acute treatment of migraine (15). Hence, the clinical potential of CGRP receptor antagonists makes it of pivotal importance to characterise CGRP receptors using such novel antagonists in human blood vessels implicated in the pathophysiology of migraine. In human cerebral arteries Edvinsson et al. (16) using this antagonist showed presence of $CGRP_1$ receptors. While we have reported CGRP receptor heterogeneity in the human coronary artery using BIBN4096BS (17), others have reported a diversity of CGRP receptors in human temporal (18) and meningeal (19) arteries. While a non-competitive behaviour of BIBN4096BS has been reported in human subcutaneous arteries (20), we have observed CGRP receptor heterogeneity in the human coronary artery using BIBN4096BS (17) and others have described a diversity of CGRP receptors in human temporal (18) and meningeal arteries (19). Regarding the latter

blood vessel, it is noteworthy that the conclusions drawn by Moreno et al. were based on the reversal of already established relaxations to CGRP by BIBN4096BS, and no experiments were performed with linear agonists such as [Cys(Et)^{2,7}]h-αCGRP.

Molecularly, $CGRP_1$ receptors consist of at least three main different entities, namely, the calcitonin receptor like receptor (CLR), receptor activity modifying protein-1 (RAMP-1) (21) and receptor component protein (RCP) (22, 23), whereas $CGRP_2$ receptors have not yet been molecularly characterized. The molecular components of $CGRP_1$ receptors that have been demonstrated in the human cerebral and meningeal artery include CLR and the RAMPs 1, 2 and 3 (11, 24); nevertheless, there is no report about RCP, which is required for the formation of a high-affinity G-protein-coupled receptor, thereby ensuring the signal transduction of CLR (25).

In view of: (i) the relevance of the meningeal artery in the pathophysiology of migraine (1, 6); (ii) the fact that homo- or heterogeneity of CGRP receptor population in this blood vessel has not yet been assessed using a classical pharmacological approach; and (iii) the $CGRP_2$ receptor selective agonist [Cys(Et)^{2,7}]h-αCGRP has not yet been studied in this blood vessel, we performed an analysis, using the Schild plot approach with the antagonists BIBN4096BS and h-α$CGRP_{8-37}$ as well as the agonists h-αCGRP and [Cys(Et)^{2,7}]h-αCGRP in this artery. This would allow a comparison of results obtained in other human blood vessels with BIBN4069BS (16, 17, 26). Since not all essential components required for a functional $CGRP_1$ receptor have yet been demonstrated in the human meningeal artery we tried to confirm the presence of mRNA of all these components.

6.2 MATERIALS AND METHODS

6.2.1 Tissue preparation

Human meningeal arteries were obtained from 22 individuals (10 male, 12 females; mean age: 50±3 years), either undergoing neurosurgical procedures (n=17) or from autopsy within 24 h of death (n=5). During the surgical procedure the dura mater together with a small piece of the meningeal artery was collected in ice-cold (0-4°C) saline and was immediately transported to the laboratory. Subsequently, the artery was placed in a cold oxygenated Krebs bicarbonate solution (composition mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4), and the surrounding tissue was removed. The meningeal arteries were used on the same day or stored overnight and used the following day for the functional experiments. The ethical committee of Erasmus MC approved this study.

6.2.2 Functional experiments

Arteries were cut into ring segments of 1-2 mm length with an internal diameter of 350-800 µm. The segments were suspended in Mulvany myographs on two parallel titanium wires and were continuously bubbled with 95% O_2 and 5% CO_2. The cyclo-oxygenase inhibitor indomethacin (0.1 µM) was added to inhibit spontaneous contractions (27). The distance between the wires was normalized to $0.9 \times I_{100}$ (I_{100} is the distance between the pins when the transmural pressure equalize 100 mm Hg) to achieve optimal conditions for active force development (28). After an initial equilibration period of 30 min, two successive challenges to KCl (30 mM) were performed to check the reproducibility of the responses. Endothelial integrity was assessed by measurement of the relaxation to substance P (10 nM) after precontraction with U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$) (10-100 nM) (17, 27, 29, 30). After 30 min, 100 mM KCl was added to determine the reference contractile response in each segment. Then, a stable contraction plateau of around 60% of the maximal contraction was obtained with KCl (25-30 mM) and the vessel segments were incubated with either vehicle, BIBN4096BS (0.1 nM-1 µM) or h-α$CGRP_{8-37}$ (0.1-10 µM) for 30 min. Cumulative concentration response curves were constructed to the CGRP re-

ceptor agonists, h-αCGRP or [Cys(Et)^{2,7}]-h-αCGRP (both: 0.1 nM-3 μM). Only one concentration response curve was constructed in each artery segment.

6.2.3 Isolation of total RNA from meningeal arteries and reverse transcriptase-polymerase chain reaction (RT-PCR) studies

The aim of the these experiment was to demonstrate the various components of the calcitonin receptor family, namely: calcitonin receptor like receptor (CLR); receptor activity modifying protein (RAMP) 1, 2 and 3; receptor component protein (RCP); and the orphan receptor originally cloned from canine thyroid cDNA (RDC-1) (Table 6.2). Meningeal arteries were isolated from the dura mater, snap frozen in liquid nitrogen and stored at 80˚C until use. Total RNA was isolated using TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) with subsequent chloroform-isopropanol extraction according to the manufacturer's instructions. Total RNA was denatured at 70°C and the first strand of cDNA was synthesised in a reaction volume of 20 μl (pH 8.3), by adding the following reagents: reverse transcription buffer (25 mM Tris HCl; 50 mM KCl; 5 mM $MgCl_2$, 2 mM dithiothreitol), 1 mM deoxy nucleotide triphosphate (dNTPs), ribonuclease inhibitor (1 U/μl), random hexamer (150 ng/μg total RNA) and, finally, moloney-murine leukaemia virus-Reverse Transcriptase (MMLV-RT) (Pro-omega, Benelux b.v., Leiden, The Netherlands). A control reaction with all of the above ingredients, except MMLVRT, was prepared. The reactions were carried out for 60 min at 42°C, extended for another 10 min at 75°C and then cooled at 4°C; the cDNA thus synthesised was stored at -20°C until used as a PCR template. The quality of cDNA was verified by PCR amplification of beta-actin (β-actin) using specific oligonucleotide primers (see later in Table 6.2). For PCR amplification, a 20 μl reaction mixture (pH 8.3), containing the following components was prepared: 1.25 mM of each dNTP, 2.5 mM $MgCl_2$, PCR buffer (1xPCR buffer: 10 mM Tris-HCl50 mM KCl), Ampli Taq GoldTM enzyme (0.5 U), 0.5 μM of each of the forward and reverse primers and 2 μl of the cDNA template. After a brief centrifugation, the enzyme was first activated for 5 min at 94°C in a PCR thermocycler (model PTC-100TM, MJ Research Inc., Watertown, MA, U.S.A.). PCR was carried out as follows: 45 sec at 94°C, 45 sec at 58°C and 45 sec at 72°C with a total of 40 cycles. Finally, the reaction was extended for an additional 10 min. An aliquot of PCR reaction product was analysed on 2% agarose gel, visualised under UV light and digitally photographed.

6.2.4 Compounds

The compounds used in the present study (obtained from the sources indicated) were: h-αCGRP, [Cys (Et)^{2,7}]h-αCGRP and h-αCGRP$_{8-37}$ (NeoMPS S.A., Strasbourg, France); BIBN4096BS (1-piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl] carbonyl] pentyl] amino]-1-[(3,5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-,[R-(R*,S*)]-) (gift: Dr. Henri Doods, Boehringer Ingelheim Pharma, Biberach/Riss, Germany); U46619 and substance P (Sigma Chemicals Co., Steinheim, Germany); and KCl (Merck, Darmstad, Germany). All compounds were dissolved in distilled water, except for BIBN4096BS, which was dissolved in 4% 1 N HCl to obtain a 0.01 M stock solution and was further diluted with distilled water. All peptides and antagonists were stored in aliquots at -80°C**.**

6.2.5 Data presentation and statistical analysis

The relaxant responses to the agonists are expressed as percentage relaxation of the tone produced by 25-30 mM KCl. All values are expressed as mean±s.e.m. and n represents the number of vessel segments, each segment obtained from a different donor. The concentration response curves for all agonists were analyzed using nonlinear regression analysis and the potency of each agonist is expressed as the corresponding pEC_{50} value using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The blocking potency of the antagonists was estimated by calculating the corresponding EC_{50} concentration ratios and plotting a Schild-plot using linear regression to get the slope value and pA_2 (the negative log of

the molar concentration of antagonist required to shift the concentration response curve of an agonist by two fold, with the slope not constrained to unity). Since it was not feasible to use agonist concentrations higher than 3 μM, concentration response curves in the presence of higher concentrations of antagonist did not always reach a plateau. In case the maximal response that was observed was ≤ 25% of the E_{max} in the absence of antagonist, the concentration response curve was not used for Schild regression analysis. In other cases, the concentration response curves were extrapolated, considering the maximal response in the absence of antagonist as E_{max}. Only concentration ratios >2 were included. Statistical significance was determined by unpaired Student's *t*-test, with differences considered significant at $P<0.05$.

6.3 RESULTS

6.3.1 Functional experiments

The contraction induced by 100 mM KCl in human middle meningeal arteries was 7.59±0.86 mN (n=19). Substance P (10 nM) elicited endothelium-dependent relaxant response that was 47±6% of the precontraction to U46619 (10-100 nM; n=19). h-αCGRP concentration-dependently relaxed arterial segments with no significant difference in relaxation between the vessel segments obtained perioperatively or *post mortem*, as illustrated by the similar pEC_{50} (8.50±0.19 and 8.56±0.28; n=15 and 4, respectively) and E_{max} (82±5% and 89±5%; n=15 and 4, respectively) values

Therefore, in the rest of the study, data from vessel segments obtained from both sources were pooled (pEC_{50}: 8.51±0.16, E_{max}: 83±4%; n=19). After 30 min incubation, BIBN4096BS did not significantly

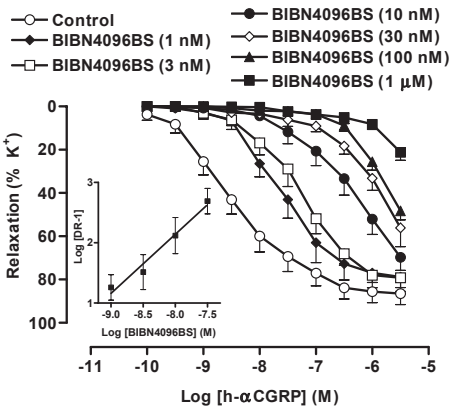

Figure 6.1. Effect of increasing concentrations of BIBN4096BS (1 nM-1 μM) on the relaxant responses to h-αCGRP in the human middle meningeal artery (n=5-19). The inset represents the average Schild plot of the concentration responses curves.

Table 6.1. Pharmacological parameters derived after nonlinear regression and Schild plot analysis of the interaction of agonists and antagonists in the human middle meningeal artery.

Agonist	E_{max}	pEC_{50}	Antagonist	pA_2	Slope
h-αCGRP	83±4 (19)	8.51±0.16 (19)	BIBN4096BS	10.59±0.54 (7)	1.10±0.31 (7)
			$h\text{-}\alpha CGRP_{8\text{-}37}$	6.46±0.16 (5)	1.04±0.13 (5)
$[Cys(Et)^{2,7}]$h-αCGRP	87±4 (9)	7.48±0.24* (9)	BIBN4096BS	9.90±0.18 (4)	1.47±0.33 (4)

E_{max} was expressed as % of the response to precontraction to KCl. All data are means±s.e.m. (n).
*, Significantly different from the corresponding value of h-αCGRP; Note that the above slopes did not significantly differ from unity.

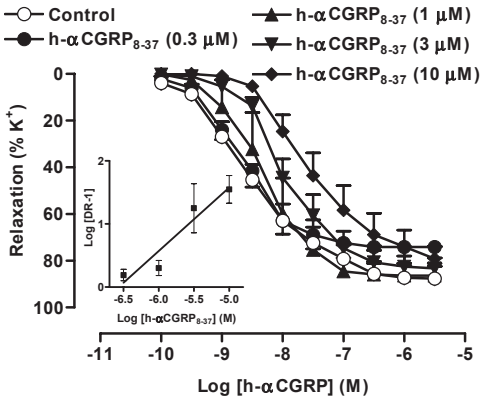

Figure 6.2. Effect of increasing concentrations of h-αCGRP$_{8-37}$ (300 nM-10 μM) on the relaxant responses to h-αCGRP in the human middle meningeal artery (n=5-19). The inset represents the average Schild plot of the concentration responses curves.

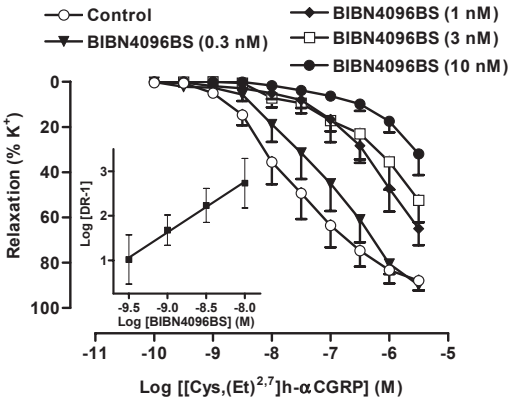

Figure 6.3. Effect of increasing concentrations of BIBN4096BS (0.3 nM-10 nM) on the relaxant responses to [Cys (Et)^{2,7}]-h-αCGRP in the human middle meningeal artery (n=4-9). The inset represents the average Schild plot of the concentration responses curves.

change the contractions induced by KCl in human middle meningeal artery segments, as compared to the control segments incubated with vehicle (data not shown). However, as expected, BIBN4096BS concentration-dependently antagonised the responses to h-αCGRP (Figure 6.1); Schild plot analysis revealed a pA_2 of 10.59±0.54, with a slope not different from unity (1.10±0.31; n=7). Likewise, h-αCGRP$_{8-37}$ also concentration-dependently blocked the relaxations to h-αCGRP (Figure 6.2). Schild plot analysis demonstrated a pA_2 value of 6.46±0.16 and a slope not significantly different from unity (1.04±0.13; n=5) (Table 6.1).

The linear agonist for $CGRP_2$ receptors, [Cys(Et)^{2,7}]h-αCGRP, was significantly less potent than h-αCGRP in relaxing human middle meningeal artery segments (pEC_{50}: 7.48±0.24; n=9), while its efficacy was similar to that of h-αCGRP (E_{max}: 87±4%; Figure 6.3). BIBN4096BS concentration-dependently blocked [Cys(Et)^{2,7}]h-αCGRP-mediated relaxations, with a pA_2 (9.90±0.18; n=4) not different from that of BIBN4096BS against h-αCGRP (P=0.38) and a slope not different from unity (Table 6.1).

Table 6.2. Detection of the molecular components relevant for CGRP receptors as well as β-actin

Components	NCBI Genebank Accession No.	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplified sequence	Size (bp)
h-CLR	AY389506	TCAAGAGCCTAAGTTGCCAAA	AATCAGCACAAATTCAATGCC	497-1057	560
h-RAMP-1	NM005855	CTGCCAGGAGGCTAACTACG	GACCACGATGAAGGGGTAGA	78-376	298
h-RAMP-2	BC040107	GGGGGACGGTGAAGAACTAT	GTTGGCAAAGTGGATCTGGT	164-391	227
h-RAMP-3	BC05385	AAGGTCTTCGCAGACATGAT	GCAGTTGGAGAAGAACTGCC	123-312	189
h-RCP	U51134	AACTGATCTGAAAGAGCAGCG	TCTTCTTCTGCTCAGCCTCTG	121-465	344
h-RDC-1	AF030297	ACGTGGTGGTCTTCCTTGTC	AAGGCCTTCATCAGCTCGTA	770-990	220
β-actin	NM_001101	TGACGGGGTCACCCACACTGTGCCCATCTA	ACTCGTCATACTCCTGCTTGCTGATCCA	468-1094	625

Each set of forward and reverse primers was designed from the nucleotide sequences reported in the NCBI Genebank. CLR, Calcitonin receptor like receptor; RAMP, receptor activity modifying protein; RCP, receptor component protein; RDC 1, the orphan receptor (originally cloned from canine thyroid cDNA); β actin (beta actin).

6.3.2 RT-PCR studies

Agarose gel electrophoresis of the PCR products from the human middle meningeal artery is shown in Figure 6.4. PCR products, corresponding in size to CLR, RAMP-1 and RCP, the essential components required for a functional $CGRP_1$ receptor, were demonstrated in this artery (Table 6.2). In addition, RAMP-2, RAMP-3, the orphan receptor RDC-1 (originally cloned from canine thyroid cDNA) and β-actin were all expressed in human middle meningeal arteries (Table 6.2). RAMP-2 and RAMP-3, when combined with the CLR, form functional adrenomedullin receptors. Furthermore, RDC has been proposed as a second molecular representative of the $CGRP_1$ receptor, beside CLR. As expected, no band was detected in the negative controls (see lane 9 in Figure 6.4).

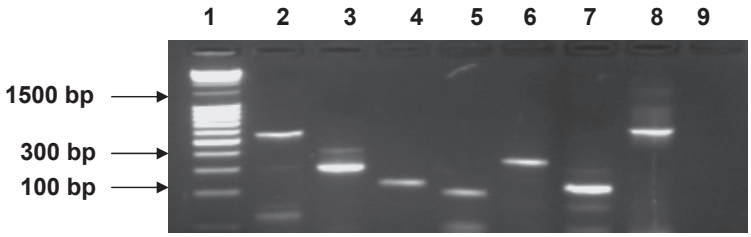

Figure 6.4. Agarose gel electrophoresis of PCR amplified products derived from cDNA obtained from segments of the human middle meningeal artery. The different lanes marked on top of the panels denote: 1 kb DNA ladder (1), CLR; 560 bp (2), RAMP-1; 298 bp (3), RAMP-2; 227 bp (4), RAMP-3; 189 bp (5), RCP; 344 bp (6), RDC-1; 220 bp (7), β-actin; 625 bp (8) and negative control (9). The size of 3 marker bands is indicated in the left margin

6.4 DISCUSSION

Since meningeal vasodilatation induced by trigeminal release of CGRP is likely to play a prominent role in the pathophysiology of migraine (1, 2, 7), our study set out to characterize the CGRP receptors mediating vasodilatation in the human isolated middle meningeal artery. Apart from the implications discussed below, our results clearly demonstrate that BIBN4096BS is a potent competitive antagonist of h-αCGRP-induced relaxations in the human middle meningeal artery. Taking into account the high potency of BIBN4096BS, it is tempting to suggest the involvement of $CGRP_1$ receptors mediating meningeal vasodilatation. The operational approach of the present study strengthens this contention as: (i) the linear agonist $[Cys(Et)^{2,7}]$h-αCGRP, which displays a higher potency at $CGRP_2$ than at $CGRP_1$ receptors (9), was found to be 10 times less potent than h-αCGRP; and (ii) the potency of BIBN4096BS against $[Cys(Et)^{2,7}]$h-αCGRP was similar to that against h-αCGRP. In keeping with this view, the corresponding Schild plot slope was not significantly different from unity in any of our experiments, a finding that supports the involvement of a homogeneous population of CGRP (most likely $CGRP_1$) receptors.

It is to be noted that the potency with which h-αCGRP produced meningeal artery relaxation in the present study is similar to that previously reported by Jansen-Olesen et al. (11); we additionally observed that pre-incubation with BIBN4096BS did not change the basal tone of KCl-elicited precontraction in meningeal arteries in comparison to the control segment. To the best of our knowledge, our study is the first to show unequivocally the competitive nature of BIBN4096BS antagonism on h-αCGRP-induced relaxations in human middle meningeal artery; however, other studies performed in subcutaneous arteries have reportedly demonstrated a non-competitive antagonism by BIBN4096BS (20). Furthermore, Moreno et al. (19) have demonstrated a biphasic interaction using BIBN4096BS for reversal of h-αCGRP-mediated dilation, whereas our results indicate only one receptor interaction as evidenced by a Schild plot slope not being significantly different from unity. Interestingly, in our study, the pA_2 value of 10.59±0.54 turns out to be the average of the previously reported two pIC_{50} values by Moreno et al. (19) for BIBN4096BS, i.e. 12.8 and 8.1 for the high (0.001-100 pM) and low (1-1000 nM) receptor interaction, respectively.

In our hands, BIBN4096BS produced a significant rightward shift of the concentration response curve to h-αCGRP at concentrations of 1 nM and above. Hence, it is clear that the concentrations that we used overlap with the highest concentration used by Moreno et al. (19) and, thus, with the relatively linear part of the concentration response curve. In line with this concentration range, we also found a slope of unity but, nevertheless, for this part of the curve Moreno et al. (19) report a pIC_{50} of 8.1 for BIBN4096BS. This discrepancy might be due to the different approaches adopted to estimate the antagonist potency of BIBN4096BS. For example, in our experiments BIBN4096BS was pre-incubated for 30 min; in contrast, in the experiments reported by Moreno et al. (19), BIBN4096BS was used to reverse h-αCGRP-evoked dilation (~75-100% of induced tone). Further, the pA_2 value of BIBN4096BS that we report in the human middle meningeal artery is very similar to the pK_b value observed in the human cerebral (10.1), temporal (10.1) and occipital (9.9) arteries (16, 26). In addition these authors also report a similar competitive behaviour of this antagonist. These findings indicate that the pharmacological profile of the CGRP receptors mediating relaxation of these arteries is similar.

It is worth noting that the conventional CGRP receptor antagonist, h-αCGRP$_{8-37}$, also concentration-dependently antagonised h-αCGRP-induced relaxations in a competitive manner. This antagonist was found to be more than 10,000-fold weaker than BIBN4096BS in human middle meningeal arteries. Despite this remarkable difference in antagonist potency, the slope derived from the Schild plot was not significantly different from one, a finding that further underlines a homogeneous CGRP receptor population in this isolated preparation. In similar experiments, Jansen-Olesen et al. (11) calculated a Schild plot slope of -0.64; however, it is not explicitly stated whether this slope differs significantly from one, and the authors used lower concentrations of h-αCGRP$_{8-37}$ (leading to concentration ratios <2 in Schild plot analysis) for calculation of the corresponding Schild plot slope.

Consistent with the above findings, the putative $CGRP_2$ receptor agonist [Cys(Et)^{2,7}]h-αCGRP (9) was more than 10 times weaker than its parent analogue h-αCGRP to elicit meningeal relaxation; hence, this finding does not seem to support the role of $CGRP_2$ receptors in this artery. In fact, [Cys(Et)^{2,7}]h-αCGRP is reported to be more potent than its other linear analogue, [Cys(ACM)^{2,7}]h-αCGRP, in various pharmacological preparations (9). It is important to note that the agonist potency of [Cys(Et)^{2,7}]h-αCGRP found in our study is similar to that previously reported for [Cys(ACM)^{2,7}]h-αCGRP in human middle meningeal arteries (11). Considering that BIBN4096BS equipotently antagonised both [Cys(Et)^{2,7}]h-αCGRP- and h-αCGRP-induced relaxations, and that the corresponding Schild plot slopes were not significantly different from unity, these findings support our contention that a homogeneous CGRP (most likely $CGRP_1$) receptor population mediates meningeal relaxation. Admittedly, the lower potency of h-αCGRP$_{8-37}$ observed in our study is in the border zone of classification of $CGRP_1$ and $CGRP_2$ receptors (32). Notwithstanding, the results obtained from our pharmacological approach, taken together, support the predominant involvement of $CGRP_1$ receptors. This conclusion is reinforced by our results obtained from RT-PCR studies in human middle meningeal arteries demonstrating the presence of all essential components required for a functional $CGRP_1$ receptor, including RCP. Although we also demonstrated the presence of RAMP-2 and RAMP-3, which constitute the adrenomedullin receptors in combination with CLR, the affinity of BIBN4096BS for adrenomedullin receptors is too low (IC_{50}: 10.3 μM, 12) for a pharmacological relevant interaction. Hence, the pharmacological parameters derived from these experiments would be minimally affected by these receptors. In contrast, in human coronary arteries we observed [Cys(Et)^{2,7}]h-αCGRP to be equipotent to h-αCGRP (17); this finding, coupled with a Schild plot slope significantly different from unity, led us to conclude that the CGRP receptors mediating human coronary relaxation represent a heterogeneous population (17).

Clearly, $CGRP_1$ receptors represent an important target for the development of antimigraine drugs. In this context, BIBN4096BS is effective in the acute treatment of migraine when given intravenously (15). Consequently, efforts are now being focussed on developing orally active $CGRP_1$ receptor antagonists. This seems to be the ideal approach in view of the predominance of $CGRP_1$ receptors in the human middle meningeal artery.

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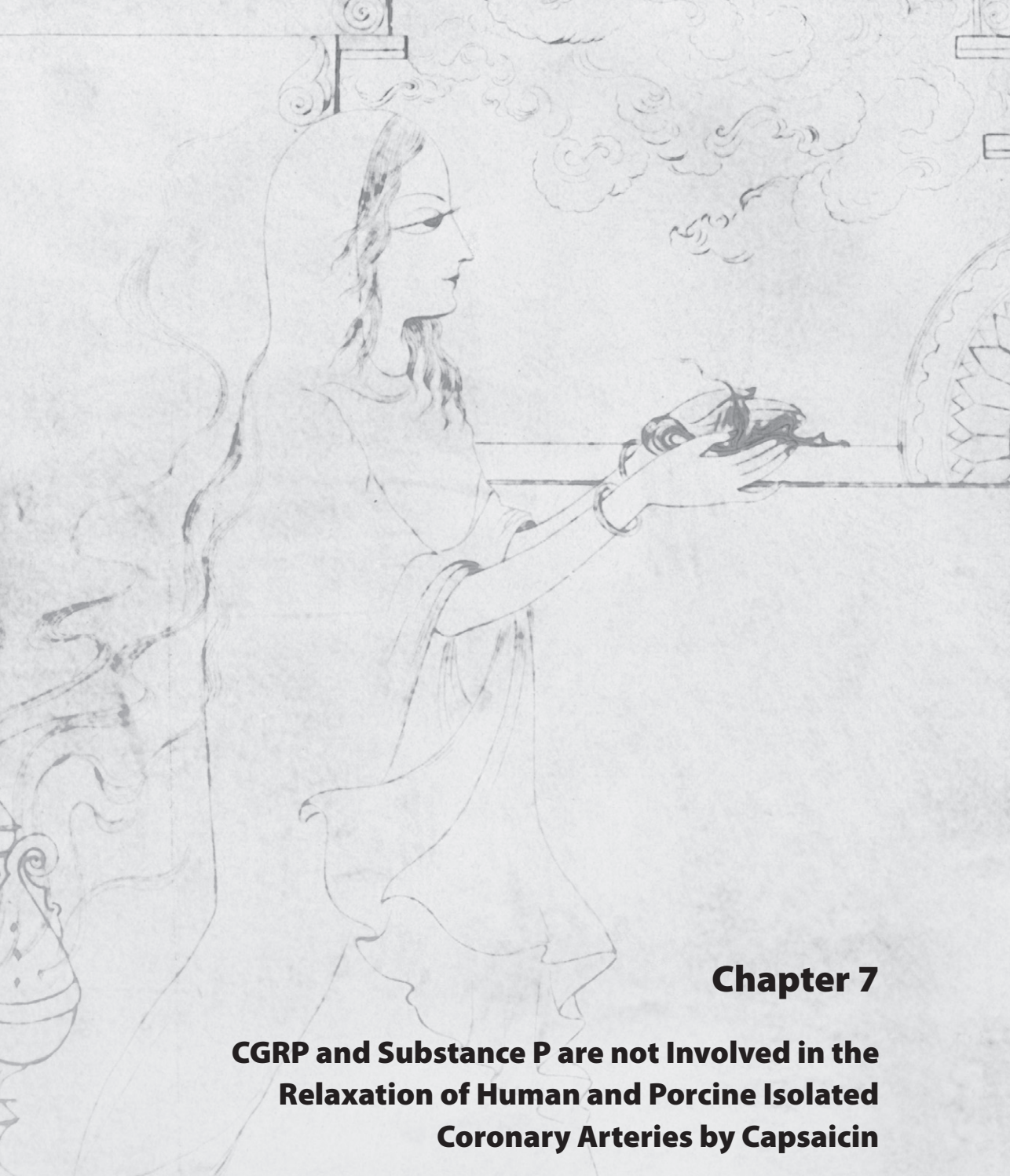
Now I believe I can hear the philosophers protesting that it can only be misery to live in folly, illusion, deception and ignorance, but it isn't – it human. (Desiderius Erasmus)



Chapter 7

CGRP and Substance P are not Involved in the Relaxation of Human and Porcine Isolated Coronary Arteries by Capsaicin

Based on: S. Gupta, L.R. Jiménez-Mena, C.M. Villalón, I.M. Garrelds, J.P. van Kats, C.J.J. Avezaat, P.R. Saxena & A. MaassenVanDenBrink, Submitted.

ABSTRACT

Capsaicin, a pungent constituent from red chilly peppers, activates sensory nerve fibres via the vanilloid receptor TRPV1 to release neuropeptides like calcitonin gene-related peptide (CGRP) and substance P. Capsaicin-sensitive nerves are widely distributed in human and porcine vasculature. In this study, we examined the mechanism of capsaicin-induced relaxations, with special emphasis on the role of CGRP, using various pharmacological tools. Segments of human and porcine proximal and distal coronary arteries, as well as cranial arteries, were mounted in organ baths. Concentration response curves to capsaicin were constructed in the absence or presence of the CGRP receptor antagonist olcegepant (BIBN4096BS, 1 μM), the neurokinin NK_1 receptor antagonist L-733060 (0.5 μM), the voltage-sensitive calcium channel blocker ruthenium red (100 μM), the TRPV1 receptor antagonist capsazepine (1 μM), the nitric oxide synthetase inhibitor L-NAME (100 μM), the gap junction blocker 18α-glycyrrhetinic acid (10 μM), as well as the RhoA kinase inhibitor Y-27632 (1 μM). Further, we also used the K^+ channel inhibitors 4-aminopyridine (1 mM), charybdotoxin (0.5 μM) + apamin (0.1 μM) and iberiotoxin (0.5 μM) + apamin (0.1 μM). The role of the endothelium was assessed by endothelial denudation in distal coronary artery segments. In these segments, we also measured levels of cAMP after exposure to capsaicin, and in human distal coronary artery segment, we in addition assessed the amount of CGRP released in the organ bath fluid after exposure to capsaicin. Capsaicin evoked concentration-dependent relaxant responses in precontracted arteries, but none of the above-mentioned inhibitors did affect these relaxations. There was no increase in the cAMP levels after exposure to capsaicin, unlike after (exogenously administered) αCGRP. Interestingly, there were significant increases in CGRP levels after exposure to vehicle (ethanol) as well as capsaicin, although this did not induce relaxant responses. In conclusion, the capsaicin-induced relaxations of the human and porcine coronary arteries are not mediated by CGRP, substance P, NO, vanilloid receptors, K^+ channels or cAMP-mediated mechanisms in human and porcine distal coronary arteries. Therefore, these relaxant responses to capsaicin are likely to be attributed to a non-specific, CGRP-independent mechanism.

7.1 INTRODUCTION

Capsaicin, a pungent constituent of red pepper, is known to activate sensory C-fibres via transient receptor potential vanilloid receptors type 1 (TRPV1) (1, 2), which are nonselective cation channels. Activation of these channels increases influx of mono- and divalent cations, which leads to an increase in the intracellular Ca^{2+} concentrations. Consequently, an array of neuropeptides like calcitonin gene-related peptide (CGRP), substance P and neurokinin A is released (3, 4). These neuropeptides play a role in the regulation of normal vascular smooth muscle tone, and are also implicated in several pathological conditions like ischemic preconditioning (5), preeclampsia (6) and migraine (7). In the pathophysiology of migraine, vasodilatation of cranial blood vessels, especially extracerebral intracranial blood vessels which are richly innervated by nerves containing number of peptides such as CGRP, seems pivotal (8). Vasodilatation of these intracranial arteries leads to activation of nociceptors, which stimulate the pain centers in the brain (9). Involvement of CGRP in the pathophysiology of migraine is further strengthened by the observation that olcegepant (BIBN4096BS, 10), a CGRP receptor antagonist, is effective in the treatment of acute migraine attacks (11). Capsaicin has been widely used in various *in vivo* models of migraine to induce cranial vasodilatation, which is attributed to endogenous release of neuropeptides, especially CGRP (12-14). The involvement of CGRP is further substantiated in all these *in vivo* setups by the fact that the capsaicin-induced vascular responses are all amenable to blockade with CGRP receptor antagonists. In addition to the *in vivo* models, also *in vitro* vascular models involving meningeal (15) and coronary (16, 17) arteries have been used in migraine research, but vasorelaxation in these models was induced by exogenously administered CGRP. Several major vascular beds, including the meningeal and coronary (18), are richly innervated with capsaicin-sensitive sensory fibres containing CGRP (19).

Interestingly, very few studies have investigated capsaicin-induced relaxations in human isolated blood vessels (20, 21). Based on CGRP-like immunoreactivity, it was claimed that capsaicin-induced relaxations are mediated by CGRP (22), but no CGRP receptor antagonists were used to unequivocally demonstrate the role of CGRP in these responses. Therefore, we were interested to study capsaicin-mediated relaxations in human and porcine isolated arteries as a model to study endogenous release of CGRP in view of its relevance in the pathophysiology of migraine. Remarkably, some recent studies indicate that capsaicin-induced relaxations are mediated by non-CGRP mediated mechanisms in guinea-pig ileum, rabbit coronary arteries and equine tracheal smooth muscle preparations (23-25), which is contrary to the belief that capsaicin-induced relaxations are mediated principally by CGRP. In these studies, relaxant responses to capsaicin were mainly attributed to different Ca^{2+}-activated K^+ channels. Therefore, in the present study we tried to characterise capsaicin-induced relaxant response in human and porcine vessels, with special emphasis on the role of CGRP, using different pharmacological tools.

7.2 MATERIALS AND METHODS

7.2.1 Tissue preparation

The study protocol was approved by the ethical committee of Erasmus MC. Human coronary artery was obtained from 'heart-beating' organ donors (13 male, 19 female; 13-65 years) who died due to non-cardiac disorders. The hearts were provided by the Heart Valve Bank, Rotterdam, The Netherlands, after donor mediation by Bio Implant Services Foundation/ Eurotransplant Foundation (Leiden, The Netherlands). The meningeal arteries were obtained from patients (4 male, 6 female; 41-75 years) undergoing craniotomy at the neurosurgical unit (n=6) or from the department of pathology (*post mortem*, n=4) within 24 h of death, at Erasmus MC, Rotterdam. During the surgical procedure or autopsy, the dura mater together with a small piece of the meningeal artery was cut and placed in a plastic container filled with ice-cold (0-4°C), physiological salt solution. The artery segment was immediately transported to the laboratory and placed in cold oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4. Excess tissue was removed. The meningeal artery (internal diameter of 350-800 µm) was used the same day or stored overnight in cold oxygenated modified Krebs solution and used the following day. Porcine hearts and heads (pigs of either sex; 6-12 months of age) were collected from a local slaughterhouse. Porcine basilar (internal diameter of 150-200 µm) as well as meningeal (internal diameter of 100-250 µm) arteries were dissected out from the skull and were placed in a cold oxygenated Krebs solution as described earlier for human meningeal arteries. In the laboratory both human and porcine proximal (internal diameter: 2-3.5 mm) and distal (internal diameter: 200-600 µm) coronary arteries were dissected out of the right ventricle, distal coronary arteries were dissected with the aid of a microscope and stored in bicarbonate solution of following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4.

7.2.2 Functional studies

Both human and porcine proximal coronary artery segments (3-4 mm length) were suspended with the help of stainless-steel hooks in 15-ml organ baths with a pretension of 15 mN (optimal tension shown in earlier experiments). Distal coronary and cranial artery segments were cut into ring segments of 1-2 mm length and were mounted in Mulvany myographs between two parallel titanium wires with a tension normalised to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development (26). Vessels were allowed to equilibrate for 30 min in Krebs solution at 37°C, a similar equilibration period was repeated after each physical or pharmacological challenge. In case of human arteries, the cyclo-oxygenase inhibitor indomethacin (0.1 µM) was added to

prevent prostaglandin synthesis during the whole experimental protocol. Two successive challenges to KCl (30 mM) were performed to test the reproducibility of responses. Endothelial integrity was assessed by observing relaxation to substance P (10 nM) after precontraction with U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$) (10 nM-1 μM), followed by washout of the agonists. KCl (18-30 mM, except where higher concentrations are indicated) or U46619 (10 nM – 1 μM), in case of the K^+ channel blockers, was used to obtain a stable contraction plateau of around 60-75% of the maximal contraction reached with KCl (100 mM); subsequently capsaicin was added in a cumulative manner in log steps. Concentration response curves were constructed in the absence or presence of the CGRP receptor antagonists olcegepant (1 μM) and $CGRP_{8-37}$ (10 μM), the neurokinin NK_1 receptor antagonist L-733060 (0.5 μM), the voltage-sensitive calcium channel blocker ruthenium red (100 μM, 45-90 mM KCl was required to get a similar precontraction as in the corresponding control segments), the nitric oxide synthetase inhibitor L-NAME (100 μM), as well as the vanilloid receptor antagonist capsazepine (5 μM).

We also investigated role of different K^+ channels by using various inhibitors, 4-aminopyridine (1 mM), a voltage-dependent K^+ channel (K_v) blocker and charybdotoxin (0.5 μM), a blocker of Ca^{2+}-dependent K^+ channels for large conductance (BK_{Ca}) and intermediate conductance (IK_{Ca}), in combination with apamin (0.1 μM), a blocker of small-conductance Ca^{2+}-dependent K^+ channels (SK_{Ca}). The effect of the RhoA kinase inhibitor, Y-27632 (1 μM) was investigated both in the absence or presence of 4-aminopyridine (1 mM). We also investigated the effect of the gap junction inhibitor 18-α-glycyrrhetinic acid (10 μM) on capsaicin-induced relaxations. Where indicated, the endothelium was removed with a human hair, and removal was confirmed by observing a relaxation of less than 10% of precontraction of U46619 after addition of substance P.

Further, in porcine distal coronary artery we also studied the effect of repeated administration of capsaicin (50 μM, four times) to verify the reproducibility of the responses in view of possible depletion of endogenous peptide pools or other agents.

7.2.3 Measurements of cAMP

Human proximal and distal, as well as porcine distal coronary artery segments were incubated in a medium containing isobutylmethylxanthine (IBMX, 0.5 mM) for 30 min in the absence or presence of olcegepant (1 μM). The arterial segments were exposed to KCl (30 mM), challenged with the ethanol (vehicle of capsaicin, final concentration in the baths 0.56%), capsaicin (10 μM) or h-αCGRP (100 nM, serving as a positive control) for 5 min and then snap frozen. The samples were stored at -80°C until cAMP assay. To determine cAMP, tissues were homogenised in 0.5 ml 0.1 M HCl using a stainless steel ultraturrax (Polytron, Staufen, Germany). Homogenates were centrifuged at 3300 *g*, and cAMP was measured in 300 μl supernatant using the ELISA kit according to the instructions of the manufacturer (R&D Systems Europe Ltd., Abingdom, U.K.).

7.2.4 Measurements of CGRP in organ bath fluid

Human and porcine distal coronary artery segments were subjected to a similar protocol as during the functional studies, while bath fluids were collected after construction of the concentration response curve. The bath fluids were collected from the segments treated with vehicle and capsaicin (100 μM) and Krebs solution was used as a control. Bath fluids were stored in tubes containing aprotinin (0.6 TIU/ml) and stored at -80°C. A competitive radioimmunoassay (Peninsula Lab INC., San Carlos, CA, U.S.A.) was used according to the instructions of the manufacturer to measure the CGRP concentrations in the bath fluid.

7.2.5 Data presentation and statistical analysis

The relaxant responses elicited by the agonists are expressed as percentage relaxation of the tone induced by 30 mM KCl or U46619 (in case of the K^+ channel blockers). All data are presented as means±S.

E.M and *n* represents the number of blood vessel segments, all obtained from different subjects. The concentration response curves for all agonists were analysed using nonlinear regression analysis and the potency of agonists was expressed as pEC_{50} using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The blocking potency of the antagonists was estimated by calculating EC_{50} ratios and apparent pK_b values were calculated for the antagonists at each given concentration, constraining the slope of the Schild plot to unity. The effect of all potential inhibitors of the relaxations to capsaicin was investigated in a paired parallel setup, i.e., relaxations in segments with inhibitors were always compared to relaxations obtained in control segments from the same subject. Statistical significance was determined by ANOVA for paired measurements followed by Dunnett's post hoc test with differences considered significant at $P \leq 0.05$, except for the measurement of CGRP in the organ bath fluids, where we could not exclude a non-Gaussian distribution due to the large degree of variability in the data. Therefore, the levels of CGRP in bath fluids were analysed by the non-parametric Kruskal Wallis test followed by Dunn's post hoc multiple comparison test. Similar as with all the other statistical tests, significance was assumed at $P \leq 0.05$.

7.2.6 Compounds

Human αCGRP and $\alpha CGRP_{8-37}$ were obtained from Polypeptide, (Wolfenbüttel Germany), olcegepant (BIBN4096BS, 1-piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl] pentyl] amino]-1-[(3,5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-, [R-(R*,S*)]-) was a gift from Boehringer Ingelheim Pharma (Biberach/Riss, Germany); 4-Aminopyridine was purchased from ICN Biomedicals (Aurora, OH, U.S.A); L-733060 was purchased from Tocris (Bristol UK); apamin, capsaicin, capsazepine, 3-isobutyl-1-methyl-xanthine, L-NAME (N^{ω}-nitro-L-arginine methyl ester HCl), charybdotoxin, ruthenium red, substance P, U46619 and Y-27632 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and KCl was obtained from Merck (Darmstad, Germany). Capsaicin was dissolved in 70% ethanol, capsazepine in methanol; olcegepant was dissolved in a small amount of 1 N HCl and then diluted with distilled water. The other compounds were dissolved in distilled water, and all compounds were stored in aliquots at -80°C.

7.3 RESULTS

7.3.1 Funcational studies in human arteries

Substance P relaxed artery segments precontracted with U46619 (10 nM-1 μM); responses were equi-efficacious in distal coronary (80±5% of contraction to U46619, n=28) and meningeal (75±8%, n=8) artery, and significantly less in proximal coronary artery (27±15%, n=4).

Both in the meningeal and distal coronary arteries, capsaicin induced concentration-dependent relaxations. In human proximal coronary artery, relaxant responses were only observed at the highest concentration of 100 μM and the maximum relaxant response (34±14% of contraction to 30 mM KCl) was significantly less than observed in the distal arteries (94±1% of contraction to 18-30 mM KCl). In human meningeal arteries there was no difference in capsaicin-induced responses between arteries obtained perioperatively or *post mortem*, therefore these data were pooled for further analysis. Capsaicin was equipotent and equiefficacious in human distal coronary (E_{max}: 94±1%, pEC_{50}: 5.27±0.12) and human meningeal artery (E_{max}: 91±5% of contraction to 18-30 mM KCl, pEC_{50}: 5.04±0.09). In human distal coronary artery segments, the lower concentrations of capsaicin (0.1 nM-1 μM) in some cases induced contractions, but in all cases we uniformly only measured the relaxant responses. The relaxations to capsaicin in proximal and distal coronary as well as meningeal segments were insensitive to blockade by the CGRP antagonist olcegepant (1 μM) (Figure 7.1, Table 7.1).

Table 7.1 Effect of various antagonists/interventions on capsaicin-induced relaxations in human isolated artery segments.

Human distal coronary artery		
Antagonist or other intervention (*n*)	**E_{max} (%)**	**pEC_{50}**
- (Control) (32)	94±1	5.27±0.12
Olcegepant (1 μM) (10)	89±4	4.74±0.12
$CGRP_{8-37}$ (1 μM) (5)	96±3	4.79±0.06
Capsazepine (5 μM) (13)	91±3	5.10±0.13
Ruthenium red (0.1 mM) (9)[#]	92±3	5.01±0.13
L-733060 (5 μM) (7)	94±2	6.03±0.78
Denuded-endothelium (5)	90±6	5.34±0.48
L-NAME (0.1 mM) (7)	94±2	5.23±0.48
18-α-Glycyrrhetinic acid (10 μM) (3)	96±2	5.08±0.28
Olcegepant (1 μM)+L-733060 (5 μM) (3)	90±6	6.04±0.23
- (Control) (10)*	97±1	5.91±0.32
4-Aminopyridine (1 mM) (6)*	95±2	5.64±0.38
Charybdotoxin (0.5 μM) + apamin (0.1 μM) (8)*	97±1	5.80±0.37
Iberiotoxin (0.5 μM) + apamin (0.1 μM) (3)*	96±2	5.94±0.65
Y-276323 (1 μM) (3)*	99±1	5.39±0.21
Y-276323 (1 μM) + 4-Aminopyridine (1 mM) (3)*	100±0	5.12±0.18
Human proximal coronary artery		
- (Control) (4)	34±14	4.30±0.14
Olcegepant (1 μM) (4)	36±16	4.40±0.17
Human meningeal artery		
- (Control) (10)	91±5	5.04±0.09
Olcegepant (1 μM) (10)	96±1	5.03±0.07
Capsazepine (5 μM) (4)	81±9	4.90±0.31
Ruthenium red (0.1 mM) (3)	74±15	5.13±0.42
L-733060 (5 μM) (4)	79±12	4.80±0.31

E_{max}: The maximum relaxant response, expressed as percentage of the respective precontraction; pEC_{50}: (-logEC_{50}, where EC_{50} is the concentration of agonist required to produce half the maximal response). The arteries were precontracted with KCl (18-30 mM) except where [#] KCl (45-90 mM) or * U46619 (10 nM-1 μM) was used to precontract arteries.

As the relaxant responses to capsaicin were small in human proximal arteries and the availability of human meningeal arteries was very limited, further experiments were only carried out in human distal coronary artery. In this preparation, the CGRP receptor antagonist

$CGRP_{8-37}$ (10 μM), the TRPV1 receptor antagonist capsazepine (1 μM) and the NK_1 receptor antagonist L-733006 (0.5 μM) also did not attenuate capsaicin-induced relaxations (Table 7.1). Similarly, there was no significant difference in relaxant responses in endothelium-intact or endothelium-denuded segments (Table 7.1). Also in the absence or presence of the NO synthase inhibitor L-NAME (100 μM) or in the presence of the gap junction blocker 18-α-glycyrrhetinic acid, capsaicin caused equipotent relaxations compared to the respective control segments (Table 7.1). Various K^+ channel blockers, namely 4-aminopyridine (1 mM), charybdotoxin (0.5 μM) + apamin (0.1 μM) and iberiotoxin (0.1 μM) + apamin (0.1 μM) were also unable to block the relaxant responses to capsaicin. The RhoA kinase inhibitor Y-27632 (1 μM) alone or in combination with 4-aminopyridine was also unable to block the relaxant responses to capsaicin.

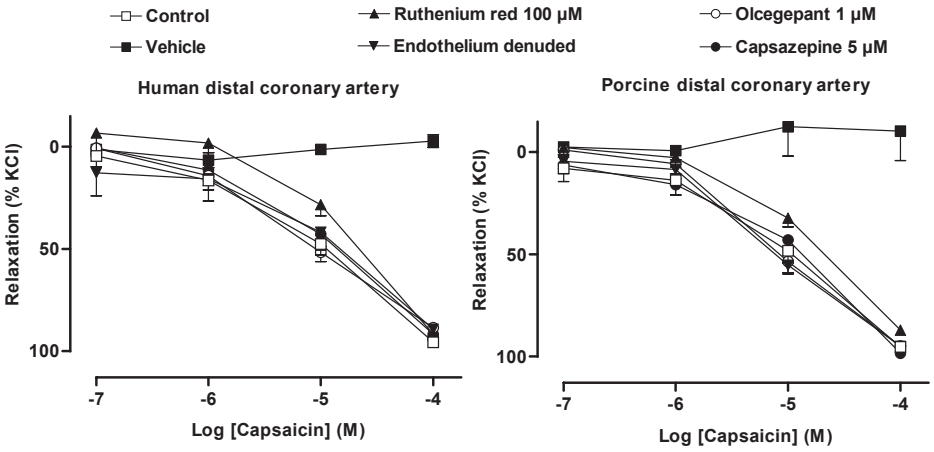


Figure 7.1. Effect of capsaicin or its vehicle in the absence or presence of various pharmacological agents in precontracted human and porcine distal coronary arteries.

Ruthenium red (100 µM), a nonselective blocker of Ca^{2+} transport through membrane channels, also did not significantly block these responses (Table 7.1). It is noteworthy that at higher concentrations, capsazepine and L-733006 significantly attenuated the responses to their respective preconstriction agents and in case of Y-27632 and ruthenium red 2 to 20-fold higher concentrations of precontracting agents were required. Additionally, the combinations of olcegepant (1 µM) + L-73360 (0.5 µM) or 4-aminopyridine (1 mM) + Y-27632 (1 µM) were also unable to block the responses to capsaicin (data not shown). The vehicle of capsaicin (0.56% ethanol) did not induce any significant relaxations. It should be noted that the potency of capsaicin was higher on a precontraction with U46619 (10 nM–1 µM, pEC_{50} of capsaicin: 5.91±0.32) than on a precontraction with KCl (18-30 mM, pEC_{50} of capsaicin: 5.27±0.12, P=0.029). However, irrespective of the precontracting agent used, the antagonists behaved in a similar fashion towards capsaicin. For example, the effects of 4-aminopyridine on capsaicin-induced relaxations were similar after precontraction with KCl (pEC_{50}: 5.01±0.05 and 5.12±0.41, *n*=7, in the absence and presence of 4-aminopyridine, respectively) and with U46619 (pEC_{50}: 6.58±0.47 and 5.64±0.38, *n*=6, in the absence and presence of 4-aminopyridine, respectively). In human meningeal artery, capsazepine, L-733006 and also ruthenium red did not block the responses to capsaicin (Table 7.1).

7.3.2 Funcational studies in porcine arteries

In porcine arteries, substance P induced relaxations in precontracted arteries to a varying degree in different vessels. The relaxations in distal coronary (64±6% of contraction to U46619, 10 nM-1 µM, n=26) basilar (44±3%, n=3) and meningeal (57±15%, n=3) artery were similar, while that in proximal coronary (8±8%, n=4) artery was significantly smaller. In porcine proximal and distal coronary arteries, capsaicin induced concentration-dependent relaxations. Unlike in human coronary arteries, the maximum response was not significantly different between porcine proximal and distal coronary arteries (Table 7.2).

In both the arteries olcegepant (1 µM) did not block the responses to capsaicin. Further experiments were carried out in distal coronary arteries and, similar as in the human distal coronary artery, capsazepine (1 µM), ruthenium red (0.1 mM), L-73360 (0.5 µM), 4-aminopyridine (1 mM), L-NAME (0.1 mM), charybdotoxin (0.5 µM) + apamin (0.1 µM), iberiotoxin (0.5 µM) + apamin (0.1 µM), Y-27632 (1 µM), 18-α-glycyrrhetinic acid (10 µM) and endothelium denudation did not affect capsaicin-induced relaxations (Figure 7.1, Table 7.2). Additionally, Y-27632 (1 µM), alone or combined with 4-aminopyridine (1 mM), was also unable to block the responses to capsaicin. Similar as in the other arteries, the vehicle did not induce a relaxation (Figure 7.1). Interestingly, in contrast to what we observed in the human distal coronary

Table 7.2 Effect of various antagonists/interventions on capsaicin-induced relaxations in porcine isolated artery segments

Porcine distal coronary artery		
Antagonist or other intervention (*n*)	**E_{max} (%)**	**pEC_{50}**
- (Control) (51)	96±1	5.32±0.10
Olcegepant (1 μM) (10)	92±2	5.26±0.11
Capsazepine (5 μM) (7)	99±1	5.15±0.26
Ruthenium red (0.1 mM) (12)#	88±3	4.89±0.09
L-733060 (5 μM) (7)	88±3	4.89±0.09
Denuded endothelium (9)	91±4	5.62±0.49
L-NAME (0.1 mM) (6)	90±8	4.84±0.15
- (Control) (15)*	99±0	5.08±0.15
18-α-Glycyrrhetinic acid (10 μM) (3)*	99±1	5.73±0.75
4-Aminopyridine (1 mM) (7)*	96±2	4.84±0.15
Charybdotoxin (0.5 μM) +apamin (0.1 μM) (11)*	99±1	5.27±0.15
Y-276323 (1 μM) (9)*	96±2	5.17±0.19
Y-276323 (1 μM) + 4-Aminopyridine (1 mM) (8)*	96±3	5.22±0.20
Porcine proximal coronary artery		
- (Control) (4)	100±0	5.33±0.42
Olcegepant (1 μM) (4)	90±6	5.79±0.16
Porcine basilar artery		
- (Control) (3)	97±1	4.70±0.05
Olcegepant (1 μM) (3)	100±0	4.97±0.24
Capsazepine (5 μM) (3)	100±0	4.80±0.01
Porcine meningeal artery		
- (Control) (3)	99±1	4.82±0.02
Olcegepant 1 μM (3)	99±1	4.88±0.04

E_{max}: The maximum relaxant response, expressed as percentage of the respective precontraction; pEC_{50}: (-logEC_{50}, where EC_{50} is the concentration of agonist required to produce half the maximal response). The arteries were precontracted with KCl (18-30 mM) except where # KCl (45-90 mM) or * U46619 (10 nM-1 μM) was used to precontract arteries.

artery, precontracting the arteries with either KCl or U46619 did not change the relaxant responses to capsaicin. Four consecutive challenges to capsaicin (50 μM) did not significantly affect the magnitude of the responses (Figure 7.2).

There were no significant differences in efficacy or potency of capsaicin in porcine basilar (E_{max}: 97±1%, pEC_{50}: 4.70±0.05, n=3) and meningeal (E_{max}: 99±1%, pEC_{50}: 4.82±0.02, n=3) arteries as compared to porcine distal coronary arteries. In both these arteries, responses to capsaicin were not affected by olcegepant (1 μM).

7.3.3 Measurements of cAMP

Capsaicin (10 μM) did not affect cAMP levels in comparison to its vehicle or the control, which was only exposed to 30 mM KCl. In contrast, αCGRP (100 nM), which was used as a positive control, significantly increased cAMP levels in both human and porcine distal coronary arteries, which was blocked after incubation with olcegepant (1 μM) (Figure 7.3).

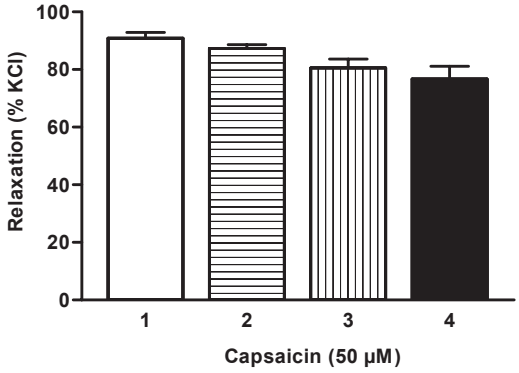


Figure 7.2. Effect of four consecutive challenges to capsaicin (50 µM) in porcine distal coronary arteries precontracted with KCl (30 mM).

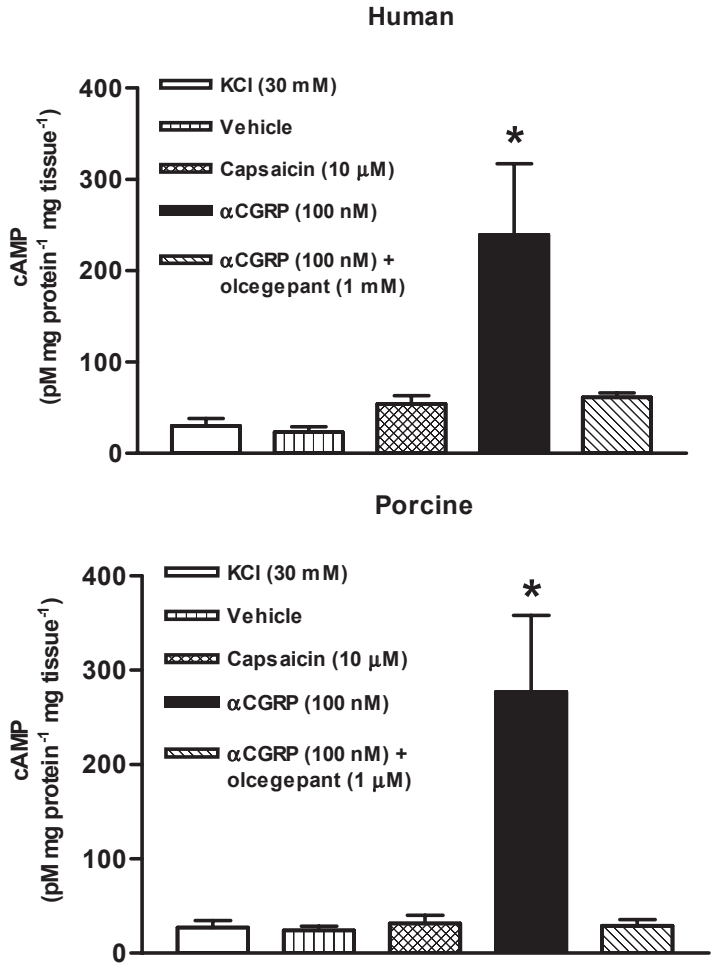


Figure 7.3. Changes in cAMP levels in human (n=4-7) and porcine (n=4-9) distal coronary artery segments after exposure to various pharmacological agents. * Significantly different ($P<0.05$) from KCl (30 mM)-treated segments.

7.3.4 Measurements of CGRP levels in organ bath fluid

Capsaicin and its vehicle induced a significant CGRP release from human distal coronary artery segments in to the organ bath fluid (Figure 7.4) as compared to levels observed in Krebs solution. There was no significant difference between CGRP levels obtained after incubation with capsaicin or its vehicle.

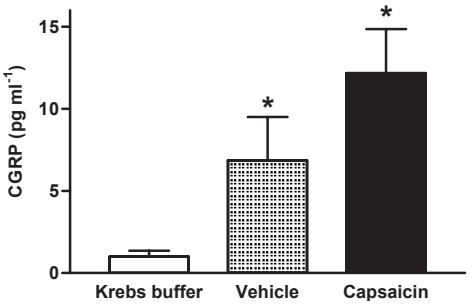


Figure 7.4. CGRP levels measured in bath fluids (Krebs buffer, control) after exposure to vehicle or capsaicin. * Significantly different (P<0.05) from Krebs buffer.

7.4 DISCUSSION

In the present study, we investigated the role of CGRP in capsaicin-induced relaxations in human and porcine isolated arteries. In all arteries investigated, there does not seem to be any relevant role of CGRP in the relaxant responses to capsaicin. Further, the effects of capsaicin appear to be mediated by non-specific mechanisms.

In all arteries investigated, capsaicin induced concentration-dependent relaxations, although these had a limited potency and efficacy in human proximal coronary artery. The responses to capsaicin were resistant to blockade with olcegepant in all tissues studied, which suggests no involvement of CGRP receptors. The potency of capsaicin is in line with what has earlier been observed in human coronary arteries (20) and guinea pig ileum (25). As expected in view of its resistance to olcegepant, $CGRP_{8-37}$ (10 μM) also did not block capsaicin-induced relaxations in human distal coronary artery segments. This is contrary to the observations by Franco-Cereceda et al. (27), where the authors claim the relaxations to capsaicin are mediated by CGRP on the basis of the observation that the sustained relaxations in the human coronary induced by capsaicin are similar to those induced by exogenous CGRP, unlike the transient relaxations to substance P, which are followed by rapid tachyphylaxis. In another publication, these authors claim involvement of CGRP in responses to capsaicin in the human isolated coronary artery on the basis of increased CGRP-like-immunoreactivity after exposure of the artery segments to capsaicin (22). It should be noted that in the above study, the authors show an increase in CGRP-like immunoreactivity in large arteries, where no functional studies were performed. Further, the classical prerequisite for demonstrating the involvement of a particular pharmacological agent, by using the corresponding antagonist to block functional responses, was lacking in this study. Admittedly, the same group did demonstrate that $CGRP_{8-37}$ inhibited responses to capsaicin in porcine coronary arteries (22).

As CGRP-induced responses are mediated by increases in cAMP (16), we measured the levels of this second messenger after exposure to capsaicin and CGRP. We observed no increase in cAMP after addition of capsaicin, in contrast to the increased levels of cAMP after exposure of the vessel segments to (exogenous) CGRP. These increased levels were, as expected, blocked by the CGRP receptor antagonist olcegepant. Interestingly, after exposure to capsaicin and its vehicle, ethanol, we observed CGRP release in the organ bath fluid where the human distal coronary arteries were mounted. Therefore, in accordance

with earlier observations (20), we observed increased CGRP levels after exposure to capsaicin. However, a similar increase was observed after administration of vehicle, while the control was not studied in the study of Franco-Cereceda et al. (20). Although capsaicin is known to activate TRPV1, there are reports that ethanol, also via activation of TRPV1, induces the release of CGRP as well (28, 29). Moreover, as obvious from Figure 7.1, the vehicle did not induce any relaxations in the precontracted arteries, and hence the released CGRP cannot account for relaxations induced by capsaicin. Interestingly, the concentration of CGRP detected in the bath fluid of about 3 pM should have been about 10,000 times higher in the vessel segments (~ 0.5 mg tissue in 5 ml organ bath fluid). Thus, the concentration of CGRP in the vessel segments should have been in the nanomolar range, which is equal to or even higher than the pEC_{50} in human distal coronary artery under similar experimental conditions (16), and should thus have induced a detectable relaxation. Therefore, it is most likely that the radioimmunoassay displayed cross-reactivity to another ligand, not CGRP. Admittedly, our observation that ruthenium red, even at a very high concentration (100 μM), did not block the responses to capsaicin, is in contrast with the observations described by Franco-Cereceda (20), where ruthenium red completely blocked the responses to capsaicin. Additional evidence for the fact that CGRP and TRPV1 are not involved in relaxant responses to capsaicin is provided by the fact that capsazepine, a competitive antagonist of TRPV1 (1), did not block responses to capsaicin in our study.

In view of reports of involvement various K^+ channels in the relaxant responses to capsaicin in various smooth muscle preparations (23, 24), we also investigated various K^+ channel inhibitors in similar or even higher concentrations, but none of these blocked responses to capsaicin. 4-Aminopyridine, a blocker of delayed rectifier K^+ channels, which is reported to block responses to capsaicin in rabbit coronary artery (24) and guinea pig ileum (25), was ineffective in either human or porcine distal coronary artery. Similarly, charybdotoxin, a BK_{Ca} and IK_{Ca} blocker, which blocked capsaicin responses in equine tracheal smooth muscle (23), did not block these responses at similar concentrations and even in combination with apamin, a SKCa blocker. We also used the RhoA kinase inhibitor Y-27632, but unlike in guinea pig ileum (25) it also did not antagonise responses to capsaicin. The capsaicin-mediated responses appear to be endothelium-independent, as denudation of the endothelium did not significantly change the capsaicin-induced responses. Further, the vehicle of capsaicin was without effect, and hence cannot account for the relaxant responses to capsaicin. Moreover, repeated administration of capsaicin in porcine coronary artery did not significantly decrease the responses, suggesting that stored neuropeptides are not responsible for the relaxations, since these would most likely be depleted after repeated challenges to capsaicin (30). Although we did not include a positive control for the various K^+ blockers in the current study, in the same set up at our laboratory relaxant responses to L-S-nitrosocysteine were blocked by the combination of charybdotoxin (100 nM) and apamin (100 nM) in porcine distal arteries (31). Further, the concentrations of the various inhibitors that we used in our study were equal to (23, 24) or even higher (24) than those employed by others.

Taken together, our observations in human and porcine distal coronary artery suggest that capsaicin-induced responses are not mediated by CGRP, substance P or TRPV1, and also do not involve various Ca^{2+}-activated K^+ channels. The relaxations to capsaicin are also not mediated by cAMP. The major component of capsaicin-induced relaxations therefore appears to be mediated by non-specific actions of capsaicin, rather than to be the result of release of neuropeptides like CGRP.

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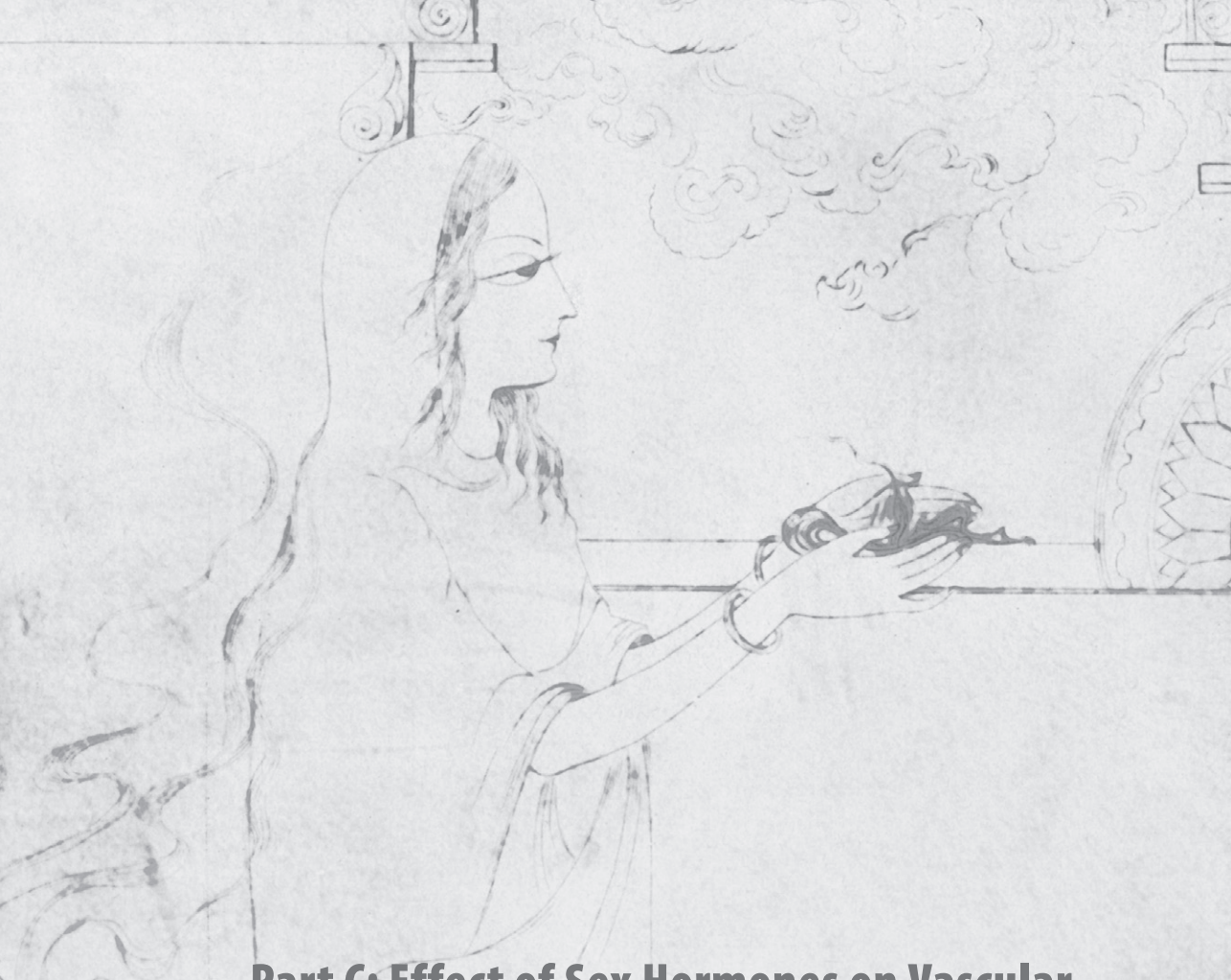

Part C: Effect of Sex Hormones on Vascular Pharmacology of Migraine

Chapter 8

Female Sex Hormones and Rat Dural Vasodilatation to CGRP, Perarterial Electrical Stimulation and Capsaicin

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ABSTRACT

The incidence of migraine is 2-3 fold higher in females than in males, and it is intricately related to the levels of female sex hormones. These hormones may regulate the synthesis and receptor expression of CGRP, which mediates neurogenic dural vasodilatation and is implicated in migraine pathogenesis. We investigated the effects of the female sex steroids, 17β-estradiol and progesterone, separately and in combination, on dural vasodilatation induced by αCGRP, periarterial electrical stimulation and capsaicin in ovariectomized rats, using intravital microscopy. Sprague-Dawley rats were ovariectomized and, 7 days later, subcutaneously implanted with 21-day release pellets of 17β-estradiol, progesterone, their combination or placebo. On day 19-21, the animals were anesthetized, overlying bone thinned to visualize the middle meningeal artery and vasodilator responses to αCGRP (10-3000 ng kg^{-1}), periarterial electrical stimulation (25-125 μA) and capsaicin (0.3-18 μg kg^{-1}) elicited. There were no significant differences in the vasodilator potency or efficacy of αCGRP or capsaicin in the different groups studied. In contrast, the vasodilator response to electrical stimulation was significantly higher in rats treated with 17β-estradiol (E_{max}: 157±19%) as compared to those observed after placebo treatment (E_{max}: 93±11%). Our results show that, in contrast to CGRP- or capsaicin-induced dural vasodilatation, 17β-estradiol enhanced neurogenic vasodilatation, suggesting increased CGRP release from perivascular nerves. This may be one of the mechanisms through which 17β-estradiol exacerbates migraine attacks in women.

8.1 INTRODUCTION

Migraine is a paroxysmal neurological disorder affecting 10-15% of the Western population, with a 2-3 fold higher prevalence in women than in men (1, 2). The prevalence of migraine before puberty is higher in males, while after puberty the prevalence increases in females (3), whose migraine attacks are aggravated during menstruation (4) and improve mostly during pregnancy (5) and after menopause (6). These findings indicate that profound changes occur in female hormone profile during these phases of life, which seem to correlate with changes in migraine attack frequency. Therefore, it is reasonable to assume that there is a hormonal factor involved in migraine pathogenesis, although very few studies have focused this area (7, 8).

It is now generally accepted that dilatation of cranial blood vessels, most probably initiated by trigeminal calcitonin gene-related peptide (CGRP) release, plays an important role in the pathophysiology of migraine (9-11). Accordingly, the newly developed CGRP receptor antagonist, BIBN4096BS (12-15), has recently been shown to be effective in the treatment of migraine (16).

The homeostasis of CGRP is strongly influenced by sex steroids. In female rats, for example, it has been shown that ovariectomy decreases plasma CGRP concentration, whilst subsequent treatment with 17β-estradiol, progesterone or their combination restores it significantly (17). Moreover, it is reported that myometrium sensitivity to CGRP increases during human pregnancy (18), and plasma concentrations of CGRP are elevated in postmenopausal women undergoing hormone replacement therapy (19). Although these findings suggest a positive correlation between plasma levels of CGRP and 17β-estradiol, estrogen withdrawal is also suggested to a be trigger for menstrual migraine (7, 8), while hormone replacement therapy has shown contradictory results in relation to the occurrence of migraine in postmenopausal women (8, 20, 21). Hence, the female sex steroids can either trigger or suppress migraine attacks. Nevertheless, taken collectively, these findings indicate that changes in the levels of female hormones, like 17β-estradiol and progesterone, may be involved in migraine pathogenesis, but an established neurobiological explanation is lacking.

As far as *in vivo* experimental models are concerned, intravital microscopy of a closed cranial window in rats is a well-established model to study the mechanisms of neurogenic dural inflammation (22) as well as neurogenic vasodilatation (23-28). Since trigeminal activation and dural vasodilatation are hallmarks of migraine pathophysiology (29, 30), this technique in rats allows monitoring changes in the diameter of

meningeal blood vessels without opening the skull. In this model, dural vasodilatation has been shown in response to exogenous CGRP (28) as well as capsaicin, which stimulates vanilloid receptors to release endogenous CGRP (31). Low intensity short electrical stimuli, preferentially activating A-δ fibers (22, 32), have been employed to cause neurogenic vasodilatation, which, being amenable to blockade by CGRP receptor antagonists $CGRP_{8-37}$ (23, 24, 33, 34) as well as BIBN4096BS (28, 35), is mediated by the release of endogenous CGRP. Other putative mediators, including substance P and neurokinin A, have a very limited role in neurogenic vasodilatation (23, 24).

On the above basis, we set out to investigate the effects of the female sex hormones, 17β-estradiol and progesterone, separately and in combination, on dural vasodilatation induced by αCGRP, periarterial electrical stimulation and capsaicin in female rats, using intravital microscopy.

8.2 MATERIALS AND METHODS

8.2.1 Animal groups and plasma 17β-estradiol and progesterone measurement

Female Sprague-Dawley rats (190-260 g), purchased from Harlan Netherlands (Horst, The Netherlands), were housed under a standard light and dark cycle and given free access to food and water. Bilateral ovariectomy or a sham operation was performed under isoflurane anesthesia and the rats were divided into five groups (n=6-9 each): (i) sham operation and ovariectomy followed on day 7 by subcutaneous implantation of pellets releasing over a 21-day period (ii) a placebo, (iii) 17β-estradiol (0.25 mg), (iv) progesterone (50 mg), and (v) a combination of 17β-estradiol (0.25 mg) and progesterone (50 mg).

On the day of experiment, in all animals a vaginal smear was made on a glass slide with the help of a cotton bud. After drying the smear, slides were stained (Giemsa, Societe Chimique Pointet Girard, Clichy, France) and analyzed for different phases of estrous cycle according to proportions of epithelial cells, cornified cells and leukocytes present in the smear[36]. The animals in the sham-operated group were in the low estrogen (estrous, metestrous or diestrous) or high estrogen (proestrous) stage. Since the number of animals was too few (2-4), we cannot state whether estrous cycle stage had any influence on the middle meningeal artery responses to CGRP, electrical stimulation or capsaicin. The smear in the ovariectomized groups showed typical patterns according to the hormone intervention, in placebo (very few leukocytes), 17β-estradiol (with copious mucous secretions, cornified epithelial cells, few nucleated epithelial cells), progesterone (few leukocytes) and 17β-estradiol and progesterone combination (cornified epithelial cells few nucleated epithelial cells).

Blood samples (700-800 µl) were collected from the tail vein under isoflurane anesthesia three times: just before ovariectomy, 7 days after ovariectomy before pellet implantation, and 14 days after ovariectomy. The samples were mixed with EDTA, centrifuged at 10,000 rpm for 10 min and the resultant plasma was stored at -80 °C until the samples were analyzed for 17β-estradiol and progesterone, using radioimmunoassay kits obtained from Diagnostic Products Corporation Nederland B.V. (Breda, The Netherlands).

8.2.2 Intravital microscopy

On day 18-21 after sham operation or ovariectomy, the rats were anaesthetized with intraperitoneal sodium pentobarbital (60 mg kg^{-1} bolus, followed by 18 mg kg^{-1} h^{-1}). The trachea was cannulated and connected to a pressure ventilator (Small animal ventilator SAR-830 series, CWE, Inc., Ardmore, PA, USA). The left femoral vein and artery were cannulated for intravenous (i.v.) administration of drugs and continuous monitoring of the blood pressure, respectively. Two or three samples of blood were withdrawn via the femoral artery to monitor blood gases, which were kept between normal values (pH: 7.35-7.48; pCO_2: 35-48 mmHg; pO_2: 100-120 mmHg). The body temperature of animals was monitored via a rectal thermometer and maintained throughout the experiment between 36.5 and 37.5 °C by a homeothermic

blanket system for rodents (Harvard Instruments, Edenbridge, Kent, UK). The rats were then placed in a stereotaxic frame and the bone overlying a segment of the middle meningeal artery was carefully drilled thin, constantly applying ice-cold saline until the artery was clearly visible. As the drilling of the skull induces vasodilatation, we allowed the animal to recover for 1 h before proceeding with the experimental protocol.

The drilled area was covered with mineral oil in order to prevent drying and facilitate the visualization of meningeal artery. The artery was captured with an intravital microscope (model MZ 16; Leica microsystem Ltd., Heerbrugg, Switzerland) using a cyan blue filter on a cold source of light and a zoom lens (80-450x magnification) and a camera were used to display images on a standard television monitor. The blood vessel diameter (30-40 µm at baseline) was continuously monitored and measured with a video dimension analyzer (Living Systems Instrumentation Inc., Burlington, VT, USA) and the effects of αCGRP, periarterial electrical stimulation and capsaicin were studied as specified below. The resulting data were displayed and recorded using a WINDAQ data acquisition system (Version 2.54; DataQ Instruments Inc., Akron, OH, USA).

8.2.3 Experimental Protocols

8.2.3.1 Effect of αCGRP

First, a bolus injection of saline was given to analyze the effect of vehicle. After 10 min, increasing doses of r-αCGRP (rat-αCGRP) were administered in quarter log dose steps (10-3000 ng kg^{-1}). The first four doses were given every 5 min, while for the last five doses an interval of 10 min was chosen for the values to return to baseline. The peak increase in arterial diameter and peak decrease in mean arterial blood pressure were measured after every dose.

8.2.3.2 Effect of periarterial electrical stimulation

In rats where periarterial electrical stimulation was employed to evoke dural blood vessel dilatation, a bipolar electrode (model NE 200X, Clark Electromedical, Edenbridge, Kent, UK) was placed on the surface of the bone, approximately within 200 µm from the vessel of interest to stimulate at 5 Hz, 1 ms for 10 s with a Grass Stimulator (model S88; Grass Instrument Co., Quincy, MA, USA). Neurogenic dural vasodilatation was induced by increasing the current intensity, monitored on an oscilloscope (model 54601A; Hewlett-Packard, Palo Alto, CA, USA), from 25 to 125 µA with steps of 25 µA. The peak increase in dural diameter after stimulation was measured and a 10-min interval was given between successive electrical stimulations. Electrical stimulation was carried out 30 min after finishing administration of αCGRP protocol in all rats.

8.2.3.3 Effect of capsaicin

First, a bolus injection of vehicle was given, then increasing doses of capsaicin, in quarter log dose steps (0.3-18 µg kg^{-1}), were administered; the first and last four doses were given at an interval of 5 and 10 min, respectively, as at higher doses it required more time for the blood vessel diameter to return to baseline. The peak increases in vessel diameter and peak decreases in mean arterial blood pressure were recorded. The protocol of capsaicin administration was started in all the groups half an hour after the electrical stimulation protocol had been concluded.

It is important to note that repeated infusions (3-7 times) of αCGRP, capsaicin or periarterial electrical stimulations produce similar changes in dural vessel diameter and blood pressure in this model[28,31]. In our hands also, the responses to αCGRP (560-1000 ng kg^{-1}) repeated after finishing the whole protocol were not different from responses obtained earlier (data not shown).

8.2.4 Data analysis

The effects of αCGRP, periarterial electrical stimulation and capsaicin on the dural vessel diameter were calculated as a percentage increase from the baseline diameter just before injection or electrical stimulation. The dose- (αCGRP and capsaicin) or current intensity- (periarterial electrical stimulation) response curves were analyzed to establish the maximum response (E_{max}) and dose (ED_{50}) or current intensity (EI_{50}) required to increase dural vessel diameter by 50% of E_{max}. The changes in mean arterial blood pressure were expressed in absolute values (mmHg). All data are presented as mean±s.e.m. At every dose (αCGRP and capsaicin) or current intensity (electrical stimulation), ANOVA was conducted between all groups, followed by post hoc Dunnett's multiple comparisons test, with $P<0.05$ considered statistically significant. The differences in the body weight, hormonal concentrations, E_{max} and ED_{50} were also evaluated in the same manner.

8.2.5 Compounds

The compounds used in the present study (obtained from the sources indicated) were: capsaicin (Sigma Chemicals Co., Steinheim, Germany); r-αCGRP (NeoMPS S.A., Strasbourg, France). Capsaicin (1 mg ml^{-1}) was dissolved in a mixture of tween 80, ethanol 70% and water (1:1:8), whereas the rest of the compounds were dissolved in isotonic saline. All compounds were stored in aliquots at -80°C, until required. Just before use, the stock solutions were further diluted to the appropriate concentration in isotonic saline for injection. The hormone pellets were purchased from Innovative Research of America (Sarasota, FL, USA).

8.3 RESULTS

8.3.1 Changes in body weight

The body weight and its changes in the different groups of rats are listed in Table 8.1. After 7 days of ovariectomy, there was significantly less increase in the body weight in the sham group as compared to the placebo group. Further, on day 14 after pellet implantation the body weight gain in the sham, 17β-estradiol- or combination-treated groups was significantly lower ($P<0.05$) than the corresponding body weight gain in the placebo group (see Table 8.1). In contrast, the body gain weight in the progesterone-treated group did not significantly differ from that in the placebo group.

8.3.2 Plasma levels of 17β-estradiol and progesterone

The concentrations of both hormones decreased significantly from day 1 (i.e. before ovariectomy; 17β-estradiol: 35±5 pg ml^{-1}, progesterone: 18±5 ng ml^{-1}) as compared to those on day 7 after ovariectomy (17β-estradiol: 21±4 pg ml^{-1}, progesterone: 5±2 ng ml^{-1}). On day 21 (i.e. 14 days after treatment with respective hormones pellets), plasma concentrations of 17β-estradiol (143±31 pg ml^{-1}) as well as progesterone (19±3 ng ml^{-1}) were significantly increased as compared to plasma concentrations at 7 days after ovariectomy (see above) or in placebo-treated animals (17β-estradiol: 11±2 pg ml^{-1}, progesterone: 9±1 ng ml^{-1}). In sham-operated animals, the hormone concentrations were similar at the different 3 time points (days 1, 7 and 21; data not shown).

8.3.3 Effect of αCGRP, periarterial electrical stimulation and capsaicin on meningeal artery diameter

Vehicle (saline) did not induce any significant effect on blood vessel diameter. αCGRP (10-3000 ng kg^{-1}) induced dose-dependent middle meningeal artery dilatations (Figure 8.1). There were no significant differences in the potency (EC_{50}) or maximum responses (E_{max}) between the different groups (Table 8.2). However, the response to CGRP at the 560 ng kg^{-1} dose, but not at other doses, was significantly higher

Table 8.1 Body weight (g) and body weight changes (Δ; g) in rats after different interventions (n=5-9).

	Sham-ovariectomized rats	Ovariectomized rats treated with			
		Placebo	17β-Estradiol	Progesterone	17β-Estradiol + Progesterone
Day 1 (before sham or ovariectomy)	206±3	228±6	218±5	217±7	203±3
Day 7 (after sham or ovariectomy)	212±5	244±4	239±5	238±7	222±2
Δ Body weight at Day 7	6±3*	22±3	21±3	21±3	19±1
Day 21 (after sham or pellet implantation)	225±5	297±7	230±5	281±8	203±2
Δ Body weight at Day 21	19±4*	78±5	12±4*	65±5	0.5±1.2*

*Significantly different from the corresponding value in placebo-treated rats.

Table 8.2 Vasodilator responses to r-αCGRP, periarterial electrical stimulation and capsaicin in rats after different interventions (n=5-9).

Pharmacological parameters		Sham-ovariectomized rats	Ovariectomized rats treated with			
			Placebo	17β-Estradiol	Progesterone	17β-Estradiol + Progesterone
r-αCGRP	E_{max} (%)	126±5	98±11	125±16	107±17	130±14
Periarterial electrical stimulation	ED_{50} (ng kg^{-1})	402±67	406±67	387±32	417±77	285±32
	E_{max} (%)	112± 8	93±11	157±19*	100±8	110±19
Capsaicin	EI_{50} (μA)	60±7	49±7	46±6	43±6	59±8
	E_{max} (%)	139±14	103±8	122±20	118±11	106±6
	ED_{50} (μg kg^{-1})	3.0±0.2	4.3±0.8	4.4±0.5	3.7±0.6	4.1±0.7

E_{max}, Maximum % increase from the corresponding baseline value; ED_{50}, dose required to increase vessel diameter by 50% of E_{max}; EI_{50} (current intensity required to increase vessel diameter by 50% of E_{max}. *Significantly different ($P < 0.05$) from the corresponding value in placebo-treated rats.

($F_{4,24}$=5.50, P=0.0027) in the sham-operated and combination-treated animals as compared to the placebo-treated group (Figure 8.1).

Periarterial electrical stimulation (25-125 μA) of the meningeal artery produced a characteristic response, which consisted of an initial transient constriction, followed by a longer-lasting dilatation. Figure 8.2 shows that the dural arterial dilatation increased with increasing current intensity in all groups. In this respect, the 17β-estradiol-treated group showed significantly higher dilatations than the placebo-treated group at stimulation intensities of 50 μA and higher (ANOVA: $F_{4,27}$=3.98, P=0.015), except at 75 μA where the difference just did not reach statistical significance (P=0.06). Likewise, the maximal response was also significantly higher in the 17β-estradiol-treated group. The responses observed in other treatment groups were not significantly different from those in the placebo-treated group (Figure 8.2, Table 8.2).

As described with periarterial electrical stimulation, i.v. administration of capsaicin (0.3-18 μg kg^{-1}) also induced a transient vasoconstriction followed by a longer-lasting dilatation of the meningeal artery. The lower doses of capsaicin (0.3-1.8 μg kg^{-1}) induced dural vasoconstriction in some animals, but the over-

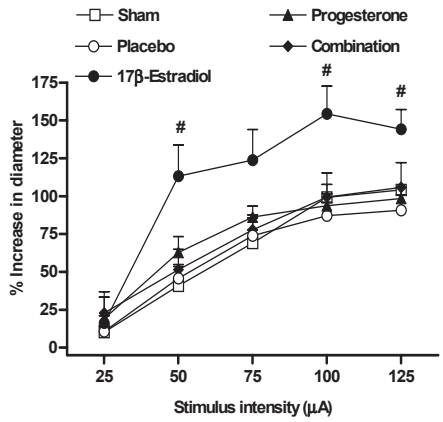

Figure 8.1. Effect of increasing doses of r-αCGRP on middle meningeal artery diameter in sham-operated or ovariectomized rats receiving placebo or hormone treatments (n=6-9). #Significantly higher (P<0.05) than the corresponding value in the placebo group.

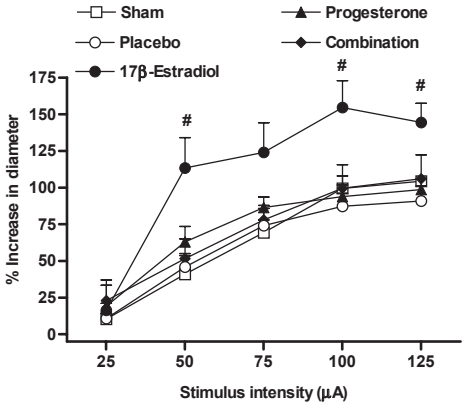

Figure 8.2. Effect of increasing intensities of periarterial nerve stimulation on middle meningeal artery diameter in sham-operated or ovariectomized rats receiving placebo or hormone treatments (n=6-7). #Significantly higher (P<0.05) than the corresponding value in the placebo group.

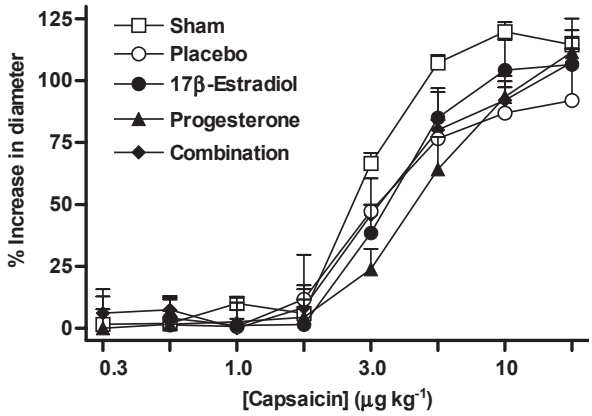

Figure 8.3. Effect of capsaicin on middle meningeal artery diameter in sham-operated or ovariectomized rats receiving placebo or hormone treatments (n=5-7). No significant changes were observed.

whelming response was that of a dose-dependent vasodilatation (Figure 8.3). There were no significant differences in the EC_{50} or E_{max} amongst the different groups (Table 8.2).

8.3.4 Effect of αCGRP, periarterial electrical stimulation and capsaicin on mean arterial blood pressure

The baseline values (mm Hg) in the different groups of animals were: sham ovariectomized rats (85±5) and ovariectomized rats treated with placebo (84±14), 17β-estradiol (86±8), progesterone (94±4) or 17β-estradiol + progesterone (84±6). Vehicle (saline) did not induce any significant effect on mean arterial blood pressure. Administration of α.CGRP (10-3000 ng kg^{-1}) produced similar dose-dependent decreases in mean arterial blood pressure in all the groups (maximum ≅40 mm Hg, Table 8.3). There were no significant changes in mean arterial blood pressure after electrical stimulation in any of the treatment groups. Further, the highest doses of capsaicin produced a similar vasodepressor response (~30 mmHg) across the treatment groups.

Table 8.3 Changes in blood pressure (mm Hg) by i.v. bolus injections of r-αCGRP in rats after different interventions (n=5-9).

r-αCGRP (ng kg^{-1})	Sham-ovariectomized rats	Ovariectomized rats treated with			
		Placebo	17β-Estradiol	Progesterone	17β-Estradiol + Progesterone
30	0.8±1.1	-0.8±1.0	0.6±1.1	-2.6±1.0	4.4±2.5
56	2.4±2.7	-1.7±8.5	-0.2±0.8	2.1±2.3	0.8±1.2
100	7.0±2.6	2.7±3.1	3.4±1.4	3.2±2.6	3.8±2.4
178	8.7±2.9	1.7±3.2	6.9±2.8	8.0±2.1	5.3±2.4
300	12.1±4.8	9.6±2.2	9.3±2.7	16.4±4.0	12.3±3.0
560	24.7±4.2	22.1±4.7	20.1±3.8	17.4±1.7	22.3±4.5
1000	30.1±6.1	26.3±4.5	27.8±4.2	27.6±4.6	25.6±4.2
1780	34.9±5.7	35.4±17.0	30.0±4.0	38.5±42.3	29.7±4.4
3000	47.3±3.2	42.5±4.3	46.8±10.7	42.3±4.6	41.9±4.2

No significant differences ($P < 0.05$) from the corresponding value in placebo-treated rats were observed.

8.4 DISCUSSION

Our study demonstrates that 17β-estradiol increased dural vasodilator responses induced by periarterial electrical stimulation in the ovariectomized rats, whilst the other hormonal interventions or placebo treatment did not have any significant effect. The vasodilator responses to αCGRP, except at one dose (560 ng kg-1), as well as to capsaicin were not affected by ovariectomy or by hormonal replacement. Within 7 days after ovariectomy, there was a significantly higher weight gain in ovariectomized rats as compared to that in sham-operated animals; this is in line with earlier observations where ovariectomy in rats was characterized by enhanced obesity due to an increase in adipose tissue lipoprotein lipase (37)Additionally, 17β-estradiol replacement decreased (38, 39), while progesterone replacement increased lipoprotein lipase activity (40, 41). Thus, earlier observations are in full agreement with our finding of increased weight gain in animals treated with progesterone or placebo, but not with 17β-estradiol.

The dural vasodilatation elicited by αCGRP in the sham-operated group (E_{max}: 126±5%, ED_{50}: 402±67 ng kg^{-1}; Table 8.2) was comparable to that reported by Petersen et al.(28) (E_{max}: 125%, ED_{50} calculated from their data: ~300 ng kg^{-1}) and overall the CGRP-induced meningeal artery dilatation was not significantly different amongst the various groups studied. Further, we did not observe any difference in hypotensive responses to αCGRP between the groups. This seems to be in contradiction with the observations made by Grewal et al. (42) in ovariectomized rats treated with 17β-estradiol, where an increased sensitivity to the depressor responses induced by αCGRP was observed. This apparent inconsistency may be due to differences in the experimental protocol: 10 min prior to administration of αCGRP, the ganglion blocker pentolinium was administered, followed by a continuous infusion of phenylephrine in order to increase vascular tone (42). In contrast, in our experimental set-up, the rats were anesthetized with sodium pentobarbital throughout the experimental protocol and no additional pharmacological intervention, other than i.v. administration of αCGRP, was carried out.

Periarterial electrical stimulation elicited stimulus-dependent neurogenic dilator responses in the rat middle meningeal artery. There was a significant increase in the vasodilator response in ovariectomized rats treated with 17β-estradiol as compared to the placebo-treated group. Earlier studies have shown that low intensity of stimulation for a short period employed in the present study is believed to selectively activate CGRP-containing A-δ sensory fibers resulting in vasodilatation, rather than C-fibers that predominantly contain substance P and cause plasma extravasation (22, 32). Indeed, rat dural periarterial nerves stain for CGRP (33, 43-45) and electrical stimulation of the dural surface causes a depletion of CGRP-immunoreactive fibers, suggesting CGRP release (33). Moreover, the neurogenic vasodilator response is antagonized by CGRP receptor antagonists $CGRP_{8-37}$ and BIBN4096BS (23, 24, 28, 33-35), but not by neurokinin receptor antagonists (23, 24). Therefore, the higher vasodilatation observed in the 17β-estradiol-treated group is most probably due to an increased release of CGRP from perivascular nerve endings innervating the rat middle meningeal artery. In keeping with this view, 17β-estradiol has been reported to increase CGRP-immunoreactive sensory innervations in rat arteries (46, 47). Further, CGRP synthesis in dorsal root ganglion is upregulated in ovariectomized rats treated with ovarian steroid hormones (48, 49). Hence, the observed increase in the vasodilatation appears to be due to an enhanced CGRP innervations, which may, in turn, lead to an augmented release of this neuropeptide in dural arteries of ovariectomized rats treated with 17β-estradiol. Interestingly, there were no significant differences in the vasodilator responses induced by electrical stimulation in rats treated with placebo, progesterone or the combination of progesterone and 17β-estradiol; this finding suggests that progesterone may attenuate the effects of 17β-estradiol and that it does not have an effect on neurogenic vasodilatation *per se* in this model. Although we have no clear-cut explanation for this phenomenon, other authors have also reported opposing actions of 17β-estradiol and progesterone (50, 51). Also, in rats, the CGRP receptor expression is regulated in divergent ways by these female sex steroids (52). In addition, progesterone and its metabolites also act as agonists on $GABA_A$ receptors and also suppress

electrical stimulation-induced plasma protein extravasation in rats (53). Alloprogesterone (a progesterone metabolite) is also reported to decrease the expression of c-fos, a molecular marker of migraine, after trigeminal activation with capsaicin (54).

Our observations showing that capsaicin produced a dose-dependent dural vasodilatation in all treatment groups (with no significant differences in either E_{max} or ED_{50} between the various groups) is in line with previous observations in this model (31, 55). In addition, our study provides for the first time an ED_{50} value for this vanilloid receptor agonist in its ability to produce dural vasodilatation, which has been previously shown to be predominantly mediated by CGRP release in this model (31). We observed no significant differences in either E_{max} or ED_{50} between the various groups.

Notably, the fact that no significant differences were observed in the capsaicin-induced vasodilatation between groups studied implies that there is no difference in the amount of CGRP released, which seems to be inconsistent with the higher E_{max} observed in the 17β-estradiol-treated group in electrical stimulation induced vasodilatation. This discrepancy may be due to the fact that i.v. administration of capsaicin produces a systemic release of CGRP; since dural arteries are free of a blood brain barrier, the vasodilatation induced by capsaicin is not just a consequence of the local release of CGRP, but also of the CGRP released in the whole vascular system. Although 17β-estradiol is reported to upregulate CGRP synthesis in rats (47), this is not a generalized phenomenon involving an overall increase in sensory vasodilator innervations. Remarkably, estrogens have been reported to selectively increase the sensory nociceptor innervations of arterioles in female rats (46); since the rat dural arteries are innervated by sensory nociceptors, this may account for the differences observed between the vasodilator responses induced by electrical stimulation and capsaicin in 17β-estradiol treated rats. In addition, capsaicin not only releases αCGRP, but also other vasodilator (substance P) and contractile agents (56). Indeed, we and others (57) also observed a triphasic blood pressure responses to administration of capsaicin, underlining that capsaicin releases an array of vasoactive agents.

In the ovariectomized rats treated with 17β-estradiol pellets, the corresponding plasma levels of the hormone was ~2.5 times the highest levels encountered in normal oestrous cycle in rats, which is a potential limitation of this study. Additional studies, which fall beyond the scope of the present investigation, will be required to further analyze the role of 17β-estradiol in migraine. Such studies may include experiments in a model of 17β-estradiol withdrawal after priming with high concentrations of 17β-estradiol, which is considered as a main trigger for menstrual migraine (7, 8), as well as experiments to elucidate the cellular and molecular mechanisms involved in the increased vasodilator responses to electrical stimulation in 17β-estradiol-treated ovariectomized rats. Studies of meningeal blood vessel in female rat in different phases of oestrous cycle might also give insight in menstrual migraine. Interestingly, CGRP is not merely a vasodilator agent, but it also has a neuronal action facilitating pain transmission (58, 59). Hence, increased CGRP levels may also be able to accentuate pain experience in migraine at the central level.

In conclusion, our study seems to provide the first line of evidence showing that 17β-estradiol can increase the neurogenic dural vasodilatation in rats. Since this experimental model is instrumental for investigating migraine pathophysiology (24-28,60), the enhanced dural vasodilatation induced by 17β-estradiol may be one of the mechanisms by which this hormone exacerbates migraine attacks in women.

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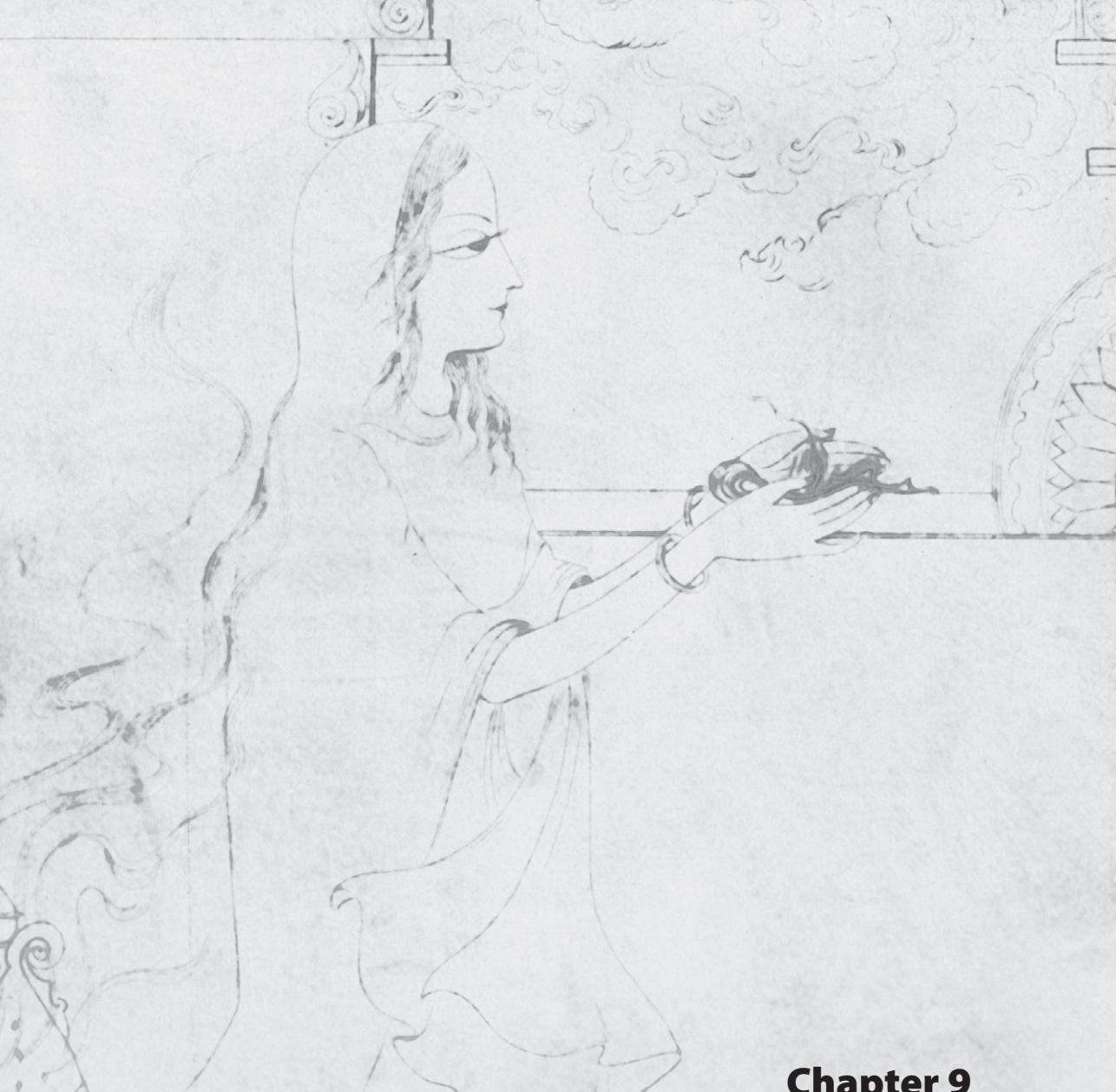
Khudee ko kar buland itna
ke har taqdeer se pehle
Khudaa bandey se khud poochhe
bataa teri raza kya hai…

Make yourself competent enough such that…
Before God decides the fate God asks your consent…



Chapter 9

The Effect of Female Sex Hormones on the Responses to CGRP, Acetylcholine and 5-HT in Rat Isolated Arteries

Based on: S. Gupta, S. Mehrotra, C.M. Villalón, R. de Vries, I.M. Garrelds, P.R. Saxena, A. MaassenVanDenBrink, Headache, In press

ABSTRACT

Female sex hormones are implicated in the modulation of reactivity of a wide range of blood vessels in physiological as well as pathological conditions. Migraine, a neurovascular syndrome, is three times more prevalent in women during their reproductive period than in men. This study sets out to investigate the effects of the female sex steroids, 17β-estradiol and progesterone (separately and in combination) on vasoactive responses to calcitonin gene-related peptide (CGRP), acetylcholine and 5-hydroxytryptamine (5-HT) in rat isolated mesenteric, caudal and basilar arteries. Female Sprague-Dawley rats were ovariectomized (Day 0) and 7 days later subcutaneously implanted with pellets releasing over a 21-day period 17β-estradiol (0.25 mg), progesterone (50 mg), their combination or placebo. On Day 25-28, the animals were killed, arteries isolated and mounted in Mulvany myographs and cumulative concentration response curves to CGRP, acetylcholine and 5-HT were constructed. The relaxant responses to CGRP were significantly potentiated in mesenteric and caudal arteries from rats treated with 17β-estradiol as compared to the placebo-treated rats. Acetylcholine-induced relaxations were potentiated in the caudal artery from rats treated with the combination of 17β-estradiol and progesterone, as compared to that from placebo-treated rats. The 5-HT-induced contractions in the three arteries were not significantly different in efficacy or potency. Our results show that 17β-estradiol potentiates CGRP-induced relaxations in the mesenteric and caudal arteries, whilst the combination treatment enhances acetylcholine-induced relaxations in the caudal artery. Although these *in vitro* experiments have been carried out in rats and a direct extrapolation to migraine in humans is not possible, our results may provide a new avenue to study the effects of sex steroids on vascular reactivity.

9.1 INTRODUCTION

Migraine, a neurovascular syndrome, is about three times more common in women than in men at peak prevalence (1, 2) and its incidence fluctuates according to reproductive milestones in female life (3-5). Interestingly, the occurrence of migraine before puberty is higher in men than in women, while it reverses after puberty (6). In women, the migraine attacks are aggravated during menstruation (3), while most migraineurs experience improvement during pregnancy (4) and after menopause (5). These observations suggest a role for female hormones in migraine pathogenesis.

Calcitonin gene-related peptide (CGRP) is implicated in the pathophysiology of migraine as a dilator of cranial blood vessels and, hence, activation of pain-sensitive nociceptors in these blood vessels (7-10). Accordingly, the CGRP receptor antagonist, BIBN4096BS (11), has been reported to be effective in the acute treatment of migraine (12). Moreover, in the past one and half decade, triptans (5-$HT_{1B/1D}$ receptor agonists) have been developed and proven effective in alleviating migraine headache (7, 13, 14). The antimigraine activity of triptans is, at least partly, attributed to constriction of dilated intracranial extracerebral arteries (7, 13, 14). These observations provide evidence for a strong vascular component in the pathophysiology of migraine.

Vascular tone is controlled by an interplay of a number of factors and female sex hormones are one of the significant determinants (15, 16). In this respect, 17β-estradiol and progesterone cause vasorelaxation *per se* at high non-physiological concentrations (rapid non-genomic pathways), but they may also manifest their actions via slow genomic actions (15, 17, 18). In addition, sex steroids modulate the synthesis of and/or sensitivity to various vasoactive agents. In fact, it seems that CGRP homeostasis is strongly influenced by sex steroids as: (i) ovariectomy in rats decreases, whilst subsequent treatment with female hormones increases, CGRP plasma levels (19), (ii) the expression of CGRP receptors is augmented during pregnancy, while it decreases after parturition (20), (iii) pregnancy in women augments myometrium sensitivity to CGRP (21), and (iv) there is a positive correlation between plasma levels of CGRP and 17β-estradiol in postmenopausal women undergoing hormone replacement therapy (22). These responses are thought to be mediated by classical genomic effects following chronic exposure

to 17β-estradiol. Moreover, the enhancement of the relaxant responses by female sex steroids in various vascular beds is suggested to be due to an increased activity of endothelium-dependent pathways, such as nitric oxide or cGMP (15, 17); indeed, acetylcholine-induced endothelium-dependent relaxations are augmented in rat isolated cerebral arteries (23) as well as in aortic rings from ovariectomized rats treated with 17β-estradiol (24). In contrast to CGRP and acetylcholine, the reactivity to 5-HT has been reported to increase after ovariectomy, and to normalize after treatment with female sex hormones in primate (25) and rabbit (26) arteries; indeed, both 17β-estradiol and progesterone can attenuate the increase in intracellular Ca^{2+} and protein kinase C translocation in vascular smooth muscle cells by 5-HT (27). Similarly, acute incubation with 17β-estradiol decreases the maximum response to contractile agents, including 5-HT, in porcine and human arteries (28, 29).

On this basis, the present study has examined the effect of 17β-estradiol and progesterone, separately and in combination, on the responses to CGRP, acetylcholine and 5-HT in isolated segments of mesenteric, caudal and basilar arteries of female rats. Since dural arteries, which seem to be of key relevance in the pathogenesis of migraine headache, are too small to study *in vitro*, we decided to study, besides a cranial blood vessel (basilar artery), the mesenteric and caudal arteries, which, like dural arteries, are densely innervated by CGRP-containing fibers (mesenteric arteries (30)) and display an endothelium-independent relaxation to CGRP (mesenteric (31) and caudal arteries (32).

9.2 MATERIALS AND METHODS

9.2.1 Animal groups and isolation of blood vessels

Female Sprague-Dawley rats (body weight: 190-260 g, age: 8-11 wks) were purchased from Harlan Netherlands (Horst, The Netherlands). All animals were given free access to food and water. After one week of acclimatization (Day 0), the rats were anesthetized with isoflurane for either a sham-operation (exteriorization but not the removal of ovaries; Group 1) or bilateral ovariectomy. One week later (Day 7) and under the same anesthesia, the ovariectomized rats were subcutaneously implanted with a pellet releasing over a 21-day period either placebo (Group 2), 0.25 mg 17β-estradiol (Group 3), 50 mg progesterone (Group 4) or the combination of the two hormones (Group 5). In these animals the concentrations of both 17β-estradiol and progesterone (Day 0; 27±2 pg ml^{-1} and 21±2 ng ml^{-1}, n=43 each, respectively) decreased following ovariectomy (Day 7; 22±2 pg ml^{-1} and 7±1 ng ml^{-1}, n=35 each, respectively) and increased after implantation of hormones pellets in ovariectomized animals; for example, on Day 21 (i.e. 14 days after treatment with respective hormones pellets), plasma concentrations of 17β-estradiol and progesterone were 201±36 pg ml^{-1}, n=11 and 17±4 ng ml^{-1}, n=9, respectively.

The rats were killed on Day 25-28 under anesthesia using sodium pentobarbital (100 mg kg^{-1}, i.p.). Subsequently, second order mesenteric, caudal and basilar arteries were isolated and placed in cold oxygenated Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4; 4°C). The surrounding tissue was removed and the arteries were cut into ring segments of 1-2 mm length (internal diameters: mesenteric, 300-500 µm; caudal, 300-400 µm; and basilar, 100-125 µm). Subsequently, the segments were mounted in Mulvany myographs (Danish myo technology a/s, Aarhus, Denmark) between two parallel titanium wires with tension normalized to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development (33). The myograph chambers containing Krebs buffer solution (see above) was aerated with 95% O_2 and 5% CO_2 at 37°C. In the case of basilar artery segments, 0.1 µM indomethacin (cyclooxygenase inhibitor) was added to the Krebs buffer to inhibit small spontaneous contractions often observed in this vessel (34). Changes in tension of the arterial segments were measured with an isometric force transducer (Danish myo technology a/s, Aarhus, Denmark) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

9.2.2 Concentration response curves

After an initial equilibration period of 30 min, two successive challenges to KCl (30 mM) were performed to check the reproducibility of the responses in blood vessels obtained from all five groups of rats. After another 30-min period, 100 mM KCl was added to determine the reference contractile response in each segment. Then, a stable contraction was obtained with 1 μM 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; mesenteric), 0.1 μM U46619 (caudal) or 5 nM endothelin-1 (basilar); U46619 did not induce a stable precontraction in this tissue. In preconstricted arterial segments, cumulative concentration response curves to r-αCGRP and acetylcholine were constructed. Concentration response curves to 5-HT were constructed in the absence of any precontraction. Only one concentration response curve was performed in each artery segment and all the concentration response curves were made in half log steps. The ethical committee of Erasmus MC dealing with animal experiments approved this study protocol.

9.2.3 Data analysis

The contractile response to 100 mM KCl, expressed in mN, was used to compare the contractile force developed by arteries from different experimental groups. The relaxant responses to r-αCGRP and acetylcholine are expressed as percentage relaxation of the precontraction induced by U46619 or endothelin-1. The contractile responses to 5-HT are expressed as percentage of the contraction induced by 100 mM KCl. All values are expressed as mean±s.e.m. and *n* represents the number of vessel segments, each of which was obtained from a different rat.

The concentration response curves for all agonists were analyzed using nonlinear regression analysis to determine the maximal response (E_{max}: maximum plateau response reached with an agonist) and potency (pEC_{50}: negative logarithm of the molar concentration eliciting 50% of the E_{max}) of each agonist. A higher pEC_{50} (i.e., lower EC_{50}) value indicates a higher potency, since a lower concentration of agonist is required to elicit the same response. ANOVA was conducted between all the groups, followed by post hoc Dunnett's multiple comparisons using E_{max} or pEC_{50} values obtained from placebo-treated ovariectomized rats as the control group with differences considered significant at $P<0.05$.

9.2.4 Compounds

Apart from the anesthetic (sodium pentobarbital), the compounds used in the present study (obtained from the sources indicated) were: 5-hydroxytryptamine creatinine sulphate, U46619 and acetylcholine chloride (Sigma Chemical Co., Steinheim, Germany); endothelin-1 and rat-αCGRP (NeoMPS S.A., Strasbourg, France). All compounds were dissolved in distilled water and stored in aliquots at -80°C, until required. Just before use, the stock solutions were further diluted to the appropriate concentration in distilled water. The hormone pellets were obtained from Innovative Research of America (Sarasota, FL, U.S.A.).

9.3 RESULTS

9.3.1 Contractions induced by KCl, U46619 and endothelin-1

All segments obtained from mesenteric, basilar and caudal arteries contracted in response to KCl, U46619 and endothelin-1. There were no significant differences in contractions induced by these agents between placebo-treated animals and other treatment groups in any of the three arteries (Table 9.1).

9.3.2 Relaxations induced by αCGRP

αCGRP induced concentration-dependent relaxations in mesenteric, caudal and basilar artery segments (Figure 9.1). αCGRP was more potent in relaxing the mesenteric and caudal artery segments from ovari-

Table 9.1 Contractile responses (mN) to KCl (used as reference) and to U46619 and endothelin-1 (used for precontraction) in isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Contractile agent (concentration)	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	KCl (100 mM)	9.5±1.1 (8)	11.1±1.2 (15)	11.0±1.5 (12)	9.1±0.8 (12)	8.3±0.7 (12)
Caudal	U46619 (1 μM)	9.1±0.9 (8)	9.2±1.13 (14)	9.2±1.4 (10)	7.2±1.0 (9)	7.9±1.0 (10)
	KCl (100 mM)	6.0±0.9 (7)	7.6±0.8 (13)	8.5±1.2 (11)	6.5±1.3 (8)	8.4±0.6 (10)
Basilar	U46619 (100 nM)	7.7±1.0 (6)	8.4±1.5 (11)	8.0±0.9 (10)	6.0±2.4 (7)	7.4±0.5 (10)
	KCl (100 mM)	4.7±0.7 (9)	6.2±0.8 (12)	5.9±0.9 (14)	6.0±0.5 (10)	5.8±0.7 (11)
	Endothelin-1 (5 nM)	4.3±0.6 (9)	5.76±1.04 (12)	4.7±0.8 (13)	5.3±0.7 (10)	5.2±1.0 (10)

Table 9.2 E_{max} and pEC_{50} values of CGRP in relaxing precontracted isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Pharmacological parameters	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	E_{max}	98±1 (9)	93±2 (11)	97±2 (8)	91±4 (9)	99±1 (8)
Caudal	pEC_{50}	9.53±0.22 (9)	9.76±0.20 (11)	10.84±0.34 (8)*	9.48±0.27 (9)	9.80±0.22 (8)
	E_{max}	83±3 (6)	71±4 (8)	84±5 (8)	81±8 (7)	81±5 (9)
Basilar	pEC_{50}	8.17±0.10 (6)	8.23±0.05 (8)	8.69±0.17 (8)*	8.33±0.14 (7)	8.25±0.04 (9)
	E_{max}	40±5 (8)	38±7 (9)	48±5 (11)	50±6 (9)	48±6 (6)
	pEC_{50}	8.30±0.09 (8)	8.25±0.07 (9)	8.18±0.23 (11)	8.37±0.11 (9)	8.45±0.11 (6)

E_{max}, Maximum response, expressed as % of precontraction induced by U46619 (mesenteric and caudal arteries) or endothelin-1 (basilar artery)
pEC_{50}, (-logEC_{50}, where EC_{50} is the concentration of the agonist required to produce half the maximal response)
*Significantly different ($P < 0.05$) from the corresponding value in placebo-treated rats

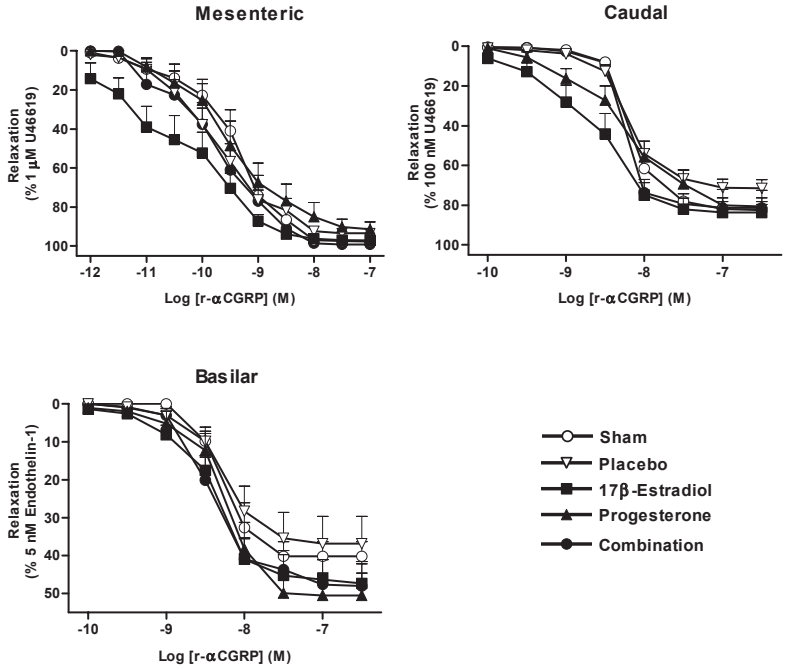


Figure 9.1. Relaxations induced by αCGRP in isolated precontracted mesenteric, caudal and basilar artery segments obtained from sham-ovariectomized rats, as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=8-11).

ectomized rats treated with 17β-estradiol than those from placebo-treated rats, but the other groups did not differ significantly from placebo (Table 9.2). In basilar artery, no differences in potency were observed between the groups. Similarly, the maximal responses to αCGRP were not different between the groups in either mesenteric, caudal arteries or basilar artery.

9.3.3 Relaxations induced by acetylcholine

Acetylcholine, like CGRP, caused concentration-dependent relaxations in all arterial segments studied. In mesenteric and basilar arteries, there were no significant differences in the E_{max} or potency of acetylcholine (Figure 9.2, Table 9.3). Further, in the case of the basilar artery, the intra-group variations were relatively high and maximal responses were less as compared to the mesenteric and caudal arteries. In the caudal artery, the responses to acetylcholine were significantly potentiated in rats treated with the combination of 17β-estradiol and progesterone ($P<0.05$) and possibly, albeit not significantly, in rats treated with 17β-estradiol alone ($P=0.06$), as compared to the placebo-treated group.

There were no significant differences in the maximal response to acetylcholine in caudal artery segments (Figure 9.2, Table 9.3).

9.3.4 Contractions induced by 5-HT

Since 2-3 mesenteric artery segments from each treatment group did not respond to 5-HT (maximal response <5% of KCl 100 mM), the data from these animals were excluded from further analysis. In the rest of the arterial segments, 5-HT produced a concentration-dependent contractile response, where neither the E_{max} nor the potency (pEC_{50}) differed amongst the study groups (Figure 9.3, Table 9.4). Similarly, there were no significant differences in the E_{max} or potency of the responses to 5-HT in the caudal and basilar artery segments (Figure 9.3, Table 9.4).

Table 9.3 E_{max} and pEC_{50} values of acetylcholine in relaxing precontracted isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Pharmacological parameters	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	E_{max}	82±6 (6)	89±3 (9)	91±3 (7)	94±2 (8)	89±5 (8)
Caudal	pEC_{50}	7.74±0.29 (6)	7.46±0.27 (9)	7.50±0.13 (7)	7.71±0.21 (8)	7.69±0.13 (8)
	E_{max}	48±7 (7)	65±5 (8)	67±10 (6)	72±4 (6)	76±6 (8)
Basilar	pEC_{50}	6.39±0.23 (7)	6.22±0.17 (8)	6.93±0.33 (6)	6.54±0.23 (6)	7.10±0.18 (8)*
	E_{max}	38±7 (5)	37 ±10 (7)	37±8 (5)	48±11 (7)	59±13 (5)
	pEC_{50}	7.50±0.52 (5)	7.21±0.35 (7)	6.78±0.16 (5)	6.57±0.25 (7)	7.42±0.33 (5)

E_{max}, Maximum response, expressed as % of precontraction induced by U46619 (mesenteric and caudal arteries) or endothelin-1 (basilar artery)
pEC_{50}, (-logEC_{50}, where EC_{50} is the concentration of the agonist required to produce half the maximal response)
*Significantly different ($P<0.05$) from the corresponding value in placebo-treated rats

Table 9.4 E_{max} and pEC_{50} values of 5-HT in contracting isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Pharmacological parameters	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	E_{max}	157±8 (7)	187±14 (9)	140±11 (10)	174±15 (7)	158±24 (9)
Caudal	pEC_{50}	6.32±0.07 (7)	6.33±0.14 (9)	6.39±0.16 (10)	6.42±0.22 (7)	6.35±0.16 (9)
	E_{max}	199±15 (7)	190±15 (10)	178±15 (9)	200±13 (7)	168±19 (8)
Basilar	pEC_{50}	6.96±0.13 (7)	6.95±0.15 (10)	7.12±0.10 (9)	6.53±0.20 (7)	7.07±0.11 (8)
	E_{max}	140±13 (6)	128±9 (9)	145±17 (8)	139±13 (8)	95±11 (6)
	pEC_{50}	6.88±0.15 (6)	7.21±0.17 (6)	7.14±0.09 (6)	6.88±0.18 (6)	7.21±0.23 (6)

E_{max}, Maximum response as percentage of the contraction induced by 100 mM KCl.
pEC_{50}, (-logEC_{50}, where EC_{50} is the concentration of the agonist required to produce half the maximal response)
*Significantly different ($P<0.05$) from the corresponding value in placebo-treated rats

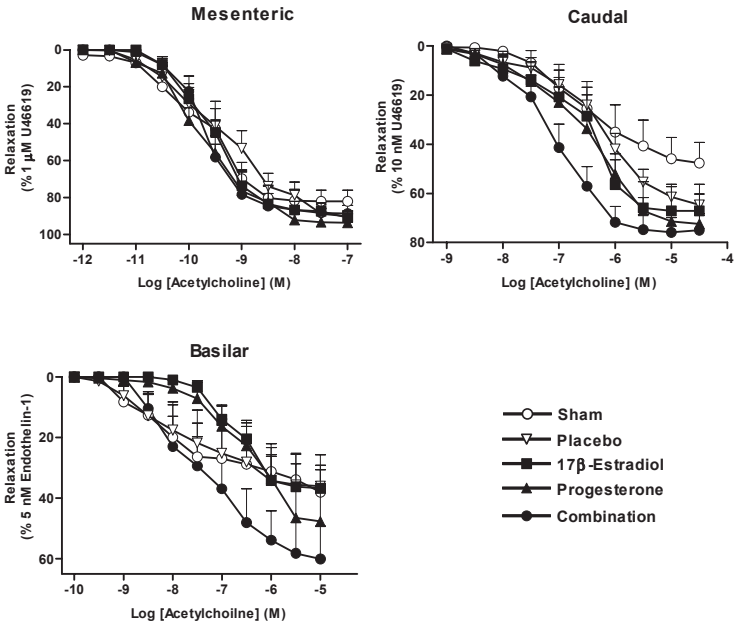


Figure 9.2. Relaxations induced by acetylcholine in isolated precontracted mesenteric, caudal and basilar artery segments obtained from sham-ovariectomized rats, as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-9).

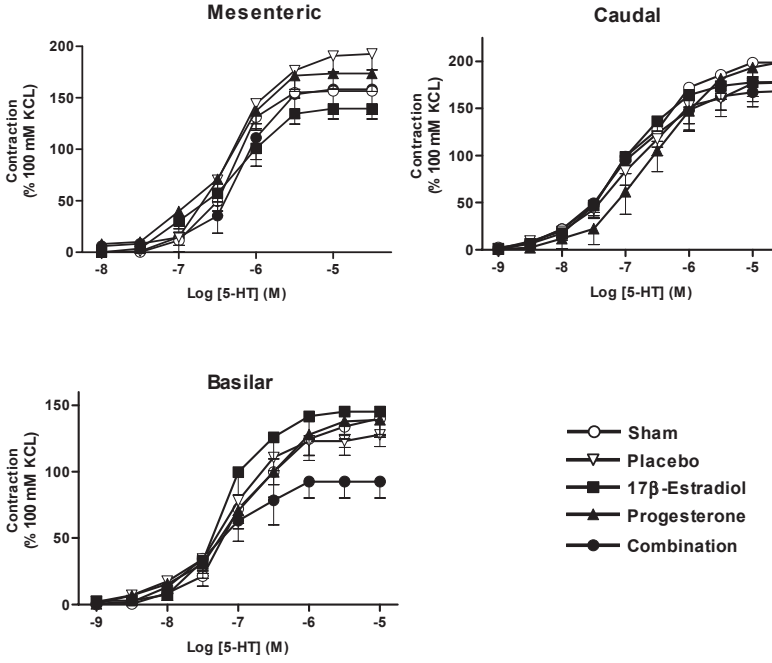


Figure 9.3. Contractions to 5-HT in mesenteric, caudal and basilar arteries segments obtained from sham-ovariectomized rats, as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=6-10).

9.4 DISCUSSION

Our results demonstrate that ovariectomy and subsequent treatment with 17β-estradiol and progesterone affect the vasoactive responses in the arteries studied. The observed changes were not generalized with respect to a particular agonist, a specific type of blood vessel or even the kind of effect being investigated (relaxant or contractile responses). The reference contractile responses to 100 mM KCl and the contractions induced by U46619 or endothelin-1 were not significantly different amongst the various treatment groups in the mesenteric, caudal or basilar artery. Thus, the observed differences in the responses to the agonists cannot be attributed to differences in the blood vessel muscle mass or a general, non-specific mechanism.

The maximum relaxation induced by CGRP was highest in the mesenteric artery, followed by that in the caudal and basilar arteries. Similarly, CGRP was a more potent relaxant (i.e. it induced relaxations at lower concentrations) in the mesenteric artery as compared to the other two arteries. This may be due to the fact that CGRP is reported to be more efficacious and potent in resistance blood vessels (35), such as the second order mesenteric arteries, which are vital in regulating total vascular tone, than in conducting blood vessels like caudal and basilar arteries. The potency of CGRP in both mesenteric and caudal arteries was significantly increased in 17β-estradiol-treated rats as compared to placebo-treated rats. In keeping with these findings, Gangula et al (36) have also reported that ovariectomy decreases the CGRP potency; moreover, in pregnancy, where 17β-estradiol levels are increased, the potency of CGRP is augmented (36). It is important to highlight that the CGRP-induced relaxations are endothelium-independent in the rat mesenteric (31) and caudal (32) arteries, similar to the observations in the human cerebral(37), meningeal (37) and coronary (38) arteries. Interestingly, unlike the mesenteric and caudal arteries, the basilar artery did not exhibit any increase in the responses to CGRP, although the potency of CGRP is in line with the previous reports in the rat basilar artery (39). This might be because the second-order mesenteric artery is resistance artery whereas the basilar is a conducting artery and in conducting arteries the potency of CGRP is less (35, 38, 40). Indeed, the enhanced responses to CGRP by 17β-estradiol do not seem to be a systemic phenomenon, as estrogen is reported to selectively increase the sensory nociceptor innervations of mesenteric arterioles in female rats (30). In a recent study in human vascular smooth muscle cells, dexamethasone was reported to increase mRNA expression of RAMP1 and CRLR (41), which are essential components of $CGRP_1$ receptors. Hence, steroidal hormones may regulate the synthesis as well as the receptor expression for CGRP. These findings clearly suggest that 17β-estradiol increases the responses to CGRP. Further, when the rats were treated with the combination of 17β-estradiol and progesterone, the effect of the latter was reversed, whilst the sham-operated rats or those treated only with progesterone did not significantly differ from the placebo-treated animals. Similarly, other authors have also reported opposing actions of 17β-estradiol and progesterone (42, 43). Also, the CGRP receptor expression in rats is regulated in divergent ways by these female sex steroids (44).

The responses to acetylcholine in mesenteric and basilar arteries were not significantly different amongst various treatment groups. This seems contrary to the fact that female sex hormones have been reported to (specifically) affect the vasoactive responses in blood vessels by enhancing the synthesis of nitric oxide, and by a direct Ca^{2+} antagonistic effect on vascular smooth muscle (45-47), hence facilitating endothelium-dependent responses. However, it has recently been shown in the rat mesenteric artery that relaxations mediated by nitric oxide, which is released by acetylcholine, are decreased and, in contrast, there is an increase in the part of the acetylcholine response mediated by endothelial-derived relaxing factor (EDRF). Hence, overall, no changes were observed in acetylcholine-induced relaxations(17), as also reinforced in the present study. Further, as the potency of CGRP was enhanced in the mesenteric artery, the mechanism involved seems to be independent of endothelium-dependent relaxations. In the case of the caudal artery, there was a significant increase in the potency of acetylcholine in the rats treated with

the combination of 17β-estradiol and progesterone. In fact, a similar tendency was observed in the rats treated with 17β-estradiol alone; this could be due to endothelium-dependent mechanisms.

In mesenteric arteries, the potency of 5-HT amongst the different treatment groups was in line with the previously reported values in rats (48). But, no significant differences were observed in the E_{max} and the potency of 5-HT in the various groups. In the basilar artery, there were no significant differences in the efficacy or potency of 5-HT in the rats exposed to different hormonal interventions; this is in accordance with previous observations in the rat isolated anterior cerebral arteries, where female sex steroids did not alter the functional activity of 5-HT (23). In contrast, in the rabbit basilar artery estrogen withdrawal may result in hypersensitivity to 5-HT (49), and an acute incubation with estrogen is reported to decrease the sensitivity to 5-HT and other contractile agents (28, 29).

The plasma levels of 17β-estradiol in the ovariectomized rats treated with 17β-estradiol pellets were ~2.5 times the highest levels encountered in normal estrous cycle in rats; this may be a potential limitation of this study. Further, ideally we would have performed our study in dural arteries. However, as pointed out above, these are too small to be studied in a myograph. Therefore, we chose blood vessels that may represent the meningeal artery based on their innervations by CGRP-containing nerves, endothelium-independent relaxant responses to CGRP, or their cranial location. Finally, since we performed our study on rat tissues, the results obviously may not directly be extrapolated to the human situation.

It is important to note that several studies addressing the effect of sex steroids on vascular responses have used acute incubation with these hormones in arteries obtained from pigs (28), rhesus monkeys (27) and even humans (29), where suppression of the contractile effects and potentiation of the vasodilator responses was observed (50). These effects are attributed to rapid non-genomic mechanisms, whereas in our experimental set-up the arteries were isolated and suspended in Krebs solution free of female sex hormones. Therefore, we assume that the effects observed in the present study are the result of a chronic treatment with the female sex hormones, mediated by a classical genomic mechanism. In this respect, it is essential to distinguish between the genomic and non-genomic actions of sex steroids; as the non-genomic actions are induced rapidly, it can thus not be excluded that in the absence of these hormones the effects will revert to the basal state (51, 52). Future studies on the effects of estrogen withdrawal, which clearly fall beyond the scope of the current study, may shed more light on the balance between the rapid, non-genomic effects and the slower, genomic effects of sex steroids (53).

Overall, the treatment with 17β-estradiol augments the vasorelaxation potency of CGRP in the mesenteric and caudal arteries, and of acetylcholine in the caudal artery. If a similar mechanism is present in meningeal arteries, our results may provide a new avenue to study the effects of sex steroids on vascular reactivity.

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Chapter 10

Rat Carotid Artery Responses to α-adrenergic Receptor Agonists and 5-HT after Ovariectomy and Hormone Replacement

Based on: S. Mehrotra, S. Gupta, C.M. Villalón, F. Boomsma, P.R. Saxena, A. MaassenVanDenBrink, Headache, Published online: 12-Jul.

ABSTRACT

We compared the contractile responses to α-adrenergic receptor agonists and 5-HT in the rat carotid artery after ovariectomy and subsequent hormone replacement with 17β-estradiol, progesterone or their combination. The prevalence of migraine is higher in females than in men, and changes in 17β-estradiol levels seem to affect the frequency of attacks in female migraineurs. However, the underlying mechanisms are not yet completely understood. Female Sprague-Dawley rats were bilaterally ovariectomized and divided into five groups; sham-operated animals and ovariectomized animals treated with placebo, 17β-estradiol, progesterone or a combination of 17β-estradiol and progesterone. Segments of carotid artery were isolated and mounted in Mulvany myographs. Blood samples were taken to measure plasma levels of epinephrine and norepinephrine. The potency of norepinephrine in ovariectomized rats was significantly reduced in animals treated with progesterone compared to those with placebo. In placebo treated ovariectomized animals, there was an noticeable response to α_2-adrenceptors on comparing with sham operated and ovariectomized rats treated with progesterone, 17β-estradiol or the combination of these hormones. The plasma levels of norepinephrine and epinephrine were not significantly affected by either ovariectomy or subsequent replacement of 17β-estradiol, progesterone, or the combination of these hormones. The contraction to 5-HT was significantly reduced (pEC_{50}) in animals having circulating sex hormones as compared to placebo treated ovariectomized animals. Taken together, our results indicate that circulating progesterone and/or 17β-estradiol may reduce contraction of the rat carotid artery in response to norepinephrine or 5-HT. This may be one of the mechanisms through which female sex hormones may aggravate migraine in women.

10.1 INTRODUCTION

Migraine is three times more common during reproductive years in women than in men (1). This gender difference in the prevalence of migraine appears to be related to fluctuating levels of female sex hormones during the menstrual cycle (2). Plasma levels of 17β-estradiol and progesterone in women are at their lowest just before periods (3), and there seems to be an association between 17β-estradiol "withdrawal" and migraine attacks, which tend to cluster just before or during menstruation in a majority of afflicted women (4-8). In contrast, the sustained high concentration of 17β-estradiol during pregnancy frequently results in headache relief (9), although migraine with aura seems to have a higher prevalence (10). The frequency of migraine attacks increases after child birth, when 17β-estradiol levels decline (11). Moreover, in two controlled trials, percutaneous 17β-estradiol reduced the frequency of migraine headaches (12, 13). Thus, migraine severity correlates with changes in estrogen levels and it is reasonable to assume that there may be a causal relationship.

It is now well recognized that migraine is a neurovascular syndrome, where the headache phase seems to be associated with cranial extracerebral vasodilatation (14-17). Although the meningeal artery is thought to play a major role in the pathophysiology of migraine (18, 19), animal experimental models based on carotid vasoconstriction in the anesthetized animals, including the cat (20, 21), dog (16, 22), pig (23, 24) and rabbit (25), clearly show the effectiveness of antimigraine compounds, particularly those acting via 5-hydroxytryptamine or α-adrenergic receptors. Moreover, *in vitro* studies show that the rat (26, 27) as well as rabbit (28, 29) isolated carotid artery contracts in response to both 5-HT(26, 28) and α-adrenergic receptor agonists (27, 29). Interestingly, female sex hormones are known to influence the vasoconstriction elicited by these agonists. For instance: (i) the contractile responses to 5-HT and α-adrenergic receptor agonists in the rat (30) and rabbit (31) isolated aorta decrease after treatment with 17β-estradiol; (ii) in ovariectomized rats, 17β-estradiol replacement reduces the vasoconstriction to α_2-adrenergic receptor activation (32); and (iii) in ovariectomized rabbits, 17β-estradiol depresses the contractions mediated by α_2-adrenergic receptors in the femoral artery, but not in the saphenous vein (33). Furthermore, 17β-estradiol (34) and progesterone (35) have

been shown to decrease the sympathetic tone, and it has been suggested that migraineurs have a low sympathetic tone (36).

In the light of these observations, the present study sets out to investigate the influence of female sex hormones on contractile responses to norepinephrine, 5-HT and the α_1-adrenergic receptor agonists phenylephrine and A61603 in isolated carotid arteries obtained from sham-operated and ovariectomized rats; the latter were subsequently treated with either placebo, 17β-estradiol, progesterone or the combination of both hormones.

10.2 MATERIALS AND METHODS

10.2.1 Animal groups

Female Sprague-Dawley rats (body weight: 190-260 g, age: 8-11 wks) were purchased from Harlan Netherlands (Horst, The Netherlands). All animals were given free access to food and water. After one week of acclimatization (Day 0), the rats were anesthetized with isoflurane anesthesia for either a sham-operation (exteriorization but not the removal of ovaries; Group 1) or bilateral ovariectomy. One week later (Day 7) and under the same anesthesia, the ovariectomized rats were subcutaneously implanted with a pellet releasing over a 21-day period either placebo (Group 2), 0.25 mg 17β-estradiol (Group 3), 50 mg progesterone (Group 4) or the combination of the two hormones (Group 5). As reported recently (37), in these animals the concentrations of both 17β-estradiol and progesterone (Day 0; 25±6 pg ml^{-1} and 24±6 ng ml^{-1}, *n*=36 each, respectively) decrease following ovariectomy (Day 7; 23±4 pg ml^{-1} and 6±2 ng ml^{-1}, *n*=26 each, respectively) and increase after implantation of hormones pellets in ovariectomized animals; for example, on Day 21 (i.e. 14 days after treatment with respective hormones pellets), plasma concentrations of 17β-estradiol and progesterone were 187±45 pg ml^{-1} and 17±4 ng ml^{-1}, *n*=5-7 each, respectively.

10.2.2 Measurements of plasma concentration of norepinephrine and epinephrine

Blood samples were collected from the tail vein under isoflurane anesthesia three times: Day 0 (before ovariectomy), Day 7 (only in ovariectomized animals) and Day 21 (21 days after sham-operation or 14 days after pellet implantation in ovariectomized animals). After collection in chilled heparinized tubes containing 3 mg glutathione S-transferase, blood was immediately centrifuged (3000 g, 15 min, 4°C) to separate plasma, which was stored at -80°C until the catecholamines were assayed using high performance liquid chromatography with fluorimetric detection (38). The values obtained in the different groups of animals on Day 0 (before sham operation or ovariectomy) and on Day 7 in ovariectomized animals (before pellet implantation) were combined, while the values obtained on Day 21 in the 5 groups are presented separately.

10.2.3 Isolation of the carotid artery

Rats were sacrificed on Day 25-28 (i.e. 25-28 days after sham operation or 18-21 days after pellet implantation in ovariectomized animals) by intraperitoneal injection of sodium pentobarbital (100 mg kg^{-1}). The right carotid artery was isolated and kept in Krebs buffer solution (composition: 119 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.1 mM glucose; pH 7.4; 4°C). Immediately after removal, the carotid artery was cut into 12 mm ring segments that were mounted in Mulvany myographs (Danish myo technology a/s, Aarhus, Denmark) between two parallel titanium wires with tension normalized to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development (39). The myograph chambers contained Krebs buffer solution (see above), which was aerated with 95% O_2 and 5% CO_2 at 37°C. Changes in tension of the rat carotid artery segments were measured with isometric force transducers (Danish myo

technology a/s, Aarhus, Denmark) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

The carotid artery segments were allowed to equilibrate for at least 30 min and were washed every 15 min. They were then challenged at 30-min intervals twice with 30 mM KCl to verify the reproducibility of responses and then with 100 mM KCl to obtain a reference contractile response for each vessel segment (see data analysis). Subsequently, the vessel segments were stabilized and concentration (10^{-10} M to $3x10^{-5}$ M) response curves to norepinephrine (α_1-, α_2- and β_1-adrenergic receptor agonist), 5-HT (vascular 5-HT_{1B} and 5-HT_2 receptor agonist), phenylephrine and A61603 (both α_1-adrenergic receptor agonists) were constructed to investigate the involvement of their respective receptors. Only one concentration response curve was constructed in each arterial segment. The concentration response curves to norepinephrine were also made in arterial segments incubated for 30 min with either: (i) vehicle, (ii) the α_1-adrenergic receptor antagonist prazosin (100 nM), or (iii) the α_2-adrenergic receptor antagonist rauwolscine (100 nM). Moreover, it may be noted that all experiments with norepinephrine were performed in the presence of cocaine (1 μM), corticosterone (3 μM) and propranolol (100 nM) to eliminate, respectively, the neuronal and extra-neuronal reuptake and the possible vasorelaxation via β-adrenergic receptors. The ethical committee of Erasmus MC dealing with animal experiments approved this study protocol.

10.2.4 Data analysis

As described earlier (40, 41), the contractile responses to all agonists were expressed as percentage of the contraction induced by 100 mM KCl in the respective segments and the data were analyzed (Graph Pad Prism 3.01, Graph Pad Software Inc., San Diego, CA, U.S.A.) to obtain, in each case, agonist E_{max} (maximum plateau response) and pEC_{50} (negative logarithm of the molar concentration eliciting 50% of the E_{max}). It must be highlighted that when a plateau was not reached with an agonist, the response at its highest concentration was considered as E_{max}, except for the experiments in the presence of an antagonist, where the E_{max} in the presence of an antagonist was assumed to be equal to the control E_{max} in case a plateau was not reached. The E_{max} and pEC_{50} values represent, respectively, the *efficacy* and *potency* of an agonist to contract the rat carotid artery. Assuming a slope of unity, the potency of the antagonists was expressed as apparent pK_b (negative logarithm of the antagonist concentration, whereby the agonist concentration eliciting a certain response is increased by a factor of 2). Statistical significance was determined by ANOVA followed by Dunnett's multiple comparison tests with differences considered significant at $P \leq 0.05$. All data are presented as means±s.e.m. and *n* represents the number of carotid artery rings used, each obtained from a different animal.

10.2.5 Compounds

The compounds used in the present study (obtained from the sources indicated) were the following: 5-hydroxytryptamine creatinine sulphate (serotonin; 5-HT), L-phenylephrine hydrochloride, norepinephrine, corticosterone, glutathione S-transferase and heparin sulfate sodium salt (all from Sigma, St. Louis, MO, U.S.A.), prazosin hydrochloride (Bufa Chemie b.v., Castricum, The Netherlands), cocaine (local pharmacy at Erasmus Medical Center, Rotterdam, The Netherlands), propranolol hydrochloride (Sigma, St. Louis, MO, U.S.A.) and rauwolscine dihydrochloride (RBI, Natick, MA, U.S.A.). Corticosterone was dissolved in dimethylsulfoxide and prazosin hydrochloride in ethanol, while all other compounds were dissolved in distilled water. The above vehicles had no effect on the agonist responses at the concentrations employed. The placebo and hormone pellets were purchased from Innovative Research of America (Sarasota, FL, U.S.A.).

10.3 RESULTS

10.3.1 Contraction to KCl

All segments contracted in response to 100 mM KCl. There were no significant differences in KCl-induced contractions between sham-operated (2.8±0.9 mN) and ovariectomized animals treated with placebo (3.3±0.6 mN), 17β-estradiol (4.3±0.7 mN), progesterone (3.7±0.6 mN) or the combination of these hormones (2.6±0.3 mN).

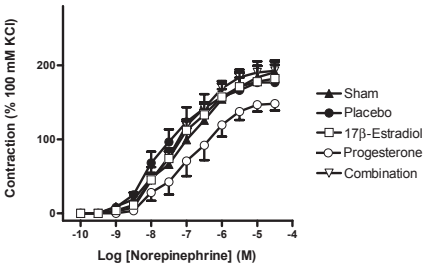

Figure 10.1. Contractions to norepinephrine in isolated carotid arteries obtained from sham-operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each)

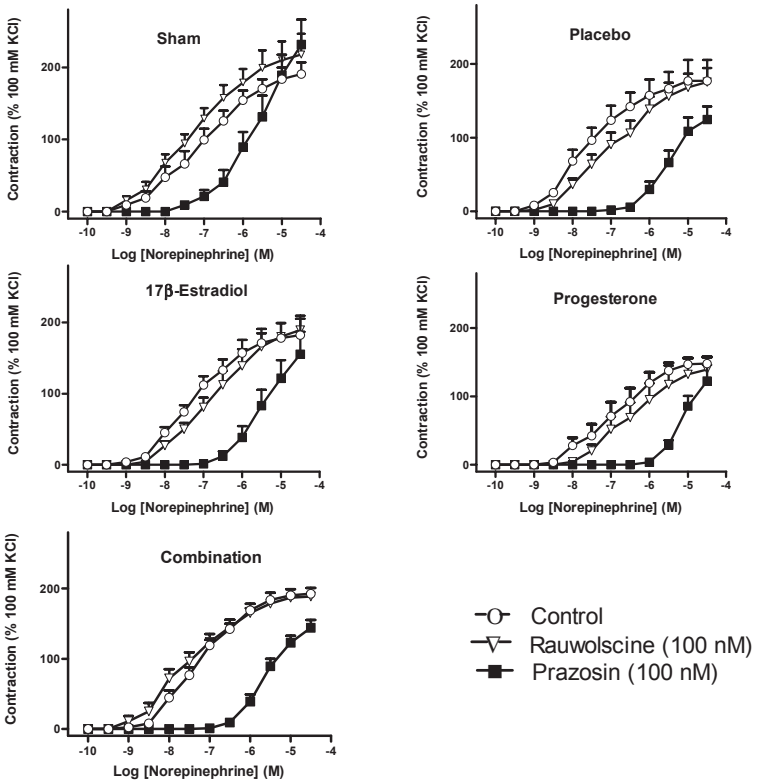

Figure 10.2. Contractions to norepinephrine in the absence (control) and in the presence of prazosin or rauwolscine (both 100 nM) in sham operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

Table 10.1 E_{max} and pEC_{50} values of norepinephrine, phenylephrine, A61603 and 5-HT in contracting isolated carotid artery segments obtained from rats after different interventions.

Pharmacological parameters		Sham-ovariectomized rats (*n*=5)	Ovariectomized rats treated with			
			Placebo (*n*=5)	17β-estradiol (*n*=5-6)	Progesterone (*n*=5-6)	Progesterone + 17β-estradiol (*n*=7)
Norepinephrine	E_{max}	189±15	146±24	182±23	147±11	193±8
Phenylephrine	pEC_{50}	7.1±0.2	7.7±0.1	7.3±0.1	7.0±0.2*	7.3±0.1
	E_{max}	195±49	244± 90	104±28	103±18	158±21
A61603	pEC_{50}	7.2±0.2	6.8±0.1	7.0±0.5	6.0±0.2	7.1±0.2
	E_{max}	88±4	108±15	66±23	78±26	133±24
5-HT	pEC_{50}	5.9±0.1	5.8±0.1	5.5±0.2	5.5±0.10	6.0±0.2
	E_{max}	172±16	210±45	83±20*	148±18	145±18
	pEC_{50}	5.8±0.2*	6.6±0.2	5.7±0.1*	5.6±0.2*	5.7±0.2*

E_{max}, Maximum response expressed as % of contraction induced by 100 mM KCl; pEC_{50}, concentration required to produce 50% of maximal response. *Significantly different ($P < 0.05$) from the corresponding value in placebo-treated rats.

10.3.2 Contractions mediated by α-adrenergic receptors

In all carotid artery segments investigated, the endogenous ligand norepinephrine induced concentration-dependent contractions (Figure 10.1). The potency (pEC_{50}) of norepinephrine to induce contractions was significantly less in vessel segments obtained from rats treated with progesterone as compared to those treated with placebo (Figure 10.1, Table 10.1). Figure 10.2 shows the concentration response curves to norepinephrine on carotid artery segments obtained

from sham-operated rats as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones, in the absence (control) or presence of the adrenergic receptor antagonists, prazosin (α_1) and rauwolscine (α_2). As compared to control responses, prazosin induced a significant rightward shift of the concentration response curve to norepinephrine in all groups; the corresponding pK_b values in the different groups, which were not significantly different from each other, were: sham-operated rats (8.38±0.35) and ovariectomized rats treated with either placebo (9.28±0.23), 17β-estradiol (9.34±0.29), progesterone (9.09±0.24) or the combination of these hormones (9.00±0.21). In contrast to the results obtained with prazosin, rauwolscine failed to induce a significant shift of the concentration response curves to norepinephrine in animals with endogenous (sham-operated) or exogenous (ovariectomized, but treated with hormone pellets) female sex steroids; interestingly, only in ovariectomized animals treated with placebo did rauwolscine significantly antagonize the contractions to norepinephrine (pK_b: 8.21±0.58; Figure 10.2). As shown in Figure 10.3, phenyl-

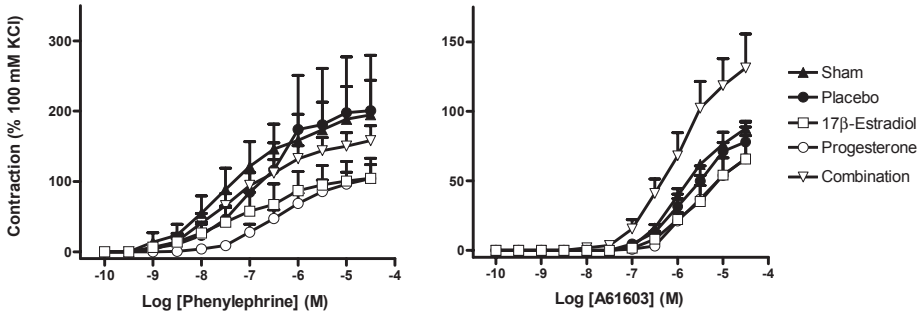

Figure 10.3. Contractions to phenylephrine and A61603 in sham operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

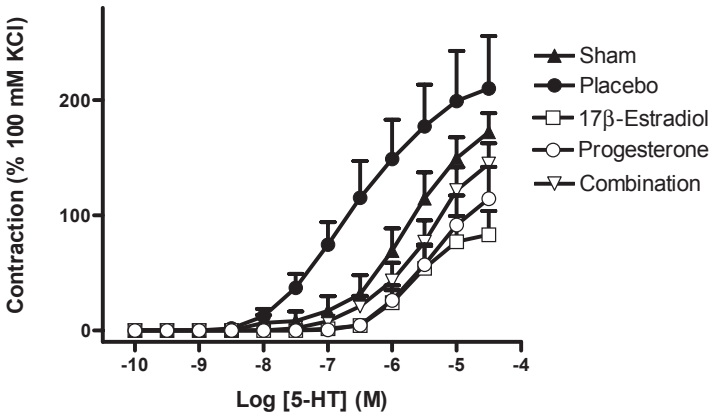

Figure 10.4. Contractions to 5-HT in sham operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

ephrine induced concentration-dependent contractions that were not different between the groups, although its potency tended to be somewhat lower in progesterone-treated animals than in the other groups (Table 10.1). Likewise, the contractions to A61603 were also not significantly different between the groups (Figure 10.3; Table 10.1).

10.3.3 Contractions to 5-HT

The contractile responses induced by 5-HT on the carotid artery rings from different groups of animals are shown in Figure 10.4. The pEC_{50} (potency) of 5-HT was greater in ovariectomized animals treated with placebo than in those treated with 17β-estradiol, progesterone, the combination of these hormones, or the sham-operated animals (Table 10.1). The E_{max} of 5-HT was significantly smaller in the ovariectomized animals treated with 17β-estradiol than in the placebo-treated group, while the E_{max} values obtained in the other groups were not different from those in the placebo group (Table 10.1).

10.3.4 Plasma concentrations of norepinephrine and epinephrine

The plasma concentrations of norepinephrine and epinephrine were not significantly different before (Day 0: 324±45 pg ml^{-1} and 242±34 pg ml^{-1}, respectively) or after (Day 7: 361±27 pg ml^{-1} and 344±54 pg ml^{-1}, respectively) ovariectomy. Similarly, the plasma levels of norepinephrine and epinephrine were not affected by treatment with 17β-estradiol and/or progesterone pellets (Figure 10.5).

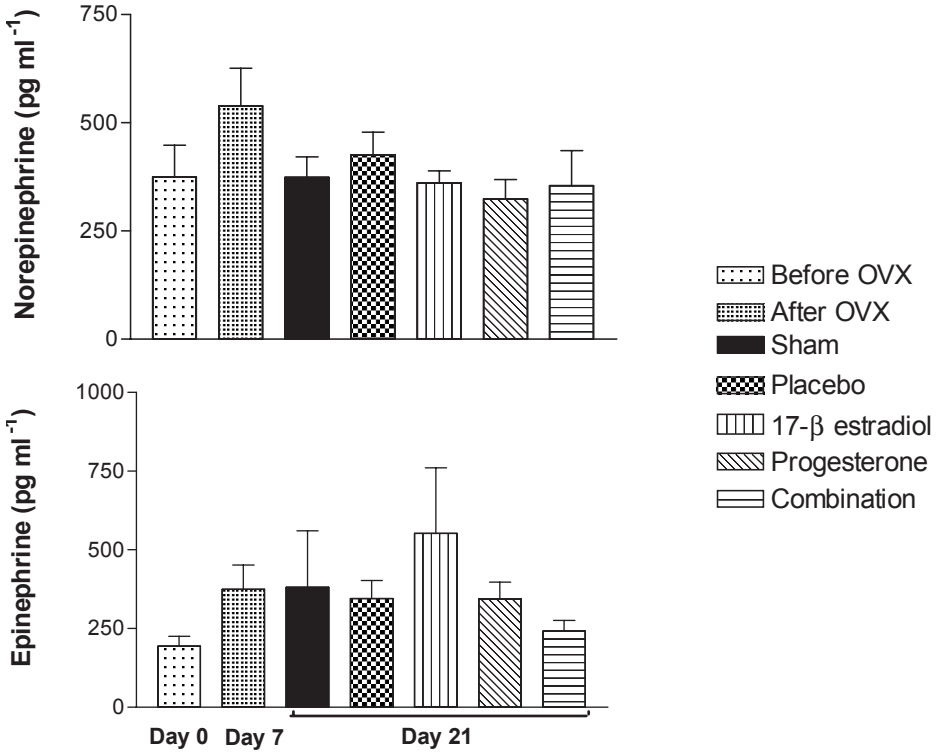

Figure 10.5. Plasma concentrations of norepinephrine and epinephrine in sham-operated or ovariectomized (OVX) rats on Day 0 (just before sham-operation or ovariectomy, *n*=7), Day 7 (7 days after ovariectomy before pellet implantation, *n*=10), and Day 21 (21 days after sham operation, *n*=4; or 14 days after implantation in ovariectomized animals of pellets containing placebo, *n*=8; 17β-estradiol, n=7; progesterone, *n*=8; or their combination, *n*=4). No value differed significantly from that in placebo-treated rats

10.4 DISCUSSION

The results of our study demonstrate that ovariectomy followed by the subsequent treatment with female sex hormones affected the contractile responses of the rat isolated carotid artery to both α-adrenergic receptor agonists and 5-HT. This effect is unlikely to be due to differences in muscle mass of the carotid artery, since we corrected for differences in muscle mass and, moreover, the contractile responses to 100 mM KCl were similar across all treatment groups. Furthermore, our results suggest that the influence of female sex hormones is dependent on the agonist used (i.e. norepinephrine or 5-HT); thus, it seems improbable that an aspecific mechanism may account for the effects that we observed in our study. Admittedly, the plasma levels of 17β-estradiol reached after pellet implantation were about twice as high as the highest levels reached in a normal menstrual cycle; future studies may shed more light on the influence of different estrous levels of 17β-estradiol on vasoactive responses.

17β-estradiol promotes vasodilatation via both genomic and non-genomic mechanisms that: (i) generate agents, such as nitric oxide, cGMP, cAMP, adenosine and prostacyclin (42, 43); and (ii) alter ion channel activity (44). Although endothelial factors might be involved, some lines of evidence suggest that steroids-induced vasodilatation is also mediated by endothelium-independent mechanisms (45-47). Furthermore, sex steroids may induce vasodilatation by inhibiting Ca^{2+} currents (48) and by activating K^{+} channels (49, 50). However, the effects of sex hormones on contraction and dilatation are divergent, which may be due to several factors, including species differences, the vascular bed under study and the experimental conditions (e.g. a specific agonist, duration and dose of steroid pretreatment).

The vasoconstrictor potency of norepinephrine in ovariectomized rats treated with progesterone, but not 17β-estradiol, was significantly attenuated. This observation seems to differ from the report that rat aorta contraction via α_1-adrenergic receptor remains unaffected by both 17β-estradiol and progesterone (51). However, 17β-estradiol and progesterone may have opposing actions (52-56). Indeed, our results are in accordance with the finding showing that pretreatment with progesterone, but not estrogen, inhibited the contractions to norepinephrine in the rat tail artery (57). The decrease in the sensitivity to norepinephrine in ovariectomized rats treated with progesterone, suggesting less availability of postsynaptic α-adrenergic receptors, may lead to cranial vasodilatation and, therefore, proneness to migraine headache (15-17). This is in line with a recent investigation showing that higher urinary progesterone metabolites were positively correlated with headache outcome measures during the luteal phase of the menstrual cycle (9). In addition, it has been reported that plasma progesterone levels show an irregular pattern in migraine patients (58). Intriguingly, it has been suggested that migraineurs have a reduction in sympathetic function as compared to nonmigraineurs (36). In addition, migraine with aura patients have a resting supine sympathetic hypofunction (59), which seems to play an important role in the maintenance of headaches (60).

Interestingly, our results also point to an increased function of α_2-adrenergic receptors after ovariectomy without hormonal replacement (placebo treatment). This is in agreement with observations that α_2-adrenergic responsiveness decreases in blood vessels obtained from ovariectomized rabbits treated with 17β-estradiol (33), As placebo-treated animals do not have circulating female sex hormones, these animals might be considered as a model for the menopause conditions in women. Keeping this in mind, this increased function of α_2-adrenoceptors may, at least partly, account for the increased blood pressure in females after menopause (61). Admittedly, this interesting finding should be explored further before any definite conclusions can be drawn. Both 17β-estradiol (34) and progesterone (35) are known to decrease the sympathetic tone, a fact that may strengthen the effects of female sex hormones on the expression of α-adrenergic receptors. Unfortunately, our study could not confirm differences in the plasma levels of norepinephrine or epinephrine amongst the different groups, probably due to the relatively small number of animals used and the variability in the measurements.

It is noteworthy that, in contrast to our findings with norepinephrine, the contractile responses to the α_1-adrenergic receptor agonists, phenylephrine and A61603 did not differ in the different groups. Although this seems to be in contrast to the report that 17β-estradiol attenuated the vasoconstrictor action of phenylephrine in the rat mesenteric artery (62), others have reported an upregulation of the α_1-adrenergic receptor in mandibular glands (63) as well as no change (saphenous vein) or a decrease (femoral artery) in the contractile response to norepinephrine (33) in the rat. In agreement with Gisclard et al. (33), our findings also suggest that changes in female sex hormones mainly perpetuate their effect via altered α_2-adrenergic receptor function in the rat blood vessels. Admittedly, additional studies using selective α_2-adrenergic receptor agonists such as BHT-933 (64), which clearly fall beyond the scope of the present investigation, may further clarify the mechanisms involved.

With regards to 5-HT, we found that the contractile response in the carotid artery was reduced in the presence of both 17β-estradiol and progesterone. This is in agreement with other studies demonstrating that the aortic contraction to 5-HT was decreased after treatment with 17β-estradiol in rats (30) as well as rabbits (31).

The difference between the responses mediated by activation of α_1/α_2-adrenergic receptors in the presence of progesterone and of 5-HT receptors in the presence of both sex hormones may be due to several factors, including differences in: (i) the density of α_1/α_2-adrenergic and 5-HT_{1B}/5-HT_{2A} receptors in the carotid artery segment; and (ii) the second messenger signaling of α_1-adrenergic and 5-HT_{2A} receptors (both involve activation of G_q proteins and phospholipase C with a resulting increase in phosphoinositol turnover and elevation of intracellular Ca^{2+}), as well as of α_2-adrenergic and 5-HT_{1B} receptors (both involve activation of $G_{i/o}$ proteins and inhibition of adenylyl cyclase with a resulting decrease in c-AMP levels). Accordingly, differences in the responsiveness of these receptors in ovariectomized animals could be mediated via the same signaling pathways.

Taken together, our results suggest that: (i) circulating progesterone, but not 17β-estradiol, reduces the potency of norepinephrine to contract the rat isolated carotid artery, while the absence of progesterone and 17β-estradiol most probably leads to an increased function of α_2-adrenergic receptors; and (ii) progesterone, 17β-estradiol as well as their combination attenuate the contraction to 5-HT. These findings, if true for human cranial blood vessels, could possibly provide a mechanism through which female sex hormones may aggravate migraine in women.

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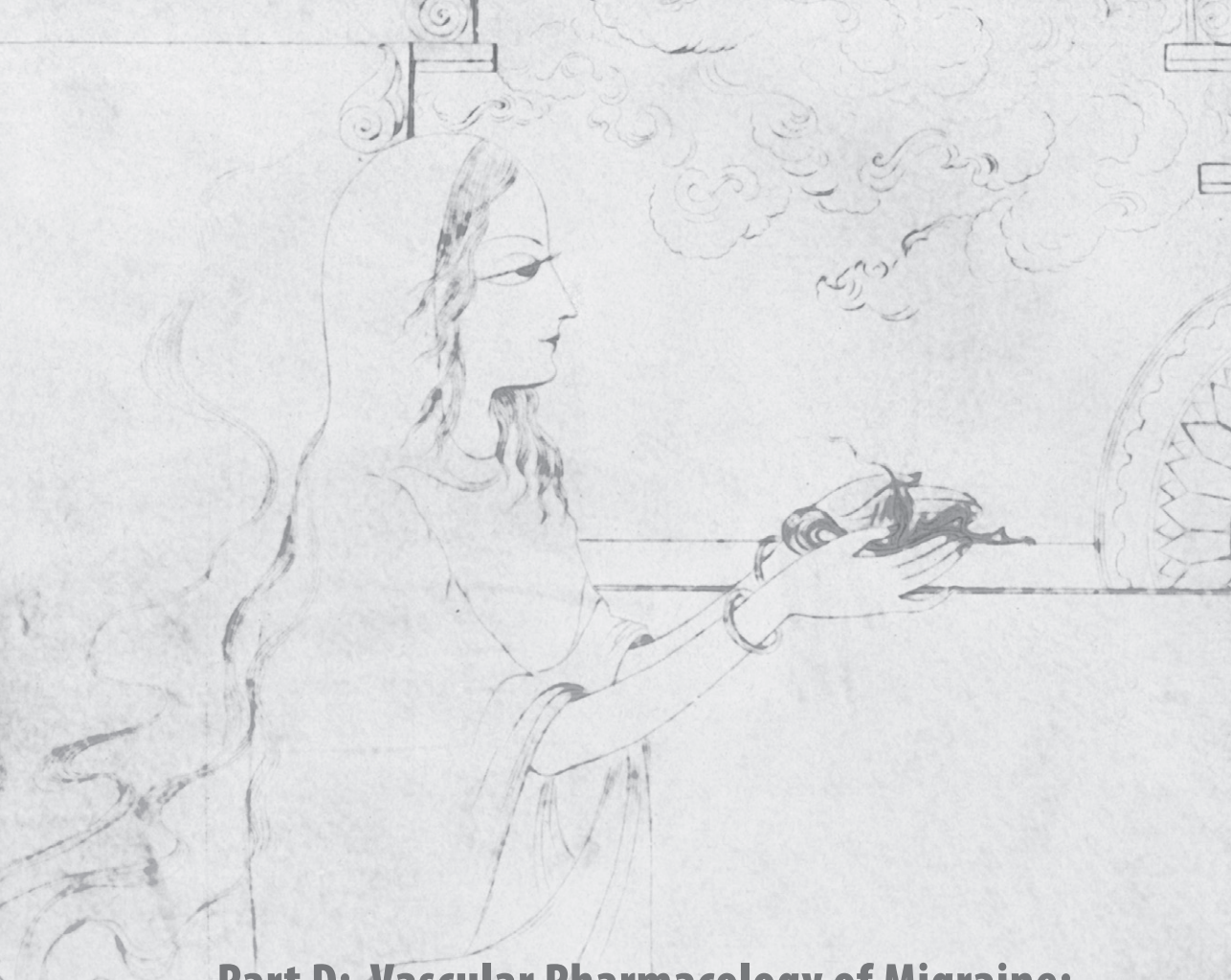

Part D: Vascular Pharmacology of Migraine: *In vivo* Approach

Chapter 11

Intravital Microscopy on a Closed Cranial Window in Mice: a Model to Study Trigeminovascular Mechanisms Involved in Migraine

Based on: S. Gupta, S. Akerman, A.M.J.M. van den Maagdenberg, P.R. Saxena, P.J. Goadsby, A. MaassenVanDenBrink, Cephalalgia, In press

ABSTRACT

The purpose of the study was to develop a mouse model to study trigeminovascular mechanisms using intravital microscopy on a closed cranial window. In addition, we studied exogenous and endogenous calcitonin gene-related peptide (CGRP)-mediated vasodilatation in dural arteries. Arteries in C57BL/6Jico mice were constricted with endothelin-1, which reduced the baseline diameter by 65-75%. Subsequently, vasodilatation was induced by α-CGRP, capsaicin or transcranial electrical stimulation of perivascular trigeminal nerves in the absence or presence of different concentrations of BIBN4096BS or sumatriptan. α-CGRP and capsaicin both induced vasodilatation in preconstricted arteries. Transcranial electrical stimulation also induced current-dependent relaxations of dural arteries with 100 μA producing maximal dilatation in the control group. BIBN4096BS blocked the responses evoked by α-CGRP, capsaicin as well as electrical stimulation, whereas sumatriptan only attenuated vasodilatation induced by electrical stimulation. This model is likely to prove useful in dissecting elements of the trigeminovascular system and for exploring pathophysiological aspects of migraine, especially in future studies using transgenic mice with mutations relevant to those observed in patients with migraine.

11.1 INTRODUCTION

Migraine is a common, chronic, episodic and disabling neurovascular disorder with largely unknown pathophysiology. It is widely believed that the headache phase of migraine involves distension of cranial blood vessels and activation of trigeminovascular nociceptive pathways (1, 2), or at least the perception of that activation (3). Electrical and chemical stimulation of the trigeminal innervation releases a range of vasoactive peptides, such as calcitonin gene-related peptide (CGRP) and substance P, resulting in dilatation of these arteries. Based on this principle, the closed cranial window model in rats was developed by Williamson *et al*. (4). This innovative technique in rats involves thinning of parietal bone, thus allowing a continuous monitoring of meningeal blood vessels without opening the skull. Any changes in vessel diameter as a result of administration of various pharmacological agents or by transcranial electrical stimulation can be studied. This approach offers the possibility of directly studying various receptor systems implicated in modulating the trigeminovascular system and thus their potential role in migraine. The use of intravital microscopy in rats has led to a better understanding of the underlying mechanism of trigeminal activation (4-6) and dural vasodilatation (7-9), which are hallmarks of migraine pathophysiology. The current experimental setup of intravital microscopy uses wild-type animals (e.g. rats), whereas more advanced animal models, preferably with a genetic predisposition to disease, would be preferred in view of recent advances in genetic research in migraine (10). Until now, three migraine genes have been identified in families with familial hemiplegic migraine (FHM), a rare autosomal dominant subtype of migraine with aura (11-13). FHM genes CACNA1A, ATP1A2 and SCN1A are all involved in ion transport and encode subunits of $Ca_v2.1(\alpha_{1A})$ voltage-dependent P/Q type calcium channels, Na^+/K^+ pumps, or neuronal voltage-gated $Na_v1.1$ sodium channels, respectively (11-13). However, the importance of the FHM genes in common migraine still needs validation.

The identification of migraine gene mutations in patients provides unique opportunities to generate transgenic mice with specific pathogenic mutations (e.g. knock-in mice), and thus a genetic predisposition to the disease. The recently described transgenic knock-in mouse model expressing the human R192Q pure FHM-1 mutation showed pure gain of functional effects (e.g. an increased calcium influx through mutant calcium channels, an increased neurotransmitter release, and a reduced threshold for cortical spreading depression, the underlying cause of the migraine aura) and appears a promising model to study migraine pathophysiology (14).

Using these mutant mice *in vivo* in an intravital experimental setup clearly seems a step forward in understanding the role of these mutations in the pathogenesis of migraine, but requires modification of the rat intravital microscopy model to the other species. Therefore, to develop and standardise this

technique we studied the role of exogenous and endogenous CGRP-induced vasodilatation in wild-type mice. CGRP is a potent vasodilator of dural arteries, and it is well established that levels of this neuropeptide are increased during migraine (15, 16). Moreover, the role of CGRP in migraine pathogenesis (17, 18) is further strengthened by the fact that infusion of CGRP induces headache or migraine attacks in humans (19). In the present study, we used BIBN4096BS, a potent and selective CGRP receptor antagonist (20-22) that has been shown to be effective in aborting acute migraine attacks in a phase-II study (23). We also included in this study the conventional antimigraine drug sumatriptan, a 5-$HT_{1B/1D}$ receptor agonist (24).

11.2 MATERIALS AND METHODS

11.2.1 Surgical preparations

Male C57BL/6Jico mice (Charles River Laboratories, St. Germain sur l'Abresle, France, 20-30 g) were anaesthetised throughout the experiment using pentobarbital sodium (80 mg kg^{-1}, i.p. and then 20 mg $kg^{-1}h^{-1}$, i.p.). The trachea was cannulated and connected to a pressure ventilator (small animal ventilator SAR-830 series, CWE, Inc., Ardmore, PA, U.S.A.). The jugular vein was cannulated for intravenous administration of drugs and the femoral artery was cannulated for continuous monitoring of blood pressure. Throughout surgery and in subsequent experiments, the core temperature of the animals was monitored via a rectal thermometer and maintained between 36.5 and 37.5 °C by a blanket (homeothermic blanket system for rodents, Harvard Instruments, Kent, U.K.). Subsequently, the mouse was placed in a stereotaxic frame; the skull was drilled thin till the dural arteries were clearly visible. As the mouse skull is very thin, care was taken to drill carefully with a constant application of ice-cold saline. In about 15% of the mice, bleeding was observed underneath the skull making the visualisation of the artery difficult; such animals were excluded from the study.

11.2.2 Intravital microscopy

11.2.3 Effect of α-CGRP, capsaicin, BIBN4096BS and sumatriptan

The effect of α-CGRP (10 μg kg^{-1}) was studied in the presence or absence of the CGRP receptor antagonist BIBN4096BS (10, 30 and 100 μg kg^{-1}) or sumatriptan (3 mg kg^{-1}), whereas the effect of capsaicin (30 μg kg^{-1}) was studied in the absence or presence of BIBN4096BS (100 μg kg^{-1}). Five min before the administration of ET-1, vehicle (control experiments), BIBN4096BS or sumatriptan were administered as an i.v. bolus. Two min after the administration of ET-1, α-CGRP or capsaicin were administered i.v. as a fast bolus or slowly in 30 s, respectively. Arterial diameter was recorded for another 5 min.

11.2.4 Transcranial electrical stimulation and the influence of BIBN4096BS and sumatriptan on neurogenic vasodilatation

In the preparations where transcranial electrical stimulation was used to evoke dilatation of the dural blood vessels, a bipolar stimulating electrode (NE 200X, Clark Electromedical, Edenbridge, Kent, U.K.) was placed on the surface of the cranial window approximately within 200 μm from the vessel of interest. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 s (Stimulator model S88, Grass Instruments, West Warwick, RI, U.S.A.). For neurogenic dural vasodilatation, we initially started with a current intensity (monitored on an oscilloscope, model 54601A, Hewlett Packard, Palo Alto, CA, U.S.A.) of 50 μA and increasing with 50 μA steps until a maximal level of dilatation was achieved, usually at 150 μA. In view of the fact that the contractile response to ET-1 began to fade off after about 7 min, transcranial stimulations were performed at intervals of 1.5 min, starting 2 min after the administration of ET-1. We also investigated the effect of pre-treatment of BIBN4096BS (30 or 100 μg kg^{-1}) or sumatriptan (3 mg kg^{-1})

on the vasodilatation induced by transcranial electrical stimulation. Both BIBN4096BS and sumatriptan were administered 5 min before the administration of ET-1.

11.2.5 Data analysis

The dural vessel diameter has been expressed in arbitrary units (AU) and reported as mean±s.e.m. Diameter measurements were done in AU, because the experimental setup changed depending on the portion of the artery segment studied and magnification of the camera to get the best image to monitor the diameter. The changes in the mean arterial blood pressure were expressed as Δ mmHg. Within each group, ANOVA was conducted to compare the changes in arterial diameter after each experimental intervention, followed by post hoc Dunnett's multiple comparisons test, using the arterial diameter 2 min after the administration of ET-1 as the control values.

11.2.6 Compounds

The compounds used in the present study (obtained from the sources indicated) were: human α-CGRP and human ET-1 (both from NeoMPS S.A., Strasbourg, France), BIBN4096BS (gift: Dr. Henri Doods, Boehringer Ingelheim Pharma K.G., Biberach, Germany), sumatriptan succinate (gift: GlaxoSmithKline, Stevenage, U.K.) and capsaicin (Sigma chemicals Co., Steinheim, Germany). α-CGRP, ET-1 and sumatriptan were dissolved in water. Capsaicin (1 mg ml^{-1}) was dissolved in a mixture of Tween-80, ethanol 70% and water (1:1:8). BIBN4096BS was dissolved in 4% HCl (1 N) to obtain a 0.01 M stock solution, which was further diluted with distilled water. All stock solutions were stored at -80°C, until required. Just before use, the stock solutions were further diluted to appropriate concentrations in isotonic saline for injection.

11.3 RESULTS

11.3.1 Representative recordings of vessel diameter

Figure11.1 depicts original dural arterial images obtained from two mice treated with vehicle or BIBN4096BS (100 μg kg^{-1}). Using the video dimension analyser, we found that the diameter of the dural arteries that we studied varied between 20-30 μm in the non-constricted state (baseline). In both mice, ET-1 (10 μg kg^{-1}) elicited a clear reduction of vessel diameter and CGRP (10 μg kg^{-1}) reversed the constrictor effect of ET-1 in the animal treated with vehicle. The vasodilator response to CGRP was not observed in the mouse treated with BIBN4096BS Also, it may be noted that the cerebral vessel (Figure 11.1, top panels) did not respond to ET-1 or CGRP.

11.3.2 Endothelin-1-induced dural artery constriction

Pilot experiments revealed that doses of ET-1 lower than 7.5 μg kg^{-1} produced variable and unstable constriction of dural arteries, making the experimental window unpredictable. ET-1 in doses of 7.5-10 μg kg^{-1} induced 65-75% reduction in the baseline diameter. Higher doses of ET-1 (15-20 μg kg^{-1}) caused a total collapse of the artery and these arteries did not dilate sufficiently in response to α-CGRP, even in the absence of antagonists. Doses of ET-1 (7.5-10 μg kg^{-1}) provided a stable baseline up to 7 min, which was sufficient to perform the protocols for the current study. ET-1 significantly reduced the baseline diameter from 60.9±4.8 to 14.3±2.6 AU 2 min after administration (overall significance across the cohort: $F_{3,26}$=6.88, P<0.0015, n=8). The diameter of vessels did not change significantly up to 7 min (23.1±3.0 AU), but after 10 min the diameter significantly increased to 32.3±2.4 AU as compared to the vessel diameter 2 min after the administration of ET-1 (Figure 11.2). The percentage decreases in diameter by ET-1 as compared to baseline were 76%, 74%, 64% and 48% after 2, 4, 7 and 10 min, respectively. Also, the mean arterial blood pressure compared to the baseline value (67±8 mmHg) increased significantly to 70±6, 61±4, 56±4 and 48±5 mmHg at 2, 4, 7 and 10 min after administration of ET-1, respectively.

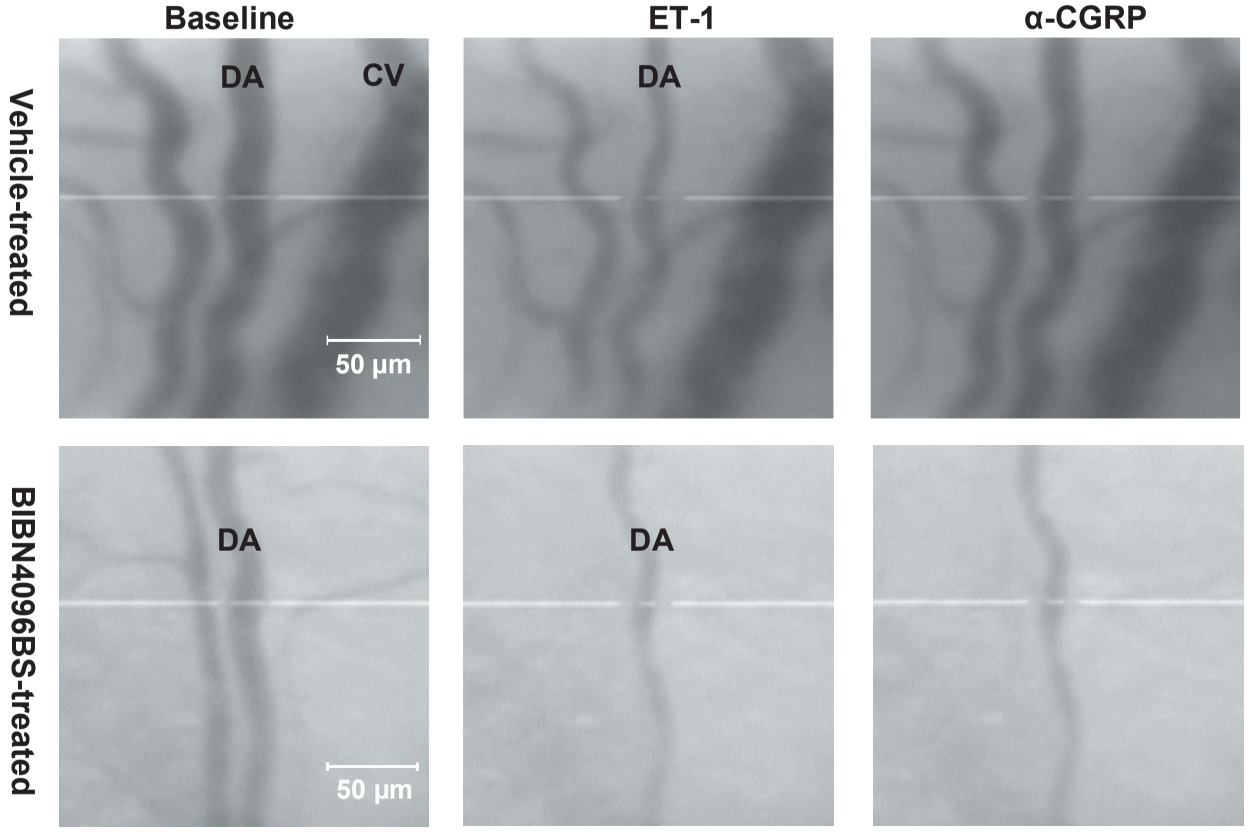

Figure 11.1. Representative video-microscopic images of dural arteries (DA) in two mice treated with vehicle (top panels) or 100 µg kg^{-1} BIBN4096BS (bottom panels). Left panels, 5 min after the administration of vehicle or BIBN4096BS (baseline); middle panels, 2 min after the administration of ET-1 (10 µg kg^{-1}); and right panels, 2 min after the administration of CGRP (10 µg kg^{-1}). The white line in the middle of each panel tracks vessel diameter. Note that ET-1 constricts dural arteries in both mice, while CGRP induced vasodilatation in the vehicle-treated, but not in BIBN4096BS-treated mouse. The cerebral vessel (CV) to the right of the dural artery in the top panels does not respond to ET-1 or CGRP.

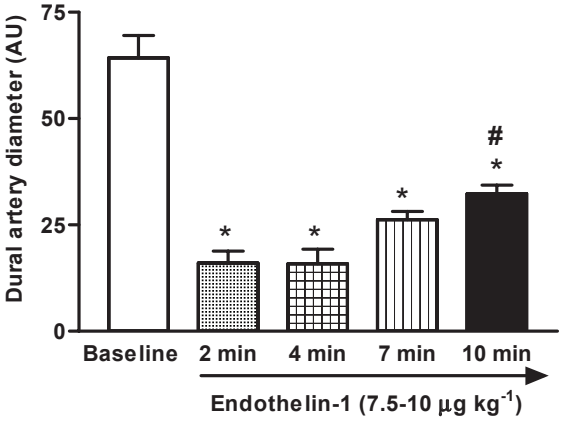

Figure 11.2. Effect of ET-1 on dural artery diameter at different time points after its administration. *, Significant difference versus the baseline diameter (P<0.05); #, significant difference versus diameter 2 min after ET-1 (P<0.05).

11.3.3 Responses to CGRP

A bolus injection of α-CGRP (10 µg kg^{-1}) induced dural artery dilatation (overall significance across the cohort: $F_{2,63}$=55.93, P<0.0001, n=22). There was a significant reduction in dural blood vessel diameter after ET-1 (7.5-10 µg kg^{-1}) injection (Table 11.1). Two min after α-CGRP injection, there was a significant increase in dural blood vessel diameter to 44.6±2.8 AU (P<0.01). After 5 min the dural blood artery diameter had significantly increased further to 57.0±2.5 AU (P<0.01, Figure 11.3) as compared to the diameter 2 min after ET-1 administration. α-CGRP decreased the mean arterial blood pressure from 111±10 mmHg to 42±8 and 48±11 mmHg at 2 and 5 min post-administration, respectively.

Pretreatment with BIBN4096BS (10, 30 and 100 µg kg^{-1}) did not cause any significant change *per se* in the dural artery diameter (Table 11.1) or mean arterial blood pressure (before: 78±6 mmHg; after 100 µg kg^{-1} BIBN4096BS: 83±6 mmHg). When a low dose of BIBN4096S (10 µg kg^{-1}) was administered, it did not antagonize α-CGRP-induced relaxations.

However, higher doses of BIBN4096BS (30 and 100 µg kg^{-1}) produced a significant blockade of α-CGRP-induced vasodilatation, as there was no increase in vessel diameter 2 min after CGRP administration; after 5 min the diameter increased significantly in both groups (Figures 11.1 and Figure 11.3, Table 11.1). The decrease in the mean arterial blood pressure at the highest dose of BIBN4096BS was 26±6 and 29±4 mmHg after 2 and 5 min after administration of α-CGRP, respectively. Administration of sumatrip-

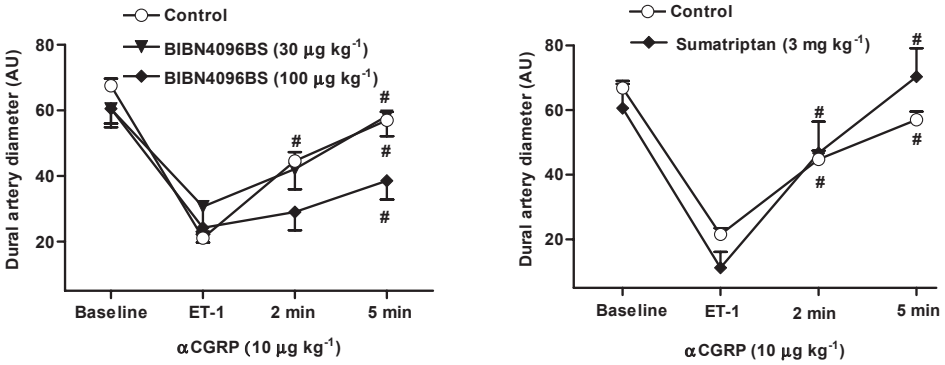

Figure 11.3. Effect of BIBN4096BS (left panel) and sumatriptan (right panel) on α-CGRP-induced dilatation of dural artery preconstricted with ET-1 (7.5-10 µg kg^{-1}). #, Significant difference versus dural artery diameter after ET-1 (P<0.05).

Table 11.1 Dural artery vasodilator responses to α-CGRP, capsaicin and transmural electrical stimulations in mice treated with vehicle, BIBN4096BS or sumatriptan

Pretreatment		Dural artery diameter (AU)					
Compound	μg kg^{-1} (n)	Before pre-treatment	5 min after pretreat-ment	2 min after ET-1	α-CGRP (10 μg kg^{-1})		
					2 min	5 min	
Vehicle	- (22)	-	67.6 ± 2.2	21.1 ± 2.0*	44.6 ± 2.8#	57.0 ± 2.5#	
BIBN4096BS	10 (5)	74.4 ± 6.5	67.0 ± 5.0	32.8 ± 10.0*	61.7 ± 8.9#	67.2 ± 10.5#	
BIBN4096BS	30 (7)	62.2 ± 5.0	60.5 ± 5.6	30.6 ± 8.4*	42.1 ± 6.2	58.3 ± 6.1#	
BIBN4096BS	100 (10)	63.0 ± 2.5	60.5 ± 4.5	24.2 ± 4.4*	29.0 ± 5.7	38.5 ± 5.6#	
Sumatriptan	3000 (5)	63.9 ± 6.5	60.5 ± 7.5	10.0 ± 5.3*	46.7 ± 9.1#	70.3 ± 8.9#	
					Capsaicin (30 μg kg^{-1})		
					2 min	5 min	
Vehicle	- (8)	-	56.9 ± 5.4	17.3 ± 2.6*	39.6 ± 3.3#	47.8 ± 2.6#	
BIBN4096BS	100 (7)	57.9 ± 6.6	56.7 ± 7.0	16.7 ± 3.7*	21.9 ± 3.71	30.8 ± 4.2#	
					Transmural electrical stimulation		
					50 μA	100 μA	150 μA
Vehicle	- (7)	-	57.4 ± 3.0	21.1 ± 2.9*	21.6 ± 2.8	44.0 ± 4.5#	44.6 ± 3.2#
BIBN4096BS	30 (5)	56.3 ± 1.9	54.8 ± 3.7	16.3 ± 1.2*	16.8 ± 3.2	29.2 ± 6.2	36.8 ± 5.3#
BIBN4096BS	100 (7)	57.1 ± 4.5	54.3 ± 7.3	16.0 ± 4.6*	18.6 ± 4.5	23.2 ± 5.8	34.8 ± 5.5
Sumatriptan	3000 (4)	46.1 ± 3.0	45.0 ± 3.8	10.7 ± 4.6*	11.6 ± 5.5	21.9 ± 4.0	32.3 ± 3.7#

*, Significant difference versus diameter 5 min after pretreatment (P<0.05); #, significant difference versus diameter 2 min after ET-1 (P<0.05). n, Number of animals studied.

tan (3 mg kg^{-1}) neither changed the mean arterial blood pressure (60±6 and 63±5 mmHg before and after administration of sumatriptan, respectively), nor did it affect α-CGRP-induced relaxations, as there was a significant vasodilatation after 2 min of administration similar to the control group (Figure 11.3).

11.3.4 Responses to capsaicin

Capsaicin (30 μg kg^{-1}) was administered slowly during 30 s, 2 min after administration of ET-1. Capsaicin induced a significant increase in the vessel diameter across the cohort ($F_{2,21}$=31.1, P<0.001) and after 2 min of administration arteries dilated significantly (Table 11.1). The vehicle of capsaicin did not affect the dural artery diameter or blood pressure (data not shown). After treatment with capsaicin the blood pressure decreased by 8±4 and 20±6 mmHg after 2 and 5 min, respectively. BIBN4096BS (100 μg kg^{-1}) pre-treatment significantly blocked capsaicin-induced relaxations (Figure 11.4).

11.3.5 Responses to transcranial electrical stimulation

Transcranial electrical stimulation induced neurogenic dural vasodilatation; the arterial diameter increased with increasing current intensity across the cohort (overall significance across the cohort: $F_{3,21}$=7.64, P<0.001). There was little change in diameter after 50 μA current, as compared to the diameter after ET-1. After 100 μA current, there was a significant increase in dural artery diameter as compared to that after ET-1 (P<0.01), but a current of 150 μA did not increase the vessel diameter further (Figure 11.5, Table 11.1).

When mice were pretreated with BIBN4096BS (30 μg kg^{-1}), there was no significant increase in vessel diameter after 100 μA current, although at a higher current intensity of 150 μA the increase was significant. At the highest dose of BIBN4096BS (100 μg kg^{-1}) there was no significant increase in the vessel

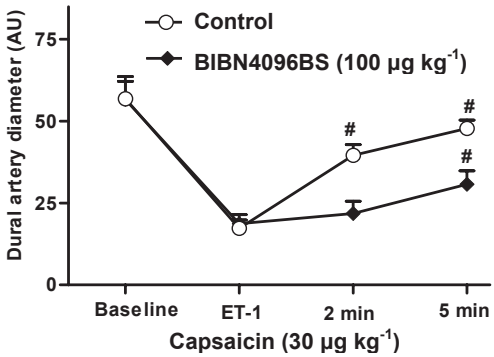

Figure 11.4. Effect of BIBN4096BS on capsaicin-induced dilatation of dural artery preconstricted with ET-1 preconstricted with ET-1 (7.5-10 µg kg^{-1}). $^{\#}$, Significant difference versus dural artery diameter after ET-1 ($P<0.05$).

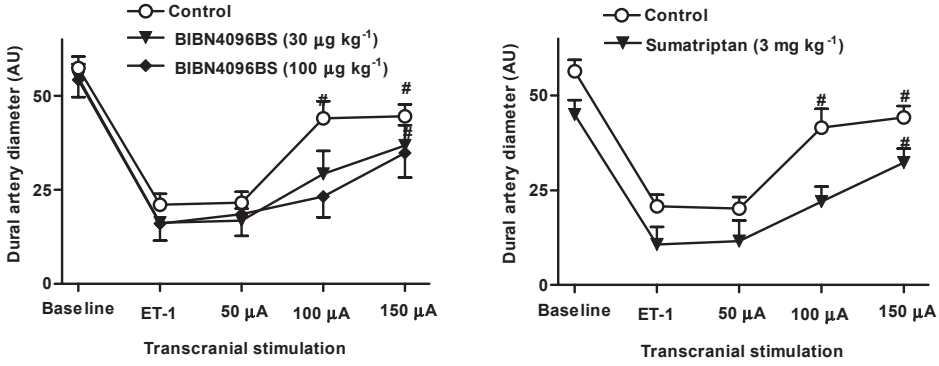

Figure 11.5. Effect of BIBN4096BS (left panel) and sumatriptan (right panel) on neurogenic vasodilatation in the preconstricted dural artery. $^{\#}$, Significant difference versus dural artery diameter after ET-1 ($P<0.05$).

diameter even at 150 µA. Sumatriptan also significantly blocked the responses to transcranial electrical stimulation at 100 µA, although at the higher intensity of 150 µA there was a significant increase in the arterial diameter of the constricted blood vessels (Figure 11.5, Table 11.1). There were no significant changes observed in the mean arterial blood pressure after the electrical stimulation.

11.4 DISCUSSION

This study demonstrates that intravital microscopy of a closed cranial window in mice is a reliable model to study the dural component of the trigeminovascular system. This is the first study in mice, which directly measures the dural artery diameter *in vivo*.

Unlike in the rat closed cranial window model, but similar to what has previously been observed in guinea-pigs (6), in the mouse model the meningeal arteries require preconstriction in order to observe subsequent vasodilatation. We used ET-1 for preconstriction since it induced a rapid vasoconstriction and provided a stable experimental window for approximately 7 min. This is a relative disadvantage in comparison to the rat model as it introduces an additional, but necessary, variable in the experimental conditions. α-CGRP-induced relaxations in the preconstricted arteries are in accordance with previous observations in an *in vivo* mouse skin vasculature assay (25). In this study, a relatively high dose of α-CGRP was used to induce dilatations, probably because of the pretreatment with ET-1 to constrict the

dural vessels. Further, it should be kept in mind that human α-CGRP is less potent in rodents (26) than in human blood vessels (27). BIBN4096BS did not induce vasoconstriction on its own, nor did it affect the mean arterial blood pressure, even at the highest dose used; this is in agreement with previous findings in rat (9, 28) and human *in vivo* studies (29, 30). α-CGRP-induced vasodilatation was dose-dependently blocked by BIBN4096BS, in line with a recent intravital microscopy study in the rat (9). In our experiments, the 5-$HT_{1B/1D}$ receptor agonist sumatriptan did not block relaxations induced by exogenously-administered α-CGRP. Similarly, rizatriptan also had no effect on the vasodilatation in response to exogenous CGRP in guinea-pig (6). Furthermore, when administered 5 min prior to ET-1, sumatriptan did not induce any significant vasoconstriction, indicating that this 5-$HT_{1B/1D}$ receptor agonist is devoid of vasoconstrictor property in the mouse meningeal artery. In the human middle meningeal artery *in vitro* sumatriptan exerts a potent vasoconstrictor effect (31), but it does not decrease vessel diameter in rats (4). As sumatriptan is able to reach the dural vasculature in the mouse without hindrance of blood brain barrier, the discrepancy in responses to sumatriptan probably can be attributed to relative differences in distribution of 5-HT receptors in cranial vasculature or receptor affinity.

Capsaicin, a vanilloid ($TRPV_1$) receptor agonist, also dilated the preconstricted arteries in the present study. Capsaicin has been widely used in animal models to induce cranial vasodilatation (32, 33) and it acts by releasing endogenous CGRP. Also in the present study, the capsaicin-induced relaxation was mainly mediated via the release of CGRP as demonstrated by the blockade with the CGRP receptor antagonist, BIBN4096BS at 2 min. The fact that the vasodilator response to capsaicin was not significantly inhibited by BIBN4096BS at 5 min could be caused by a further increase of the effect of capsaicin after 2 min, as observed in the control experiments. Thus, the dose of BIBN4096BS applied might not have been sufficiently high to completely block the higher response observed at 5 min. Further, besides CGRP, capsaicin may release other vasodilators, such as substance P (34). Finally, there is a tendency for the diameter to increase at the highest time points, although the baseline diameter did not significantly change during the experimental time window that we used (see Figure 11.2).

Transcranial electrical stimulation of the closed cranial window induced current-dependent dilatation of the meningeal arteries. Earlier studies on a closed cranial window in rats used the voltage that induced maximal dilatation, while we employed a stepwise increase in voltage, yielding currents of 50, 100 and 150 μA. This improvement over the existing model provides the flexibility to study the meningeal vessels at increasing current intensities and thus differences in the threshold stimulation between various animals may be detected. Further, a stepwise increase in stimulus intensity allows a more detailed assessment of the effects of inhibitors of the response to transcranial electrical stimulation; for example, 30 μg kg^{-1} of BIBN4096BS inhibited the vasodilator response to a stimulus intensity of 100 μA, but not to the supramaximal stimulus obtained with 150 μA. In contrast, the higher dose of BIBN4096BS (100 μg kg^{-1}) inhibited vasodilator responses to both 100 μA and 150 μA. In view of the dose-dependent blockade by BIBN4096BS, it can be concluded that, similar to the rat and guinea-pig models (6), endogenous release of CGRP from perivascular nerves is a major component mediating vasodilatation induced by transcranial electrical stimulation. Indeed, a low stimulus intensity during a short period, as employed in the present study, has been reported to selectively activate Aδ sensory fibres, predominantly releasing CGRP (4). Relatively high doses of BIBN4096BS (30 and 100 μg kg^{-1}) were required to inhibit vasodilatation, in line with the reported lower potency of this CGRP antagonist in rodents (9, 22). In addition, as mentioned earlier regarding the blockade of vasodilator responses to capsaicin by BIBN4096BS, the inhibitory actions of BIBN4096BS and sumatriptan at the highest stimulus intensity (i.e., at later time points) may have been underestimated because of a non-significant decline of the vasoconstrictor response to ET-1. Other agents, such as nitric oxide, may also contribute to neurogenic vasodilatation as observed in rats and cats (35, 36) and, indeed, such a dilatation would be resistant to BIBN4096BS blockade. After electrical stimulation as well as α-CGRP the dural artery diameter did not recover completely; this might be due to

persistence of the constrictor effect of ET-1 (Figure 11.2). The other possibility might be that the stimulus intensity and the dose of CGRP were not high enough to dilate artery to the preconstriction level.

Sumatriptan blocked the responses to transcranial electrical stimulation at 100 μA, compared to the control group. This may be due to the action of sumatriptan on presynaptic 5-$HT_{1B/1D}$ receptors, blocking the exocytosis of endogenously stored neuropeptides, including CGRP, as sumatriptan *per se* did not cause any change in vessel diameter. Similarly, in the rat cranial window model, 5-$HT_{1B/1D}$ receptor agonists have been shown to block neurogenic vasodilatation (4, 6).

In conclusion, this mouse model of a closed cranial window can be used to further explore the mechanism of action and pharmacology of existing and putative antimigraine drugs. The main advantage of the mouse compared to the rat model is that this model provides the potential to use genetically modified mice. This is especially relevant because recent advances in migraine genetics made it possible to generate transgenic knock-in mice harbouring pathogenic mutations observed in migraine patients (10, 14). This mouse model may provide an important insight into the effect of these mutations on dural artery vasodilatation and, thus, offers an additional avenue to investigate the pathophysiology of migraine.

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Simplex Sigellum Veri: The Simple is the Seal of Truth

Chapter 12

Clonidine Inhibits the Canine External Carotid Vasodilatation to Capsaicin by $\alpha_{2A/2C}$-Adrenoceptors

Based on: L.R. Jiménez-Mena, S. Gupta, E. Muñoz-Islas, Araceli Sánchez-López J. Lozano-Cuenca, A. Sánchez-López, D. Centurión, S. Mehotra, A. MaassenVanDenBrink, C.M. Villalón, European Journal of Pharmacology, 2006; 543:68-76.

ABSTRACT

Migraine is a disorder associated with increased plasma concentrations of calcitonin gene-related peptide (CGRP). CGRP, a neuropeptide released from activated trigeminal sensory nerves, dilates cranial blood vessels and transmits vascular nociception. Moreover, several antimigraine drugs inhibit the dural neurogenic vasodilatation to trigeminal stimulation. Hence, this study investigated in anaesthetized dogs the effects of the α_2-adrenoceptor agonist, clonidine, on the external carotid vasodilator responses to capsaicin, αCGRP and acetylcholine. 1-min intracarotid infusions of capsaicin (10, 18, 30 and 56 μg/min), αCGRP (0.1, 0.3, 1 and 3 μg/min) and acetylcholine (0.01, 0.03, 0.1 and 0.3 μg/min) produced dose-dependent increases in external carotid conductance without affecting blood pressure or heart rate. Interestingly, the carotid vasodilator responses to capsaicin, but not those to αCGRP or acetylcholine, were partially inhibited after clonidine (total dose: 24.4 μg/kg, i.v.); in contrast, equivalent volumes of saline did not affect the responses to capsaicin, αCGRP or acetylcholine. The inhibitory responses to clonidine were antagonized by i.v. administration of the α_2-adrenoceptor antagonists rauwolscine ($\alpha_{2A/2B/2C}$; 300 μg/kg), BRL44408 (α_{2A}; 1000 μg/kg) or MK912 (α_{2C}; 100 and 300 μg/kg), but not by imiloxan (α_{2B}; 1000 μg/kg). These results suggest that clonidine inhibits the external carotid vasodilator responses to capsaicin by peripheral trigeminovascular and/or central mechanisms; this inhibitory response to clonidine seems to be predominantly mediated by α_{2A}-adrenoceptors and, to a much lesser extent, by α_{2C}-adrenoceptors.

12.1 INTRODUCTION

Though the precise mechanisms involved in the pathophysiology of migraine remain elusive (1, 2), this neurovascular disorder has been associated with cranial vasodilatation and release of calcitonin gene-related peptide (CGRP) resulting from activation of perivascular trigeminal sensory nerves (3, 4); this cranial vasodilatation, in turn, stimulates sensory nerve transmission (5). In keeping with these findings, the antimigraine action of triptans and ergots has been attributed to: (i) a normalization of the increased levels of CGRP during migraine (6), via an inhibition of the trigeminal release of CGRP (7); and (ii) a vasoconstriction of cranial arteries (4, 8-10).

The release of CGRP can be experimentally induced by either antidromic electrical stimulation of afferent nerves (7) or chemical stimulation with capsaicin (11). In view that cranial blood vessels are innervated by CGRP-containing trigeminal sensory nerves (5) a number of strategies have attempted to induce the release of CGRP from capsaicin-sensitive trigeminal sensory nerves in experimental models of migraine (2). For example, in anaesthetized pigs, the intracarotid infusion of capsaicin induces a marked vasodilatation in the carotid circulation which is: (i) associated with an increase in plasma levels of CGRP (12); and (ii) markedly antagonized by the $CGRP_1$ receptor antagonist, BIBN4096BS (12), at the same doses that antagonized the carotid vasodilatation to exogenous α-CGRP (13).

Table 12.1 Binding affinity constants (pK_i) of several antagonists for cloned human α_2-adrenoceptor subtypes.

Antagonist	α_{2a}	α_{2b}	α_{2c}
Rauwolscine	9.5[a]	9.4[a]	9.9[a]
	8.9[b]	8.9[b]	9.3[b]
BRL44408	8.2[c]	6.2[c]	6.8[c]
	7.6[d]	6.0[d]	6.4[d]
Imiloxan	5.8[d]	6.9[d]	6.0[d]
	6.5[b]	7.2[b]	6.8[b]
MK912	8.9[c]	8.9[c]	10.2[c]
	9.1[b]	9.1[b]	10.2[b]

Data taken from: [a], (38); [b], (50); [c], (51); [d], (40).

On the other hand, dihydroergotamine has been shown to induce: (i) external carotid vasoconstriction in anaesthetized dogs by stimulation of 5-HT1B receptors and $\alpha_{2A/2C}$-adrenoceptors (8); and (ii) inhibition of neurogenic vasodilatation induced by trigeminal activation, via presynaptic mechanisms (14). These findings suggest that, apart from cranial vasoconstriction, inhibition of CGRP-induced neurogenic vasodilatation may play a role in the antimigraine efficacy of some agents (14, 15). Considering the above, we decided to investigate whether selective activation of α_2-adrenoceptors is capable of inhibiting the external carotid vasodilatation induced by capsaicin in an *in vivo* experimental model of migraine. Therefore, the present study in vagosympathectomized dogs set out to analyze: (i) whether clonidine, an α_2-adrenoceptor agonist with antihypertensive properties (16) is capable of inhibiting the vasodilator responses to capsaicin, αCGRP and acetylcholine in the external carotid circulation; and (ii) the specific α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and/or α_{2C}) involved in this response by investigating the effects of the α_2-adrenoceptor antagonists rauwolscine ($\alpha_{2A/2B/2C}$), BRL44408 (α_{2A}), imiloxan (α_{2B}) or MK912 (α_{2C}) (see Table 12.1).

12.2 MATERIALS AND METHODS

12.2.1 General

Experiments were carried out in a total of 43 male mongrel dogs (15-20 kg) that were anaesthetised with sodium pentobarbitone (30 mg/kg, i.v.) and additional amounts (1 mg/kg, i.v.) were provided when required. All dogs were intubated with an endotracheal tube and artificially respired with room air, using a Palmer ventilator pump (20 strokes/min; stroke volume: 13-16 ml/kg) (17). Catheters were placed in: (i) the right femoral vein for the administration of vehicle, antagonists or clonidine; and (ii) the femoral artery, connected to a Statham pressure transducer (P23 ID), for the measurement of arterial blood pressure. After administration of vehicle or antagonists, the venous catheter was flushed with 3 ml of saline. Mean blood pressure (MBP) was calculated from the systolic (SAP) and diastolic (DAP) arterial pressures: MBP=DAP+(SAP-DAP)/3. Heart rate was measured with a tachograph (7P4F) triggered from the arterial blood pressure signal. The right common carotid artery was dissected free and the corresponding internal carotid and occipital arteries were ligated. Thereafter, an ultrasonic flow probe (4 mm, R-series), connected to an ultrasonic T206 flowmeter (Transonic Systems Inc., Ithaca, NY, USA), was placed around this artery; thus, the flow through this bed was considered as the external carotid blood flow (18). Bilateral cervical vagosympathectomy was systematically performed in order to prevent possible baroreceptor reflexes produced by the intracarotid infusions of capsaicin, CGRP, acetylcholine and phenylephrine. Subsequently, a catheter was introduced into the right cranial thyroid artery for the intracarotid infusions of capsaicin, αCGRP and acetylcholine. Since carotid arterioles are dilated under our experimental conditions, we had to produce a carotid preconstriction by a continuous infusion of phenylephrine (an α_1-adrenoceptor agonist). For this purpose, a needle (0.5 mm diameter), connected to a catheter, was inserted into the right common carotid artery for the infusion of phenylephrine by another motor-driven syringe. This phenylephrine-induced vasoconstriction, which allows to obtain greater vasodilator responses (19), was compared to that elicited by i.v. infusions of clonidine or saline. Capsaicin, αCGRP and acetylcholine (1 ml/min for 1 min) as well as phenylephrine (0.3 ml/min continuously) were infused into the carotid artery by WPI model sp100i pumps (World Precision Instruments Inc., Sarasota, FL, USA) (for further details, see below). Arterial blood pressure, heart rate and external carotid blood flow ere recorded simultaneously by a model 7D polygraph (Grass Instrument Co., Quincy, MA, USA). The body temperature of the animals was maintained between 37-38ºC.

12.2.2 Experimental protocol

After a stable haemodynamic condition for at least 60 min, baseline values of mean blood pressure, heart rate and external carotid blood flow were determined. Then, the 43 animals were divided into three groups (n=8, 9 and 26, respectively) which were subsequently subdivided on the basis of treatment with different compounds (see Figure 12.1).

The first group (n=8) received 1-min intracarotid infusions of capsaicin (10, 18, 30 and 56 µg/min), αCGRP (0.1, 0.3, 1 and 3 µg/min) and acetylcholine (0.01, 0.03, 0.1 and 0.3 µg/min). Then, as shown in Figure 12.1, this group was subdivided into 2 subgroups that subsequently received, throughout the experiment, a continuous intracarotid infusion of, respectively: (i) vehicle (0.3 ml/min of physiological saline; n=4); and (ii) phenylephrine (6.8 µg/min; rate: 0.3 ml/min; n=4), which produces a decrease in external carotid conductance similar to that elicited by the last infusion of clonidine (see below). 20 min later, the responses to the above doses of capsaicin, αCGRP and acetylcholine were elicited again *during* the continuous infusion of saline or phenylephrine.

The second group (n=9) received a continuous intracarotid infusion of phenylephrine (6.8 µg/min; rate: 0.3 ml/min) and, 20 min later, the above doses of capsaicin, αCGRP and acetylcholine were analyzed. Then, as shown in Figure 12.1, this group was subdivided into 2 subgroups, so that the infusion of phenylephrine was: (i) stopped in the first subgroup (n=5); and (ii) kept continuous throughout the experiment in the second subgroup (n=4). Subsequently, by the use of another motor-driven syringe with a needle inserted into the femoral vein, the first and second subgroups received sequential continuous i.v. infusions (rate: 0.5 ml/min) of, respectively: (i) clonidine (0.01, 0.03, 0.1, 0.3 and 1 µg/kg.min; n=5) during 10 min, except the last dose which was maintained throughout the experiment in order to keep a constant external carotid vasoconstriction (similar to that by phenylephrine); and ii) equivalent volumes of physiological saline. 20 min after the start of the infusion of the last dose of clonidine or saline, the responses to the above doses of capsaicin, αCGRP and acetylcholine were elicited again (see

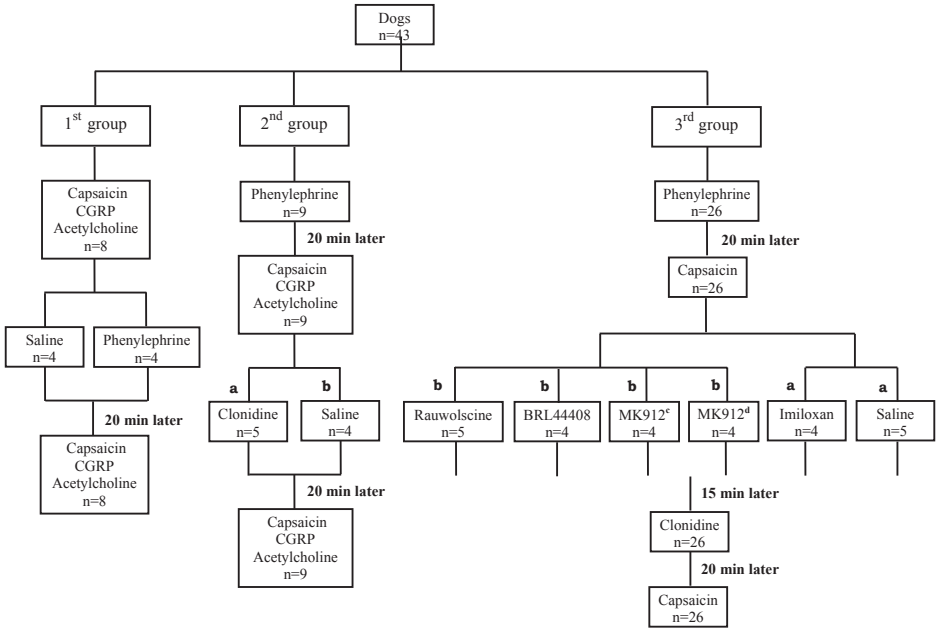

Figure 12.1. Experimental protocols followed in the 3 main groups of animals and their corresponding subdivision into different subgroups. [a], The infusion of phenylephrine was stopped at this point; [b], Continuous infusion of phenylephrine throughout the experiment; [c], MK912 (100 µg/kg); [d], MK912 (300 µg/kg).

Figure 12.1). With this infusion scheme, clonidine reached a total dose of 24.4 μg/kg just before starting the administration of capsaicin.

Lastly, the third group (n=26) received an infusion of phenylephrine as previously described and, 20 min later, the responses to the above doses of capsaicin were elicited. Then, as shown in Figure 12.1, this group was subdivided into 6 subgroups that received i.v. bolus injections of the α_2-adrenoceptor antagonists: (i) rauwolscine ($\alpha_{2A/2B/2C}$, 300 μg/kg; n=5); (ii) BRL44408 (α_{2A}, 1000 μg/kg; n=4); (iii) imiloxan (α_{2B}, 1000 μg/kg; n=4); (iv) MK912 (α_{2C}, 100 μg/kg; n=4); (v) MK912 (α_{2C}, 300 μg/kg; n=4); or (vi) an equivalent volume of physiological saline (0.15 ml/kg; n=5). After 15 min, each subgroup received sequential i.v. infusions of clonidine as previously described for the second group. It is important to note that after administration of rauwolscine, BRL44408 or MK912, but not of imiloxan or saline, the clonidine-induced external carotid vasoconstriction was blocked (results obtained from preliminary experiments; not shown). Therefore, in order to maintain the external carotid circulation under a vasoconstriction state similar to that before the administration of these compounds, the infusion of phenylephrine was: (i) kept continuous throughout the experiment in the subgroups receiving rauwolscine, BRL44408 or MK912; and (ii) interrupted just before the administration of imiloxan or saline (see Figure 12.1). 20 min after the start of the infusion of the last dose of clonidine (total dose: 24.4 μg/kg, as previously described), the responses to the above doses of capsaicin were elicited again.

The dose-intervals between the different doses of capsaicin, αCGRP and acetylcholine (given sequentially as they produced transient responses) ranged between 5 and 20 min, as in each case we waited until the external carotid blood flow had returned to baseline values. The doses of these compounds were selected from preliminary experiments, in which reproducible and dose-dependent increases in external carotid blood flow were elicited with no changes in blood pressure or heart rate.

12.2.3 Drugs

Apart from sodium pentobarbitone, the compounds used in this study (obtained from the sources indicated) were: capsaicin, rat αCGRP, acetylcholine chloride, clonidine hydrochloride, (l)-phenylephrine hydrochloride and rauwolscine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); BRL44408 maleate (2-[2H-(1-Methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole maleate) and imiloxan hydrochloride (Tocris Bioscience, Ellisville, MO, USA); and MK912 ((2S-trans)-1,3,4,5′,6,6′,7,12b-Octahydro-1′,3′-dimethyl-spiro[2H-benzofuro[2,3-a]quinolizine-2,4′(1′H)-pyrimidin]-2′(3′H)-one L-657,743) (gift: Dr. W.L. Henckler; Merck & Co., New Jersey, NJ, USA). The compounds were dissolved in physiological saline except capsaicin, which was dissolved in 5% v/v ethanol; this vehicle had no effect (when given by i.v. or intracarotid routes) on the systemic or carotid haemodynamic variables (not shown). The doses of all the agonists refer to their free base, whereas those of the antagonists refer to their salt. The experimental protocol of this investigation was approved by the Ethical Committee of our institution (CICUAL).

12.2.4 Data presentation and statistical analysis

All data in the text and figures are presented as mean±s.e.m. The external carotid vascular conductance was calculated by dividing blood flow (ml/min) by mean blood pressure (mmHg) and multiplied by hundred. The peak changes in external carotid conductance were expressed as percent change from baseline. The difference between the variables within one subgroup of animals was compared by using a two-way repeated measures analysis of variance (randomised block design) followed by the Student-Newman-Keuls' test (20). Statistical significance was accepted at $P<0.05$ (two-tailed).

12.3 RESULTS

12.3.1 Systemic and carotid haemodynamic variables

Baseline values of heart rate, mean blood pressure and external carotid conductance in the 43 dogs were: 189±5 beats/min, 149±5 mmHg and 158±9 ml/min.mmHg, respectively. These values were not significantly modified after i.v. administration of vehicle or the antagonists BRL44408 (1000 µg/kg), imiloxan (1000 µg/kg) or MK912 (100 µg/kg) (Table 12.2). Moreover, rauwolscine (300 µg/kg) significantly increased mean blood pressure and heart rate, whilst MK912 (300 µg/kg) significantly increased heart rate (Table 12.2).

On the other hand, the continuous i.v. infusions of clonidine: (i) dose-dependently decreased the external carotid conductance, particularly during the infusion of the last three doses (155±9 ml/min.mmHg before and 130±58, 125±56, 96±43*, 70±9* and 48±5* ml/min.mmHg after, respectively 0.01, 0.03, 0.1, 0.3 and 1 µg/kg.min of clonidine; *, P<0.05); (ii) significantly decreased heart rate (184±13 beats/min before and 136±9 beats/min after 1 µg/kg.min of clonidine); and (iii) significantly increased mean blood pressure (146±12 mmHg before and 182±24 mmHg after 1 µg/kg.min of clonidine).

Moreover, the continuous intracarotid infusion of phenylephrine (6.8 µg/min; 20 min after starting the infusion) significantly decreased the external carotid conductance (165±14 ml/min.mmHg before and 55±8 ml/min.mmHg during treatment) without significant changes in mean blood pressure (159±10 mmHg before and 164±7 mmHg during treatment) or heart rate (195±7 beats/min before and 191±7 beats/min during treatment). It is to be noted that the decreased external carotid conductance during the infusion of phenylephrine (55±8 ml/min.mmHg; equivalent to an approximate decrease of 67%) did not significantly differ from that during the infusion of 1 µg/kg.min of clonidine (48±5 ml/min.mmHg; equivalent to an approximate decrease of 70%). That is why the enhanced vasodilator responses to capsaicin, αCGRP and acetylcholine during the continuous intracarotid infusion of phenylephrine were considered as the control responses when compared to those elicited during the highest infusion dose of clonidine (see below).

Table 12.2 Changes in mean blood pressure (MBP), heart rate (HR) and external carotid conductance (ECC) induced by i.v. administration of vehicle or several antagonists in anaesthetized dogs.

Treatment group	n	MBP (mmHg)		HR (beats/min)		ECC (ml/min.mmHg)	
		Before	After	Before	After	Before	After
Vehicle (0.15 ml/kg)	4	189±20	182±42	215±19	224±21	117±18	139±41
Rauwolscine (300 µg/kg)	5	149±12	176±14[a]	186±10	218±7[a]	104±27	100±27
BRL444008 (1000 µg/kg)	4	161±29	190±25	193±5	174±11	120±45	80±12
Imiloxan (1000 µg/kg)	4	137±17	125±4	211±21	139±42	131±15	143±5
MK912 (100 µg/kg)	4	157±17	180±22	180±8	210±5	114±34	121±39
MK912 (300 µg/kg)	4	157±14	151±17	168±4	202±8[a]	126±14	127±17

[a], P<0.05 before *vs*. after treatment.

12.3.2 Effect of vehicle (saline), phenylephrine or clonidine on the external carotid vasodilator responses to capsaicin, αCGRP and acetylcholine

1-min intracarotid infusions of capsaicin, αCGRP and acetylcholine produced, respectively, dose-dependent increases in external carotid conductance as follows: 17±2, 23±2, 35±6 and 47±10% after 10, 18, 30 and 56 μg/min of capsaicin; 20±8, 26±11, 44±15 and 78±24% after 0.1, 0.3, 1 and 3 μg/min of αCGRP; and 4±3, 14±3, 39±12 and 57±12% after 0.01, 0.03, 0.1 and 0.3 μg/min of acetylcholine. These vasodilator responses to capsaicin, αCGRP and acetylcholine were reproducible as they remained unaffected during a continuous intracarotid or i.v. infusion of physiological saline (data not shown).

In contrast, as shown in Figure 12.2, the continuous intracarotid infusion of phenylephrine (6.8 μg/min) significantly enhanced the external carotid vasodilator responses to the 2 highest doses of capsaicin (Figure 12.2A), αCGRP (Figure 12.2B) and acetylcholine (Figure 12.2C) when compared with their

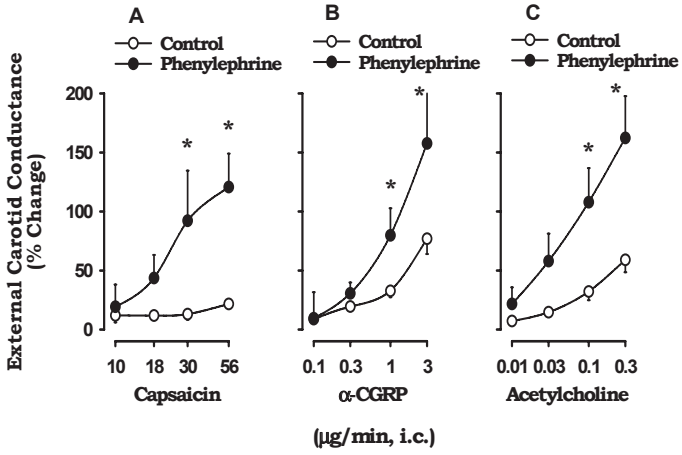

Figure 12.2. External carotid vasodilator responses to: (A) capsaicin; (B) α-CGRP; or (C) acetylcholine before (control; without phenylephrine) and during a continuous intracarotid (i.c.) infusion of phenylephrine (6.8 μg/min, 20 min later) throughout the experiment (n=4). *, P<0.05 *vs*. the corresponding control response.

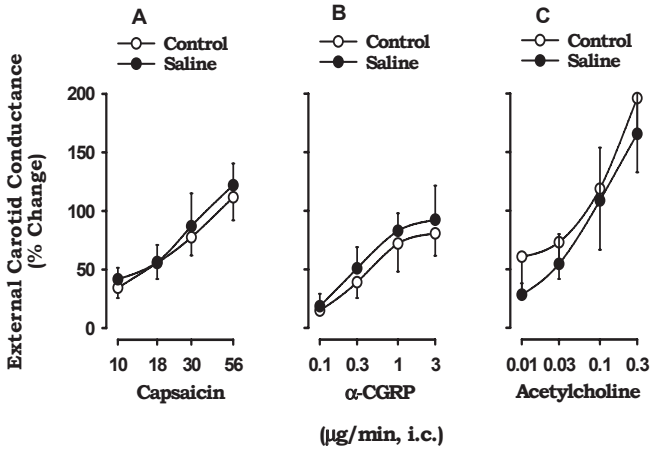

Figure 12.3. Reproducibility analysis of the external carotid vasodilator responses to: (A) capsaicin; (B) α-CGRP; or (C) acetylcholine during a continuous intracarotid (i.c.) infusion of phenylephrine (6.8 μg/min, 20 min later) before (control; without physiological saline) and during a continuous i.v. infusion of physiological saline (0.5 ml/min, 20 min later) (n=4).

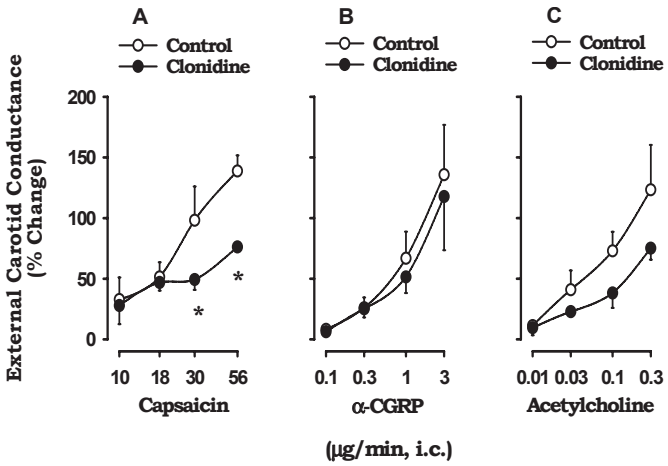

Figure 12.4. External carotid vasodilator responses to: (A) capsaicin; (B) α-CGRP; or (C) acetylcholine before (control; during a continuous intracarotid [i.c.] infusion of phenylephrine 6.8 µg/min, 20 min later) and during a continuous i.v. infusion of clonidine (1.0 µg/kg.min, 20 min later) throughout the experiment (n=5). *, $P<0.05$ *vs*. the corresponding control response.

respective control responses. The duration of action of the vasodilator responses to capsaicin and αCGRP (between 5 and 20 min) was longer-lasting than that to acetylcholine (between 1 and 5 min). Moreover, as shown in Figure 12.3, during the continuous intracarotid infusion of phenylephrine, the enhanced responses to capsaicin (Figure 12.3A), αCGRP (Figure 12.3B) and acetylcholine (Figure 12.3C) remained unchanged when elicited again during the continuous i.v. infusion of physiological saline. Hence, the enhanced vasodilator responses to capsaicin, αCGRP and acetylcholine during the infusion of phenylephrine were considered as the control responses when compared to those elicited during the highest infusion dose of clonidine; the latter produced a decrease in external carotid conductance similar to that by the infusion of phenylephrine (see above).

In contrast with the above, Figure 12.4 shows that the external carotid vasodilator responses to the last 2 doses of capsaicin (Figure 12.4A), but not those to αCGRP (Figure 12.4B) or acetylcholine (Figure 12.4C), were significantly – and specifically – inhibited by the continuous i.v. infusion of clonidine; the latter had reached a total dose of 24.4 µg/kg just before the administration of capsaicin (see Experimental protocol section).

12.3.3 Effect of vehicle (saline), rauwolscine, BRL44408, imiloxan or MK912 on the inhibition produced by clonidine of the capsaicin-induced vasodilator responses

Since clonidine inhibits capsaicin-induced external carotid vasodilatation, the potential involvement of α_2-adrenoceptors was investigated by using rauwolscine, a selective α_2-adrenoceptor antagonist (see Table 12.1). Hence, Figure 12.5 shows that the inhibition by clonidine was: (i) completely antagonized by rauwolscine (300 µg/kg, i.v.; Figure 12.5B); and (ii) unaffected by an equivalent i.v. volume of saline (Figure 12.5A). Based on these findings, further experiments were carried out in order to identify the specific subtypes (α_{2A}, α_{2B} and/or α_{2C}) involved in the inhibition by clonidine; for this purpose, the selective antagonists BRL44408 (α_{2A}; 1000 µg/kg), imiloxan (α_{2B}; 1000 µg/kg) or MK912 (α_{2C}; 100 and 300 µg/kg) were investigated (see Table 12.1). As shown in Figure 12.6, clonidine-induced inhibition on the external carotid vasodilator responses to capsaicin was: (i) completely antagonized by BRL44408 (Figure 12.6A); (ii) partially (100 µg/kg; Figure 12.6C) or completely (300 µg/kg; Figure 12.6D) antagonized by MK912; and (iii) resistant to antagonism by imiloxan (Figure 12.6B).

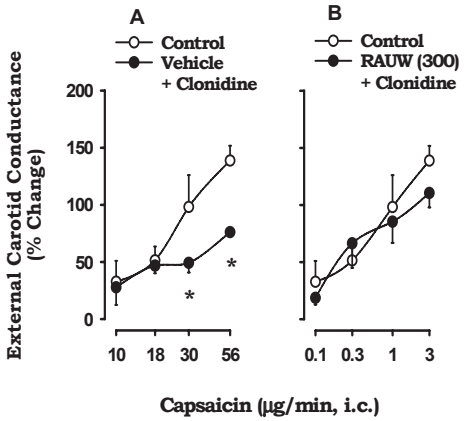

Figure 12.5. External carotid vasodilator responses to capsaicin before (control; during a continuous intracarotid [i.c.] infusion of phenylephrine 6.8 µg/min, 20 min later) and during a continuous i.v. infusion of clonidine (1.0 µg/kg.min, 20 min later) throughout the experiment in dogs previously administered i.v. with either: (A) vehicle (saline: 0.15 ml/kg; n=5); or (B) rauwolscine (RAUW, 300 µg/kg; n=5). *, P<0.05 *vs*. the corresponding control response.

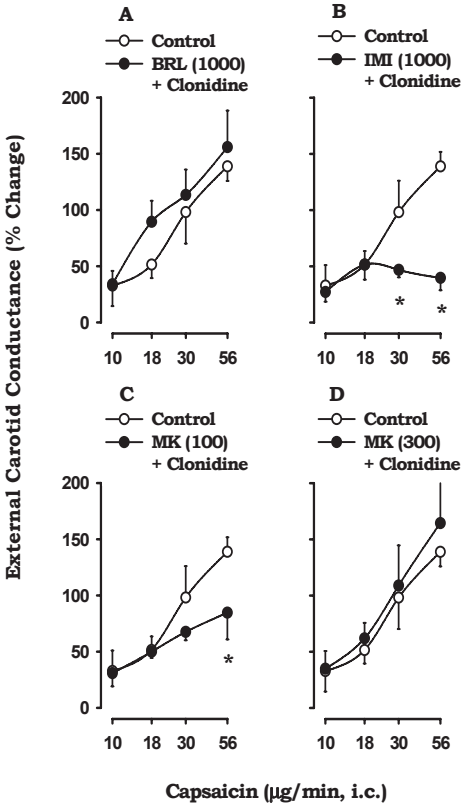

Figure 12.6. External carotid vasodilator responses to capsaicin before (control; during a continuous intracarotid [i.c.] infusion of phenylephrine 6.8 µg/min, 20 min later) and during a continuous i.v. infusion of clonidine (1.0 µg/kg.min, 20 min later) throughout the experiment in dogs previously administered i.v. with either: (A) BRL44408 (BRL, 1000 µg/kg; n=4); (B) imiloxan (IMI, 1000 µg/kg; n=4); (C) MK912 (MK, 100 µg/kg; n=4); or (D) MK912 (MK, 300 µg/kg; n=4). *, P<0.05 *vs*. the corresponding control response.

12.4 DISCUSSION

12.4.1 General

Trigeminal ganglion stimulation increases cerebral blood flow associated with the release of vasoactive neuropeptides, including CGRP (21). This vasodilatation can also be produced by chemical stimulation with capsaicin in a number of vascular experimental models (11, 22, 23). Consistent with the latter findings our results show, in the first instance, that administration of capsaicin and αCGRP produced dose-dependent external carotid vasodilator responses, as previously reported in the carotid circulation of anaesthetized pigs (12, 13). Indeed, capsaicin-induced porcine carotid vasodilatation, which is associated with an increase in plasma levels of CGRP, can be antagonized by the $CGRP_1$ receptor antagonist, BIBN4096BS (12). Although it is tempting to speculate from our results that capsaicin-induced canine external carotid vasodilatation is also mediated by release of CGRP, this hypothesis is unproven by our study. Admittedly, further experiments would be required to corroborate whether capsaicin-induced canine external carotid vasodilatation is: (i) associated with an increase in plasma concentrations of CGRP; and/or (ii) amenable to blockade by a CGRP receptor antagonist (e.g. BIBN4096BS).

Irrespective of the above unproven hypothesis, our study clearly demonstrates that clonidine specifically inhibited the external carotid vasodilator responses to capsaicin, but not those to αCGRP or acetylcholine. This inhibition by clonidine, being blocked by the α_2-adrenoceptor antagonists rauwolscine ($\alpha_{2A/2B/2C}$), BRL44408 (α_{2A}) or MK912 (α_{2C}), but not by imiloxan (α_{2B}) (see Table 12.1), is mediated by α_2-adrenoceptors, predominantly the α_{2A}- and, to a much lesser extent, the α_{2C}-adrenoceptor subtypes.

12.4.2 Systemic and carotid haemodynamic changes produced by the different treatments

The increase in mean blood pressure and heart rate produced by rauwolscine (Table 12.2) may be explained by blockade of presynaptic sympatho-inhibitory α_2-adrenoceptors, with a resulting increase in noradrenaline release (24). A similar line of reasoning could account for the increase in heart rate produced by MK912 (300 µg/kg; Table12.2), which is equipotent to rauwolscine to block α_{2A}- and α_{2C}-adrenoceptors (Table 12.1).

Moreover, in contrast to phenylephrine, the continuous i.v. infusion of clonidine, which can easily cross the blood-brain barrier (25), produced bradycardia and a vasopressor response. The clonidine-induced bradycardia, which cannot be explained by activation of a baroreceptor reflex mechanism since the animals were vagosympathectomized, may be attributed to stimulation of sympatho-inhibitory α_2-adrenoceptors located on: (i) cardiac sympathetic neurons; and/or (ii) the rostroventrolateral medulla. This, in turn, would result in inhibition of the sympathetic discharge (26). Furthermore, the clonidine-induced vasopressor response may be due to activation of $\alpha_{1/2}$-adrenoceptors on vascular smooth muscle (27, 28).

On the other hand, the continuous infusions of phenylephrine (6.8 µg/min, given intracarotidly) and clonidine (1 µg/kg.min, i.v.) produced a sustained vasoconstriction in the external carotid circulation, most likely due to stimulation of, respectively, α_1- (29)and α_2- (9) adrenoceptors. It is noteworthy that phenylephrine (given intracarotidly) and clonidine (i.v.) were administered by different routes because, in preliminary experiments, we observed that clonidine given intracarotidly produced a marked external carotid vasoconstriction (a decrease of 70% in external carotid conductance) at very low doses (up to 0.5 µg/kg.min during 1 min). This low dose of clonidine did not inhibit capsaicin-induced external carotid vasodilatation probably because the plasma levels of this imidazoline were not high enough to reach (and have discernible effects in) the central nervous system. Consequently, we designed an experimental approach to produce a similar decrease in external carotid conductance (48±5 ml/min.mmHg; equivalent to an approximate decrease of 70%) by administering a continuous i.v. infusion of 1 µg/kg.min of clonidine. With this i.v. infusion scheme, clonidine reached a total dose of 24.4 µg/kg just before starting

the administration of capsaicin; this dose of clonidine (which is about 50 times higher than that given intracarotidly; see above) is high enough to reach the central nervous system.

12.4.3 Reproducibility of the canine external carotid vasodilator responses to capsaicin, αCGRP and acetylcholine

The vasodilator responses to capsaicin, αCGRP and acetylcholine did not significantly differ before and during the continuous i.v. infusion of saline, either in control dogs (lower responses; see Results section) or in dogs receiving a continuous intracarotid infusion of phenylephrine (enhanced responses; Figure 12.3); this finding indicates that the above vasodilator responses are highly reproducible. Moreover, the fact that these responses were enhanced during the continuous intracarotid infusion of phenylephrine (Figure 12.2) is attributed to the decrease in external carotid conductance resulting from an increase in the non-neurogenic vascular tone; under these conditions, there is a wider window for eliciting vasodilator responses. Since the highest dose of clonidine (1 μg/kg.min) also produced a similar decrease in external carotid conductance (see Results section), the enhanced vasodilator responses to capsaicin, αCGRP and acetylcholine during the infusion of phenylephrine were considered as the control responses when compared to those elicited during the infusion of clonidine (1 μg/kg.min). Consequently, any effect of clonidine on capsaicin-, αCGRP- or acetylcholine-induced external carotid vasodilatation should be attributed to a direct interaction of this imidazoline with its respective receptors, rather than to a decrease in baseline external carotid vascular conductance.

12.4.4 Mechanisms involved in the responses to capsaicin, αCGRP and acetylcholine

The fact that capsaicin, αCGRP and acetylcholine produced dose-dependent increases in external carotid blood flow without modifying blood pressure or heart rate suggests a local vasodilator action. Regarding the mechanisms involved in these vasodilator responses, other lines of pharmacological evidence in the carotid circulation have previously shown the involvement of: (i) CGRP release and activation of BIBN4096BS-sensitive $CGRP_1$ receptors for capsaicin (12) (ii) BIBN4096BS-sensitive $CGRP_1$ receptors for αCGRP (13); and (iii) atropine-sensitive muscarinic receptors located on the vascular endothelium for acetylcholine (18).

12.4.5 Specific inhibition by clonidine on the vasodilator responses to capsaicin

The fact that the vasodilator responses to capsaicin, but not to αCGRP or acetylcholine, were inhibited during the infusion of clonidine (see Figure 12.4) indicates that this inhibition is: (i) specific; and (ii) unrelated to a postsynaptic interaction with CGRP receptors.

12.4.6 Possible locus of the receptors involved in the inhibitory action of clonidine

Since clonidine can cross the blood-brain barrier (25), the inhibition by clonidine in our study may involve central and/or peripheral mechanisms. This suggestion is consistent with previous studies showing that: (i) dihydroergotamine and ergotamine inhibit CGRP release after trigeminal ganglion stimulation by presynaptic mechanisms (7, 30); and (ii) UK 14,304, an α_2-adrenoceptor agonist, inhibited the dural neurogenic plasma extravasation produced by trigeminal stimulation (31). Thus, we cannot categorically exclude an action of clonidine on receptors located on: (i) trigeminal vascular neurons; (ii) trigeminal ganglia; and/or (iii) trigeminal nucleus caudalis. The latter is strengthened by the fact that α_2-adrenoceptors are present in trigeminal nucleus caudalis (32).

12.4.7 Role of α_2-adrenoceptors in the inhibitory action of clonidine: close pharmacological resemblance to the $\alpha_{2A/2C}$-subtypes

Some studies suggest that the release of substance P and CGRP from nerve endings can be inhibited by activation of presynaptic α_2-adrenoceptors (31, 33). In keeping with these findings, the inhibitory action of clonidine on the vasodilatation to capsaicin was abolished by rauwolscine (Figure 12.5B) at a dose high

enough to completely – and selectively – antagonize α_2-adrenoceptors in the canine external carotid circulation (29). Although, admittedly, clonidine can also interact with imidazole binding sites (34), rauwolscine does not block these sites at concentrations that completely antagonize α_2-adrenoceptors (35, 36). Therefore, the above lines of evidence, taken together, support our contention that α_2-adrenoceptors mediate the inhibition by clonidine in our study.

α_2-adrenoceptors exist in three pharmacologically and structurally distinguishable receptor subtypes, namely $\alpha_{2A,}$ α_{2B} and α_{2C} (28, 37, 38). Hence, we further investigated the role of these subtypes in the above inhibition by clonidine employing antagonists with moderate to high subtype selectivity (see Table 12.1): BRL44408 (α_{2A}; 1000 µg/kg), imiloxan (α_{2B}; 1000 µg/kg) and MK912 (α_{2C}; 100 and 300 µg/kg) at doses high enough to completely antagonize their respective receptor subtypes in the canine external carotid circulation (39). In the first instance, it is noteworthy that the blockade produced by BRL44408 and MK912 on the inhibition by clonidine (Figure 12.6) was selective, as these compounds failed to antagonize the external carotid vasoconstriction to phenylephrine (39). Moreover, it must be pointed out that vascular α_{2C}-adrenoceptors are completely antagonized by 100 µg/kg of MK912 (39), but this dose partially blocked (Figure 12.6C), whilst 300 µg/kg abolished (Figure 12.6D), the inhibition by clonidine in our study. In this respect, although MK912 displays a very high affinity for the α_{2C} subtype (pK_i: 10.2): (i) it cannot selectively discriminate amongst the three subtypes (see Table 12.1); and (ii) no information is available on the selectivity of this subtype "selective" antagonist at canine α_2-adrenoceptor subtypes. In fact, the *in vitro* α_{2A}- versus α_{2C}-selectivity of BRL44408 and MK912 is small (40), leaving very little room for *in vivo* selectivity. These findings, coupled to the antagonism by BRL44408 (Figure 12.6A) and the inactivity of imiloxan (Figure 12.6B) on the inhibition by clonidine, lead us to suggest: (i) that mainly α_{2A}-adrenoceptors and, to a much lesser extent, α_{2C}-adrenoceptors are involved; and (ii) no role for α_{2B}-adrenoceptors. This suggestion is in agreement with: (i) the affinity of BRL44408 and MK912 for α_{2A}- and α_{2C}-adrenoceptor subtypes (Table 12.1); and (ii) the expression of α_{2A}- and α_{2C}-adrenoceptors, but not α_{2B}-adrenoceptors, in trigeminal ganglion neurons (41, 42)and in the trigeminal nucleus caudalis (43, 44).

12.4.8 Possible transductional mechanisms involved in α_2-adrenoceptor-induced inhibition of the vasodilator responses to capsaicin

Admittedly, our study provides no direct evidence of the transductional mechanisms involved in clonidine-induced inhibition of the vasodilator responses to capsaicin. Nevertheless, it is important to emphasize that α_2-adrenoceptors are predominantly coupled to the inhibitory heterotrimeric GTP-binding protein which: (i) inhibits the activity of adenylyl cyclase and the opening of voltage-gated Ca^{2+} channels; and (ii) activates K^+ channels (45). These are signal transduction systems usually associated with a decrease in the release of neurotransmitters (28, 46, 47).

12.4.9 Possible clinical implications

It has been proposed that neurogenic dural vasodilatation (produced by an increase in the trigeminal release of CGRP) is likely to be involved in migraine pathophysiology. Hence, inhibition of this mechanism may result in antimigraine action (14, 15). Interestingly, our study shows that clonidine specifically inhibits capsaicin-induced external carotid vasodilatation (Figure 12.4), but clinical trials indicate that the antimigraine efficacy of this imidazoline does not differ from that of placebo (48). Therefore, these findings may shed further light on the mechanisms involved in the antimigraine efficacy of some agents with complex pharmacology including ergots (8, 9) and isometheptene (49). Amongst other properties, these agents activate α_2-adrenoceptors, but their antimigraine actions within the bounds of α_2-adrenoceptor activity could be mainly attributed to cranial vasoconstriction rather than inhibition of neurogenic vasodilatation.

In conclusion, the above results show that clonidine specifically inhibited the canine external carotid vasodilator responses to capsaicin. This inhibitory action of clonidine, which involves the activation of rauwolscine-sensitive α_2-adrenoceptors, seems to be predominantly mediated by α_{2A}-adrenoceptors and, to a much lesser extent, by α_{2C}-adrenoceptors.

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Part E: Vascular Pharmacology of Preeclampsia

Chapter 13

Functional Reactivity of 5-HT Receptors in Human Umbilical Cord and Maternal Subcutaneous Fat Arteries after Normotensive or Preeclamptic Pregnancies

Based on: S. Gupta, L.M. Hanff, E.A.P. Steegers, P.R. Saxena., A.G. Vulto, W. Visser, A. MaassenVanDenBrink, Journal of Hypertension, 2006; 24:1345-1353

ABSTRACT

We investigated the functional reactivity of 5-hydroxytryptamine (serotonin; 5-HT) receptors in foetal umbilical cord arteries (UCA) and maternal subcutaneous fat resistance arteries (SFA) in normotensive and preeclamptic pregnancy. Study groups were divided based on the presence or absence of preeclampsia and duration of gestation. Segments of UCA and SFA were mounted in tissue baths and concentration response curves to 5-HT and sumatriptan (5-$HT_{1B/1D}$ receptor agonist) were constructed in the absence or presence of ketanserin (5-HT_{2A} receptor antagonist) or GR125743 (5-$HT_{1B/1D}$ receptor antagonist). Both 5-HT and sumatriptan contracted all UCA segments studied. The responses to 5-HT and the potency of ketanserin in UCA were not different between the study groups, indicating a similar profile of the 5-HT_{2A} receptor. In contrast, the potencies of sumatriptan and GR125743 were significantly higher in normotensive full term pregnancies than in normotensive pre term pregnancies in UCA. The response to sumatriptan in UCA arteries was not significantly different between preeclamptic and normotensive pregnancies. However, the potency of both sumatriptan and GR125743 were positively correlated to the gestational age in the normotensive group, while this relationship was absent in the preeclamptic group. In SFA, responses to 5-HT and sumatriptan were not different between the preeclamptic patients and normotensive controls. In both UCA and SFA, 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors mediate vasoconstriction. The sensitivity of 5-$HT_{1B/1D}$ receptors increases in the last trimester in the UCA in normal pregnancies, which seems to be expedited in preeclamptic patients. Hence, further studies on 5-$HT_{1B/ID}$ receptors will give new insights into the foetal development and pathophysiology of preeclampsia.

13.1 INTRODUCTION

Preeclampsia affects about 5% of all pregnancies and is a major cause of morbidity and mortality to both mother and foetus (1). The etiology of preeclampsia is still largely unknown, although it has been linked to abnormalities in trophoblast invasion into the placental bed (2). The syndrome is clinically characterized by maternal hypertension and proteinuria, and diagnosed after 20 weeks of gestation. The usual physiologic adaptations in response to increased fluid volume that are observed in normal pregnancies are attenuated in preeclampsia, resulting in increased vascular resistance (3). This higher vascular resistance of the maternal vasculature in preeclampsia may be attributed to *increased plasma concentrations* of contractile agents (4-7), but could alternatively be attributed to an *increased sensitivity* of the arteries to vasoconstrictor agents like angiotensin II (8, 9), 5-hydroxytryptamine (5-HT; serotonin) (7) and noradrenaline (10), combined with a decreased response to vasodilating peptides such as calcitonin gene-related peptide (11) and acetylcholine (12). Vascular hyperreactivity in preeclamptic women has been demonstrated by, for example, an increased vasoconstrictive response to the cold pressor test, which is mediated by α-adrenergic receptors (13). Obviously, increased plasma levels of a vasoconstrictor, in combination with an increased sensitivity of the respective receptors, may synergistically lead to increased vascular resistance and hence hypertension.

It is well established that in pregnancy the umbilicoplacental circulation lacks autonomic innervations (14) and thus, the regulation of vascular reactivity is mainly dependent on local autocrine and circulating vasoactive substances in the blood. 5-HT potently constricts human umbilical blood vessels (15) and causes platelet aggregation, which leads to further release of 5-HT; both these properties may thus synergistically impede normal placental blood flow in conditions like preeclampsia (7). 5-HT receptors have been classified into 7 main groups (5-HT_1-5-HT_7 receptors) with several established subtypes in the first two groups (16). Studies in umbilical cord reveal that the contractile response to 5-HT involves 5-HT_{1B} and 5-HT_2 receptors (17). 5-HT_7 receptors are also believed to mediate relaxation in human arteries (18), but information regarding their role in the regulation of hemodynamics in umbilicoplacental vessels is lacking. Maternal circulatory concentrations of 5-HT are significantly increased in women with preeclampsia (5-7). High levels of circulating factors like 5-HT in preeclampsia may induce endothelial

dysfunction in the resistance arteries of the mother (19), where 5-HT acts as a potent vasoconstrictor (20), which may further contribute to the increased vascular resistance observed in preeclampsia. In addition, the increased plasma levels of 5-HT may also affect 5-HT receptors in umbilicoplacental and maternal resistance vessels. On this basis, the present study was undertaken to study the differences in the functional reactivity of 5-HT receptors between normotensive pregnant and preeclamptic women. We studied umbilical cord arteries (UCA) as a representative of foetal blood vessels and subcutaneous fat arteries (SFA), representing maternal resistance arteries.

13.2 MATERIALS AND METHODS

13.2.1 Funcational studies

The Ethics Committee of the Erasmus MC approved this study. Umbilical cord and subcutaneous abdominal fat (in case of caesarean section) were obtained after informed consent from pregnant women admitted to the Obstetrics Department of Erasmus MC, St. Franciscus Gasthuis or Ikazia Ziekenhuis (all Rotterdam, The Netherlands). Preeclamptic patients (diastolic blood pressure $\geq$ 90 mm Hg and protein/creatinine ratio (PCR) $\geq$ 30 mg protein/mmol creatinine), as well as normotensive females of different gestational ages were included in the study. Patients were classified as pre-term at a gestational age < 37 weeks; all patients with higher gestational age were classified as full-term. The preeclamptic patients were treated with different antihypertensive drugs like α-methyldopa, dihydralazinee, ketanserin, labetalol and nicardipine. Women suffering from diabetes and normotensive women with intrauterine growth retardation (IUGR, neonate weight is < 10^{th} percentile weight for his/her age in weeks corrected for gestational age, parity and foetal sex (21) and in the preeclamptic group patients suffering from preexisting hypertension were not included in the study. Segments of umbilical cord and maternal subcutaneous fat tissues were collected in cold Krebs bicarbonate solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4), transported to laboratory and stored in carbogenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution at 4˚C. UCA were isolated from the umbilical cord after removing the Wharton's jelly and subcutaneous arteries were isolated after removing adhering subcutaneous fat. Functional experiments were performed on the same or the subsequent day. In pilot experiments, we did not observe any differences in responses of UCA to KCl, 5-HT or sumatriptan between experiments that were performed on the same day or the next day.

UCA segments of 3-4 mm length (internal diameter: 1.5-2.0 mm) were suspended with the help of stainless-steel hooks in 15-ml organ baths. These segments were set at a pretension of 25 mN as determined to be the optimal tension in pilot experiments (data not shown). Segments showing bulging or macroscopic undulations were not used in experiments. SFA segments were cut into rings of 1-2 mm length with an internal diameter of 150-500 µm. Artery segments were suspended in Mulvany myographs on two parallel titanium wires. Subsequently, the distance between the wires was normalized to $0.9 \times I_{100}$ (I_{100} is the distance between the pins when the transmural pressure equalizes 100 mm Hg) to achieve optimal conditions for active force development. For both UCA and SFA, the vessel segments were continuously bubbled with 95% O_2 and 5% CO_2 and the temperature was maintained at 37˚C. After an initial equilibration period of 45 min, two successive challenges with KCl (30 mM) were performed to verify the reproducibility of the response. Subsequently, KCl (100 mM) was added to determine the reference contractile response of the artery segments to compensate for small differences in the muscle mass of the artery segments. In both UCA and SFA, endothelial function was evaluated by observing the relaxant response to substance P (100 nM) after precontraction with U46619 (9,11-dideoxy-11α, 9α-epoxy, methanoprostaglandin $F_{2\alpha}$ 10-300 nM). Cumulative concentration response curves to 5-HT were constructed in a parallel setup in the presence of vehicle (saline) or after 30-min incubation with the $5\text{-}HT_{2A}$ receptor antagonist, ketanserin (10 nM, 100 nM or 1 µM). Similarly, concentration response

curves to sumatriptan were constructed in a parallel setup in the presence of vehicle or three increasing concentrations (10 nM, 100 nM or 1 μM) of the 5-$HT_{1B/1D}$ receptor antagonist, GR125743 (N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride) (22). In UCA, we also investigated the relaxant response to 5-HT mediated by putative 5-HT_7 receptors. For discerning 5-HT_7 responses in UCA, artery segments were incubated with both ketanserin and GR125743 (100 nM, each), the segment was precontracted with KCl (30 mM) and then 5-HT was added in a cumulative manner. Due to the limited number of vessel segments that could be isolated from the small samples of subcutaneous fat that were obtained during caesarean sections, experiments were performed using only one concentration (100 nM, each) of ketanserin and GR125743 and no experiments on the role of 5-HT_7 receptors were performed. In both UCA and SFA, only single concentration responses curve was constructed in each artery segment.

13.2.2 Chemicals

5-HT, U46619, substance P (Sigma Chemicals Co., Steinheim, Germany), KCl (Merck, Darmstad, Germany), ketanserin tartrate (Janssen, Beerse, Belgium), sumatriptan succinate (GlaxoSmithKline, Stevenage, U.K.) and (N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride (GR125743) (Pfizer Limited, Sandwich, Kent, U.K.) were all dissolved in distilled water and stored in aliquots at -80˚C.

13.2.3 Statistical analysis

The contractile response to 100 mM KCl, expressed in milli-Newton (mN), was used to compare the contractile force developed by arteries from different experiment groups. All contractile responses to the agonists are expressed as percentage of the contraction induced by 100 mM KCl. All values are expressed as mean±s.e.m. and n represents the number of segments, each segment obtained from a different patient. Since it was not always possible to study all compounds in segments obtained from one patient, the number of segments for each experimental condition may differ from the total number of patients in a group. Concentration response curves were analyzed using nonlinear regression analysis using GraphPad Prism 3.01 (GraphPad Software Inc., San Diego, CA, U.S.A.). The efficacy of the agonists was expressed as E_{max} (maximal response) and their potency as pEC_{50} (-logEC_{50}, where EC_{50} is the concentration of the agonist required to produce half the maximal response). Since 5-HT activates different 5-HT receptor subtypes, the blocking potency of ketanserin (apparent pK_b) was estimated by calculating concentration-ratios between the EC_{50} of agonist in the presence and absence of antagonist and plotting a Schild plot, assuming a slope of unity. The antagonism of the response to sumatriptan by GR125743 only reached significance at concentrations of 100 nM or higher. Therefore, only two concentration points of antagonist were available, which prohibits the calculation of a Schild slope and pA_2 (concentration of the antagonist required to shift the concentration responses curve of agonist by two fold to right hand side). For that reason, the antagonist potency of GR125743 was also expressed as apparent pK_b, assuming a slope of unity. In case of UCA, the pK_b was calculated at 100 nM to allow a uniform comparison with data obtained with SFA, where only one concentration of the antagonists could be studied. The influence of the way of delivery (vaginal or caesarean section) on the response to KCl, 5-HT, sumatriptan or the antagonist was assessed by analysis of covariance (ANCOVA), using the way of delivery as a factor and gestational age as a covariant. Group means were compared by using unpaired Student t-test, with differences considered significant at $P<0.05$. Correlation analyses were carried out using Pearson's coefficient of correlation between gestational age or neonatal weight and responses to 5-HT, ketanserin, sumatriptan or GR125743.

13.3 RESULTS

The demographic details of all the subjects from whom the umbilical cord artery, subcutaneous fat arteries, or both were obtained, are presented in Table 13.1. The KCl (100 mM)-induced contractions were not different between UCA obtained from normotensive and preeclamptic women. There were no differences between the responses to KCl, 5-HT, sumatriptan or the antagonists in the UCA obtained from vaginal deliveries and cesarean sections; therefore, the results were pooled in further analysis. Within the preeclamptic group, patients with or without IUGR did not differ significantly in the responses to KCl, 5-HT, sumatriptan or their antagonists, hence their results were pooled in further analyses. In none of the UCA investigated, we observed an endothelium-dependent relaxation to substance P. The results obtained from UCA are presented in four main groups namely: the normotensive pre-term, the normotensive full-term delivery, the preeclamptic pre-term and the preeclamptic full-term delivery group.

Table 13.1. Demographic details of the patients from whom the umbilical cord artery and subcutaneous fat arteries were obtained.

	Normotensive pre-term	Normotensive full-term	Preeclamptic pre-term	Preeclamptic full-term
Number	14	48	34	12
Maternal age (y)	30.6±1.5	33.0±0.7	32.9±1.2	31.5±1.6
Gestational age at delivery (wk)	30.6±1.1	39.3±0.1	30.2±0.7	38.8±0.4
Diastolic BP (mm Hg)	69.0±0.9	74.7±1.4	104.2±2.5	98.2±1.8
Systolic BP (mm Hg)	118.5±0.9	121.1±1.8	167.0±5.9	144.8±4.9
PCR (mg/mmol)	Not determined	Not determined	967±293	699±423
Birth weight (g)	1555±193	3443±73	1161±99	3138±131
Vaginal delivery	11	25	2	5
Cesarean section	3	23	32	7
Intrauterine growth restriction	Not included	Not included	11	1

BP: Blood pressure, PCR: Protein/creatinine ratio

13.3.1 Responses to 5-HT in umbilical cord artery

5-HT induced contractions in all UCA studied. The E_{max} and pEC_{50} of 5-HT were not significantly different between the groups (Figure 13.1, Table 13.2). Ketanserin antagonized the responses to 5-HT in a concentration-dependent manner in all groups with an average pK_b of around 7.6; there was no difference in the potency of ketanserin between the study groups. We did not observe any relaxations to 5-HT, putatively mediated by 5-HT_7 receptors, in precontracted UCA in the presence of ketanserin and GR125743 (both 100 nM, data not shown).

13.3.2 Responses to sumatriptan in umbilical cord artery

Sumatriptan also induced contractions in all UCA studied (Figure 13.2, Table 13.2). In the pre-term normotensive group, the pEC_{50} of sumatriptan (5.71±0.23, n=12) was significantly lower than in the normotensive full-term group (6.62±0.12, n=30). The E_{max} values between the groups were not significantly different. Within the preeclamptic group, there were no significant differences in E_{max} and potency of sumatriptan between pre-term and full-term in UCA. In the preeclamptic pre-term group, the potency of sumatriptan (6.26±0.19, n=22) tended to be higher compared to the normotensive pre-term group, although this difference did not reach significance (P=0.08). GR125743 significantly antagonized contractile responses to sumatriptan at concentrations ≥ 100 nM, which is in accordance with previous observations in human coronary artery and saphenous vein (23). Similar as observed with the potency of

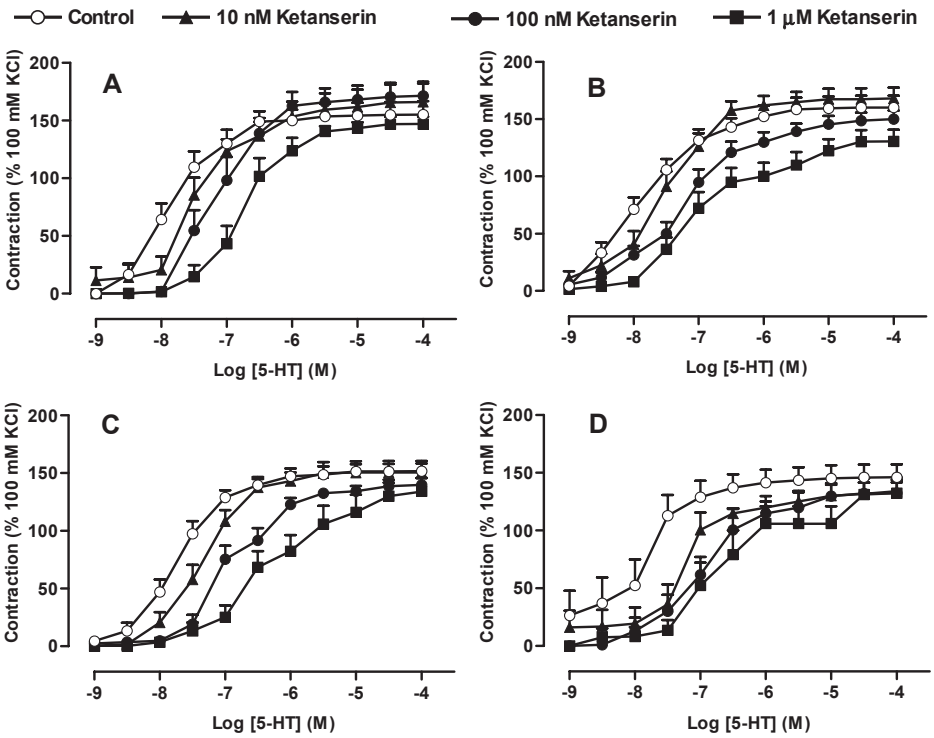

Figure 13.1. Concentration response curves to 5-HT in the absence or presence of increasing concentrations of the 5-HT_{2A} receptor antagonist, ketanserin (10 nM-1 μM), in umbilical cord artery segments. Panel A: normotensive pre-term, panel B: normotensive full-term, panel C: preeclamptic pre-term and panel D: preeclamptic full-term deliveries.

sumatriptan, the potency of GR125743 in the preeclamptic pre-term group (8.09±0.22, n=17) tended to be higher compared to the normotensive pre-term group (P=0.06), while the potency of GR125743 was similar in the preeclamptic pre-term and preeclamptic full-term groups. In addition, the pK_b of GR125743 was significantly lower in normotensive pre-term deliveries (7.28±0.37, n=7) compared to that in normotensive full-term deliveries (8.05±0.13, n=18) in UCA, while such a difference was absent in UCA obtained from preeclamptic patients.

13.3.3 Relationship between foetal development and functional response to 5-HT, ketanserin, sumatriptan and GR125743 in umbilical cord artery

The fact that sumatriptan-induced responses were different in the UCA obtained from the pre-term and full-term normotensive deliveries with mean gestational ages of 31 and 39 weeks, respectively, suggests that 5-$HT_{1B/1D}$ receptors are still in a development phase during the third trimester. To study the effect of foetal development in further detail on the various pharmacological parameters obtained in the normotensive (both pre-term and full-term deliveries) and preeclamptic groups, we performed a correlation analysis with gestational age. We did not observe any significant correlation between the gestational age and the E_{max} or pEC_{50} of 5-HT or the pK_b of the 5-HT_{2A} receptor antagonist, ketanserin, in either group (Table 13.3). In contrast, there was a significant correlation between the gestational age and the potency of the 5-$HT_{1B/1D}$ receptor agonist, sumatriptan (r=0.456, P=0.002), as well as the potency of its antagonist, GR125743 (r=0.486, P=0.012), in UCA obtained from normotensive controls. In UCA obtained from patients suffering from preeclampsia, the potency of sumatriptan and its antagonist GR125743 did not correlate with gestational age.

Table 13.2. Pharmacological parameters derived from umbilical cord arteries obtained from normotensive pre-term and full-term pregnancies and pregnancies complicated by pre-term preeclampsia.

	Normotensive pre-term (n=7-14)		Normotensive full-term (n=18-39)		Preeclamptic pre-term (n=17-28)		Preeclamptic full-term (n=10-12)	
	5-HT	Sumatriptan	5-HT	Sumatriptan	5-HT	Sumatriptan	5-HT	Sumatriptan
E_{max} (% KCl)	155±12	121±12	160±10	122±8	152±7	132±10	146±11	138±7
pEC_{50}	7.89±0.16	5.71±0.23*	7.80±0.14	6.62±0.12	7.76±0.11	6.26±0.19	7.93±0.19	6.48±0.20
pK_b ketanserin	7.64±0.24		7.77±0.17		7.50±0.13		7.67±0.21	
pK_b GR125743		7.28±0.37*		8.05±0.13		8.09±0.22		8.19±0.24
KCl (mN)	26.3±3.1		20.4±1.6		27.6±2.5		22.7±2.8	

pK_b values of ketanserin and GR125743 determined at 100 nM; KCl: response to 100 mM KCl; n: number of UCA segments, each segment obtained from a different woman; *, Significantly different ($P<0.05$) from normotensive full-term.

Table 13.3 Pearson's coefficient of correlation between gestational age or neonatal weight and the agonist potency (pEC_{50}) of 5-HT and sumatriptan as well as antagonist potency (pK_b) of ketanserin and GR125743 to their respective agonists in umbilical cord artery segments obtained from normotensive controls (NT) and preeclamptic patients (PE).

Pearson coefficient	pEC_{50} 5-HT		pK_b Ketanserin		pEC_{50} Sumatriptan		pK_b GR125743	
	NT	PE	NT	PE	NT	PE	NT	PE
Gestational age	-0.111	0.008	0.013	-0.092	0.456**	0.287	0.486*	0.146
Neonatal weight	-0.022	0.139	-0.028	-0.053	0.540***	0.260	0.424*	0.091
Number of patients	42	37	32	33	44	34	26	27

*$P<0.05$, **$P<0.01$ and ***$P<0.001$

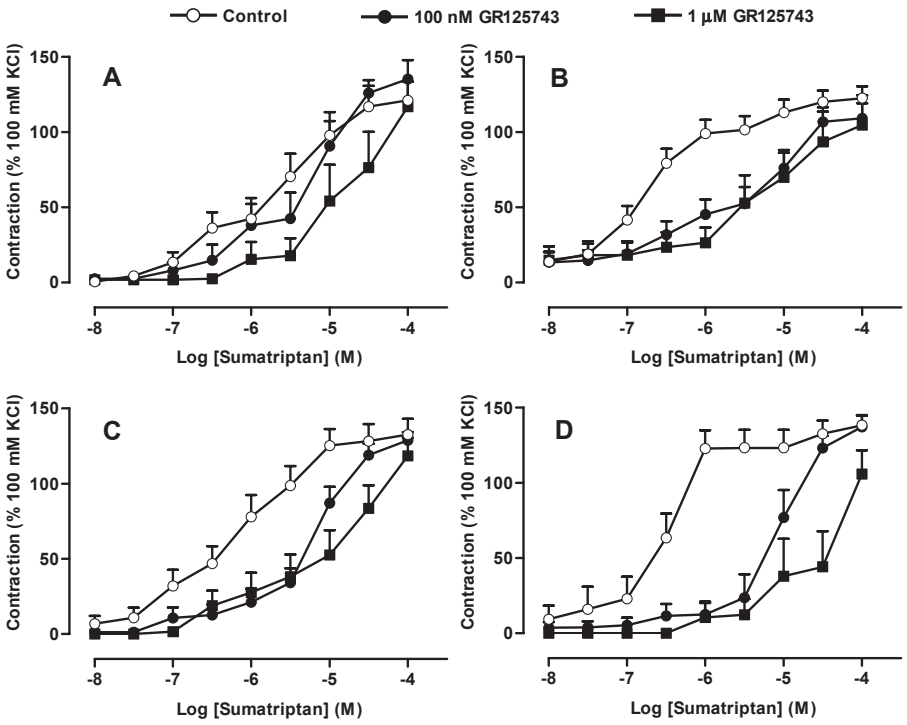

Figure 13.2. Concentration response curves to sumatriptan in the absence or presence of increasing concentrations of the 5-$HT_{1B/1D}$ receptor antagonist, GR125743 (10 nM-1 µM), in umbilical cord artery segments. Panel A: normotensive pre-term, panel B: normotensive full-term, panel C: preeclamptic pre-term and panel D: preeclamptic full-term deliveries.

Since neonatal weight has been also described a marker for foetal development (24), we also analyzed the correlation between neonatal weight (that strongly correlated with gestational age, r=0.940, P<0.001) and the responses to the agonists and antagonists. In the preeclamptic group, there were twelve neonates with IUGR; we performed our correlation analysis either including or excluding these patients. As with the results obtained with gestational age, there was no significant correlation between the responses to 5-HT or ketanserin and neonatal weight in either of the groups. The potency of sumatriptan again significantly correlated with neonatal weight in the normotensive group (r=0.540, P<0.001), but not in the preeclamptic group (r=0.260, P=0.137 and r=0.233, P=0.262), including or excluding the patients with IUGR, respectively). There was a significant correlation between pK_b of GR125743 (r=0.424, P=0.031) and neonatal weight in the normotensive group, but not in the preeclamptic group (r=0.091, P=0.651 and r=0.188, P=0.415, including or excluding the patients with IUGR, respectively). Further, there was a significant negative correlation between the gestational age and contractile responses to 100 mM KCl in UCA in normotensive group (r=0.289, P=0.044, n=49), but not in the preeclamptic group (r=0.211, P=0.203, n=38).

13.3.4 Responses to 5-HT and sumatriptan in subcutaneous fat arteries

One SFA in the normotensive group and three in the preeclamptic group did not respond to 5-HT or sumatriptan. These patients were not included in further analysis because the potency of the antagonists could not be assessed in these specimens. Because of the limited occurrence of deliveries by caesarean sections in pre-term normotensive patients, all the SFA from normotensive patients were analysed as one group, irrespective of their gestational age, unlike for UCA. There was no significant difference in

Table 13.4. Pharmacological parameters derived from subcutaneous fat arteries obtained from normotensive pregnancies and pregnancies complicated by preeclampsia.

	Normotensive (n=8-19)		Preeclamptic (n=8-16)	
	5-HT	Sumatriptan	5-HT	Sumatriptan
E_{max} (% 100 mM KCl)	109±15	70±13	115±18	59±15
pEC_{50}	7.18±0.12	6.15±0.26	7.39±0.12	5.96±0.25
pK_b Ketanserin (100 nM)	8.32±0.36		8.43±0.56	
pK_b GR125743 (100 nM)		8.82±0.24		8.01±0.25*
Response to 100 mM KCl (mN)	4.72±0.89		6.46±1.32	

n: number of UCA segments, each segment obtained from a different woman. *, Significantly different (P<0.05) from normotensive controls.

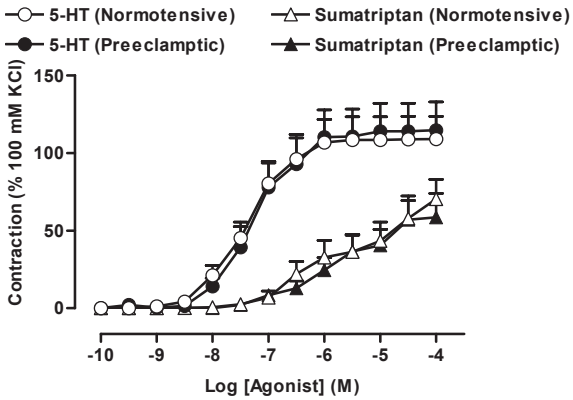

Figure 13.3. Concentration response curves to 5-HT and sumatriptan in subcutaneous fat resistance arteries obtained from normotensive and preeclamptic deliveries

the endothelium-dependent relaxations between SFA obtained from preeclamptic patients (46±9% precontraction with U46619) and those obtained from normotensive women (37±6%). Neither the maximal response or pEC_{50} of 5-HT, nor the potency of ketanserin was different between the preeclamptic and normotensive group (Figure 13.3, Table 13.4). Similarly, the responses to sumatriptan were not different between the two groups. In contrast, the $5\text{-HT}_{1B/1D}$ receptor antagonist, GR125743, was less potent in arteries obtained from preeclamptic patients than in normotensive controls.

13.3.5 Relationship between foetal development and responses to 5-HT, ketanserin, sumatriptan and GR125743 in subcutaneous fat arteries

The gestational age in the preeclamptic group (32.0±1.2 weeks) was significantly less than that of the normotensive controls (38.9±0.2 weeks). There was no significant correlation between gestational age or neonatal weight and responses to 5-HT or ketanserin in either of the groups. There was a significant correlation between the pEC_{50} of sumatriptan and neonatal weight, but not with gestational age, in the normotensive group. This correlation was absent in the SFA obtained from the preeclamptic patients. The potency of antagonist also did not have any significant correlation with neonatal weight or gestational age. The correlation data should be interpreted with caution in view of the limited number of patients in the study group.

13.3.6 Comparison between subcutaneous fat arteries and umbilical cord arteries responses

In SFA, we observed endothelium-dependent relaxations, whereas in UCA these responses were absent. The maximal response and potency of 5-HT and sumatriptan were significantly lower in SFA than in UCA. In contrast, the potency of the antagonist ketanserin was higher in SFA than in UCA in both groups. In both UCA and SFA the maximal response to sumatriptan was less than the 5-HT response and in case of SFA this difference was more pronounced.

13.4 DISCUSSION

In the current study, we investigated vasoreactivity in foetal and maternal arteries obtained from pre-eclamptic and normotensive pregnancies. As pregnancies complicated by preeclampsia are often associated with premature delivery, it is vital to take into account differences in gestational age while comparing with normotensive controls. Certain receptor systems are still in the developmental phase in the umbilicoplacental circulation in the third trimester of pregnancy. For example, in umbilical and placental veins, the reactivity of 5-HT and histamine tends to increase throughout this period (24). Therefore, we included a correlation analysis between various pharmacological parameters obtained and markers of foetal development (gestational age and neonatal weight).

In UCA segments, obtained both from preeclamptic patients and normotensive controls, 5-HT and sumatriptan induced contractions that were sensitive to antagonism by ketanserin and GR125743, respectively. These results confirm the role of 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in mediating vasoconstriction in UCA. Although we did not use selective 5-HT_{1B} or 5-HT_{1D} receptor antagonists, vasoconstriction to sumatriptan is most likely mediated via the 5-HT_{1B} receptor (25). Even though 5-HT is not a selective 5-HT_{2A} receptor agonist, we chose 5-HT as an agonist in this study as it encompasses the study of all 5-HT receptor subtypes and provides the possibility to study the 5-HT_{2A} receptors using ketanserin. Further, the use of 5-HT allows comparison of our results with the other studies on 5-HT receptors in UCA (15, 17, 24, 26). We did not observe any difference in the 5-HT-induced contractions in UCA between the preeclamptic and the normotensive group, which seems to be in contrast to the finding by Bertrand *et al*. (24). However, the higher E_{max} induced by 5-HT in UCA from preeclamptic patients in their study (24) is merely based on higher contractile responses to KCl, and disappears when the responses are corrected for the differences in absolute contractile force between the groups. Indeed, others also did not report any significant differences in the reactivity to various vasoconstrictors, including 5-HT, in UCA obtained from normotensive and preeclamptic patients (27, 28) and some studies have even reported decreased responses to 5-HT in UCA obtained from severe preeclamptic patients (27). In accordance with previous reports (24), we did also not observe any correlation between foetal development and the potency of 5-HT or ketanserin in UCA obtained from normotensive and preeclamptic patients. Taken together, our findings suggest that 5-HT_{2A} receptors are not affected in preeclampsia, and that these receptors are already fully developed early in the third trimester of pregnancy.

In the study by Lovren *et al*. (17), a sub-threshold concentration of a vasoconstricting agent was required for observing the activity of 5-$HT_{1B/1D}$ receptors (only three out of ten vessels responded to sumatriptan without prestimulation), whereas in our study sumatriptan produced contractile responses in all UCA without prestimulation. This discrepancy might be explained by higher levels of endogenous vasoconstrictors such as thromboxane A_2 in our preparations, which could then 'unmask' responses to sumatriptan (29, 30) or could eventually be due to the higher pretension (25 mN) used in our study as used (20 mN) in the study by Lovren *et al*.

In our study, the potency of sumatriptan in UCA was about 10-fold higher in the full-term deliveries as compared to pre-term deliveries within the normotensive group, suggesting that 5-$HT_{1B/1D}$ receptors are still developing in the last trimester. This observation was strengthened by the significant positive correla-

tion between foetal development (expressed as either gestational age or neonatal weight) and the pEC_{50} of sumatriptan in the normotensive group, whereas this correlation was absent in preeclamptic group. Similarly, the pK_b value of GR125743 only correlated with foetal development in the normotensive controls and not in the preeclamptic group. Additionally, unlike in the normotensive group, in the preeclamptic group the potency of sumatriptan does not seem to increase in full-term pregnancies, compared to the pre-term group. Finally, the pEC_{50} of sumatriptan and the pK_b of GR125743 in the normotensive pre-term control group appear to be lower than that in preeclamptic pre-term group, although this differences did not reach significance (P=0.06-0.08). Taken together, our observations suggest that in the preeclamptic group the normal development of 5-$HT_{1B/1D}$ receptors is compromised, and that the response to these receptors may already be on higher strata at an earlier gestational period. This higher sensitivity may be explained by a larger number of 5-$HT_{1B/1D}$ receptors and/or a more efficient coupling of these receptors with the second messenger pathway in UCA of preeclamptic patients. The cause of this higher sensitivity could be increased plasma levels of 5-HT in preeclampsia (15), or, alternatively, foetal factors induced by umbilicoplacental vasoconstriction, which may contribute to maternal hypertension. Future studies, like binding experiments of 5-$HT_{1B/1D}$ receptors in foetal and maternal blood vessels at different gestational ages may give more insight into role of these receptors in foetal development and preeclampsia.

A more prominent role of 5-$HT_{1B/1D}$ receptors in preeclampsia seems to gain relevance because contractile responses mediated by 5-$HT_{1B/1D}$ receptors may be augmented by other vasoconstrictive agents like thromboxane A_2 (26, 29), and indeed plasma levels of both 5-HT and thromboxane A_2 are known to be increased in preeclampsia (4). Interestingly, preeclampsia usually is manifested clinically in the third trimester, which is the same period where we observed an increase in the activity of 5-$HT_{1B/1D}$ receptors, suggesting that these receptors might have a pathophysiological role in preeclampsia. Antagonism of 5-HT receptors has been explored earlier as a therapeutic option for the treatment of preeclampsia using the 5-HT_{2A} receptor antagonist ketanserin (31, 32), but in a substantial number of patients the antihypertensive response is insufficient (33). However, the combination of ketanserin and aspirin, inhibiting the synthesis of thromboxane A_2, is beneficial in the prevention of preeclampsia in women with mild to moderate hypertension (31). Since the augmentation of contractile responses to 5-HT by thromboxane A_2 is mainly mediated by 5-$HT_{1B/1D}$, and not by 5-HT_{2A} receptors (34), 5-$HT_{1B/1D}$ receptor antagonism might have an additive therapeutic value in the treatment of preeclampsia, especially by increasing the already compromised umbilicoplacental blood flow.

In SFA we did not observe any difference in substance P induced-relaxations between preeclamptic patients and normotensive controls, which seems to be at variance with earlier observations (35). However, in a recent study performed on subcutaneous and myometrial resistance arteries, relaxations to substance P were not different between normotensive and preeclamptic subjects (12), which is in accordance with our observations. In UCA, we did not observe any relaxations to substance P, which is also in line with previous reports (36). Both 5-HT and sumatriptan elicited contractions that were sensitive to antagonism by ketanserin or GR125743, respectively. Thus, our results demonstrate the presence of 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in SFA. There were no significant differences in the reactivity to 5-HT or sumatriptan between the normotensive and the preeclamptic group. It should be noted that although vascular hyperreactivity has been described in preeclampsia (5), increased vascular sensitivity is not a generalized phenomenon in this syndrome, as we pointed out above (12, 27, 37) and as observed in the present study. Contractile responses to 5-HT and sumatriptan in SFA were significantly smaller than the responses observed in UCA, underscoring that humoral factors play a more dominant role in contraction of umbilical cord arteries, which are not innervated. As in most studies in patients, preeclamptic patients were treated with antihypertensive drugs, which might induce an underestimation of differences between the preeclamptic and the normotensive pregnant women in our study.

Admittedly, our data suggest that antagonism of 5-$HT_{1B/1D}$ receptors would have a more prominent effect on the umbilicoplacental circulation than on maternal hypertension. However, since the umbilico-

placental blood flow tends to decrease after antihypertensive treatment of the mother (38), resulting in foetal distress, and factors released in response to a reduced blood flow to the foetus may also contribute to maternal hypertension, it is pivotal to maintain a sufficiently high umbilicoplacental blood flow. In conclusion, we demonstrate that 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors mediate contraction in foetal (UCA) and maternal (SFA) arteries. The functional profile of 5-HT_{2A} receptors does not change during the third trimester, nor is it different between preeclamptic pregnancies and normotensive controls. In contrast, the sensitivity of 5-$HT_{1B/1D}$ receptors increases during the third trimester in UCA in normotensive pregnancies, while this development is expedited in preeclamptic patients. Further studies on the role of 5-$HT_{1B/1D}$ receptors will give more insight into the role of 5-HT in foetal development and the pathophysiology of preeclampsia.

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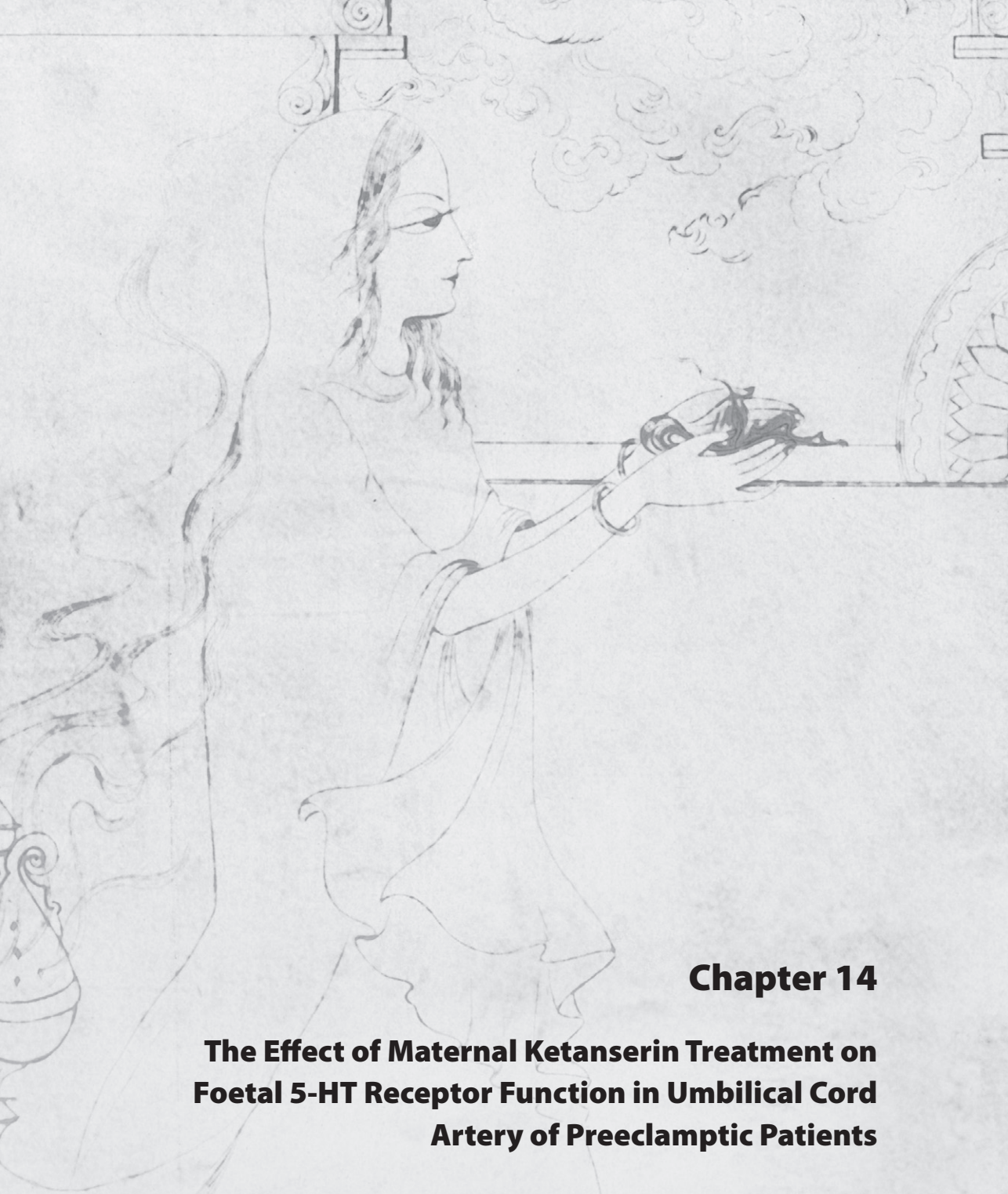
When you want something, all the universe conspires in helping you achieve it. (Paulo Coelho)



Chapter 14

The Effect of Maternal Ketanserin Treatment on Foetal 5-HT Receptor Function in Umbilical Cord Artery of Preeclamptic Patients

Based on: L.M. Hanff, S. Gupta, A. MaassenVanDenBrink, E.A.P. Steegers, P.R. Saxena, A.G. Vulto, W. Visser, Submitted.

ABSTRACT

Maternal treatment with the 5-HT_{2A} receptor antagonist ketanserin in preeclamptic patients is associated with a high placental transmission of ketanserin, resulting in pharmacologically active levels of ketanserin in umbilical cord artery (UCA) and neonate. To study whether exposure to ketanserin influences the characteristics of foetal 5-HT receptors, functional studies were performed on 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in UCA from preeclamptic patients treated with ketanserin. UCA were obtained, immediately after delivery, from preeclamptic patients (n=7), treated antenatally with intravenous ketanserin. Preeclamptic patients (n=13), not treated with ketanserin (non-ketanserin), were included as a control group. Segments of UCA were prepared and mounted in tissue baths and isometric force changes were determined. Cumulative concentration-response curves to 5-HT and to the 5-$HT_{1B/1D}$ receptor agonist sumatriptan, were constructed in the absence or presence of the 5-HT_{2A} receptor antagonist ketanserin or the 5-$HT_{1B/1D}$ receptor antagonist GR125743, respectively. All UCA segments showed contractile responses to both 5-HT and sumatriptan, and the concentration-response curves showed a rightward shift with increasing concentrations of ketanserin and GR125743, respectively, indicating the presence of functional 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in the foetal tissue. No significant differences were found in maximum response E_{max} (expressed in % of response on 100 mM KCl), or potency (pEC_{50}) of 5-HT in the two groups (E_{max} 141±7.7%, pEC_{50} 7.67±0.26 in ketanserin-treated group and E_{max} 162±12.6%, pEC_{50} 7.69±0.14 in non-ketanserin treated group, respectively). No significant differences were found in the potency of the antagonist ketanserin in both study groups (pK_b 7.65±0.31 in ketanserin group and 7.46±0.17 in non-ketanserin group, respectively). Similarly, with sumatriptan, no significant differences were found between ketanserin-treated patients and non-ketanserin treated patients (E_{max} 142±16.2 and 140±14.7%, respectively, pEC_{50} 6.17±0.37 and 6.41±0.28% respectively, pK_b of GR125743 7.83±0.48 and 8.43±0.29, respectively). In conclusion, foetal exposure to ketanserin in preeclamptic patients does not significantly influence the functional characteristics of 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in the umbilical cord artery.

14.1 INTRODUCTION

Preeclampsia is a disease occurring in 2-5% of the pregnant women and it forms one of the leading causes of maternal and neonatal mortality and morbidity during pregnancy (1). The pathophysiology of the disease is not yet fully understood, but impaired trophoblast invasion and endothelial dysfunction are considered to be important factors in the pathogenesis (1, 2). The main clinical characteristics of preeclamptic patients are elevated blood pressure, proteinuria and oedema, occurring after the twentieth week of gestation. Treatment with antihypertensive drugs is indicated to stabilise the patient and prevent maternal complications like organ failure or haemorrhages in retina or brain. Preterm delivery of, often severely growth restricted, neonates occurs frequently in early-onset preeclamptic patients (3, 4).

In the past years, prolonging the pregnancy of early-onset preeclamptic patients ("temporising management"), using potent antihypertensive drugs, has been undertaken to improve neonatal outcome (5-7). One of the antihypertensive drugs used most often in The Netherlands in the treatment of preeclampsia, is the 5-HT_{2A} receptor antagonist ketanserin. The drug is thought to act by blocking the vasoconstrictive response to 5-HT in the blood vessels (8). An increased vasoconstrictive response to 5-HT, leading to increased peripheral resistance, has been implicated as one of the mechanisms involved in preeclampsia. Hence, the use of a 5-HT antagonist such as ketanserin, is considered a rational approach in the treatment of preeclampsia (9, 10). However, for temporising management in severe, early-onset preeclamptic patients, high intravenous dosages of ketanserin are needed for prolonged periods of time, resulting in extensive foetal exposure to ketanserin. Indeed, concentrations of ketanserin equal to maternal levels, were found in the umbilical cord (26-373 ng/ml) and in the neonate (71-302 ng/ml) after maternal use of ketanserin [11]. Whether these pharmacologically active concentrations of ketanserin, being a 5-HT_{2A} receptor antagonist, influence the characteristics and possibly cause desensitisation of

foetal 5-HT receptors, is unknown. In animal studies, chronic blockade of 5-HT_{2A}-receptors by ketanserin has been shown to lead to an unexpected down-regulation of 5-HT_{2A} receptors (12, 13).

5-HT is known to be one of the earliest neurotransmitters produced during foetal brain development (14, 15). It can be speculated that foetal exposure to 5-HT receptor blocking drugs such as ketanserin, can cause harmful effects on the foetus, especially considering the abundant presence of 5-HT receptors in the foetal brain. Although human studies have not yet shown clinical adverse effects in neonates, clearly attributable to maternal ketanserin treatment (8, 11, 16) studying foetal 5-HT receptors in functional studies will yield more detailed information regarding foetal development of these receptors and may lead to a better assessment of the risk of long-term effects in the neonate after maternal ketanserin treatment. In our study, we selected the umbilical cord artery as representative of foetal vessels, knowing that 5-HT_{2A} and 5-$HT_{1B/1D}$ are the major receptors mediating 5-HT induced vasoconstriction in human umbilical cord artery (17).

Based on the results of animal studies (12, 13), we hypothesise that prolonged exposure of tissue and blood vessels to the 5-HT_{2A} receptor antagonist ketanserin influences the functionality of foetal 5-HT receptors, by downregulation of the 5-HT_{2A} receptor. In the present study, functional responses of 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in UCA from preeclamptic patients treated with ketanserin were compared with responses from preeclamptic patients not treated with ketanserin.

14.2 MATERIALS AND METHODS

Twenty preeclamptic patients, admitted to the antenatal ward of the Erasmus MC were included in the study in the period 2002-2004. The Ethics Committee of the Erasmus MC approved the protocol and all patients gave informed consent prior to inclusion. Preeclampsia was defined as the occurrence, after 20 weeks of gestation, of a diastolic blood pressure ≥ 110 mmHg and a protein/creatinin ratio ≥ 30 mg/mmol creatinin, or the occurrence of a repetitive diastolic blood pressure ≥ 90 mmHg in combination with the HELLP (haemolysis, elevated liver-enzymes, low platelet-count) syndrome. Patients were divided into two study groups; one study group was treated with ketanserin before delivery and the other study group treated with other antihypertensive drugs (dihydralazine, nifedipine or nicardipine) without exposure to ketanserin. All patients in the ketanserin group and the majority of patients (85%) in the non-ketanserin group used methyldopa orally. Ketanserin treatment consisted of an intravenous bolus injection of 10 mg followed by a continuous infusion of ketanserin at 4 mg/h. According to the blood pressure, the infusion rate of ketanserin was increased with 2 mg/h every 20 min to a maximum of 20 mg/h. Each increment was preceded by an intravenous loading bolus injection of 10 mg ketanserin. Drug treatment was targeted at an intra-arterial diastolic blood pressure of ≤ 90 mmHg. Antihypertensive treatment was continued as long as foetal and/or maternal condition did not warrant delivery, as judged by the attending obstetrician. The umbilical cords were collected immediately after caesarean or vaginal deliveries.

Umbilical cord was collected in Krebs solution at 4 °C (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1, pH 7.4), transported to the laboratory and UCA was isolated from the umbilical cord. Functional experiments were performed on the same or subsequent day. Segments of UCA of 3-4 mm length and 1.5-2 mm internal diameter were suspended with help of stainless-steel hooks in 15 ml organ baths filled with carbogenated (95% O_2 / 5% CO_2) Krebs solution at 37°C. Each segment was set under a tension of 25 mN, as determined to be the optimal tension in pilot experiments. The segments were washed after every 15 min and were allowed to equilibrate for 45 min, to ensure that no maternal ketanserin was present in the vessels before start of the experiments. Two successive challenges to KCl (30 mM, Merck, Darmstad, Germany) were performed to check the reproducibility of the response. 100 mM KCl was subsequently added to determine the reference contractile

response of the segment. Serotonin (5-HT, Sigma Chemicals Co., Steinheim, Germany) and sumatriptan (Pfizer Ltd, Sandwich Kent, U.K.) were added to different segments in a cumulative manner in the absence or presence of antagonists.

Concentration response curves to 5-HT or to sumatriptan were constructed in a parallel set-up in presence of vehicle or after 30 min of incubation with the 5-HT_{2A} antagonist ketanserin (10 nM, 100 nM or 1μM) (Pfizer Ltd, Sandwich Kent, U.K.) or the 5-$HT_{1B/D}$ antagonist GR125743 (10 nM, 100 nM or 1 μM) (Pfizer Ltd, Sandwich Kent, U.K.) (18), respectively. All agonists and antagonists were dissolved in distilled water and stored in aliquots at -80°C. Only a single concentration-response curve was constructed in each artery segment.

14.2.1 Data and statistical analysis

Clinical characteristics between the two groups were compared using Wilcoxon's rank sum test. All contractile responses to the agonists were expressed as percentage contraction of the tone induced by 100 mM KCl. All values were expressed as mean ± s.e.m. The concentration response curves for the agonists were analysed using non-linear regression analysis (Graph pad Prism 3.01, Graph pad Software Inc., San Diego, CA, U.S.A). The potency of agonist was expressed as pEC_{50} (-log(EC_{50})) and the blocking potency of the antagonists (pK_b) was estimated by calculating concentration-ratios between EC_{50}-values of agonist in the presence and in the absence of antagonists and plotting a Schild-plot (19), assuming a slope of unity. Statistical analysis was performed using SPSS (version 11.5, SPSS Inc, Chicago, USA). Statistical significance was determined by the Students t-test, with differences considered significant at $p < 0.05$. A post hoc power analysis was performed to verify whether sufficient patients were investigated. Correlation coefficients between $pEC_{50,}$ E_{max} or pK_b and duration of ketanserin treatment, cumulative dosage or maximum dosage was calculated according to Pearson's coefficient of correlation (r).

14.3 RESULTS

The demographic and clinical characteristics of the seven ketanserin-treated and thirteen non-ketanserin treated patients included in this study, are summarized in Table 14.1. The groups did not differ significantly with respect to age, gestational age at admission or delivery, blood pressure at admission, neonatal weight or way of delivery. In all ketanserin-treated patients, initially adequate blood pressure control was achieved with ketanserin, but in two patients alternative intravenous antihypertensive drugs (nicardipine n=1, dihydralazine n=1) were added to maintain adequate blood pressure control. In one patient in the ketanserin-treated group, ketanserin treatment was stopped two hours before delivery and one patient stopped ketanserin treatment 45 hours before delivery. These patients were included in the analysis in the ketanserin group because possible effects of maternal ketanserin treatment on umbilical cord receptors were assumed to persist at least several days, considering the half-life of 5-HT_2 receptors of 3 to 5.5 days (20) and the elimination half-life of ketanserin of 13-18 h (21).

The response to 100 mM KCl did not differ significantly between the study groups in the ketanserin-treated group (36 mN ± 16) versus the non-ketanserin-treated group (24 mN ± 10). 5-HT and the 5-$HT_{1B/1D}$ agonist, sumatriptan, induced potent contractions in the UCA in both groups, which did not differ with respect to E_{max} and pEC_{50} (Figure 14.1, Table 14.2). The concentration-response curves to 5-HT showed a rightward shift after exposure to increasing concentrations of the 5-HT_{2A} antagonist ketanserin (Table 14.2, Figure 14.2a and 14.2b). Similarly, 30 min incubation with the 5-$HT_{1B/1D}$ antagonist, GR125743, resulted in a rightward shift of the concentration-response curves to sumatriptan (Table 14.2). No statistical differences were found between the pK_b values of 5-HT receptors in UCA of preeclamptic patients treated with ketanserin or those not treated with ketanserin. Although our sample size was limited, a post hoc power analysis (α=0.05, β=0.8) showed that the group size was sufficiently large to detect a

Table 14.1 Clinical characteristics of preeclamptic patients, treated with ketanserin before delivery (n=7) and treated with other drugs (nifedipine, dihydralazine or nicardipine) before delivery (n=13). Data are expressed as median (range) or number (%).

	Preeclamptic patients treated with ketanserin (n=7)	Preeclamptic patients not treated with ketanserin (n=13)
Age (years)	34 (25-41)	31 (18-44)
BP diastolic at admission (mmHg)	105 (85[1]-120)	100 (90-120)
BP systolic at admission (mmHg)	180 (140-220)	160 (110-200)
Protein/creatinin ratio (mg/mmol)	325 (80-1856)	470 (127-1257)
Gestational age at admission (weeks)	28 (25 4/7 – 35)	29 4/7 (24 4/7 –34 6/7)
Gestational age at delivery (weeks)	31 (26 4/7 – 36 2/7)	30 6/7 (26 3/7 – 36 6/7)
Way of delivery		
- Caesarian (n)	7 (100%)	12 (92%)
- Vaginal (n)	0	1 (8%)
Twin gestation (n)	0	1 (8%)
Ketanserin use		
Duration of treatment (hr)	54 (17-399)	-
Maximum dosage (mg/hr)	9 (6-18)	-
Cumulative dosage (mg)	379 (174-3055)	-
Neonatal weight (g)	1035 (750-1985)	1205 (650-2165)
below 10th percentile (n)	2 (28%)	6 (46%)
below 2.3th percentile (n)	1 (14%)	1 (8%)

1 One patient with severe HELLP-syndrome

difference in pEC_{50} of 0.7, corresponding to a five-fold difference in potency, which we consider to be clinical relevant.

No correlations were found for 5-HT for E_{max} or pEC_{50}, with duration of ketanserin treatment (for E_{max}, r=-0.539, p=0.212; for pEC_{50}, r=-0.297, p=0.518), cumulative dosage (for E_{max}, r=-0.516, p=0.236; for pEC_{50}, r=-0.379, p=0.401) and maximum dosage respectively (for E_{max}, r=-0.024, p=0.959; for pEC_{50}, r=-0.333, p=0.465). Similarly, no correlations were found for sumatriptan for E_{max} or pEC_{50}, with duration of ketanserin treatment (for E_{max}, r=0.021, p=0.964; for pEC_{50}, r=-0,058, p=0.902), cumulative dosage (for E_{max},

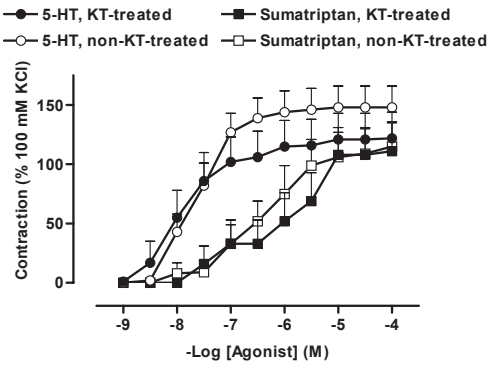


Figure 14.1. Concentration response curves to 5-HT in umbilical cord artery segments of ketanserin treated patients (●) (n = 7) and of patients, not treated with ketanserin (○) (n = 13) and concentration response curves to sumatriptan in umbilical cord artery segments of ketanserin treated patients (■) (n = 7) and of patients, not treated with ketanserin (□) (n = 13).

Table 14.2. 5-HT receptor characteristics, expressed as mean±s.e.m of E_{max}, pEC_{50} and pK_b from umbilical cord arteries obtained from preeclamptic patients treated with ketanserin and from preeclamptic patients not treated with ketanserin. 5-HT and sumatriptan were used as agonists in absence or presence of ketanserin (100 nM) and GR125743 (100 nM) as their respective antagonists.

	Preeclamptic patients treated with ketanserin		Pre-eclamptic patients not treated with ketanserin	
	5-HT	Sumatriptan	5-HT	Sumatriptan
E_{max} [1]	141 ± 7.7 (n=7)	142 ± 16.2 (n=7)	162 ±12.6 (n=11)	140 ± 14.7 (n=11)
pEC_{50}	7.67 ± 0.26 (n=7)	6.17 ± 0.37 (n=7)	7.69 ± 0.14 (n=11)	6.41 ± 0.28 (n=11)
pK_b	7.65 ± 0.31 (n=5)	7.83 ± 0.48 (n=4)	7.46 ± 0.17 (n=10)	8.43 ± 0.29 (n=8)

[1]expressed as percentage of the response induced by 100 mM KCl.

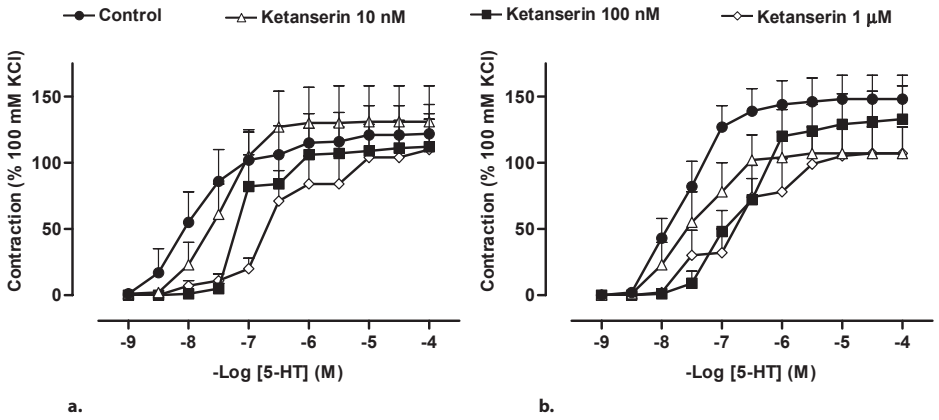


Figure 14.2. Concentration response curves to 5-HT in the absence (●) or presence of increasing concentrations of the 5-HT_{2A} receptor antagonist, ketanserin 10 nM (■), 100 nM (△) and 1 µM (◇), in umbilical cord artery segments of ketanserin treated patients (n=7) (a) and of patients, not treated with ketanserin (n=13) (b).

r=0.105, p=0.822; for pEC_{50}, r=0.015, p=0.974) and maximum dosage respectively (for E_{max}, r=0.524, p=0.227; for pEC_{50}, r=-0.272, p=0.555).

14.4 DISCUSSION

The presence of 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in utero-placental vessels of normotensive patients has been described by several authors (22-24). Increased concentrations of 5-HT have been reported at birth in maternal and placental circulation (25), indicating a role for 5-HT in maintaining vascular tone at birth. In preeclampsia, Middelkoop et al (26) have shown an increase in maternal circulation of 5-HT in pre-eclamptic patients, suggesting a role for 5-HT in the aetiology of preeclampsia.

In agreement with these studies, we found that 5-HT and sumatriptan induced contractions in all UCA tested, indicating the presence of 5-HT receptors. Ketanserin and GR125743 moved the 5-HT and sumatriptan curves rightwards in concentration-dependent manner, confirming the presence of functional 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in umbilical cord artery. The pK_b values (7.65 and 7.46) of ketanserin against 5-HT are one log unit lower than the pA_2 values of 8.7-8.9 described by other authors for umbilical cord artery (17, 27). This might be explained by the fact that the blocking activity of ketanserin is being underestimated in our experiments because 5-HT will have functional affinity for 5-HT_1 receptor subtypes as well. Even though 5-HT is not a selective 5-HT_{2A} receptor agonist, we chose 5-HT as an agonist in this

study as it encompasses the study of all 5-HT receptor subtypes and allows comparison of our results with other studies on 5-HT receptor functionality (17, 28, 29). Furthermore, our results are in line with previously reported values of pA_2 of 7.7-7.85 for 5-HT_{2A} receptor in animal tissue (30, 31). The pK_b value of GR125743 (7.83-8.43) for sumatriptan is in accordance with previously observed pA_2 values of 8.18 in human coronary artery and 8.34 in human saphenous vein (32).

The 5-HT_{2A} receptor antagonist ketanserin has been used increasingly in recent years as an antihypertensive drug in preeclamptic patients. However, the substantial transplacental transmission and subsequently high foetal exposure to ketanserin (11) may lead to adverse effects on foetal 5-HT receptors. The early appearance and continued expression of foetal 5-HT receptors during gestation has been demonstrated in animal studies (14). Lauder et al (14) showed in mouse embryos that both structural as well as functional damage occurred after foetal exposure to high dosages of the 5-$HT_{2A/2C}$ receptor antagonist, mianserin and the 5-$HT_{2A/2B/2C}$ receptor antagonist, ritanserin. No malformations were found after foetal exposure to ketanserin. Whitaker et al (33) demonstrated in animal studies that foetal exposure to the 5-HT-agonist, 5-methoxytryptamine, or exposure to a decreased level of maternal 5-HT (achieved by adding a tryptophan hydroxylase inhibitor antagonist) resulted in downregulation and upregulation of 5-HT receptors in new born offspring, respectively. In humans, only data on foetal effects after use of selective serotonin re-uptake inhibitors (SSRI's) during pregnancy are available. These drugs have shown to affect foetal 5-HT regulation, resulting in adverse effects on neonatal behaviour (tremor, restlessness, rigidity postnatally) following gestational exposure (33, 34).

Based on the aforementioned data on adverse foetal effects after maternal drug use, we studied the effect of maternal ketanserin treatment on foetal 5-HT receptor characteristics in preeclamptic patients. We hypothesized, based on animal studies (12, 13), that foetal exposure to ketanserin might influence the functionality of 5-HT_{2A} receptor, but not of the 5-$HT_{1B/D}$ receptor. Our data show no significant differences in 5-HT- or sumatriptan-induced vasoconstrictive responses of the UCA between the preeclamptic group treated with ketanserin and the preeclamptic group without exposure to ketanserin, indicating that exposure to ketanserin does not influence foetal 5-HT_{2A} and 5-$HT_{1B/1D}$ receptor characteristics in UCA.

14.4.1 Dosage and duration of treatment

It can be expected that high dosages of ketanserin and long-term treatment will exert a more pronounced effect on receptor characteristics than lower dosages, although all dosages used in this study were in the pharmacologically active range and have been known to cause high umbilical cord plasma levels of ketanserin (11). These plasma levels are in the same range as the concentrations ketanserin used in our *in vitro* experiments, indicating that results obtained with our experiments are of clinically relevance.

In our study no relationship between dosage and pEC_{50}, E_{max} or pK_b could be established. It should be born in mind that the number of patients in our study was relatively small, because many early-onset preeclamptic patients need alternative treatment before delivery after the maximum dosage of ketanserin is reached. However, power analysis established that statistical power in our study was sufficient to detect clinically relevant differences.

14.4.2 Gestational age

Limited information is available with respect to the influence of gestational age on the response of the umbilical artery to 5-HT. Bertrand et al (29) showed an increase in umbilical and placental veins sensitivity to serotonin throughout the third trimester in normotensive patients, whereas this change was not seen in preeclampsia. In our study the study groups did not differ with respect to gestational age, therefore a possible confounding factor of gestational age on the results was excluded. However, it can be speculated that clinical effects of maternal ketanserin treatment on receptor population will (partly) depend on gestational age, if sensitivity to 5-HT in UCA indeed changes with gestational age. Our results, suggesting a lack of effect of maternal ketanserin treatment on functionality of 5-HT receptors in UCA

support the currently available clinical data, showing that use of ketanserin in preeclamptic patients is safe for foetus and neonate.

In conclusion, prolonged foetal exposure to ketanserin in preeclamptic patients does not seem to influence the functional characteristics of 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors in the umbilical cord artery.

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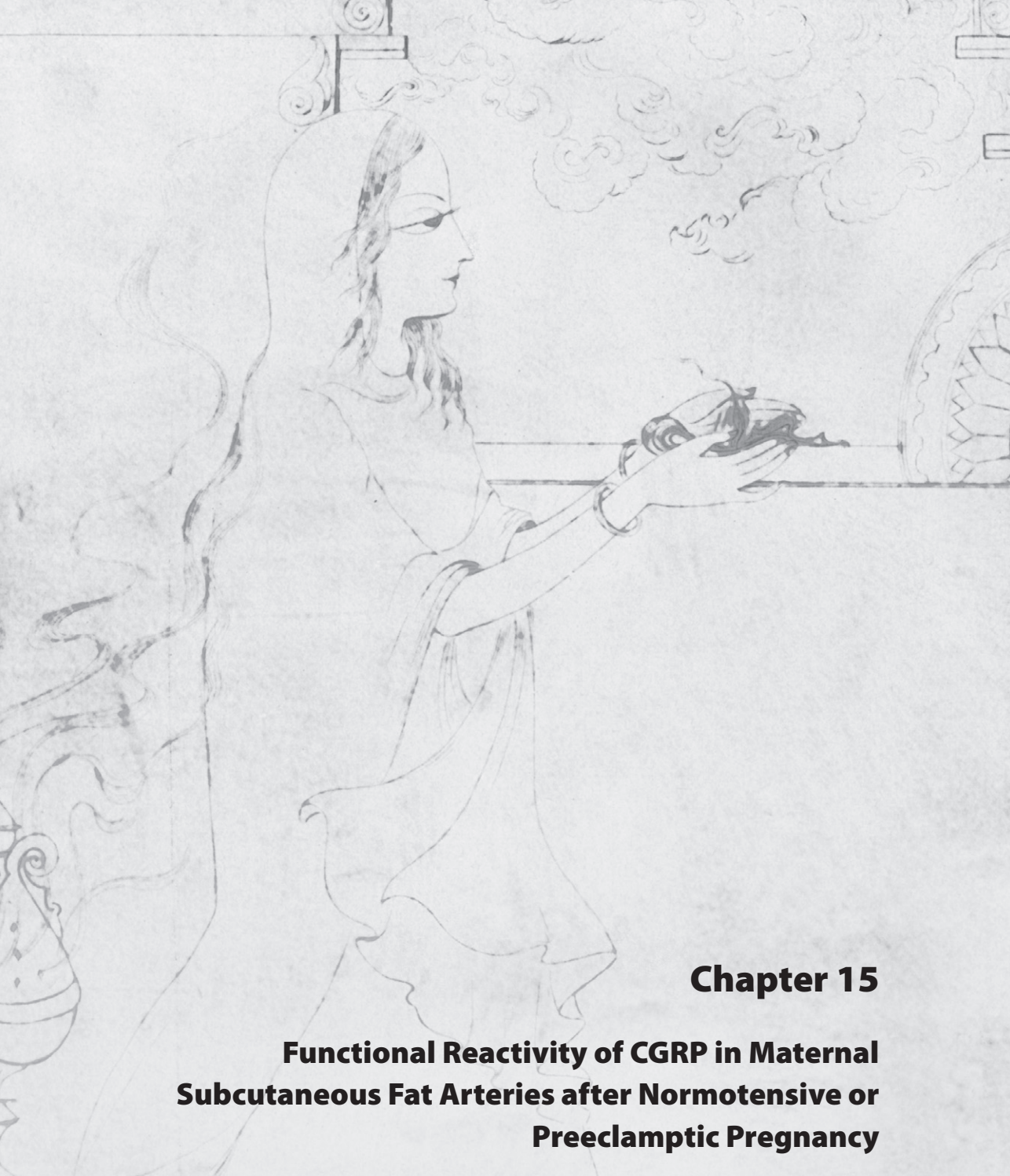

Chapter 15

Functional Reactivity of CGRP in Maternal Subcutaneous Fat Arteries after Normotensive or Preeclamptic Pregnancy

Based on: S. Gupta, W. Visser, P.R. Saxena, E.A.P Steegers, Antoinette MaassenVanDenBrink. To be submitted

ABSTRACT

We investigated the functional reactivity of CGRP receptors in maternal subcutaneous fat resistance arteries in normotensive and preeclamptic pregnancy. Study groups were divided based on the presence or absence of preeclampsia. Segments of subcutaneous fat resistance arteries were mounted in Mulvany myographs and concentration-response curves to αCGRP were constructed in the absence or presence of BIBN4096BS (1 nM). In addition, blood was collected from both the normotensive and preeclamptic subjects and plasma CGRP levels were measured. CGRP dose-dependently relaxed the all the artery segments. There was no significant difference in either efficacy (maximal response) or potency (pEC_{50}) of αCGRP in subcutaneous fat arteries obtained from the preeclamptic patients (E_{max}: 88±2.8% and pEC_{50}: 8.84±0.26) as compared to those obtained from normotensive controls (E_{max}: 77±5.2% and pEC_{50}: 8.69±0.17). Similarly, there was no difference in the potency of BIBN4096BS between preeclamptic and normotensive women (pK_B: 10.12±0.45 and 9.73±0.36, respectively). Similarly, there was no significant difference between plasma levels of αCGRP between the normotensive and preeclamptics (7.65±4.9, 7.42±2.7 pg ml^{-1} respectively). CGRP caused equi-efficacious relaxations in the subcutaneous fat resistance arteries in preeclamptics as compared to the normotensive pregnant women. Therefore, we did not find a role of CGRP preeclampsia in maternal vasculature. Future studies should asses the plasma concentration and functional responses of CGRP in preeclamptic and normotensive women of the same gestational age.

15.1 INTRODUCTION

Preeclampsia affects about 5% of all pregnancies and is a major cause of morbidity and mortality to both mother and foetus (1). The etiology of preeclampsia is still largely unknown, although it has been linked to abnormalities in trophoblast invasion into the placental bed (2). The syndrome is clinically characterized by maternal hypertension and proteinuria, and diagnosed after 20 weeks of gestation. The usual physiologic adaptations in response to increased fluid volume that are observed in normal pregnancies are attenuated in preeclampsia, resulting in increased vascular resistance (3). This higher vascular resistance of the maternal vasculature in preeclampsia may be attributed to increased plasma concentrations of contractile agents (4-7), but there may also be an increased sensitivity of the arteries to vasoconstrictor agents, like angiotensin II (8, 9), 5-hydroxytryptamine (5-HT; serotonin) (7) and noradrenaline (10) and/or a decreased response to vasodilating peptides, such as calcitonin gene-related peptide (CGRP) (11) and acetylcholine (12).

Plasma levels of CGRP are significantly increased in pregnancy and are implicated in the adaptive response to adjust the increased volume associated with this condition (13). Plasma CGRP levels are reported to decrease in preeclampsia (14) and pregnancy-induced hypertension (15), which may contribute to the development and maintenance of hypertension during pregnancy. However, others have reported that there is no difference in placental and plasma concentrations of CGRP in preeclamptic women as compared to normotensive controls (16). CGRP mediates its actions in humans predominantly through $CGRP_1$ receptors although other receptors, such as $CGRP_2$ receptors seem to present in various animal (17-19) and human (20) tissues. The $CGRP_1$ receptor is a multi component entity consisting of the calcitonin receptor like receptor (CLR), receptor activity modifying protein-1 (RAMP-1) and receptor component protein (RCP) (18, 21) which are also reported to decrease in preeclampsia (11, 22). Further, a compromised responsiveness to CGRP in foetoplacental vessels has been observed in preeclamptic pregnancies (11). As in preeclampsia, the responses to endogenous ligands are altered in both foetal and maternal vasculature; this may subsequently affect hemodynamics and hence may contribute to the hypertension observed in this syndrome. Therefore, in the present study we investigated the response to CGRP in subcutaneous fat arteries (SFA) representing maternal resistance arteries from preeclamptic patients in comparison with normotensive pregnant control subjects.

15.2 MATERIALS AND METHODS

The Ethics Committee of the Erasmus MC approved this study. Subcutaneous abdominal fat was obtained during caesarean section after obtaining informed consent from pregnant women admitted to the Obstetrics Department of Erasmus MC (Rotterdam, The Netherlands). Preeclamptic patients (diastolic blood pressure $\geq$ 90 mm Hg and protein/creatinine ratio (PCR) $\geq$30 mg protein/mmol creatinine), as well as normotensive females of different gestational ages were included in the study. The preeclamptic patients were treated with different antihypertensive drugs, like α-methyldopa, dihydralazinee, ketanserin, labetalol and nicardipine. Women suffering from diabetes and normotensive women with intrauterine growth retardation (IUGR, neonate weight $\leq 10^{th}$ percentile weight for his/her age in weeks corrected for gestational age, parity and foetal sex) (23), as well as preeclamptic women suffering from preexisting hypertension were not included in the study. Maternal subcutaneous fat tissues were collected in cold Krebs bicarbonate solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4), transported to the laboratory and stored in carbogenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution at 4˚C. Subcutaneous arteries were isolated after removing adhering subcutaneous fat. Functional experiments were performed on the same or the subsequent day. SFA segments were cut into rings of 1-2 mm length with an internal diameter of 150-500 µm. Artery segments were suspended in Mulvany myographs on two parallel titanium wires. Subsequently, the distance between the wires was normalized to $0.9 \times I_{100}$ (I_{100} is the distance between the pins when the transmural pressure equalizes 100 mm Hg) to achieve optimal conditions for active force development. The vessel segments were continuously bubbled with 95% O_2 and 5% CO_2 and the temperature was maintained at 37˚C. After an initial equilibration period of 45 min, two successive challenges with KCl (30 mM) were performed to verify the reproducibility of the response. Subsequently, KCl (100 mM) was added to determine the reference contractile response of the artery segments to compensate for small differences in the muscle mass of the artery segments. The endothelial function was evaluated by observing the relaxant response to substance P (100 nM) after precontraction with U46619 (9,11-dideoxy-11α, 9α-epoxy, methanoprostaglandin $F_{2\alpha}$, 10-300 nM). Cumulative concentration response curves to CGRP were constructed in the presence of vehicle (saline) or after a 30-min incubation with CGRP receptor antagonist BIBN4096BS (1 nM). The arteries were precontracted with 18-25 mM KCl to obtain a precontraction amounting 60-70% of the reference contractile response to 100 mM KCl. Only one single concentration response curve was constructed in each artery segment.

For determination of plasma levels of CGRP, 5 ml blood was collected in tubes containing EDTA and aprotinin (0.6 TIU/ml). Blood was centrifuged at 15000 rpm, for 10 min at 4°C, and plasma was stored at -80°C untill further analysis. Competitive radioimmunoassay (Peninsula Lab INC., San Carlos, CA, U.S.A.) was used for measuring the CGRP concentrations in the plasma.

Chemicals

The compounds used in the present study (obtained from the sources indicated) were U46619, substance P (Sigma Chemicals Co., Steinheim, Germany), KCl (Merck, Darmstad, Germany), h-αCGRP, (NeoMPS S.A., Strasbourg, France); BIBN4096BS (1-piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl] carbonyl] pentyl] amino]-1-[(3,5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-,[R-(R*,S*)]-) (gift: Dr. Henri Doods, Boehringer Ingelheim Pharma, Biberach/Riss, Germany) were all dissolved in distilled water and stored in aliquots at -80˚C.

Statistical analysis

The contractile response to 100 mM KCl, expressed in milli-Newton (mN), was used to compare the contractile force developed by arteries from different experimental groups. The relaxant responses elicited by CGRP are expressed as percentage relaxation of the tone induced by 18-25 mM KCl. All values are

expressed as mean±s.e.m. and n represents the number of segments, each segment obtained from a different patient. Concentration response curves were analyzed using nonlinear regression analysis using GraphPad Prism 3.01 (GraphPad Software Inc., San Diego, CA, U.S.A.). The efficacy of the agonists was expressed as E_{max} (maximal response) and their potency as pEC_{50} (-logEC_{50}, where EC_{50} is the concentration of the agonist required to produce half the maximal response). The blocking potency of BIBN4096BS (apparent pK_b) was estimated by calculating concentration-ratios between the EC_{50} of agonist in the presence and absence of antagonist and plotting a Schild plot, assuming a slope of unity. Group means were compared by using unpaired Student t-test, with differences considered significant at $P<0.05$. Correlation analyses were carried out using Pearson's coefficient of correlation between gestational age or neonatal weight and responses to CGRP and BIBN4096BS.

15.3 RESULTS

The demographic details of all the subjects from whom the umbilical cord artery, subcutaneous fat arteries, or both were obtained, are presented in Table 15.1. The KCl (100 mM)-induced contractions were not different between subcutaneous fat arteries obtained from normotensive and preeclamptic women.

Table 15.1 Demographic details of the patients from whom subcutaneous fat arteries were obtained.

	Normotensive	Preeclamptics
Number	13	10
Maternal age (y)	32.2±1.5	30.9±2.2
Gestational age at delivery (wk)	38.5±0.8	31.4±1.3*
Diastolic BP (mm Hg)	70.9±3.2	108.1±6.5*
Systolic BP (mm Hg)	115.0±3.8	160.6±7.9*
PCR (mg/mmol)	Not determined	359±185
Birth weight (g)	3165±114	1390±228*
Preterm deliveries	1	7
Intrauterine growth restriction	none	3

BP: Blood pressure, PCR: Protein/creatinine ratio. *Statistically significant from corresponding value in normotensive group.

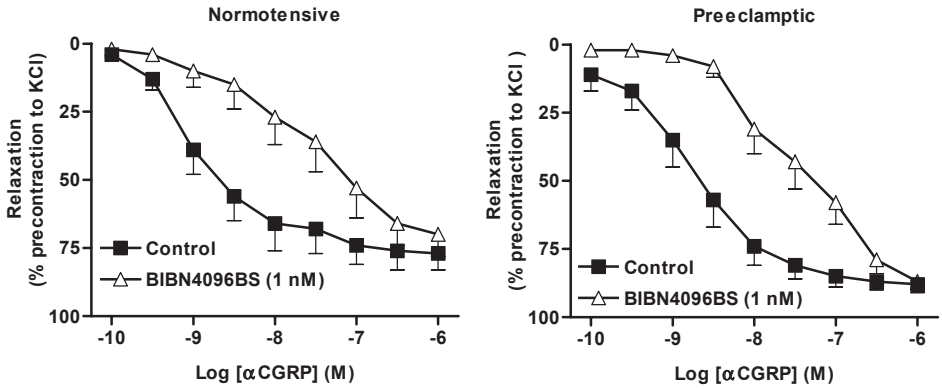

Figure 15.1. Concentration response curves to αCGRP in subcutaneous fat resistance arteries obtained from normotensive and preeclamptic pregnant women in the absence or presence of BIBN4096BS (1 nM).

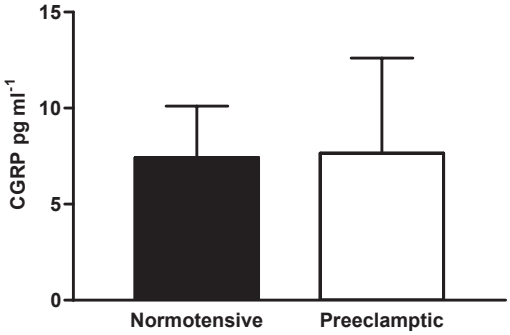

Figure 15.2. CGRP levels measured in plasma of normotensive pregnant controls and preeclamptic patients.

CGRP induced concentration-dependent relaxations in all the artery segments. The E_{max} of CGRP (maximal response expressed as percentage of the KCl-induced precontraction) in subcutaneous fat arteries obtained from the preeclamptic patients (88±2.8%) was not significantly different from than those obtained from normotensive controls (77±5.2%). There were no significant differences in the potency of CGRP between the preeclamptic patients (pEC_{50}: 8.84±0.26) and normotensive controls (pEC_{50}: 8.69±0.17). BIBN4096BS (1 nM) significantly blocked the relaxant responses to CGRP in both groups (Figure 15.1) and was equipotent in antagonizing CGRP-mediated responses in preeclamptic (apparent pK_b: 10.12±0.45) and normotensive (apparent pK_b: 9.73±0.36) subcutaneous fat artery segments.

There were no significant differences in plasma levels of CGRP between preeclamptic and normotensive subjects (Figure 15.2).

15.4 DISCUSSION

In the present study, we demonstrated that CGRP-induced relaxations in human subcutaneous fat artery segments from preeclamptic women and normotensive controls are equi-efficacious and equipotent. Similarly, there were no significant differences in the potency of the CGRP antagonist, BIBN4096BS, in these maternal artery segments. CGRP induced concentration-dependent relaxations in these resistance arteries with a potency that is in line with previous studies in human subcutaneous arteries (24, 25). The plasma levels of CGRP did not differ between preeclamptic and normotensive women. This is in contrast with reports of decreased CGRP plasma levels in preeclamptics (14) and pregnancy induced hypertension (15, 26), but similar levels of CGRP in preeclamptics and normotensive pregnant women have also been reported by others (16).

The similar efficacy of CGRP in preeclamptic patients and normotensive pregnant women fits well in view of the similar plasma levels of CGRP observed between the two groups in the present study. Interestingly, responses to CGRP are reported to be augmented in human subcutaneous fat arteries from hypertensive patients as compared to controls (24), and the authors speculate that this might be an adaptive mechanism in response to increased blood pressure. It should be noted that unlike in hypertensive patients, in pregnancy there is also a concurrent increase in female sex steroids such as 17β-estradiol and progesterone, which are reported to enhance the hypotensive effects of CGRP (27). Similarly, the expression of CGRP receptor components (28) and CGRP sensitivity (29) in the mesenteric arteries of pregnant rats is significantly increased. Thus, it is feasible that any small difference in the efficacy or potency of CGRP between preeclamptic and normotensive pregnant women is eclipsed by the effect of increased sex steroid levels in these pregnant subjects. However, our observations seem to be in contrast to reports of attenuated CGRP responses in preeclamptic patients in umbilical cord artery and chorionic artery (11).

In addition, there are reports of reduced CGRP receptor expression in human foeto-placental tissues (11) and reduced CGRP and adrenomedullin sensitivity in umbilical and chorionic arteries obtained from preeclamptic patients (11). It is also reported that CGRP mRNA expression in human foeto-placental is decreased in preeclampsia and HELLP syndrome (22). As the earlier studies investigated the responses in foeto-placental arteries and we investigated maternal arteries, this difference may account for the differences in the CGRP responses. Therefore, decreased CGRP responses or attenuated CGRP receptor expression does not seem to be a generalized phenomenon in fetal and maternal arteries.

Admittedly, a shortcoming of our study is that tissue and plasma samples were obtained from pre-eclamptic women at a shorter gestational age (31.4 weeks) than the normotensive controls (38.5 weeks). Therefore, to draw unequivocal conclusions about the role of CGRP in preeclampsia, pregnant women of a similar gestational age should be studied.

CGRP is one of the most potent endogenous vasodilators, which is widely distributed in the human body. Therefore, it is likely to play a significant role in maintaining the physiological vascular tone. Although administration of CGRP has been reported to be beneficial in animal models of preeclampsia (30), this may be due to a decrease in the elevated blood pressure rather an direct effect on pathology of preeclampsia.

In conclusion in maternal vasculature we did not find a prominent role of CGRP in the pathophysiology of preeclampsia.

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Ragon mein daudte phirne ke ham nahin khayel
Jo aankh hi say na tapka who lahu kya hai (Mirza Galibh)

Part F: Summary and General Discussion

Chapter 16

Summary and general discussion

16.1 SUMMARY

16.1.1 Introduction to migraine (Chapter 1)

It is now well accepted that migraine is a neurovascular syndrome, where the headache is mainly due to cranial vasodilatation with a concurrent activation of the trigeminal system. Several experimental migraine models, based on vascular and neuronal involvement, have been developed. Obviously, these migraine models do not entail all facets of this clinically heterogeneous disorder, but their contribution at several levels (molecular, *in vitro*, *in vivo*) has been crucial in the development of novel antimigraine drugs and in the understanding of migraine pathophysiology. After preliminary screening using *in vitro* models on isolated arteries from experimental animals, the *in vitro* models on human arteries, such as human isolated meningeal and coronary arteries can be used to further evaluate the vasoactive potential of a new agent and to pharmacologically characterize new receptor system, which might serve as a target for future antimigraine activity. However, as the pathophysiology of migraine definitely seems to involve multiple physiological systems, comprehensive *in vivo* models, which may mimic more aspects involved in migraine, seem to be vital. Various *in vivo* models are used, such as the porcine model measuring arteriovenous anastomotic blood flow. The other models utilize electrical stimulation of the trigeminal ganglion/nerve to study neurogenic dural inflammation, while the superior sagittal sinus stimulation model takes into account the transmission of trigeminal nociceptive input in the brainstem. The integrated models, namely electrical stimulation of the trigeminal ganglion or systemic administration of capsaicin, allow study of the activation of the trigeminal system and its effect on the cranial vasculature; these models hence provide a more comprehensive view on the pathophysiology of migraine than the models focusing on only vascular or neuronal aspects. The aforementioned migraine models have advantages obviously also. An integrative approach that involves the use of several models (rather than only one) will enable the advantages to add up to the progress, while the limitations are minimized. Overall, migraine models have provided significant insights into pathophysiological mechanisms in this complex disorder, and are imperative in further progress in migraine research.

16.1.2 Potential role of sex hormones in the pathophysiology of migraine (Chapter 2)

Migraine is three times more in females than in males in its peak prevalence, and migraine severity and occurrence fluctuate according to the reproductive milestones of a woman's life. Therefore, it seems logical that the female sex steroids, 17β-estradiol and progesterone, which fluctuate during different phases of the female life cycle, play role in migraine pathogenesis. Sex steroids, particularly 17β-estradiol, affect the synthesis, release as well as the receptor expression of a number of vasoactive agents implicated in migraine. The synthesis of calcitonin gene-related peptide (CGRP), a potent vasodilator of cranial arteries, in the dorsal root ganglion is positively related to 17β-estradiol. Further, in vascular beds containing nociceptors, CGRP innervations are increased in concurrence with increases in 17β-estradiol levels. Similarly, plasma levels of CGRP and CGRP receptor expression are increased in ovariectomised rats when they are treated with 17β-estradiol, although in certain vascular beds this phenomenon is not observed. In this sense, it is tempting to suggest that the decrease in peripheral vascular resistance by sex steroids may contribute, at least in part, to the pathophysiology of cranial vessels related in migraine. Thus, the dysfunction of these vessels causes hyperreactivity to endogenous vasorelaxants. Like CGRP, the activity of nitric oxide activity is also enhanced by 17β-estradiol in various animal models. This increase may be attributed to enhanced eNOS (endothelial-nitric oxide synthetase) activity. Similarly, estrogen is reported to decrease $[Mg^{2+}]$ intracellularly and extracellularly. Interestingly in migraine patients, this divalent cation is reported to be deficient and this ion has been used for migraine prophylaxis. Adrenoceptor expression also fluctuates with 17β-estradiol, but varies according to the vessel type under study, which also is the case with 5-HT receptor activity.

Although the experimental evidence in animals seems to suggest that with increases in estrogen there is a higher propensity for getting a migraine attack, literature from human studies indicates that the withdrawal of estrogen is the main trigger. However, both statements seem to oversimplify the actual situation. Firstly, it is not just the withdrawal of estrogen that is important, but prior to this decrease there should be a 'priming' with sufficiently high concentrations of estrogen. Secondly, probably not just the absolute levels of the both progesterone and estrogen matter, but the relative rate of the change of these steroids seems to be the culprit. Research on the role of sex steroids is further complicated by the fact that these hormones do not only have their genomic action via their classical nuclear receptor mechanisms, but also a rapid action tentatively mediated by a membrane receptor via non-genomic pathways. Taken together, sex steroids definitively seem to be involved in the pathophysiology of migraine, but there is a long road ahead before we can unequivocally explain this in the fair sex.

16.1.3 Introduction to preeclampsia (Chapter 3)

Preeclampsia is a multi-system disorder with an incidence in pregnancy varying between 2% and 7%, and is the leading cause of maternal mortality during pregnancy in The Netherlands. The disorder is characterised by its occurrence after the 20th week of gestation, elevated maternal blood pressure and proteinuria. In severe cases, preeclampsia can lead to serious maternal complications such as HELLP-syndrome (haemolysis, elevated liver enzymes, low platelet counts), pulmonary edema, acute renal failure, liver failure or haemorrhage, *abruptio placentae* and eclampsia. Preeclampsia is also associated with increased perinatal morbidity and mortality. The higher vascular resistance of the maternal vasculature in preeclampsia may be attributed to increased plasma concentrations of contractile agents, but could alternatively be attributed to an increased sensitivity of the arteries to vasoconstrictor agents like angiotensin II, 5-hydroxytryptamine (5-HT; serotonin) and noradrenaline combined with a decreased response to vasodilating peptides such as CGRP and acetylcholine (ACh). The increased sensitivity to contractile agents could be because of upregulation of the respective receptors and/or more efficient coupling with their second messenger systems, while vice versa for endogenous vasorelaxing agents. The pathophysiology of preeclampsia is still far from clear, but it is widely accepted that immunological maladaptations, which are thought to be the result of a putative misalliance of foetal trophoblasts with maternal tissue, as well as endothelial cell activation or dysfunction, are major players. Therefore, research to divulge the factors involved in the pathogenesis of preeclampsia is vital. In the same context, we tried to explore the vascular mechanisms that might contribute to this syndrome.

16.1.4 Characterisation of CGRP receptors (Chapters 5-6)

These studies were carried out to characterise calcitonin gene-related peptide (CGRP) receptors in human and porcine isolated proximal and distal coronary arteries, as well as human meningeal arteries using BIBN4096BS. BIBN4096BS is a dipeptide derivate, a very potent and selective human CGRP receptor antagonist. Human (h)-αCGRP-induced relaxations were blocked by BIBN4096BS in all arteries studied. In contrast to the other vessels, the slope of the Schild plot in the human distal coronary artery segments was significantly less than unity and BIBN4096BS potently blocked these responses. In the same preparation, h-αCGRP$_{8-37}$ behaved as a weak antagonist of h-αCGRPinduced relaxations, also with a Schild plot slope less than unity. The linear agonists, [ethylamide-Cys^{2,7}]h-αCGRP ([Cys(Et)^{2,7}]-h-αCGRP) and [acetimidomethyl-Cys^{2,7}]h-αCGRP ([Cys(Acm)^{2,7}]-h-αCGRP), had a high potency, suggesting the presence of CGRP$_2$ receptors, while the potent blockade by BIBN4096BS points to the presence of CGRP$_1$ receptors. Using RT-PCR, mRNAs encoding for the essential components of functional CGRP$_1$ receptors were demonstrated in both human proximal and distal coronary artery, as well as meningeal artery. The above results demonstrate the presence of CGRP$_1$ receptors in all coronary artery segments investigated, but the human distal coronary artery segments seem to have an additional population of CGRP receptors not complying with the currently classified CGRP$_1$ or CGRP$_2$ receptors.

h-αCGRP relaxed meningeal arterial segments more potently than [Cys(Et)^{2,7}]h-αCGRP, unlike in human distal coronary artery, while the maximal responses to these agonists were not significantly different. h-αCGRP$_{8-37}$ also antagonised the response to h-αCGRP, with a Schild plot slope not different from unity. Considering the high antagonist potency of BIBN4096BS, coupled to the lower agonist potency of [Cys (Et)^{2,7}]h-αCGRP, it is reasonable to suggest a predominant role of CGRP$_1$ receptors in the human middle meningeal artery. This view is reinforced by Schild plot analysis, which revealed a slope of unity in all experiments, giving further evidence for a homogeneous CGRP receptor population in this vascular preparation.

16.1.5 Capsaicin-induced relaxations in isolated arteries (Chapter 7)

Capsaicin activates sensory nerve fibres via the vanilloid VR1 receptor, leading to the release of neuropeptides such as CGRP and substance P. Capsaicin-sensitive nerves are present in coronary as well as meningeal arteries. Therefore, we examined the role of CGRP in capsaicin-induced relaxations in both these arteries. Capsaicin relaxed both human and porcine vessels in a concentration-dependent manner. The removal of endothelium or the blockade of CGRP or NK$_1$ receptors, calcium channels or cyclic GMP did not affect the relaxant responses to capsaicin. Similarly, various K^+ channel antagonists, gap junctions blockers and NO synthase inhibitors were also not able to block capsaicin-mediated responses. In conclusion, the capsaicin-induced relaxations in the human and porcine isolated coronary artery, as well as in the human meningeal artery, are not mediated by CGRP, and are thus not a suitable model to study CGRP release through activation of perivascular nerves. Capsaicin-induced relaxation in isolated arteries might be attributed to a non-specific phenomenon.

16.1.6 Effect of sex steroids on the vascular pharmacology of migraine (Chapters 8, 9 and 10)

We investigated the effects of the female sex steroids, 17β-estradiol and progesterone, separately and in combination, on dural vasodilatation induced by αCGRP, periarterial electrical stimulation and capsaicin in ovariectomized rats, using intravital microscopy. There were no significant differences in the vasodilator potency or efficacy of αCGRP or capsaicin in the dural arteries of different groups studied. In contrast, the vasodilator response to electrical stimulation was significantly higher in rats treated with 17β-estradiol as compared to that observed after placebo treatment. In similar groups, we also studied vasoactive responses to CGRP, ACh, 5-HT and adrenaline in various isolated artery segments mounted in organ baths. The relaxant responses to αCGRP were significantly potentiated in rat mesenteric and caudal artery treated with 17β-estradiol as compared to the placebo-treated group. In carotid artery, our results indicate that circulating progesterone and/or 17β-estradiol may reduce contraction of the rat carotid artery in response to noradrenaline or 5-HT. Taken together, our results show that 17β-estradiol enhanced the electrically-induced dural vasodilatation, which might be due to an increased release of neuropeptides like CGRP from perivascular nerves, whereas contractions to 5-HT seemed to be attenuated by increased 17β-estradiol. These mechanisms in tandem may exacerbate migraine attacks in women.

16.1.7 Intravital microscopy model in mice (Chapter 11)

The purpose of the study was to develop a mouse model to study trigeminovascular mechanisms using intravital microscopy on a closed cranial window. The main difference in the mouse model compared to the rat model, which was developed earlier by others, was the necessity to constrict the dural arteries before any comprehensible dural dilatations could be discerned. After developing the model, we studied vasodilatation induced by exogenous and endogenous CGRP in dural arteries. h-αCGRP and capsaicin both induced vasodilatation in preconstricted arteries, and electrical stimulation induced current-dependent relaxations in the dural arteries. BIBN4096BS blocked responses evoked by αCGRP, capsaicin and electrical stimulation, whereas sumatriptan blocked electrical stimulation-induced vasodilatation

only. This murine model will be useful in dissecting various aspects of the trigeminovascular system, especially in exploring the role of various ion channel mutations found in migraine patients using transgenic mice with these mutations.

16.1.8 Evidence showing that clonidine inhibits the canine external carotid vasodilatation to capsaicin by $\alpha_{2A/2C}$-adrenoceptors (Chapter 12)

The role of CGRP in the pathophysiology of migraine seems to be pivotal, as this peptide is perceived to activate trigeminal sensory nerves, dilates cranial blood vessels and transmits vascular nociception. Moreover, several antimigraine drugs decrease the dural vasodilatation induced by trigeminal ganglion stimulation. On this basis, the present study has investigated in vagosympathectomized dogs the effects of the prophylactic antimigraine drug, clonidine (a classical α_2-adrenoceptor agonist), on the external carotid vasodilator responses to capsaicin, CGRP and acetylcholine. Intracarotid infusions of capsaicin, CGRP and acetylcholine produced dose-dependent increases in external carotid conductance without affecting blood pressure or heart rate. Interestingly, the external carotid vasodilator responses to capsaicin, but not those to CGRP or acetylcholine, were partially inhibited after i.v. administration of clonidine, but not by equivalent volumes of physiological saline. The inhibitory responses to clonidine were antagonized by i.v. administration of the α_2-adrenoceptor antagonists rauwolscine ($\alpha_{2A/2B/2C}$), BRL44408 (α_{2A}) or MK912 (α_{2C}) but not by imiloxan (α_{2C}). These results suggest that clonidine inhibits the canine external carotid vasodilator responses to capsaicin probably through a peripheral trigeminovascular and/or central mechanism, mainly mediated by α_{2A}-adrenoceptors and, to a lesser extent, by α_{2C}-adrenoceptors.

16.1.9 Vascular pharmacology of preeclampsia (Chapters 13, 14, 15)

We investigated the functional reactivity 5-HT receptors in umbilical cord arteries and maternal subcutaneous fat arteries in normotensive and preeclamptic pregnancy of different gestational age. There were no significant differences in the pharmacological profile of the 5-HT_{2A} receptor, whereas the sensitivity of 5-$HT_{1B/1D}$ receptors was significantly increased in the full term delivery as compared to preterm delivery only in normotensive pregnancies. In contrast, the sensitivity of 5-$HT_{1B/1D}$ receptors seems to be expedited in preterm preeclamptic patients. In subcutaneous fat arteries there were no differences in 5-HT_{2A} and 5-$HT_{1B/1D}$ receptor profile, and also the endothelium-dependent relaxations between two groups was similar. Hence, further studies on 5-$HT_{1B/ID}$ receptors will give new insights into foetal development and the pathophysiology of preeclampsia.

In the preeclamptic patients, we also investigated the effect of ketanserin treatment on 5-HT receptor profile (E_{max} and pEC_{50}) in umbilical cord arteries and found that there was no difference in 5-$HT_{1B/1D}$ and 5-HT_{2A} receptor profile. Thus, foetal exposure to ketanserin in preeclamptic patients does not significantly influence these 5-HT receptors. In conjunction with other literature reports, our results support that ketanserin is a relatively safe drug when used in preeclamptic patients. In maternal subcutaneous fat resistance arteries, we also investigated vasoactive responses to CGRP in normotensive and preeclamptic pregnancies. There was no difference in efficacy or potency of CGRP between the two groups.

16.2 GENERAL DISCUSSION AND FUTURE STUDIES

16.2.1 Basic research in migraine

The high prevalence of migraine, 15-20% (1) in general western populations, combined with the fact that the pathophysiology of this disorder is still unraveling, makes migraine research an exciting and challenging topic. However, a prerequisite for any basic research is a reliable and predictive model. In migraine, there are quite a few models, but none of them is completely satisfactory, as all these models are based on only a part of the aspects known to be involved in migraine pathophysiology. Therefore, these models

should be used in combination (in series or parallel) to divulge more robust and predictable information from the various hypotheses. The *in vitro* models, like brain slice cultures and isolated artery segments, can give us vital clues and a direction to select more appropriate *in vivo* models, which are essential keeping in view the multitude of symptoms observed in migraineurs. The integrated models are a safer bet for basic research as it is evident that both neuronal and vascular mechanisms are involved, although the relative contribution of each of these is still debated. Further, with recent discoveries of various genetic mutations in ion channels in a subgroup of migraine patients (familial hemiplegic migraine), expression of these mutations in mice have provided a new avenue in migraine research. Therefore, future studies should focus on:

i. Investigating the effect or contribution of these mutations *in vivo* on vascular and neuronal components. This could be feasible by using the mouse model of intravital microscopy (2) to investigate vascular parameters, and by developing a trigeminal ganglion stimulation model (3) in mice. Further, it should be studied whether the parameters like CGRP and/or electrical stimulation-induced vasodilatations are affected by sex, age or different phases of the oestrous cycle.
ii. Exploring other mechanisms implicated in migraine pathogenesis. For example, pilot experiments on the antagonism of NMDA receptors seem promising in the rat model of intravital microscopy. We intend to explore the potential of current (ketamine, magnesium) NMDA receptor modulators, as well as compounds that are currently in development (4) to affect vasodilatation induced by electrical stimulation and capsaicin in the rat intravital microscopy model.

16.2.2 CGRP, its receptors and migraine

CGRP was discovered in 1982 (5) and increased plasma levels of CGRP in migraineurs have been reported back since 1988 (6). Increased plasma levels of CGRP and subsequent vasodilatation are now thought to be one of the major contributors to the headache phase of migraine, and thus CGRP receptor antagonists may act as antimigraine agents. The classification of CGRP receptors is still in an early stage. Although $CGRP_1$ receptors have been characterized in a number of species, including humans, the existence of $CGRP_2$ receptors is still precarious, and the $CGRP_2$ receptors have not yet been molecularly identified. An inherent problem of classification of receptors of large molecular weight peptides is that the agonists and antagonist are usually also in the similar high weight range as the parent molecule. Further, because of its peptide nature and large molecular weight, antagonists of CGRP receptors ought to be administered parenterally. This makes the compliance of these agents (like BIBN4096BS) difficult for migraine patients, and hence less appealing to research and development in pharmaceutical industry. Therefore, there are only a few compounds available for characterising CGRP receptors, and thus the classification of these receptors has been painstakingly slow. Interestingly, CGRP is extensively distributed in the human body and has a number of diverse actions. In addition, wide range of affinity estimates have been reported in both human as well as animal tissues, which cannot be explained by only one single CGRP receptor subtype (7). The success of BIBN4096BS as antimigraine treatment in a small clinical trial (8) has given an impetus for further research in this field. Therefore, future studies may be directed:

i. To further explore the nature of the atypical CGRP receptor population in human distal coronary artery. cAMP measurements in these segments may establish whether the heterogeneity that we observed on a functional level is also present at the second messenger level. Further characterization may offer more selective targets for prospective antimigraine drugs.
ii. To investigate the side-effect potential of CGRP antagonists. This seems highly relevant in view of the widespread physiological distribution and functions of CGRP. It is already known that CGRP has a protective effect in ischemic preconditioning in isolated perfused rat hearts (9), and CGRP could also have a similar protective role in the brain circulation. As mentioned above, the development of more selective targets may enable the development of CGRP receptor antagonists without harmful

side effects. The lessons learnt from the development of triptans should be kept in mind, as compounds that are more selective will provide fewer side effects.

16.2.3 Sex steroids and migraine

In chapters 8 and 9 in this thesis, we investigated the relationship between CGRP, which is one of the mediators implicated in migraine, and female sex steroids. There is evidence from both *in vitro* and *in vivo* studies, that there is a positive relation between female sex steroids and this potent vasodilator peptide. However, the exact mechanism involved still has to be unravelled. Further, the importance of our observations in the pathophysiology of migraine, as well as the role of the sex steroids in other pathways in the pathophysiology of migraine, needs to be studied.

Essentially, in most experimental models, stable levels of sex hormones are used, while clinical evidence indicates that not only absolute levels of female sex steroids seem to be of relevance, but rather their (rate of) change. This is evident from the fact that in conditions like menstruation and the perimenopausal period where, hormone levels are very unstable, migraine severity is also enhanced. Disregarding the limitations of basic research, we will inevitably need a multidisciplinary approach, also encompassing clinical observations, to understand the influence of female sex steroids on migraine. Such a holistic approach will also be relevant to understand why migraine without aura seems to be predominantly triggered by a decline in estrogen levels, while migraine with aura seems to be prompted by high levels of estrogen. While migraine with and without aura are often considered as two entities of the same disease, the differential effect of female sex steroids on these two forms of migraine suggests that, at least in some aspects, they represent separate entities. Another interesting dimension to this ever increasing enigma is the so-called mismatch between the slow genomic and rapid non-genomic actions of sex steroids (10). As the changes during the menstrual cycle are drastic and rapid, the balance between excitatory and inhibitory stimuli might be disturbed, and could thus could be responsible for the increased susceptibility to migraine in females during this period. Thus, future studies can focus on these following aspects:

i. Investigation of the exact mechanism involved in 17β-estradiol-induced increases in cranial vasodilatation following periarterial electrical stimulation. This may be done by using CGRP receptor antagonists, immunohistochemistry, or by measuring total CGRP content in the dura mater. Further, CGRP measurements in rat plasma should be performed to determine the effect of various hormonal interventions on plasma levels of CGRP.
ii. Study the effect of withdrawal of sex steroids, as well as differences between various phases of the estrous cycle on the sensitivity to CGRP in the rat. This could be performed by treating ovariectomized rats with estrogen and removing the hormone-releasing pellets a short period before the experiment, thus conducting intravital microscopy experiments in the falling phase of hormone. Similarly, rats in different phases of estrous cycle should be investigated using the intravital microscopy model.
iii. Study the effects of male sex steroids on CGRP-induced vasodilatations in the rat intravital microscopy model. This could be realized by treating oopherictomized rats with various hormonal interventions, followed by intravital microscopy experiments.
iv. Investigate the effects of sex steroids on the sensitivity to CGRP in humans. This could be done by studying the functional responses to exogenous CGRP in arteries (e.g., obtained from gluteal biopsies) of transsexuals (male to female as well as female to male), before, during and after their sex-change procedure, which will be in parallel with hormone-treatment. In addition, the responses to CGRP could be measured (both *in vivo* and *in vitro*) in blood vessels from female migraine patients or healthy subjects during different phases of the menstrual cycle.

16.2.4 Preeclampsia and its pathophysiology

Like migraine, the pathophysiology of preeclampsia is also still enigmatic in nature. It is now widely believed that in the development of preeclampsia, an inadequate placental trophoblast invasion of the maternal uterine spiral arteries results in poor placental perfusion leading to placental ischemia. This is thought to result in the release of factors into maternal circulation which cause activation and/or dysfunction in the maternal endothelium (11). In addition, the metabolism of eicosanoids, as well as circulating lipid peroxides with the resultant oxidative stress advance the pathophysiology of preeclampsia as an excessive maternal inflammatory response to pregnancy. Usually, the therapeutic interventions in preeclampsia are limited to the use of antihypertensive drugs like nicardipine, hydralazine, labetalol, or nifedipine. Ketanserin, which is also used for the treatment of preeclampsia, did not alter the 5-HT receptor profile in patients, but unfortunately has a limited success rate in decreasing blood pressure, even at high doses.

We investigated the vascular aspects of preeclampsia, which affect the main symptom, i.e., the increased blood pressure. We demonstrated that 5-$HT_{1B/1D}$ receptors develop earlier in the preeclamptics as compared to normotensive pregnant females, which opens the possibility of usage of 5-$HT_{1B/1D}$ receptor antagonists in preeclampsia if these observations are further substantiated by other studies, which can be designed on following points:

i. Study whether the sensitivity or number of 5-$HT_{1B/1D}$ receptors in preeclampsia differs from that in normotensive controls at various phases of foetal development. These studies could be performed in umbilical cord artery as well as in maternal subcutaneous arteries by employing real time PCR and/or binding studies.

16.2.5 A relation between migraine and preeclampsia?

The prevalence of migraine in women rises after the average age of menarche, and peaks before the average age of menopause, affecting women most frequently during their childbearing years. Migraine has long been hypothesized to be associated with various vascular diseases, but epidemiological reports on this topic are contradictory. Although the aetiology of both migraine and preeclampsia is not totally understood, both have a strong vascular component, increased platelet aggregation and a high underlying cardiovascular risk profile in the pathophysiology. Hence, a relationship between migraine and preeclampsia seems plausible (12). There have been a handful of studies in this area, and in eight out of ten studies a positive association between migraine and preeclampsia was reported, although there is very little empirical evidence for the same (12). Therefore, prospective clinical studies should be planned to explore this relation and inclusion of patients should be based on presently accepted criteria for both migraine and preeclampsia. Only if there is unequivocal evidence of an association between these syndromes, then only future studies should be planned on this topic.

A multidisciplinary research involving a large diversity of disciplines, including epidemiology, obstetrics, neurology, endocrinology, and pharmacology may prove beneficial in increasing the depth and breadth of knowledge on the topics of migraine, pregnancy and its maternal complications, including preeclampsia.

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16.4 SAMENVATTING

16.4.1 Introductie migraine (Hoofdstuk 1)

Het is bekend dat migraine een neurovasculair syndroom is, waarbij de hoofdpijn voornamelijk veroorzaakt wordt door het verwijden van bloedvaten in het hoofd, gepaard gaand met een gelijktijdige activering van het trigeminale systeem. Er zijn verscheidene onderzoeksmodellen voor migraine ontwikkeld, gebaseerd op de betrokkenheid van bloedvaten en neuronen. Uiteraard bevatten deze migrainemodellen niet alle facetten van deze klinisch heterogene aandoening, maar hun bijdrage op verschillende niveaus (moleculair, *in vitro*, *in vivo*) is cruciaal geweest in de ontwikkeling van nieuwe geneesmiddelen tegen migraine en het onderzoeken van de pathofysiologie van migraine. Na een eerste screening in geïsoleerde bloedvaten van proefdieren met behulp van *in vitro* modellen kunnen de *in vitro* modellen met humane bloedvaten, zoals humane geïsoleerde meningeale en coronaire bloedvaten, gebruikt worden om het vasoactieve potentieel van een nieuw middel verder te evalueren en om een nieuw receptor systeem farmacologisch te karakteriseren, wat zou kunnen dienen als een 'target' voor toekomstige antimigraine middelen. Gezien het feit dat er zeker meerdere fysiologische systemen bij de pathofysiologie van migraine betrokken zijn, lijken *in vivo* modellen die meerdere bij migraine betrokken aspecten nabootsen van vitaal belang. Er zijn verschillende *in vivo* modellen in gebruik, zoals het varkensmodel om de arterioveneuze anastomotische bloedstroom te meten. De andere modellen maken gebruik van elektrische stimulatie van het ganglion of neuron trigeminale om neurogene durale ontsteking te bestuderen, terwijl het sinus sagittalis superior model rekening houdt met de transmissie van trigeminale nociceptieve input in de hersenstam. De geïntegreerde modellen, voornamelijk elektrische stimulatie van het ganglion trigeminale of systemische toediening van capsaicine, maken het mogelijk om de activatie van het trigeminale systeem en het effect hiervan op de bloedstroom in het hoofd te bestuderen; deze modellen geven hierdoor meer inzicht in de pathofysiologie van migraine dan de modellen die zich toespitsen op alleen de vasculaire of neuronale aspecten. De eerder genoemde migrainemodellen hebben uiteraard ook duidelijke voordelen. Een integrale aanpak waarbij meerdere modellen gebruikt worden (in plaats van slechts één model) zal bijdragen aan de vooruitgang van het onderzoek, terwijl de beperkingen geminimaliseerd worden. In het geheel genomen hebben de migraine modellen belangrijke inzichten verschaft in de pathofysiologische mechanismen in deze complexe ziekte, en zijn ze noodzakelijk voor verdere vooruitgang in het onderzoek naar migraine.

16.4.2 De potentiële rol van geslachtshormonen in de pathofysiologie van migraine (Hoofdstuk 2)

Migraine komt drie keer zoveel voor bij vrouwen als bij mannen tijdens de piek van de prevalentie, en de hevigheid en de verschijning van de migraine fluctueren al naar gelang de reproductieve mijlpalen in een vrouwenleven. Het lijkt daarom logisch dat de vrouwelijke geslachtshormonen, 17β-oestradiol en progesteron, die fluctueren gedurende de verschillende fasen van de levenscyclus van de vrouw, een rol spelen in de pathogenese van migraine. Geslachtshormonen, vooral 17β-oestradiol, hebben invloed op de synthese, het vrijkomen en de receptorexpressie van een aantal vasoactieve stoffen die betrokken zijn bij migraine. De synthese van *calcitonin gene-related peptide* (CGRP), een potente vasodilator van craniële arteriën, in het dorsale wortel ganglion is positief gerelateerd aan 17β-oestradiol. Verder zijn CGRP-gemedieerde innervaties verhoogd, parallel aan verhogingen in plasmaspiegels van 17β-oestradiol, in vaatbedden die nociceptoren bevatten. Analoog zijn plasmaspiegels van CGRP en de expressie van CGRP receptoren verhoogd in geovariectomeerde ratten wanneer deze behandeld worden met 17β-oestradiol, hoewel dit fenomeen in sommige vaatbedden niet aanwezig is. In deze context is het verleidelijk te suggereren dat de vermindering in perifere vaatweerstand door geslachtshormonen (tenminste gedeeltelijk) bijdraagt aan de pathofysiologie van bij migraine betrokken craniële bloedvaten. De dysfunctie van deze bloedvaten veroorzaakt zo hyperreactiviteit in respons op endogene vasorelaxantia. Net als CGRP wordt de activiteit van stikstofoxide ook verhoogd door 17β-oestradiol in verschillende

diermodellen. Deze verhoging kan worden toegeschreven aan verhoogde eNOS (endotheliaal stikstofoxide synthetase) activiteit. Van oestrogeen is verder bekend dat het de intracellulaire en extracellulaire [Mg^{2+}] verlaagt. Interessant genoeg is dit divalente kation deficiënt bij migraine patiënten, en wordt dit ion ook gebruikt voor migraine profylaxe. Adrenoceptor expressie fluctueert ook met 17β-oestradiol, maar varieert al naar gelang het type bloedvat, hetgeen ook het geval is met de receptor activiteit van 5-HT.

Hoewel het experimentele bewijs in dieren erop lijkt te wijzen dat een toename van oestrogeen een hogere kans op een migraine aanval geeft, suggereert de literatuur betreffende humane studies dat de onttrekking van oestrogeen de grootste trigger is. Beide zienswijzen lijken echter de situatie zoals deze in de realiteit plaatsvindt teveel te versimpelen. Ten eerste is het niet alleen de onttrekking van oestrogeen die belangrijk is, maar voorafgaand aan deze afname moet er een 'priming' zijn met voldoende hoge concentraties van oestrogeen. Ten tweede gaat het waarschijnlijk niet alleen om de absolute waarden van zowel progesteron en oestrogeen, maar lijkt de relatieve mate van de verandering van deze hormonen de boosdoener te zijn. Onderzoek naar de rol van geslachtshormonen wordt verder bemoeilijkt door het feit dat deze hormonen niet alleen hun genomische activiteit hebben via hun klassieke nucleaire receptor mechanismen, maar ook een snelle activiteit, die waarschijnlijk gemedieerd wordt door een membraanreceptor via non-genomische mechanismen. Samenvattend kan gezegd worden dat geslachtshormonen zeker betrokken lijken te zijn bij de pathofysiologie van migraine bij de vrouw, maar dat er nog een lange weg te gaan is voordat men de betrokken mechanismen duidelijk kan verklaren.

16.4.3 Introductie preeclampsie (Hoofdstuk 3)

Preeclampsie is een multi-systeem afwijking die voorkomt tijdens 2% tot 7% van alle zwangerschappen; het is de hoofdoorzaak van het overlijden van zwangere vrouwen in Nederland. De afwijking wordt gekarakteriseerd door het voorkomen na de 20^{e} week van de zwangerschap, verhoogde maternale bloeddruk en proteinurie. In ernstige gevallen kan preeclampsie leiden tot zorgwekkende complicaties voor de moeder zoals het HELLP-syndroom (haemolyse, verhoogde leverenzymen, lage waarden van bloedplaatjes), pulmonaire oedeem, acuut nierfalen, leverfalen of bloedingen, *abruptio placentae* en eclampsie. Preeclampsie is geassocieerd met verhoogde perinatale morbiditeit en sterfte. De hogere weerstand van de bloedvaten van de maternale vasculatuur in preeclampsie kan toegeschreven worden aan verhoogde plasmaconcentraties van contractiele stoffen, maar zou ook toegeschreven kunnen worden aan een verhoogde gevoeligheid van de bloedvaten voor vasoconstrictoire stoffen zoals angiotensine II, 5-hydroxytryptamine (5-HT; serotonine) en noradrenaline in combinatie met een verminderde reactie op vasodilatoire peptiden zoals CGRP en acetylcholine (ACh). De verhoogde gevoeligheid voor contractiele stoffen kan veroorzaakt worden door een upregulatie van de respectievelijke receptoren en/of een meer efficiënte binding met hun second messenger systemen, en het omgekeerde voor endogene vasodilatoire stoffen. De pathofysiologie van preeclampsie is nog steeds verre van duidelijk, maar het wordt algemeen aangenomen dat immunologische maladaptaties, die vermoedelijk het resultaat zijn van een misalliantie van foetale trophoblasten met maternale weefsels, evenals endotheliale celactivatie of dysfunctie, een grote rol spelen. Daarom is onderzoek naar de factoren die betrokken zijn bij de pathogenese van preeclampsie van vitaal belang, en probeerden wij de vasculaire mechanismen die bij kunnen dragen aan dit syndroom te onderzoeken.

16.4.4 Karakterisatie van CGRP receptoren (Hoofdstukken 5-6)

Deze studies zijn uitgevoerd om de calcitonin gene-related peptide (CGRP) receptoren in geïsoleerde proximale en distale coronair arteriën van de mens en het varken te karakteriseren, alsmede in humane meningeaal arteriën met gebruik van BIBN4096BS. BIBN4096BS, een dipeptide derivaat, is een zeer potente en selectieve humane CGRP receptor antagonist. Relaxaties geïnduceerd door humaan (h)-αCGRP werden geblokkeerd door BIBN4096BS in alle bloedvaten die bestudeerd zijn. In tegenstelling tot de

andere vaten was de helling van de Schild plot in de humane distale coronaire vaatsegmenten significant kleiner dan één, terwijl BIBN4096BS deze responsen potent blokkeerde. In hetzelfde preparaat gedroeg h-αCGRP$_{8-37}$ zich als een zwakke antagonist van door h-αCGRP geïnduceerde relaxaties, ook met een Schild plot helling kleiner dan één. De lineaire agonisten, [ethylamide-Cys^{2,7}]h-αCGRP ([Cys(Et)^{2,7}]-h-αCGRP) en [acetimidomethyl-Cys^{2,7}]h-αCGRP ([Cys(Acm)^{2,7}]-h-αCGRP), hadden een hoge potentie, wat lijkt te wijzen op de aanwezigheid van CGRP$_2$ receptoren, terwijl de potente blokkade door BIBN4096BS op de aanwezigheid van CGRP$_1$ receptoren wijst. Gebruikmakend van RT-PCR werden mRNAs aangetoond die coderen voor de essentiële componenten van functionele CGRP$_1$ receptoren in zowel de humane proximale als distale coronaire arterie als in de meningeaal arterie. Bovengenoemde resultaten tonen de aanwezigheid van CGRP$_1$ receptoren aan in alle segmenten van de coronair arterie die onderzocht zijn, maar de segmenten van de humane distale coronaire vaten lijken een extra populatie van CGRP receptoren te bevatten die niet overeenkomt met de CGRP$_1$ of CGRP$_2$ receptoren zoals die op dit moment geclassificeerd zijn.

h-αCGRP relaxeerde de meningeaal arterie potenter dan [Cys(Et)^{2,7}]h-αCGRP, in tegenstelling tot de situatie in de humane distale coronair arterie, terwijl de maximale respons op deze agonisten niet significant anders was. h-αCGRP$_{8-37}$ antagoneerde ook de response op h-αCGRP, met een Schild plot helling niet verschillend van één. Gezien de hoge antagonist potentie van BIBN4096BS en de lagere agonist potentie van [Cys (Et)^{2,7}]h-αCGRP, is het voor de hand liggend dat in de humane meningeaal arterie voornamelijk CGRP$_1$ receptors actief zijn. Deze zienswijze wordt versterkt door de Schild plot analyse die een helling van één voortbracht in alle experimenten, wat verdere aanwijzingen voor een homogene CGRP receptor populatie in dit bloedvat levert.

16.4.5 Door capsaicine geïnduceerde relaxaties in geïsoleerde arteriën (Hoofdstuk 7)

Capsaicine activeert sensorische zenuwvezels via de vanilloid VR1 receptor, wat leidt tot het vrijkomen van neuropeptiden zoals CGRP en substance P. Zenuwen die gevoelig zijn voor capsaicine komen zowel in coronaire als meningeale bloedvaten voor; daarom onderzochten wij de rol van CGRP in capsaicine-geïnduceerde relaxaties in deze beide bloedvaten. Capsaicine relaxeerde bloedvaten van zowel mensen als varkens op concentratie-afhankelijke wijze. De verwijdering van endotheel of de blokkade van CGRP of NK$_1$ receptoren, calcium kanalen of cyclisch GMP had geen invloed op de relaxerende responsen op capsaicine. Verschillende K^+ kanaal blokkers, gap junctions remmers en NO synthase remmers waren ook niet in staat om door capsaicine gemedieerde responsen te blokkeren. In conclusie zijn de door capsaicine geïnduceerde relaxaties in de geïsoleerde coronaire bloedvaten van mens en varken, evenals in de humane meningeaal arterie, niet gemedieerd door CGRP, en zijn deze geïsoleerde bloedvaten dus geen geschikt model voor de bestudering van het vrijkomen van CGRP door activatie van perivasculaire zenuwen. Door capsaicine geïnduceerde relaxaties in geïsoleerde arteriën moeten worden toegeschreven aan een niet-specifiek fenomeen.

16.4.6 Het effect van geslachtshormonen op de vasculaire farmacologie van migraine (Hoofdstukken 8, 9 en 10)

We onderzochten de effecten van de vrouwelijke geslachtshormonen 17β-oestradiol en progesteron, zowel apart als in combinatie, op durale vasodilatatie geïnduceerd door αCGRP, periarteriële elektrische stimulatie en capsaicine in geovariectomeerde ratten met gebruik van intravitale microscopie. Er waren geen significante verschillen tussen de vasodilatoire potentie of effectiviteit van αCGRP of capsaicine in de durale arteriën in de verschillende groepen die bestudeerd zijn. In tegenstelling hiermee was de vasodilatoire respons op elektrische stimulatie aanzienlijk hoger in ratten die behandeld waren met 17β-oestradiol in vergelijking met wat gevonden werd bij behandeling met placebo. In vergelijkbare groepen hebben we ook gekeken naar de vasoactieve responses op CGRP, ACh, 5-HT end adrenaline in verscheidene segmenten van geïsoleerde arteriën die in orgaanbadjes gehangen waren. De relaxatie respons op αCGRP was significant gepotentieerd in mesenteriale en caudale arteriën van ratten die

behandeld waren met 17β-oestradiol vergeleken met de groep die met placebo behandeld was. In de a. carotis duiden onze resultaten er op dat circulerend progesteron en/of 17β-oestradiol de contractie in respons op noradrenaline en 5-HT zou kunnen verlagen. Samenvattend tonen onze resultaten aan dat 17β-oestradiol de door elektrische stimulatie geïnduceerde durale vasodilatatie verhoogt, wat waarschijnlijk veroorzaakt wordt door een toegenomen afgifte van neuropeptiden zoals CGRP uit perivasculaire zenuwen, terwijl contracties in respons op 5-HT verlaagd lijken te worden door verhoogde concentraties van 17β-oestradiol. Deze mechanismen, die elkaar kunnen versterken, zouden migraine aanvallen bij vrouwen kunnen verhevigen.

16.4.7 Intravitale microscopie model in muizen (Hoofdstuk 11)

Het doel van de studie was om een muizenmodel te ontwikkelen om trigeminovasculaire mechanismen te bestuderen met gebruik van intravitale microscopie via een venster in een gesloten schedel. Het voornaamste verschil tussen het muizenmodel en het rattenmodel, dat al eerder door anderen ontwikkeld is, is het feit dat het in het muizenmodel noodzakelijk is eerst een constrictie te induceren in de durale arteriën voordat er durale vasodilatatie kan worden gedetecteerd. Na het model ontwikkeld te hebben, bestudeerden we durale vasodilatatie die geïnduceerd werd door exogeen en endogeen CGRP. h-αCGRP en capsaicine induceerden beide vasodilatatie in bloedvaten na preconstrictie, en elektrische stimulatie induceerde stroomafhankelijke relaxaties in de durale bloedvaten. BIBN4096BS blokkeerde responsen veroorzaakt door αCGRP, capsaicine en elektrische stimulatie, terwijl sumatriptan alleen de vasodilatatie blokkeerde die door elektrische stimulatie geïnduceerd werd. Dit muizenmodel zal bruikbaar zijn voor het bestuderen van verschillende aspecten van het trigeminovasculaire systeem, met name in het onderzoek naar de rol van ion kanaal mutaties die gevonden zijn bij migraine patiënten, waarbij gebruik gemaakt zal worden van transgene muizen met deze mutaties.

16.4.8 Clonidine remt vasodilatatie van a. carotis externa van de hond in respons op capsaicine via α2A/2C-adrenoceptoren (Hoofdstuk 12)

De rol van CGRP in de pathofysiologie van migraine lijkt essentieel omdat wordt aangenomen dat dit peptide trigeminale sensorische zenuwen activeert, craniële bloedvaten verwijdt en een transmitter voor vasculaire nociceptie is. Bovendien verminderen verscheidene antimigraine geneesmiddelen de durale vasodilatatie gegenereerd door stimulatie van het ganglion trigeminale. Op basis hiervan zijn in de huidige studie in gevagosympathectomeerde honden de effecten onderzocht van het profylactische antimigraine middel clonidine (een bekende α_2-adrenoceptor agonist) op vasodilatoire responsen op capsaicine, CGRP en acetylcholine in de a. carotis externa. Intracarotide infusies van capsaicine, CGRP en acetylcholine resulteerden in een dosis-afhankelijke toename van de conductantie van de a. carotis externa zonder verandering in bloeddruk of hartfrequentie. Interessant genoeg werden de vasodilatoire responsen van de a. carotis externa op capsaicine, maar niet op CGRP of acetylcholine, gedeeltelijk geremd na i.v. toediening van clonidine, maar niet door dezelfde hoeveelheden fysiologische zoutoplossing. De remmende effecten van clonidine werden geantagoneerd door i.v. toediening van de α_2-adrenoceptor antagonisten rauwolscine ($\alpha_{2A/2B/2C}$), BRL44408 (α_{2A}) of MK912 (α_{2C}), maar niet door imiloxan (α_{2B}). Deze resultaten suggereren dat clonidine de vasodilatoire respons van de a. carotis externa van de hond op capsaicine remt door een perifeer trigeminovasculair en/of centraal mechanisme, voornamelijk door middel van α_{2A}-adrenoceptors en, in mindere mate, α_{2C}-adrenoceptoren.

16.4.9 Vasculaire farmacologie van preeclampsie (Hoofdstukken 13, 14, 15)

We onderzochten de functionele reactiviteit van 5-HT receptoren in de bloedvaten van de navelstreng en maternale subcutane vet arteriën in normotensieve en preeclamptische zwangerschappen op verschillende momenten tijdens de zwangerschap. Er waren geen significante verschillen in het farmacologische profiel van de 5-HT_{2A} receptor, terwijl de gevoeligheid van $5\text{-HT}_{1B/1D}$ receptoren aanmerkelijk toegeno-

men was bij de bevalling na een voldragen zwangerschap in vergelijking met te vroeg geboren baby's na normotensieve zwangerschappen. In contrast hiermee lijkt de ontwikkeling van 5-$HT_{1B/1D}$ receptoren in preeclamptische zwangeren die vroegtijdig baren versneld te zijn verlopen. In de subcutane vet arteriën waren er geen verschillen tussen het 5-HT_{2A} en 5-$HT_{1B/1D}$ receptor profiel en de endotheel-afhankelijke relaxaties tussen de twee groepen was gelijk. Toekomstige studies van 5-$HT_{1B/ID}$ receptoren zullen nieuwe inzichten verschaffen in de ontwikkeling van de foetus en de pathofysiologie van preeclampsie.

In de preeclamptische patiënten hebben we ook het effect onderzocht van de behandeling met ketanserine op het 5-HT receptor profiel (E_{max} en pEC_{50}) in de bloedvaten in de navelstreng. Omdat wij geen verschil in het 5-$HT_{1B/1D}$ en 5-HT_{2A} receptor profiel vonden, heeft de blootstelling van de foetus aan ketanserine in preeclamptische patiënten geen duidelijke invloed op deze 5-HT receptoren. In overeenstemming met andere gepubliceerde literatuur tonen onze resultaten aan dat ketanserine een relatief veilig geneesmiddel is voor preeclamptische patiënten. In de maternale subcutane weerstands arteriën hebben wij tevens de vasoactieve reacties op CGRP onderzocht bij normotensieve en preeclamptische zwangerschappen. Er was geen verschil tussen deze twee groepen in effectiviteit of potentie van CGRP.

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ABOUT THE AUTHOR

Saurabh Gupta was born on March 15, 1976 in Palampur, India. He completed his high school (Central Board of Secondary Education) in 1995. He studied pharmacy in the Government College of Pharmacy, Bangalore, India. After successfully obtaining his Bachelors degree in Pharmacy in 1998, he joined the National Institute of Pharmaceutical Education and Research, Mohali, India. Here he pursued his Masters in Pharmacology under the supervision of Dr. S.S Sharma and he established a focal ischemia model in rats working towards his dissertation. After obtaining his Masters degree in Pharmacology in 1999, he joined Dr. Reddy's Research Foundation in Hyderabad, India. He worked in the Department of Drug Metabolism and Kinetics in the capacity of Senior Biologist. In July 2002 he joined the Department of Pharmacology, Erasmus MC, Rotterdam, The Netherlands, as AIO, with Dr. Antoinette MaassenVanDenBrink as his superviser under the promotorship of Prof. Pramod R. Saxena and Prof. A. H. J. Danser. Prof. Carlos. M. Villalón (Departamento de Farmacología y Toxicologia, México D.F., México) also guided him during his PhD period. During his tenure of 4 years, he collaborated with various scientists at the Hospital Pharmacy and the Department of Obstetrics and Gynaecology, Erasmus MC. He also had the opportunity to collaborate and work with Dr. Simon Akerman (Headache Group, Queen Square, London, U.K.) and Dr. A.M.J.M. van den Maagdenberg (Department of Human Genetics and Department of Neurology, LUMC, Leiden).

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ABBREVIATIONS

5-HT	5-hydoxytryptamine
AMPA	α-amino-3-hydroxy-5-methyloxazole
ANOVA	analysis of variance
ATP1A2	Na^+ / K^+-ATPase α_2-subunit gene
AU	arbitrary units
AVA	arteriovenous anastomoses
A-V SO_2	oxygen saturation difference the arterial and external jugular venous blood samples
BIBN4086BS	olcegepant
BK_{Ca}	Ca^{2+}-dependent K^+ channels for large conductance
bp	base pair
CACNA1A	$Ca_v2.1$ (P/Q-type) Ca^{2+} channel α_1-subunit gene
c-FOS	FBJ osteosarcoma oncogene
CGRP	calcitonin gene-related peptide
$CGRP_{8-37}$	C-terminal fragment of CGRP
CLR	calcitonin receptor like receptor
CNS	central nervous system
CREB	cAMP response-element binding protein
[Cys(Acm)^{2,7}]-h-αCGRP	[acetimidomethyl-Cys^{2,7}]h-αCGRP
[Cys(Et)^{2,7}]-h-αCGRP	[ethylamide-Cys^{2,7}]h-αCGRP
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
cGMP	cyclic guanylylmonophosphate
CV	cerebral blood vessel
CRC	concentration response curve
DA	dural artery
EC_{50}	concentration required to elicit half the maximal response
E_{max}	maximum plateau response reached with an agonist
ESR1	estrogen receptor 1
ET-1	endothelin-1
FHM	familial hemiplegic migraine
GABA	γ-aminobutyric acid B
HRT	hormone replacement therapy
HELLP	haemolysis, elevated liver enzymes, low platelet counts
IBMX	isobutylmethylxanthine
IK_{Ca}	intermediate-conductance Ca^{2+}-activated K^+ channel
i.c.	intracarotid
i.p.	intraperitoneal
i.v.	intravenous
L-NAME	N^{ω}-nitro-L-arginine methyl ester HCl
µA	micro ampere
µM	micro molar
MBP	mean arterial blood pressure
min	minute
mM	milli molar
MRI	magnetic resonance imaging
NF-κB	nuclear factor-κB

NK_1	neurokinnin-1
nM	nano molar
NMDA	*N*-methyl-D-aspartic acid
NO	nitrix oxide
NOS	nitrix oxide synthase
NSAIDS	non-steroidal anti-inflammatory drugs
pA_2	the negative logarithm ($-^{10}$log) of the molar concentration of antagonist required to shift the concentration response curve of an agonist by two fold.
pEC_{50}	negative logarithm ($-^{10}$log) of EC_{50}
pK_b	antagonists potency, calculated by dose ratio (DR) of EC_{50} of agonists in presence of the antagonist by the EC_{50} of agonist in control situation and plotting $-^{10}$log (DR-1) in Schild plot while constraining the slope to unity.
pK_i	binding affinity constants
PCR	Protein/creatinine ratio
PR	progesterone receptor
PROGINS	306-bp *Alu* insertion within the progesterone receptor gene
RAMP1	Receptor activity modifying protein
RCP	Receptor component protein
RDC	the orphan receptor (originally cloned from canine thyroid cDNA)
RT-PCR	reverse transcriptase polymerase chain reaction
s	second (time)
s.e.m.	standard error of mean
SCN1A	neuronal voltage-gated sodium channel gene
SK_{Ca}	small-conductance Ca^{2+}-activated K^+ channel
SNAP	S-nitroso N-penicillamine
TRPV1	transient receptor potential vanilloid receptors type 1
TxA_2	thromboxane A_2
UCA	umbilical cord artery

Short description of various compounds mentioned and used

18-α-glycyrrhetinic acid	gap junction inhibitor
4-aminopyridine	voltage-dependent K^+ channel (K_v) blocker
A61603	α_1-adrenergic receptor agonist
Apamin	blocker of small-conductance Ca^{2+}-dependent K^+ channels (SK_{Ca})
BIBN4096BS	CGRP receptor antagonist
BRL15572	selective 5-HT_{1D} receptor antagonist
BRL44408	α_{2A}- adrenoceptors antagonist
Capsaicin	vanilloid receptor agonist
Capsazepine	vanilloid receptor antagonist
$CGRP_{8-37}$	putative $CGRP_1$ receptor antagonist
Charybdotoxin	blocker of Ca^{2+}-dependent K^+ channels for large conductance (BK_{Ca}) and intermediate conductance (IK_{Ca}),
Clonidine	α_2-adrenoceptor agonist
Cocaine	neuronal reuptake inhibitor
Corticosterone	extra-neuronal reuptake inhibitor
CP122,288	plasam protein extravasation inhibitor
[$Cys(Acm)^{2,7}$]-h-αCGRP	putative $CGRP_2$ receptor agonist
[$Cys(Et)^{2,7}$]-h-αCGRP	putative $CGRP_2$ receptor agonist

Ergotamine	$\alpha_{2A/2C}$-adrenoceptors.agonist
GR125743	reversible 5-$HT_{1B/1D}$ receptor antagonist
GR127935	irreversible 5-$HT_{1B/1D}$ receptor antagonist
Imiloxan	α_{2B}-adrenoceptors antagonist
Ketanserin	5-HT_{2A} receptor antagonist
L-733060	the neurokinin NK_1 receptor antagonist
Labetolol	selective α-adrenoceptor blocking and non-selective β-adrenoceptor blocking agent
LY2925578	glutamate R5 receptor antagonist
L-NAME	nitric oxide synthetase inhibitor
Memantine	NMDA receptor antagonist
MK801	NMDA receptor antagonist
MK912	$\alpha_{2A/2B/2C}$ antagonist
Norepinephrine	α_1-, α_2- and β_1-adrenergic receptor agonist
Phenylephrine	α_1- adrenoceptors.agonist
Prazocin	α_1- adrenoceptors.antagonist
Propranolol	β-adrenergic receptors antagonist
Rauwolscine	$\alpha_{2A/2B/2C}$- adrenoceptors.antagonist
Ruthenium red	voltage-sensitive calcium channel blocker
SB224284	selective 5-HT_{1B} receptor antagonist
Sumatriptan	5-$HT_{1B/1D}$ receptors agonist
U46619	thromboxane A_2 analogue
Y-27632	RhoA kinase inhibitor