

“Role of TRPA1 and TRPV1 in Propofol Induced Vasodilation”

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To

"My Beloved Parents"

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CHAPTER 1:

SPECIFIC AIMS & BACKGROUND

Hypothesis:

Recent findings from our lab have shown the prominent role of two transient receptor channels TRPA1 and TRPV1 cross-talk with each other in sensory neurons and also in heterologous expression systems (Wickley *et al.* 2010; Zhang *et al.* 2011). Studies from other lab have also shown that the functionality of one channel gets impaired in the absence of the other (Akopian *et al.* 2008). Though both channels can function independently but interestingly studies have demonstrated that these channels can interact with each other by heterotetramerization thereby promoting its function (Akopian *et al.* 2010). Moreover it has been also depicted that the homologous and heterologous desensitization between TRPV1 and TRPA1 responses follow calcium dependent/independent pathways (Ruparel *et al.* 2008). Ruparel *et al.* 2011 have also demonstrated that TRPA1 can be desensitized by TRPV1 agonist arachidonyl-2-ethanolamine. Studies have also illustrated the role of TRPA1 and TRPV1 in cross-desensitization and resensitization where the functionality of one channel is restored in the presence of other (Salas *et al.* 2009). Few other studies have also shown a prominent role of calcium in regulating the activity of TRPA1, but surprisingly it has been found that the modulation of TRPA1 by calcium is dependent on TRPV1 (Patil *et al.* 2010). Most of these studies were performed in the sensory neurons or in heterologous expression system. ***Very few studies to date have shown the role of these channels in the vascular bed.*** Recent reports have demonstrated that TRPA1 activation *in vivo* or *in vitro* causes a vasodepressor response. A role for TRPA1 activation by propofol as a mechanism for mediating the hypotensive response observed in the clinical setting and in other *in vitro* models of vascular reactivity has not been explored. Because of the

recent data indicating cross-talk between TRPA1 and TRPV1 channels in sensory neurons and heterologous expression systems, and the fact that propofol activates TRPA1 channels in these systems provides us with a strong rationale for our overall goal of study.

Specific Aims:

- I. To determine the role of TRPA1 and TRPV1 in propofol-induced depressor responses *in-vivo***

Rationale:

The overall goal of this aim is to investigate the role of TRPA1 and TRPV1 in propofol induced vasodepressor response and also to delineate whether nitric oxide and BK channel are the downstream mediators of this pathway. As it is known that propofol activates TRPA1 in neurons and cross-talk exists between these channels, we are interested to determine the signaling cascade by which propofol induce vasodilation. Most importantly we have investigated the role of TRPA1 and TRPV1 in vasculature using propofol as our mechanistic tool

- II. To determine the role of TRPA1 and TRPV1 in propofol induced vasodilation *in-vitro* in isolated coronary microvessels and primary aortic endothelial cells to get at cellular pathways and mechanisms involved**

Rationale:

Our *in-vivo* studies have shown a prominent role of TRPA1 in propofol induced depressor responses. But results obtained from *in-vivo* study can lend to a number of factors as in an *in-vivo* set-up there are different vascular-beds involved for example cerebral, mesenteric, renal etc; so to narrow down our findings we performed vasoreactivity studies in the heart. Heart is known to be a major regulatory organ involved in vasomotor tone; so we performed our next set of studies in isolated coronary microvessels. Recent findings have delineated that nitric oxide activates TRP channels. But none of these studies have shown the role of nitric oxide as a downstream modulator of TRP pathway. Our *in-vivo* and coronary microvessel data showed a prominent role of nitric oxide in propofol induced vasodilation. Moreover our data suggests that Nitric Oxide (NO) is a downstream mediator in this process. So to further strengthen our hypothesis we have measured NO in primary cultured endothelial cells isolated from control, TRPA1^{-/-}, TRPV1^{-/-} and TRPAV^{-/-} mice. We have also performed immunocytochemistry to determine the co localization of these channels in endothelial cells.

Background:

Endothelium:

Endothelial cells (EC's) form a unique, dynamic and heterogeneous signal-transducing site in the vascular system that lines the innermost surface of blood vessels and lymphatic vessels (Fishman *et al.* 1982).The endothelium regulates numerous processes such as fluid filtration, neutrophil recruitment, hormone trafficking, platelet

aggregation, wound healing, angiogenesis and, most importantly, control of blood flow and pressure (Cines *et al.* 1998). Detailed study of endothelial cells first became feasible with the culture of EC's *in-vitro* in the year 1970 (Jaffe *et al.* 1970; Gimbrone *et al.* 1974; Lewis *et al.* 1973). ECs are rich in various well-known markers such as platelet/endothelial cell adhesion molecule (PECAM-1), vascular endothelial cadherins etc. ECs are actively engaged in two main cellular processes such as endocytosis and transcytosis. Apart from regulation of these cellular processes, endothelial cells also perform various functional roles which are described below (Nilius *et al.* 2001; Serban *et al.* 2010).

Roles of the Endothelium:

Endothelial cells perform different functions at different locations of the body. For example arterial endothelial cells regulate oxygen transport from blood to tissue and hence control the vasomotor tone of the smooth muscle cells, blood flow and hence regulate the blood pressure. In the microvascular bed endothelial cells also regulate the transport of metabolites, macromolecules and gases and are hence involved in cell-nutrition (Jaffe *et al.* 1970; Gimbrone *et al.* 1974; Lewis *et al.* 1973). The other major functions of ECs are blood coagulation, wound healing and angiogenesis. The functionality of endothelial cells depends on various signaling cascades which can originate from blood cells such as white-blood cells, thrombocytes or the cells adhering to it such as smooth muscle cells, mast cells, fibroblasts or from other cellular mechanisms which can be mechanical, chemical or neuronal. Defects in the signaling cascade can lead to atherosclerosis, hypotension/hypertension, other injuries and inflammation (Fishman *et al.* 1982; Serban *et al.* 2010; Nilius *et al.* 2001).

Regulation of vasoreactivity:

Endothelial cell mediated vasoreactivity was first discovered by a nobel laureate Dr. Robert Furchgott in 1980. His experiments for the first time demonstrated that simple arteries can be relaxed with acetylcholine by the activation of muscarinic receptors (Furchgott *et al.* 1980; Furchgott *et al.* 1989). Later his findings led to the discovery of the vasodilator substance NO which is also sometimes referred as endothelium derived relaxing factor (EDRF). In the year 1977 nobel laureate Dr. Ferid Murad also discovered that nitroglycerin release NO and relaxes smooth muscles. Later in the year 1986 nobel laureate Dr. Louis J Ignarro concluded that EDRF was identical to NO. These series of discoveries concluded the vasoreactive properties of endothelial cells are regulated by NO. The examples of the common vasorelaxants are circulating hormones such as catecholamine and vasopressin, platelet derived products such as serotonin and adenosine diphosphate (ADP), autacoids like histamine and bradykinin, prostaglandins, thrombin, shear stress or physical exercise, unsaturated fatty acids, polyphenols present in red wine, green tea or chocolate. Apart from various vasodilators there are some common vasoconstrictors which are also released from the endothelium. The common vasoconstrictors are endothelin, endothelium derived contracting factors such as thromboxane A_2 , superoxide anions, neurotransmitters, hormones such as noradrenaline etc. (Busse *et al.* 2002; Feletou *et al.* 2006). The well-known mechanism by which the common vasodilators stimulate the process of vasorelaxation is as follows:

Agonists/Vasodilators bind to their specific receptor which leads to an influx of calcium ions that in turn activates the enzyme endothelial nitric oxide synthase (eNOS). eNOS

with the help of other co-factors catalyzes the synthesis of NO from L-Arginine. NO once produced activates soluble guanylyl cyclase which catalyzes the formation of cyclic guanosine monophosphate (cGMP) which in turn activates other downstream target molecules such as potassium channels (BK_{Ca} , K_{ATP}) thereby causing vasorelaxation (Ignarro *et al.* 1986; Luscher *et al.* 1986; Serban *et al.* 2010). In contrast, there are several NO-independent pathways which can also lead to vasorelaxation. **Fig 1** below depicts regulatory mechanisms of NO production in endothelial cells.

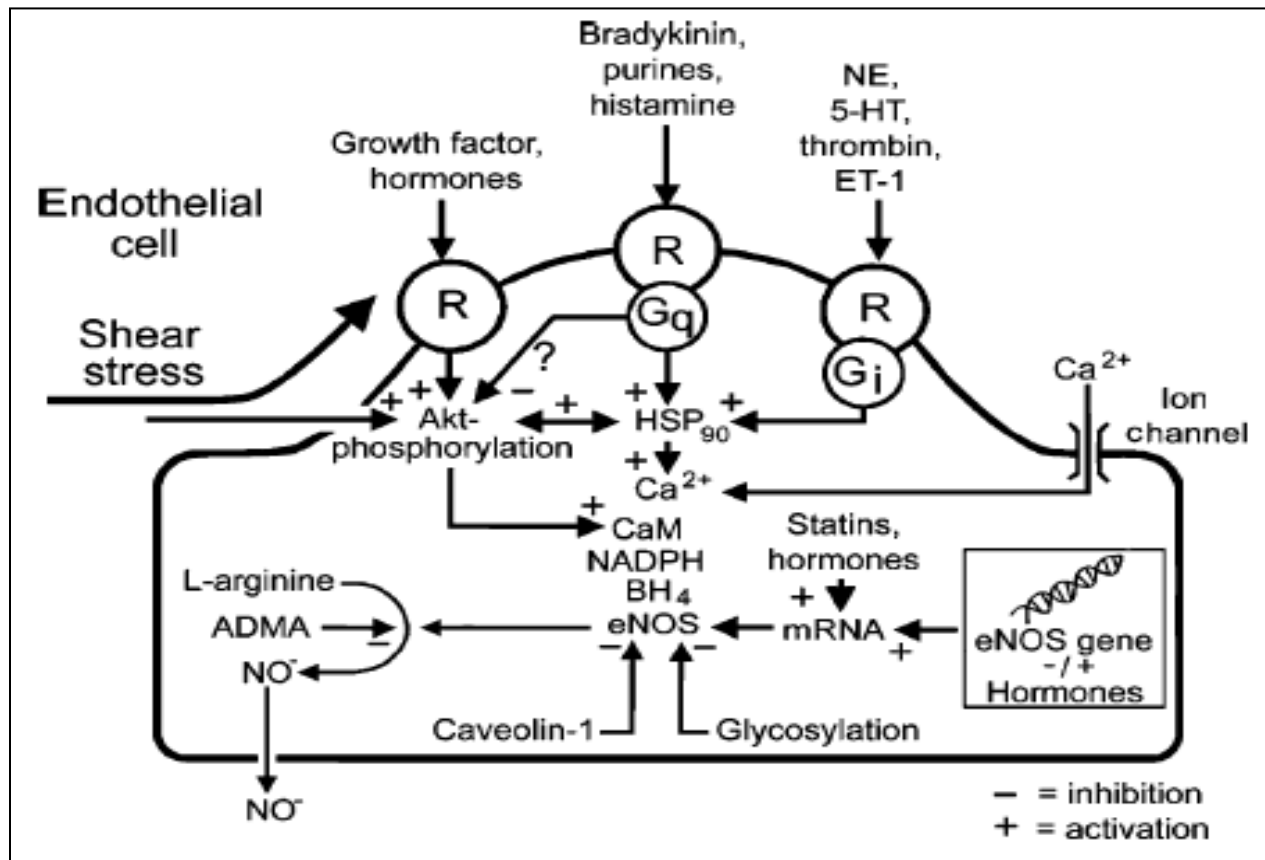


Fig 1: Schematic diagram representing the regulation of production of nitric oxide in endothelial cells (Serban *et al.* 2010).

The vasoconstriction is mediated by regulators such as endothelin-1 which is released from endothelial cells in response to various stimuli such as hypoxia, noradrenaline etc. The formation of endothelin-1 is catalyzed by endothelin-converting enzyme which is activated by some external factors. Endothelin-1 once formed activates ET-B, a type of G-protein coupled receptors present in the smooth muscle cells leading to contraction. Though endothelin is known as a potent vasoconstrictor, it can also interact with ET-A receptors on the endothelial cells triggering the release of vasodilators such as NO. Other vasoconstrictor substances such as thromboxane A_2 is also a well-known prostanoid synthesized from arachidonic acid by cyclooxygenase enzyme and are known to stimulate G-protein coupled receptors in the vascular smooth muscle cells thereby leading to vasoconstriction (Halushka *et al.* 1989). Superoxide anions are also known to scavenge NO hence inducing vasoconstriction.

Pharmacomechanical vs electromechanical regulation of vasomotor tone:

These processes play a major role in the regulation of vasoreactivity in the smooth muscle cells. Smooth muscle cells are the outermost covering of the blood vessels. The two processes referred to as pharmacomechanical and electromechanical coupling regulate the contractile properties of the blood vessels. These above mentioned processes are also regulated by the types of ion-channels/receptors present in a particular artery and also depends on the nature of activation. Pharmacomechanical coupling leads to the activation of second messengers such as cAMP, calcium influx which then leads to Ca^{2+} dependent Ca^{2+} release from intercellular stores (Somlyo *et al.* 1968). Pharmacomechanical coupling is generally induced by drugs, hormones etc. In contrast to pharmacomechanical coupling, electromechanical coupling operates through

changes in surface membrane potential. The change in membrane potential depolarizes the membrane thereby leading to opening of voltage-gated calcium channels. The influx of calcium results in depletion of intracellular stores such as endoplasmic reticulum and sarcoplasmic reticulum. Neurotransmitters or hormones or other depolarizing factors will result in contraction whereas hyperpolarizing factors such as NO will hyperpolarize the membrane and hence will cause vasorelaxation (Somlyo *et al.* 1968).

Ion channels:

Living cells are surrounded by a membrane which is highly rich in phospholipids. Due to this particular feature the membrane is highly impermeable to charged particles/ions such as sodium, potassium, calcium, etc. To maintain the cellular functionality each cell has different gates that allow particular ions to enter the cells also called channels. The inflow/outflow of these ions can hence result in change in concentration of some important second messengers such as calcium. Such change in concentration inside the cell plays a significant role in controlling various vital functions of a particular cell such as beating of heart, nervous stimulation in brain (MacKinnon *et al.* 2004; Voets *et al.* 2005). There are hundreds of ion-channels which can be again subdivided into dozens of subfamilies. The two main characteristics of ion-channels are **channel selectivity** and **channel gating**. Channel selectivity controls the particular ion that can enter the pore whereas channel gating regulates the opening and closing of the channel (MacKinnon *et al.* 2004; Voets *et al.* 2005). Role of ion channels in signal transduction is still not completely clear but has received immense importance in last few years. Ion channels are generally involved in short-term responses that in turn result in production or release of various growth factors, pro and anti-coagulants, and vasomotor regulators

such as NO, prostacyclins/PGI₂ etc. The other important regulators of signal transduction pathways are released by exocytosis. A few examples are tissue plasminogen activators (tPA), tissue factor pathway inhibitor (TFPI), von Willebrand factor (vWF). These factors are released when the ion channels are activated by various substances such as acetyl-choline, histamines, bradykinins, angiotensin, ATP, ADP, thrombin and other growth factors as well as in response to mechanical stimuli such as shear stress (Voets *et al.* 2005).

Endothelial Ion Channel Expression:

The expression of ion channels in EC's varies with particular growth and isolation conditions as they quickly adapt themselves to the external conditions (Nilius *et al.* 2001). The different types of ion-channels present in the endothelium are TRP channels, BK channels, store-operated calcium channels, sodium-calcium exchanger, chloride channels etc.

TRANSIENT RECEPTOR POTENTIAL (TRP) channels and their role in the vasculature:

Background:

The *trp* gene was originally identified in the laboratory of Cosen and Manning in the year 1969. With the help of an electroretinogram which measures the change in current potential in eye in response to light; the scientists found that unlike wild-type flies, under continuous light illumination the receptor potential of *trp* mutant flies quickly returns to base-line rather than a normal plateau like action potential (Cosen *et al.* 1969).

Interestingly under conditions of dim illumination the *trp* mutant flies behave the same like that of normal wild-type flies. Further analysis of the mutant photoreceptor cells gave some interesting insights in the field of science. The *trp* gene was later cloned and was found that this gene encodes the light-activated calcium channel, TRP in *Drosophila melanogaster* (Montell *et al.* 1989, Hardie *et al.* 1992). Interestingly it was shown that calcium depletion mimics *trp* phenotype and signifies a class of inositide-mediated calcium entry. This calcium entry regulates the excitation during continuous exposure of light in *Drosophila* (Hardie *et al.* 1992). Recent evidence has shown that chlorophyte algae group seems to have TRP like genes which may play a role in calcium signaling pathways (Wheeler *et al.* 2008). Rapid progression in research has led us to identify and characterize TRP channels in yeast, worms, insects, fish and mammals (Petersen *et al.* 1995; Wes *et al.* 1995).

Classification and Modulation of TRP channels:

TRP channels are voltage-independent, non-selective channels which are permeable to Na^+ , K^+ , Cs^+ , Li^+ , Ca^{2+} and Mg^{2+} and belong to a highly diverse group of 6-transmembrane cationic permeable ion channels. There are 28-*trp* related genes in mouse, 27 in humans, 17 in worm *C.elegans* and 13 in *Drosophila melanogaster* (Petersen *et al.* 1995; Wes *et al.* 1995). These channels are classified in 7 subfamilies as represented in the following schematic **Figure 2** taken from Nilius and Owsianik 2011.

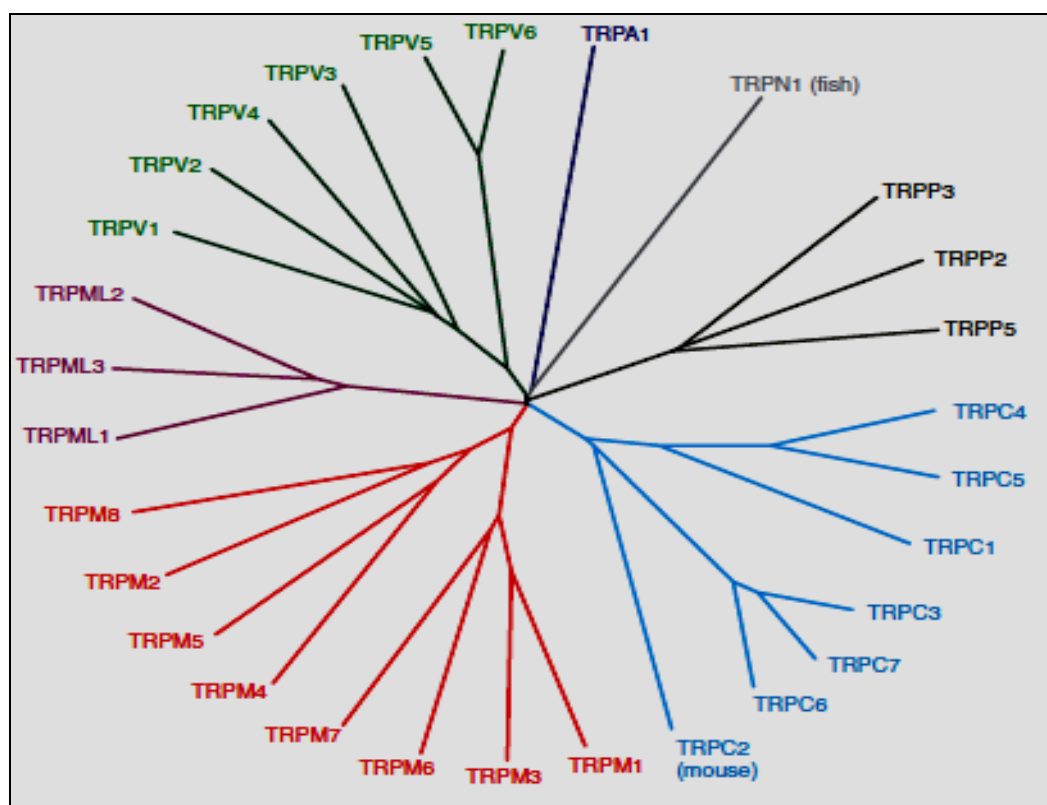


Fig 2: The following schematic diagram represents the phylogenetic tree of TRP superfamily (Taken from Nilius and Owsianik Genome Biology, 2011)

These channels are regulated by a large number of endogenous and exogenous agonists. For example TRPV1, the well know pain receptor is activated by compounds such as capsaicin (Caterina *et al.* 2001), piperine a component of black pepper (McNamara *et al.* 2005), noxious heat and protons. TRPA1 is covalently activated by reactive compounds such as isothiocyanates [active component present in mustard oil, wasabi and horseradish](Jordt *et al.* 2004), cinnamon oil, acrolein, Δ -9-tetrahydrocannabinol [the psychoactive compound in marijuana], (Hinman *et al.* 2006; Bandell *et al.* 2004; Bautista *et al.* 2005) and non-covalently activated by compounds including nicotine (Talavera *et al.* 2009), ozone (Taylor *et al.* 2010). Other examples

include TRPM8 the cold receptor, which is activated by menthol and eucalyptol. In comparison to natural plant-derived products TRP channels are also activated by synthetic compounds such as icillin which activates both TRPA1 and TRPM8 (Lee *et al.* 2008). Other highly selective synthetic compounds include olvanil for TRPV1 and 4 α -phorbol-12, 13-didecanoate (4 α -PDD), lumiphorbols, phorbol-hexonates and GSK1016790A for TRPV4 (Watanabe *et al.* 2002; Klausen *et al.* 2009; Thorneloe *et al.* 2008). The endogenous ligands include diacylglycerol which activates TRPCs. Several arachidonic acid derivatives are also known to activate TRPs. For example, arachidonoyl ethanolamide, 12,15-(S)-hydroperoxyeicosatetraenoic acid and leukotriene B₄ are known to activate TRPV1 while sphingosine is known to activate TRPM3, though the mechanism of activation of these channels by these ligands are currently not known (Kiselyov *et al.* 2009; Zygmunt *et al.* 1999; Watanabe *et al.* 2003; Grimm *et al.* 2005). Shear stress or mechanical stress can also activate several endogenous ligands that can also indirectly activate TRP channels (Corey *et al.* 2004). For example cell-swelling can stimulate phospholipase A₂ leading to the production of arachidonic acid thereby leading to activation of TRPV4 (Oike *et al.* 2004; Thorod *et al.* 1997; Vriens *et al.* 2004). Intravascular pressure results in the production of diacylglycerol (DAG) which activates PKC. PKC then activates TRPM4, whereas DAG also activates TRPC6 and TRPM4 (Nilius *et al.* 2005). Pulsatile stretch in the walls of the endothelial cells activates TRP channels such as TRPC1 and TRPV2. The ankyrin domains present in the amino terminus of TRPA1 also serve as the gate for mechanotransduction (Cordero-Morales *et al.* 2011, Sotomayor *et al.* 2005).

Regulation of TRP channels:

TRP channels are regulated by various processes such as protein-protein interactions, phosphorylation, capacitative and non-capacitative mechanisms (Nilius *et al.* 2001; Montell *et al.* 2005; Minke *et al.* 2002). TRPC3-TRPC7, TRPV1, TRPV4, TRPM4, TRPM7 are all known to be regulated by phosphorylation (Nilius *et al.* 2005; Parekh *et al.* 2005; Hisatsune *et al.* 2004). PKC, PKA, calmodulin-dependent protein kinase II, src, non-receptor tyrosine kinase Fyn and Lyn are known to either activate or sensitize different TRP channels such as TRPV1, TRPV4, TRPC6 and TRPM7 (Nagy *et al.* 2004; Xu *et al.* 2003). Interesting findings have also shown that protein phosphorylation can downregulate the activity of some TRP channels such as TRPC3, TRPC6, TRPC7 (Trebak *et al.* 2003, Zhang *et al.* 2001; Trebak *et al.* 2005). This inhibitory phosphorylation can act as an important negative feed-back loop that regulate the influx of Ca^{2+} and hence protect the cells from detrimental effects of excessive calcium influx in the cells (Kwan *et al.* 2000; Kwan *et al.* 2004). Recent studies have also proposed that TRPC1/TRPC4 channels play an important role as store-operated calcium channels (SOC).

TRP Channels in Endothelium and their role in vasculature:

Rapid progress in molecular biology has lead to the discovery of number of TRP ion channels in the endothelium. For example studies have shown that targeted mutation of TRPC4 can impair ATP and acetyl-choline induced calcium influx in endothelial cells (Yao *et al.* 2005). The huge influx of calcium can in turn enhance the release of specific endothelium derived hyperpolarizing factors such as nitric oxide, prostacyclins and can result in decrease in vasomotor tone. On the other hand, TRPs in endothelium can also

activate potassium channels thereby hyperpolarizing the smooth muscle resulting in vasorelaxation (Yao *et al.* 2005). Recent studies have also shown a prominent role of TRPV1 and TRPV4 in regulating vascular tone. Apart from regulation of TRP channels in vasomotor tone, there are evidences of TRP channels such as TRPC1, TRPC4, TRPC6 and TRPV1 having a role in regulating vascular permeability (Paria *et al.* 2004; Pocock *et al.* 2004; Poblete *et al.* 2005), vascular remodeling and angiogenesis. Angiogenic stimulators such as VEGF, FGF, PDGF induces proliferation of endothelial cells thus promoting angiogenesis. TRPC4, TRPC6, TRPM6 and TRPM7 are known to be possible regulators of angiogenesis (Montell *et al.* 2005; Fleig *et al.* 2004). The following diagram **Fig 3** demonstrates the possible role of TRP channels in endothelium.

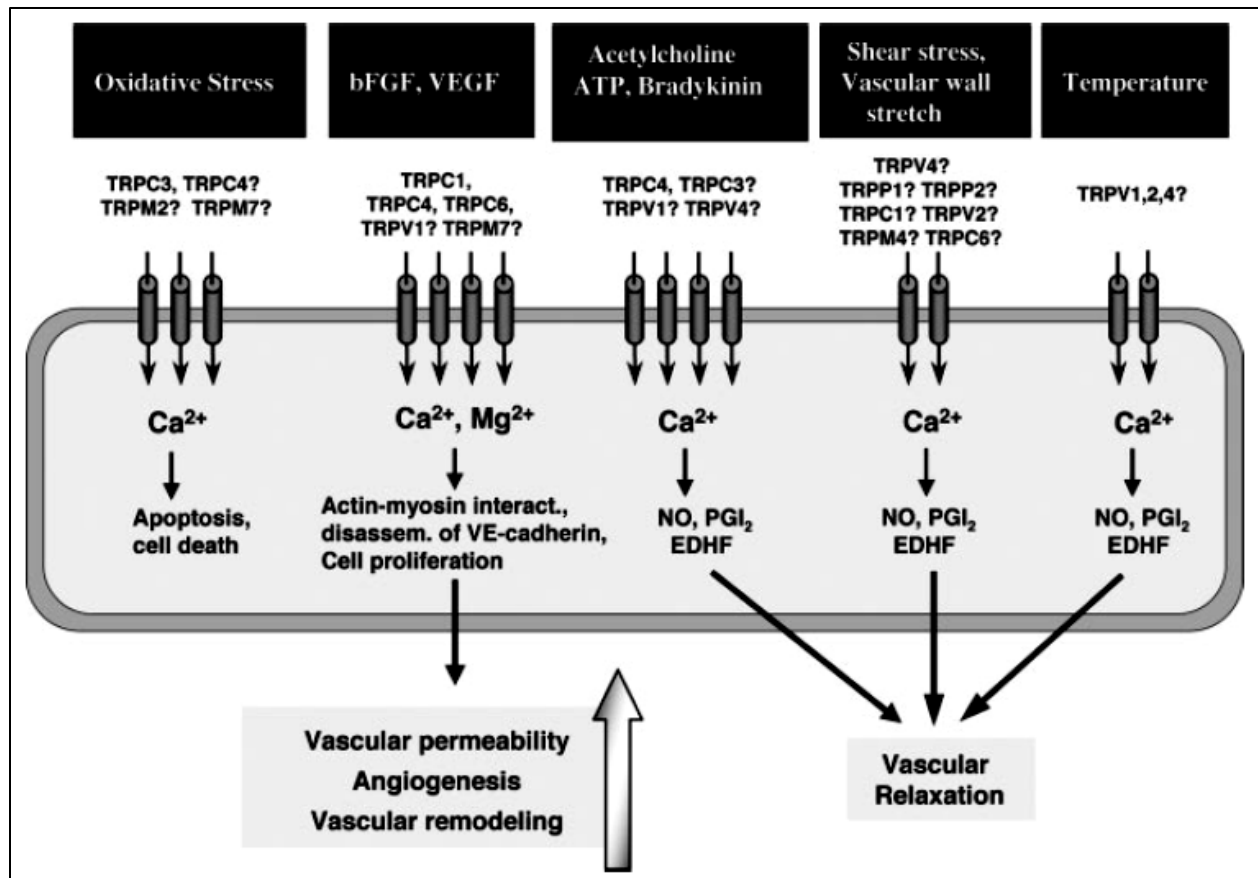


Fig 3: This figure represents the functional roles of different TRP channels in the vascular bed (Taken from Xiaoqiang Yao and Christopher J. Garland, 2005)

Role of TRP Channels in blood pressure regulation:

TRPC1 is known to be a key isoform involved in pulmonary vasoconstriction and is already known to be broadly expressed throughout different vascular beds (Kunichika *et al.* 2004). TRPC4 and TRPC6 are also known to play critical role as agonist-induced vasorelaxation. TRPM4 is expressed in different organs such as heart, kidneys, adrenal glands, vascular endothelium and smooth muscle cells and is known to play a major role in regulation of blood pressure. Studies have shown that TRPM4 knock-out mice show a profound increase in blood pressure and are hence hypertensive compared to

wild-type mice. Interestingly a specific ganglionic blocker hexamethonium abolishes the difference in blood pressure between wild-type and TRPM4 suggesting the role of autonomic nervous system in the regulation of blood pressure (Guyenet *et al.* 2006), in addition to increased plasma adrenaline levels and increase in catecholamine breakdown products in urine, suggesting a sympathetic regulation of blood pressure in these mice. On the other hand there are studies which have also shown the role of TRPM4 in myogenic constriction of cerebral arteries.

Role of TRPA1 and TRPV1 in regulation of vasomotor tone:

TRPA1 and its role in vascular bed:

TRPA1, one of the distinguished members of the TRP family has achieved a lot of attention for the last few years. TRPA1 gene was originally cloned in the year 1999 from lung fibroblasts (Jaquemar *et al.* 1999). It is also named as ANKTM1 due to the presence of 14-19 ankyrin repeats at its N-terminus (**Fig 4**) (Bessac *et al.* 2008; Story *et al.* 2003). TRPA1 gene is diversely distributed among various species such as mouse, rat, dog, chicken, zebrafish, fruit fly, worms and humans (Macpherson *et al.* 2010).

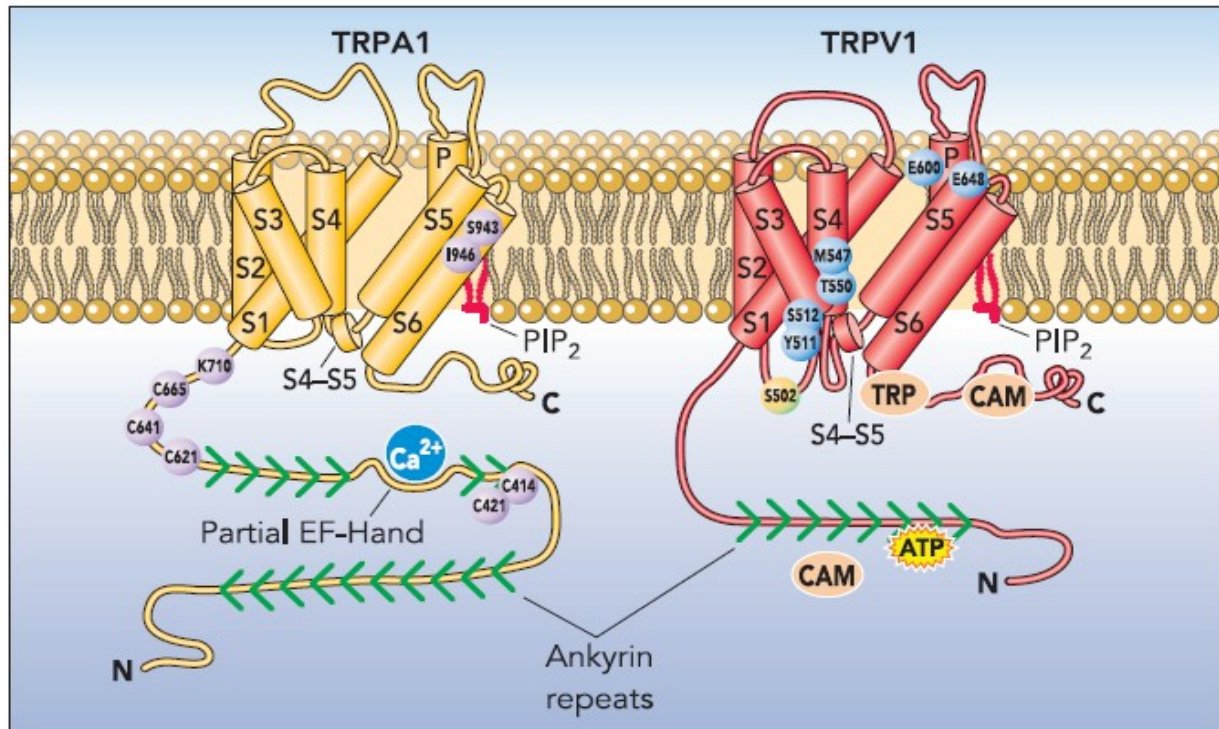


Fig 4: Schematic diagram showing the structure of two common TRP channels (Taken from Bret F. Bessac and Sven-Eric Jordt, Physiology 2008)

The structure is similar to other TRP proteins and has six transmembrane segments (S1-S6). It is known that the ankyrin repeats mediate protein-protein interaction however; recent evidence has shown that these they also play a major role in agonist stimulation (Cordero-Morales *et al.* 2011, Sotomayor *et al.* 2005). Also deletion in the ankyrin repeat leads to improper insertion of the channel to the plasma membrane (Nilius *et al.* 2011). The structural model suggests that the cysteine residues present at the N-terminus of this channel is important for covalent modification of this channel (Hinman *et al.* 2006; Macpherson *et al.* 2007). The putative EF hand motif involved in intracellular Ca^{2+} dependent activation of TRPA1 is present at the amino terminus of this channel (Zurborg *et al.* 2007). Another potential calcium-binding domain is located

at the C-terminus end of this channel (Sura *et al.* 2012). It has been shown that calcium is one of the endogenous regulators of this channel, as it both activates and potentiates this channel. Four conserved residues Glu1077, Asp1080, Asp1081 and Asp1082 are known to be important for agonist stimulation (Samad *et al.* 2011). It is still not clear, but TRPA1 may be modulated by kinases such as Cdk, PKA. Recent reports have also demonstrated that bradykinin activates this channel leading to increase in cAMP and PKA, so PKA may be involved in resensitizing and activating this channel (Bandell *et al.* 2004). TRPA1 is also known to be negatively regulated by phosphoinositides and inorganic polyphosphates by interacting with some positively charged amino acids at its C-terminus end (Kim *et al.* 2007; Kim *et al.* 2009, Nilius *et al.* 2008). TRPA1 is also known to be modulated by protein-protein interactions, so far the best example is TRPV1 (Patil *et al.* 2010, Staruschenko *et al.* 2010). TRPA1 is also known to be modulated by post-translational modification such as ubiquitinylation (Stokes *et al.* 2006). Some of the common agonists of TRPA1 are isothiocyanates, (present in mustard oil, wasabi and horseradish) (Jordt *et al.* 2004; Bandell *et al.* 2004) methyl salicylate (in wintergreen oil), cinnamaldehyde (cinnamon oil), allicin (an active component of garlic) and diallyl disulphide(a flavor component of garlic) (Bautista *et al.* 2005; Macpherson *et al.* 2005), acrolein (tear gas), (Bautista *et al.* 2006). ozone, hydrogen peroxide (Andersson *et al.* 2008), Δ -9tetrahydrocannabinol (psychoactive compound present in marijuana), ultraviolet light (Hill *et al.* 2009). Other potential activators are reactive oxygen species, reactive nitrogen species and reactive carbonyl species. Other activators are electrophilic prostaglandins and unsaturated aldehydes. Alkaline pH also activates this channel. A tissue damage release factor such as 4-

hydroxynonenal (4-HNE) and 4-oxononenal also activates this channel (Taylor-Clark *et al.* 2008). Formaldehyde and formalin are also potent activators of this channel. Other gaseous transmitters that activate this channel are nitric oxide (Miyamoto *et al.* 2009) and hydrogen sulphide (Miyamoto *et al.* 2011). The common non-electrophilic modulators are propofol, isoflurane, desflurane and etomidate (Fischer *et al.* 2010; Leffler *et al.* 2011; Matta *et al.* 2008). Lidocaine activates TRPA1 in a concentration-dependent manner (Piao *et al.* 2009). Several non-steroidal anti inflammatory drugs (NSAIDs) such as flufenamic, niflumic, mefenamic, flurbiprofen, ketoprofen, diclofenac and indomethacin reversibly activate TRPA1 (Hu *et al.* 2010; Nassini *et al.* 2010). Compounds widely present in cosmetics such as parabens, alkyl esters of hydroxybenzoate also activate this channel. Common calcium antagonists such as nifedipine, nimodipine, nicardipine and nitrendipine also act as TRPA1 activators (Komatsu *et al.* 2012). Higher alcohols such as 1-butanol, 1-hexanol also proportionately activates TRPA1. Menthol and nicotine are known as bimodal regulators of TRPA1, as lower concentration of this agonist activates this channel whereas higher concentration inhibits its activity (Karashima *et al.* 2007; Talavera *et al.* 2009). Another species-specific activator of TRPA1 is caffeine which activates mouse TRPA1 but inhibits the human TRPA1 (Nagatomo *et al.* 2008). Apart from calcium, TRPA1 is also activated by other metals such as zinc, copper and cadmium (Hu *et al.* 2009). Heat suppresses its activity while cold promotes it (Story *et al.* 2003). Hypertonic solutions also activate TRPA1. Recent studies have also shown that ajoene an interesting compound of garlic which is an antithrombotic agent also enhances the activation of TRPA1 (Yassaka *et al.* 2010).

Previous studies have shown that TRPA1 is predominantly present in dorsal root ganglion, trigeminal sensory neurons, nodose ganglia and inner ear, recent evidence has also shown that TRPA1 is also present in various other organs such as brain, heart myocytes, small intestine, pancreas, skeletal muscle, lungs and colon(Stokes *et al.* 2006; Nilius *et al.* 2012). TRPA1 is also expressed in the neurons of sympathetic superior cervical ganglia and hence known to play a significant role in autonomic nervous system. In vagal fibers which innervate the heart, TRPA1 is known to regulate vasovagal reflexes (Pozsgai *et al.* 2010). TRPA1 expression is also seen in central nervous system and is known to enhance glutamate release upon activation. Recent evidence has shown the prominent role of TRPA1 in vascular bed (Earley *et al.* 2009). It is known that TRPA1 agonists causes arterial dilation by two distinctive mechanisms. TRPA1 channels present in perivascular nerves releases calcitonin gene related peptide (CGRP) and thereby causes vasodilation, on the other hand it is also known that TRPA1 present in the myoendothelial junction sites of cerebral arteries activates small and intermediate conductance Ca^{2+} activated potassium channels thereby hyperpolarizing the adjacent smooth muscle cells (Earley *et al.* 2009). Recent findings have also shown that TRPA1 agonists can cause transient depressor responses followed by sustained increase in blood pressure and heart rate, which can be due to elevated sympathetic activity. Gasotransmitters such as hydrogen sulphide is also known to cause vasodilation due to the release of CGRP (Pozsgai *et al.* 2012). Based on the promising study that garlic activates TRPA1, it can be speculated that TRPA1 has protective effects against cardiovascular disorders. However it is now known that garlic is an important cardioprotective agent which protects against endothelial

dysfunction and hypertrophy of heart (Sun *et al.* 2006). Also interestingly it has been also found that the cardioprotective effect of garlic is due to the gaseous compound hydrogen sulphide. Red blood cells convert garlic derived polysulfides into hydrogen sulfide which leads to activation of K_{ATP} channels thereby causing vasorelaxation and hence reduces cardiac arrhythmias (Zhao *et al.* 2001; Benavides *et al.* 2007; Bian *et al.* 2006; Lefer *et al.* 2007). Another possible activator of TRPA1 is AITC (a component present in mustard oil) which is also known to have some vasodilatory effect. Interestingly studies have demonstrated that AITC induced vasodilation is neither blocked by cyclooxygenase or NOS inhibitors but is profoundly reduced by inhibiting potassium channels (Earley *et al.* 2009). Intravenously injected TRPA1 agonists also induce a transient hypotensive response which can be inhibited by a cholinergic antagonist atropine sulphate suggesting the functionality of the autonomic system leading to the precise role of vasovagal reflexes (Pozsgai *et al.* 2010). TRPA1 agonists also increase blood flow in skin. These findings show that there may be a promising role of TRPA1 in cardiovascular system (Pozsgai *et al.* 2010). Pungent TRPA1 agonists like garlic is also known to activate sensory neurons in the blood vessels thereby releasing CGRP a potent vasodilator (Bautista *et al.* 2005). Studies have also demonstrated the role of TRPA1 in bradycardia and bradypnoea (Harada *et al.* 1975). TRPA1 agonists are also known to induce adrenaline secretion via central nervous system thereby regulating vasomotor responses (Iwasaki *et al.* 2008). **Fig 5** delineates the different routes by which TRPA1 mediates vasodilation.

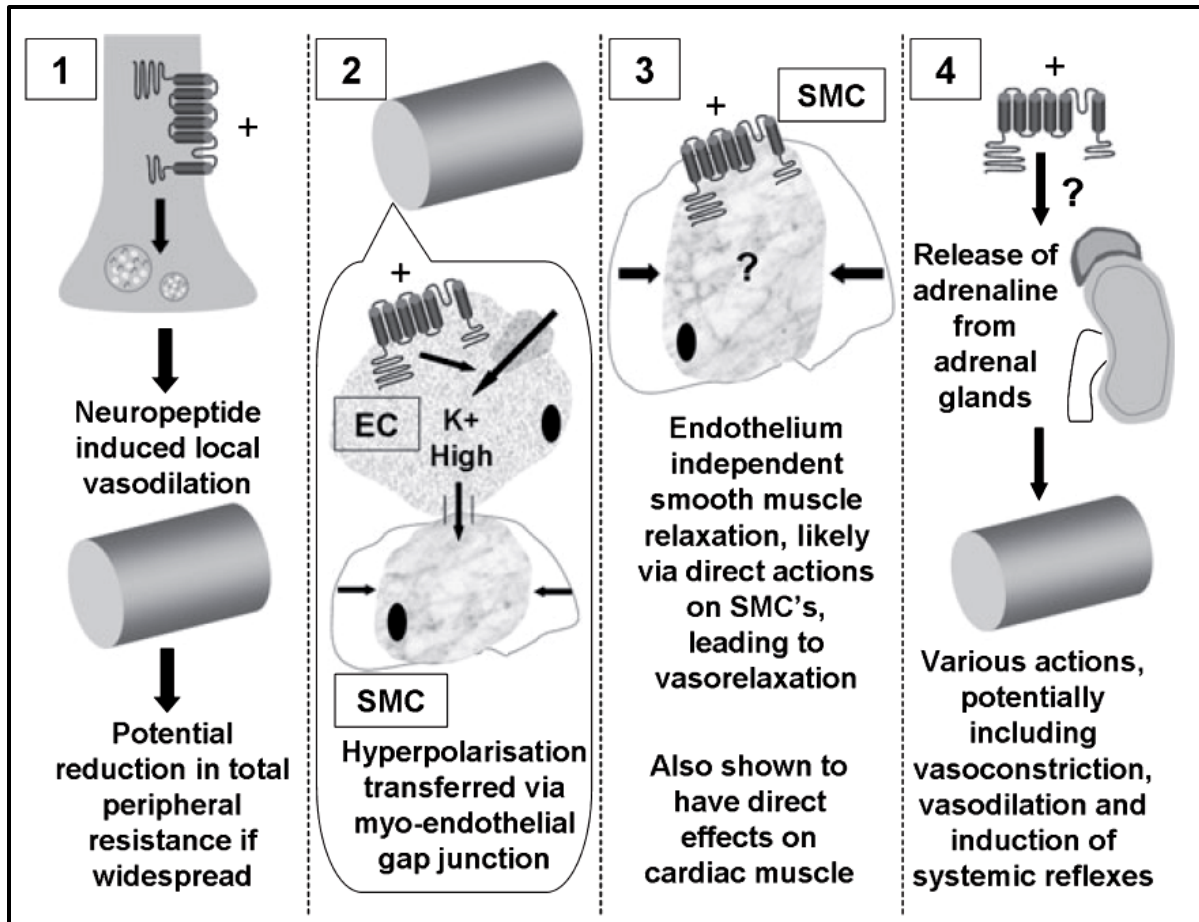


Fig 5: This figure illustrates the proposed routes/pathways by which TRPA1 mediates vasoreactivity: (Taken from JV Bodkin and SD Brain, *Acta Physiologica* 2010).

Role of TRPV1 in vascular bed:

TRPV1 has more or less the same structure like TRPA1, having 6 transmembrane domains, an amino terminus and a carboxyl terminus end. The pore region is present between S5 and S6 and is known as the channel selectivity gate for cations. The pore region may also modulate temperature sensation. The amino terminus has 6 ankyrin repeats, which are important for protein-protein interactions, tetramerization etc. (Caterina *et al.* 1997; Huang *et al.* 2006; Story *et al.* 2006; Hardie *et al.* 2007; Caterina

et al. 2007; Schaible *et al.* 2006). The carboxyl terminus is also important for tetramerization, membrane trafficking and temperature sensation. The C-terminus also has a conserved hydrophobic domain known as TRP domain which has this consensus sequence WKFQR, where W is conserved among all the members of the TRP family (Baylie *et al.* 2011). TRPV1 is activated by excessive high temperatures (>43°C), capsaicin (the active compound present in chilli peppers), endocannabinoids, protons, piperine, hypotonic and hypertonic solutions, shear stress etc (Caterina *et al.* 1997; Tominaga *et al.* 1998; McNamara *et al.* 2005; Zygmunt *et al.* 1999; Ahern *et al.* 2006; Kim *et al.* 2006; Mc. Hugh *et al.* 2006; Tzavara *et al.* 2006). TRPV1 is expressed in small to medium neurons of dorsal root ganglia trigeminal neurons, different areas of brain such as organum vasculosum of the lamina terminalis (OVLT) and supraoptic nucleus (SON) (Ciura *et al.* 2006). Recent reports have shown the presence of TRPV1 in endothelial cells and vascular smooth muscle cells (Bratz *et al.* 2008). TRPV1 is also predominantly expressed along with TRPA1 in sensory neurons and also play a major role in regulating pain-signalling pathways but interestingly recent evidences have shown a prominent role of this receptor in regulating vasomotor tone. Since this channel is found in endothelial cells, smooth muscle cells and perivascular nerves, it stimulates different downstream targets that result in vasodilation. In porcine coronary artery endothelial cells, TRPV1 relaxed arterial rings in a dose-dependent fashion. L-NAME, an inhibitor of Nitric oxide synthase and iberitoxin an inhibitor of BK_{Ca} channels inhibited the dose-dependent relaxation suggesting a downstream role of NO and BK channel in TRPV1-mediated vasodilation (Bratz *et al.* 2008). Recent findings have also shown that VSN16, a synthetic cannabinoid like compound indirectly/ directly activates TRPV1 (Hoi

et al. 2007). Results have also demonstrated that anandamide induced vasodepressor response has been completely blunted in the presence of a specific TRPV1 antagonist, capsazepine. These data thereby shows a prominent role of TRPV1 in vasorelaxation in endothelial cells (Poblete *et al.* 2005). It has been also found that in human cerebral artery endothelial cells activation of TRPV1 results in phosphorylation of vasodilator stimulated phosphoprotein (VASP) (Golech *et al.* 2004). VASP a substrate of protein kinase A and protein kinase G is a known regulator of vasodilation. Interestingly other studies have also shown that in cultured mouse aortic endothelial cells activation of TRPV1 results in influx of calcium which in turn activates endothelial nitric oxide synthase leading to phosphorylation of PKA (Yang *et al.* 2010). Capsaicin is also shown to cause endothelium dependent vasodilation in mouse mesenteric arteries. Studies have also shown that capsaicin in diet can cause relaxation of isolated mesenteric arteries. But the role of endothelial TRPV1 in regulation of blood pressure *in vivo* is not clear as the channel is ubiquitously expressed throughout the body (Yang *et al.* 2010). Recent findings have also shown that activation of this channel in both sensory nerves and endothelial cells leads to the production of CGRP thereby causing vasodilation. On the other hand TRPV1 present in the vascular smooth muscle cells results in increase in vasomotor tone thereby leading to vasoconstriction. Interestingly it has been seen that capsaicin has biphasic effects on vasculature; at lower nanomolar concentrations this drug can elicit vasodilation by activating the sensory neurons whereas at micromolar concentrations it can cause vasoconstriction by non-neuronal stimulations (Fernandes *et al.* 2012). Though it is still unclear what is the cause. However it may be possible that activation of TRPV1 by endogenous agonists can trigger vasodilation and

vasoconstriction separately depending on the location whereas application of exogenous capsaicin can result in the activation of all TRPV1 simultaneously (Fernandes *et al.* 2012). Other studies have also shown that activation of TRPV1 releases prostacyclins and endothelium-derived hyperpolarizing factors and thus causing vasodilation (Baylie *et al.* 2011). On the other hand studies have also shown that TRPV1 present in the perivascular nerves can also cause neurogenic vasodilation. It has been also known that TRPV1 plays a prominent role in regulation of the baroreceptor pathways (Sun *et al.* 2009). These findings have suggested that TRPV1 sense change in arterial pressure and play a potential role in regulating blood pressure homeostasis.

Cross-talk between TRPA1 and TRPV1:

TRPA1 and TRPV1 are co-expressed in largely overlapping subsets of nociceptors and recent evidence suggests that both TRPA1 and TRPV1 are expressed in vascular bed. Interestingly it has been shown that TRPV1 null mice show reduced responses to mustard oil suggesting that TRPA1 function is impaired in the absence of TRPV1 (Akopian *et al.* 2008). Intriguingly one of the studies has proposed that TRPA1 desensitization is also dependent on variety of cellular modulators and TRPV1 (Akopian *et al.* 2007). Recent reports also describes cross-desensitization of TRPA1 and TRPV1 *in vitro* (Ruparel *et al.* 2008), further supporting the notion that TRPA1 and TRPV1 are functionally linked in sensory neurons *in vivo*. Surprisingly other studies have also shown that TRPA1 desensitization is also regulated by an endocannabinoid which is a specific agonist of TRPV1 (Ruparel *et al.* 2011). Recent reports by Patil *et al.* 2010 have also demonstrated that TRPV1 modulates TRPA1 by calcium. Moreover

studies have also shown that there is a functional interaction between these two channels TRPA1 and TRPV1 (Staruschenko *et al.* 2010). Salas *et al.* 2009 have demonstrated that TRPA1-mediated responses in heterologous expression systems expressing only TRPA1 indicate that the features of TRPA1 are not duplicated in cells only expressing TRPA1 and, instead, can be restored only when TRPA1 and TRPV1 channels are coexpressed. Previous studies from our lab have also shown that the sensitivity of TRPV1 is restored via TRPA1 and protein kinase C (Wickley *et al.* 2010; Zhang *et al.* 2011). It has been also found that compounds like 4-oxo2-nonenal (4-ONE) activates sensory neurons via TRPA1 and TRPV1, mediates mechanical hyperalgesia, edema formation and also causes vasodilation in mice (Graepel *et al.* 2011). Interestingly, studies have also shown predominantly the role of TRPA1 in regulating blood pressure and thereby causing hypotension whereas it seems that this group has concluded that there is also a role of TRPV1 (Poszgai *et al.* 2010). But studies investigating the cross-talk between these two channels in the vascular bed are rare and the proposed studies will help us to assess the possibility.

Anesthetics and vasoreactivity:

Anesthesia, also called as “Reversible lack of awareness” was originally suggested by Oliver Wendell Holmes, Sr. in 1846. The condition is a reversible state of analgesia/painlessness, loss of skeletal muscle reflexes which can be induced by a single drug called as anesthetics or combination of other drugs such as sedatives, analgesics along with the anesthetic. Anesthetics are widely used all over the world to help the patients during surgery so that they do not feel the pain. There are different kinds of anesthetic procedures such as general anesthesia, regional anesthesia and

also dissociative anesthesia. In general anesthesia a combination of inhalational and intravenous anesthetic and also other sedatives and narcotics are used which results in complete unconsciousness and sedation due to temporary block in sensory, motor and sympathetic nerve transmission. While in case of regional anesthesia/local anesthesia, a sedative along with the local anesthetic agent is applied. Regional anesthesia can be epidural/spinal which is generally applied when procedures involve lower extremities of the body. Dissociative anesthetics such as ketamines are given to patients during short-term procedures where the patients still remain in a conscious state and can drink and swallow, this kind of anesthesia inhibit the transmission of nerve impulses between cerebral cortex and the limbic system (<http://www.scientificamerican.com/article.cfm?id=how-does-anesthesia-work>). Though anesthetics are used all the time but there are various side-effects which can be sometimes detrimental to human health. Some of the common side-effects of anesthetics include nausea, vomiting, hiccups, impair breathing, decrease blood-flow and hence hypotension, abnormal heart rate, cardiac myopathy/depression in cardiac muscle and impairment of kidney and liver function. Apart from these diverse side-effects one of the common side-effect of intravenous anesthetic is pain at the site of injection. Though researchers are working for years to minimize pain at the site of injection by pre-treating patients with other drugs such as lidocaine (Borazan *et al.* 2012) but the effectiveness of these drugs are still questionable. So scientists are still investigating on the properties of anesthetics to improve their functionality.

Propofol and its clinical relevance:

Propofol, clinically known as Diprivan is a short-acting intravenous anesthetic used during general anesthesia, endoscopic procedures and also in veterinary medicine. It is a popular anesthetic used in more than 50 countries. Recovery from this drug is very fast compared to other anesthetics. Patients show diverse effects from this drug. The common side-effects are drop in blood pressure leading to vasodilation, apnea and pain at the site of injection. So administration of this drug requires proper clinical setting. In some patients who are severely ill with acute neurological and inflammatory diseases infusion of propofol with catecholamines/corticosteroids can lead to a fatal but rare disease called as propofol infusion syndrome (Vasile *et al.* 2003). This syndrome results in cardiac failure, metabolic acidosis and renal failure. This drug also shows profound sedation at lower doses. The current formulation of propofol is as follows: 1% propofol, 10% soyabean oil, 1.2% purified egg phospholipid, 2.25% glycerol and sodium hydroxide to adjust pH, EDTA: chelating agent , sodium metabisulfite/benzyl alcohol: antimicrobial agent. The following figure depicts the chemical structure of this anesthetic (Fig 6).

Structure of propofol:

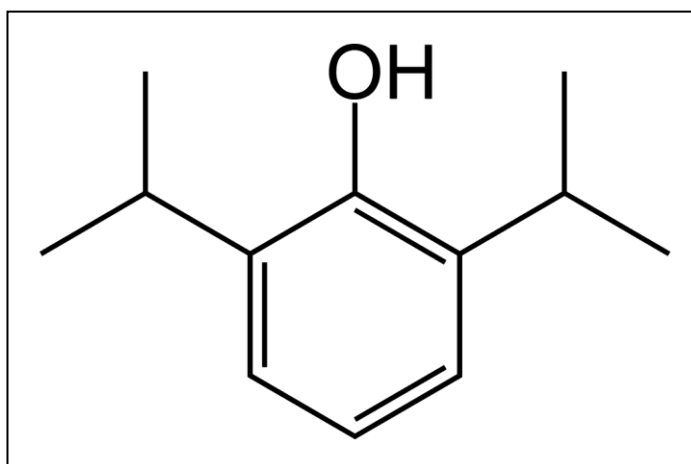


Fig 6: Chemical Structure of Propofol (taken from Hansen *et al.* 2013)

Mechanism of action:

There are several mechanisms of action of propofol such as binding of GABA_A receptor and thereby inhibiting its action (Trapani *et al.* 1998; Krasowski *et al.* 2001; Krasowski *et al.* 2002). It also acts as a sodium channel blocker (Haeseler *et al.* 2003). Recent evidences have also shown that the endocannabinoid system is playing a major role in regulating propofol's anesthetic action (Fowler *et al.* 2004). Interestingly it has been also found that this anesthetic causes a prominent reduction in the brain's information integration capacity (Schrouff *et al.* 2011). Most recent intriguing evidence also showed us a new pathway that anesthetics like propofol also activate TRPA1 (a sub-type of the well-known TRP super-family) (Matta *et al.* 2008).

Propofol and vasorelaxation:

It is well known that induction of anesthesia with most, if not all anesthetic agents, triggers a profound decrease in blood pressure when administered to patients presenting for surgery. The widely used intravenous anesthetic agent, propofol, is one such compound which elicits the vasodilatory response in humans and rodents as well as cardiac depression. The signaling pathways and cellular mechanisms by which propofol (and other anesthetic agents) cause myocardial depression and hypotension are actively being investigated and likely involve release of endothelium-derived relaxation factor (NO) however, little mechanistic evidence is available for how this occurs (Sun *et al.* 2009; Wickley *et al.* 2006, Horibe *et al.* 2000; Park *et al.* 1995; Liu *et al.* 2009; Gragasin *et al.* 2009; Petros *et al.* 1993; Doursout *et al.* 2002; Wang *et al.*

2007). On the other hand there are few studies implicating the role of different potassium channels such as BK channel (Klockgether-Radhke *et al.* 2004), ATP sensitive potassium channel (Lam *et al.* 2010) in propofol inducing hypotension. Several studies have demonstrated the vasodilatory effects of propofol in coronary arteries. Klockgether *et al.* 2000 illustrated that propofol and thiopental caused a dose-dependent vasorelaxation that is independent of endothelium-derived factors. Ouattara *et al.* 2001 have also investigated the coronary effects of propofol in rabbit hearts and concluded that propofol stimulated an increase in coronary blood flow that was not compromised during cardiac hypertrophy. Interestingly, Yamanoue *et al.* 1994 proposed that propofol-induced vasodilation in porcine coronary artery is dependent on the antagonistic properties of calcium channels.

Anesthetic activation of TRP channels:

Recent studies have indicated that several general anesthetic agents (local, intravenous and inhalational) are capable of activating or sensitizing TRPA1 and TRPV1 channels in sensory neurons and heterologous expression system (Sato *et al.* 2009; Matta *et al.* 2008; Fischer *et al.* 2010; Leffler *et al.* 2011). Other studies have also shown that general anesthetics such as isoflurane, enflurane and desflurane sensitize the vanilloid receptor, TRPV1 (Cornett *et al.* 2008). Interestingly it has been also found that lidocaine stimulated the release of CGRP via activating TRPV1 (Leffler *et al.* 2008). Studies have also shown that the intravenous anesthetic propofol activates both TRPA1 and TRPV1 (Fischer *et al.* 2010; Tsutsumi *et al.* 2001). Propofol this drug is known to cause hypotension in patients. Moreover TRPA1 and TRPV1 is known to have a role in vasculature. So we have tried to investigate that whether propofol has any role in

activation/regulation of these channels in vascular bed. Furthermore we have also tried to delineate the downstream mediators of this process.

CHAPTER2

AIM-1: To determine the role of TRPA1 and TRPV1 in propofol-induced depressor responses *in-vivo*

Background and Rationale:

TRPA1 is known to be activated by a large group of agonists and is known to play an important role in pain-inflammatory pathways. But recent reports investigated the role of TRPA1 in regulating vasodilation. Earley *et al.* 2009 have concluded for the first time the presence of TRPA1 in the myoendothelial junctions of cerebral arteries. Poszgai *et al.* 2010 have demonstrated the role of TRPA1 in regulation of blood flow and blood pressure in anesthetized mice, though the results show that the regulatory effect is endothelium-independent and is dependent on sympathetic pathway. Studies by Graepel *et al.* 2011 illustrated 4-ONE dependent activation of TRPA1 causing vasodilation *in-vivo* which is dependent on CGRP. Other studies have also demonstrated that garlic, hydrogen sulphide can also activate TRPA1 thereby promoting the neurogenic release of CGRP (Poszgai *et al.* 2012; Bautista *et al.* 2005). Studies have also shown the potential role of TRPV1 in regulating vasomotor tone. Recent findings have shown that anandamide, a well-known endocannabinoid stimulates TRPV1 mediated release of CGRP thereby causing vasodilation (Golech *et al.* 2004, Tamaki *et al.* 2012; Akerman *et al.* 2004). Peroni *et al.* 2004 have also demonstrated that estrogen dependent sensitivity of TRPV1 in rat mesenteric arteries. Agonists like methanamide and delta-9-hydrocannabinol also results in TRPV1 mediated activation of CGRP and K_{ATP} channels (Breyne *et al.* 2006, Wilkinson *et al.* 2007). Other studies have also shown the effect of anandamide on release of cyclooxygenase mediators in rat aorta by activating TRPV1 (Herradon *et al.* 2007). Interestingly other studies have also delineated the role of endothelial TRPV1 in regulating vasodilation. Milman *et al.* 2006 have illustrated that TRPV1 activation results

in phosphorylation of mitogen activated kinase and also protein kinase B/Akt. Poblete *et al.* 2005 have depicted for the first time that activation of endothelial TRPV1 present in the rat mesenteric arteries results in release of NO. Interestingly findings from Bratz *et al.* 2008 have demonstrated the presence of TRPV1 in porcine coronary endothelial cells. This group has also demonstrated for the first time that endothelial TRPV1 activation in porcine coronary arteries results in release of nitric oxide thereby activating BK_{Ca}. Similarly other studies have also shown that TRPV1 activation has resulted in the activation of BK_{Ca} in rat mesenteric arteries (Parmar *et al.* 2010). Reports from Dannert *et al.* 2007 have also demonstrated the role of TRPV1 in rat aorta. Surprisingly Yang *et al.* 2010 have illustrated that activation of TRPV1 in mouse aortic endothelial cells results in phosphorylation of PKA. This work was later supported by Xu *et al.* 2011 whose results concluded that hypertensive rats have delayed response to stroke when fed with capsaicin. These groundbreaking findings over last couple of years have provided compelling evidence of the role of endothelial TRPV1 in vasodilation. Recent report by Poszgai *et al.* 2010 have demonstrated the role of TRPV1 and TRPA1 in mediating vasodepressor responses *in-vivo*. Using cinnamaldehide as their tool the authors reported an increase in blood pressure in TRPV1 KO mouse compared to controls which suggests that there is a minimal role of TRPV1 *in vivo*. Previously our lab has used propofol, the intravenous anesthetic as a tool to study the role of TRPA1 and TRPV1 in sensory neurons (Wickley *et al.* 2010; Zhang *et al.* 2011). Propofol an intravenous anesthetic causes pain at the site of injection. Apart from propofol recent evidence have also shown that local membrane permeable anesthetic lidocaine also activates TRPA1 (Leffler *et al.* 2011). TRPA1 activation by lidocaine also results in

glutamate release (Piao *et al.* 2009). This particular anesthetic is also known to be responsible for activation and sensitization of TRPV1 which involves CGRP. Phosphatidylinositol 4,5 biphosphate is known to be a potent regulator of this process (Leffler *et al.* 2008). Other studies have delineated that pungent anesthetics such as isoflurane, desflurane induces mechanical hyperalgesia and also results in airway constriction of isolated bronchi (Eilers *et al.* 2010 ; Satoh *et al.* 2009). Most interestingly recent studies have demonstrated that propofol activates TRPA1 in sensory neurons and heterologous system (Matta *et al.* 2008; Fischer *et al.* 2010). A recent report demonstrated the activation of TRPV1 by propofol in sensory neurons, though the extent of activation is less compared to that of TRPA1 (Fischer *et al.* 2010). Most of these studies using propofol and other anesthetics were done to investigate the role of TRPA1 and TRPV1 in nociception and neurogenic inflammation. One of the major side-effect of anesthesia is that it causes hypotension in patients during surgery. The common example is propofol. None of the above mentioned studies have shown the role of TRPA1 and TRPV1 in vasculature using this anesthetic. The signaling pathways and cellular mechanisms by which propofol (and other anesthetic agents) cause myocardial depression and hypotension are actively being investigated and likely involve release of endothelium-derived relaxation factor (NO) however, little mechanistic evidence is available for how this occurs (Sun *et al.* 2009; Wickley *et al.* 2006, Horibe *et al.* 2000; Park *et al.* 1995; Liu *et al.* 2009; Gragasin *et al.* 2009; Petros *et al.* 1993; Doursout *et al.* 2002; Wang *et al.* 2007). On the other hand there are few studies implicating the role of different potassium channels such as BK channel (Klockgether *et al.* 2004), ATP sensitive potassium channel (Lam *et al.* 2010) in propofol induced

hypotension. ***However, the cellular mechanisms by which propofol activate NO Oxide and BK channels in the vasculature is not known.*** Thus the overall goal of this aim is look at the role of TRPA1 and TRPV1 in propofol induced vasodepressor response and to delineate the role of Nitric Oxide and BK channel in the response.

II. Materials and Methods:

Ethics Statement:

All experiments were conducted with the approval of Institutional Animal Care and Use Committee of the University of NEOMED and in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996).

Mice:

Mice breeding pairs were purchased from Jackson Labs (Bar Harbor, ME) and were bred in the animal facility of Northeastern Ohio Medical University (NEOMED). Mice were housed in a room with a 12:12-h light-dark cycle and maintained with a constant temperature and continuous access to food and water. Experiments were performed in 8-12 weeks old males of C57Bl6, TRPA1^{-/-}, TRPV1^{-/-} mice and double knockout of TRPV1 and TRPA1 channels (TRPAV^{-/-}) mice.

Generation of TRPAV^{-/-} mice:

TRPA1^{-/-} and TRPV1^{-/-} mice were brought from Jackson labs. TRPA1^{-/-} females were bred with TRPV1^{-/-} mice to obtain TRPAV heterozygote (TRPA1^{+/-}, TRPV1^{+/-}). These

heterozygotes were then bred to obtain the double knock-out animals. Since the probability of obtaining knock-outs by breeding the heterozygote was 1 out of 16 animals, so two mating schemes were set up. In mating scheme 1 the double knock-out were again crossed with each other to obtain more double knock-outs, but since $TRPA1^{-/-}$ males have some sub-fertility problems so a mating scheme-2 was also set up where $TRPA1^{-/+};TRPV1^{-/-}$ male was crossed with $TRPA1^{-/+};TRPV1^{-/-}$ females to generate $TRPA1^{-/-};TRPV1^{-/-}$ ($TRPAV^{-/-}$).

Genotyping:

Genotyping was performed by PCR using genomic DNA. Genomic DNA was obtained by alkaline lysis of tail pieces. The TRPA1 transgene was detected using the primers 5'-TCCTGCAAGGGTGATTGCGTTGTCTA-3' (WT forward) and 5'-TCATCTGGGCAACAATGTCACCTGCT-3' (WT reverse) and 5'-CCTCGAATCGTGGATCCACTAGTTCTAGAT-3' (mutant forward) and 5'-GAGCATTACTTACTAGCATCCTGCCGTGCC-3' (mutant reverse). Similarly the TRPV1 transgene was detected using the primers 5'-CCTGCTCAACATGCTCATTG-3' (WT forward), 5'-TGGATGTGGAATGTGTGCGAG-3' (mutant forward) and 5'-TCCTCATGCACTTCAGGA AA-3' (common reverse) primers.

In Vivo Mean Arterial Blood Pressure Measurement:

Mice were first anesthetized with 1.5-2.5% sevoflurane gas with supplemental oxygen using a Veterinary Anesthesia and Monitoring Device. They were then placed on a temperature controlled table and the core temperature was measured via a rectal probe

and maintained at 37°C. The right jugular vein was then isolated and was cannulated with PE-50 polyethylene tubing (Becton Dickinson, Oakville, ON) containing 50 U/ml heparin (50 U/ml in Dulbecco's PBS) in saline for intravenous drug infusions. Next a midline incision was made on the ventral right thigh region and the femoral nerve was isolated and pulled aside. The distal and proximal ends of the femoral artery were held with surgical sutures for temporary control of bleeding, and the distal end of the femoral artery was tied off. The femoral artery was isolated followed by cannulation with a high-fidelity microtip transducer catheter connected to a data acquisition system (PowerLab ML820, ADInstrument, Colorado Springs) through a pressure interface unit (Millar Instrument, Transducer Balance, TCB 600) which is used to measure systolic, diastolic, pulse pressure, mean arterial pressure and heart rate. The catheter was inserted deep into the femoral artery to allow mean aortic blood pressure to be recorded. All measured variables were recorded and stored on an iMAC computer using the Powerlab System (ADInstruments, Castle Hill, Australia). The blood pressure data were analyzed using AD-Instrument Chart v5.1.2 software. Following each surgery, mice were given a bolus injection of the ganglionic blocker hexamethonium (5 mg/kg; Sigma, St. Louis, MO) to eliminate reflex adjustments and focus on the primary actions of propofol. Initial studies were performed to determine the effects of continuous infusion of propofol (2.5–10 mg/kg/min) administered in an escalating fashion at the rate of 20 µl/min for 4 mins. Hemodynamic response curves were performed in all four mice groups to increasing doses of propofol in the presence and absence of the antagonists – HC-030031 (TRPA1 antagonist, 60 mg/kg/min), SB366791 (TRPV1 inhibitor, 10 µg/kg/min), LNAME (eNOS antagonist, 100 mg/kg/min), Penitrem A (BK_{Ca} channel antagonist,

50µg/kg/min). Five to ten minutes elapsed following each inhibitor before propofol infusion began to allow for MAP to stabilize. Pressures and/or HR were continuously recorded throughout the experiment. After each experiment the mice were euthanized using a lethal dose of Nembutal. To eliminate the neuronal effects we have used a specific ganglionic blocker hexamethonium which primarily inhibits the action of nicotinic acetylcholine receptors and hence our objective was to investigate the vascular signaling cascade involved propofol induced hypotension *in vivo*.

Drugs and Chemicals:

Propofol stock solution (10 mg/ml, injectable emulsion) was diluted in saline. SB366791, HC-030031 and Penitrem A (Pen A) was dissolved in stock solutions of DMSO and then diluted in saline. Hexamethonium (5 mg/ml) stock solution and L-Nitro Arginine Methyl Ester (L-NAME) was made up in saline.

Data analysis and statistics:

The mean base-line values of HR and MAP were taken at 30seconds before the infusion of drugs. The largest value after infusion of drug was taken as the maximum MAP. The percentage of change in MAP was calculated using the formula: Percent change (% change) = (Maximum-Base-line)/Base-line * 100 and the calculation of percent change was performed using Excel 2007 (Microsoft, Redmond, WA). Data are expressed as means ± SE. Statistical comparisons were performed using One-way Analysis of Variance (Simple ANOVA)/ Kruskal-Wallis One way Analysis of Variance on ranks followed by pairwise multiple comparison procedures such as Tukey/Dunn test as

appropriate. For statistical analyses, Sigma Plot 11.0 software for Windows XP (Systat Software, San Jose, CA) was utilized. A value of $p < 0.05$ was considered statistically significant.

Results:

1.1: TRPA1 channels regulate vascular tone:

Phenotypic characteristics of mice are displayed in **Table 1**. Body weight is represented as follows (in g): TRPA1^{-/-}: 29.0 ± 1.32 vs. TRPAV^{-/-}: 28 ± 2 vs TRPV1^{-/-}: 27 ± 0.7 vs control: 25 ± 0.6 . Base-line HR was significantly higher in TRPA1^{-/-} (580 ± 39 beats/min) compared with controls (400 ± 14.5 beats/min.); though no changes in base-line HR was observed in TRPAV^{-/-} (543 ± 16 beats/min.) and TRPV1^{-/-} (471 ± 27) compared to controls. Baseline MAP was recorded in all mice before any perturbation. TRPAV^{-/-} mice (94 ± 3.13) were significantly hypertensive compared with TRPV1^{-/-} (82 ± 3.4) and control mice (81.8 ± 2.8). To eliminate reflex adjustments and focus on primary actions of TRPA1 and TRPV1 channels, hemodynamic effects were examined in the mice after autonomous blockade with HEX. The average change in MAP following HEX administration in control mice was -4.5 ± 3.5 mmHg. Propofol a specific TRPA1 agonist decreased MAP in a dose-dependent manner (**Fig 7A and C**) in control mice which was attenuated in TRPA1^{-/-} and TRPAV^{-/-} but not in TRPV1^{-/-} mice suggesting a role of TRPA1 in regulating vasomotor tone (**Fig 7B and C**) *in vivo*.

Table 1: Phenotypic mouse characteristics:

	Control	TRPA1^{-/-}	TRPV1^{-/-}	TRPAV^{-/-}
Body Weight (g)	25 ± 0.6	29 ± 1.32	27 ± 0.7	28 ± 2
Basal heart rate, beats/min	400 ± 14.5	580 ± 39*	471 ± 27	543 ± 16
Systolic blood pressure, mmHg	96.8 ± 2.5	109 ± 1.7	99 ± 1.08	112 ± 3.02*
Diastolic blood pressure, mmHg	69 ± 3.2	76 ± 1.2	66 ± 2.5	78 ± 2.7*
MAP, mmHg	81.8 ± 2.8	90 ± 1.06	82 ± 1.05	94 ± 3.13*
HEX MAP, mmHg	77.8 ± 1.36	88.6 ± 3.6*	74.5 ± 2.5	90.5 ± 1.8*
Prop 2.5 MAP, mmHg	72.4 ± 2.6	86 ± 2.5*	72.5 ± 2.3	91 ± 2.10*
Prop 5 MAP, mmHg	62 ± 3.2	81 ± 3.2*	66 ± 3.1	85 ± 2.3*
Prop 10 MAP, mmHg	55 ± 3.5	78 ± 2.2*	57 ± 3.3	81 ± 2.2*

Values are represented as Mean±SE. Body weights are recorded before anesthesia [Control: n=26; TRPA1^{-/-}: n=12; TRPV1^{-/-}: n=12 and TRPAV^{-/-}: n=12]. MAP= Mean Arterial Pressure; HEX= Hexamethonium ; Prop= Propofol. * denotes significance from control.

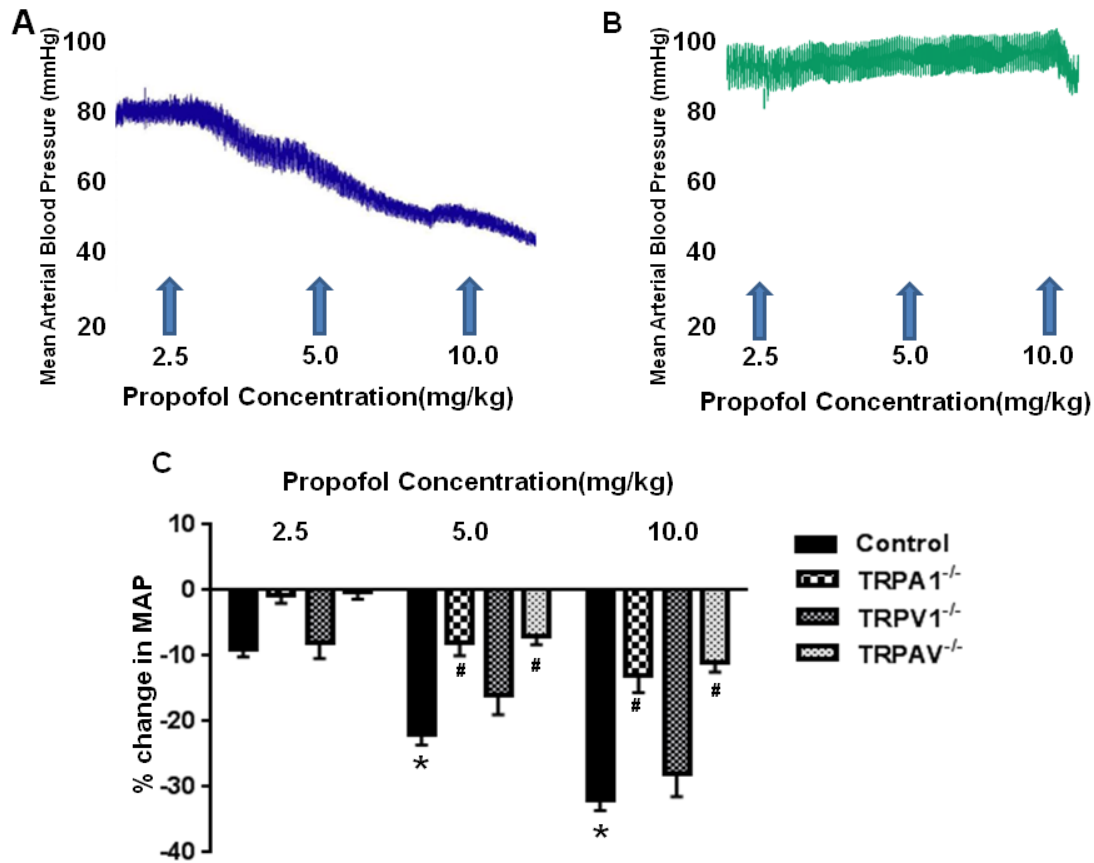


Fig 7: Propofol induced changes in mean arterial pressure (MAP). *A* and *B*: representative traces of blood pressure decreases in response to propofol in control and TRPA1^{-/-} mice. *C*: Summary data of dose-dependent decreases in MAP in control mice (n=26), TRPA1^{-/-} (n=12), TRPV1^{-/-} (n=12) and TRPAV^{-/-} (n= 12) at different concentrations of propofol. * *P*<0.05 vs. baseline. # *P*<0.05 vs. control at that concentration.

1.2: Propofol induced dose-dependent depressor response was blunted in control mice in the presence of a specific TRPA1 antagonist HC-030031:

After successful completion of surgery, propofol was administered to control animals. HC-030031(30mg/kg) was then injected intravenously to these mice followed by propofol administration. In the presence of a specific TRPA1 antagonist propofol induced depressor response was attenuated as shown in the **Figure 8** below. These data suggested a prominent role of TRPA1 in propofol-induced depressor response.

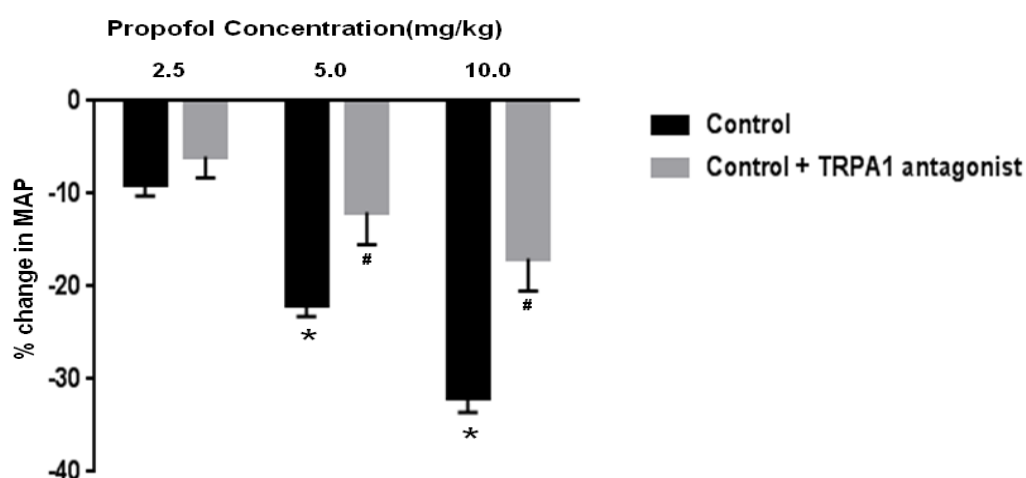


Fig 8: Propofol induced changes in mean arterial pressure (MAP) in control mice. Summary data of dose-dependent decreases in MAP in control mice (n=12) in response to propofol which was significantly attenuated in the presence of a specific TRPA1 antagonist HC-030031(n=8). * $P < 0.05$ vs. baseline. # $P < 0.05$ vs. control at that concentration.

1.3: Propofol induced depressor response is regulated by NO and BK channel in control mice:

To further determine the potential role of NO in propofol induced depressor response *in-vivo*, a specific inhibitor LNAME (100mg/kg) was used to block the endothelial nitric oxide synthase. On the other hand to investigate the role of big conductance calcium-activated potassium channel (BK_{Ca}) channel in propofol induced depressor response, Pen A (50µg/kg) was also used to block the action of this channel. LNAME (difference from base-line: 13 ± 3.16 mmHg) significantly attenuated the response to propofol in control mice (**Fig 9A**) suggesting the role of NO in propofol induced depressor response. Furthermore Pen A (difference from base-line: -1 ± 3.5 mmHg) significantly blunted propofol induced depressor response in control mice (**Fig 9B**). Interestingly the combination of L-NAME and Pen A (difference from base-line: 14.4 ± 3.2 mmHg) further attenuated propofol-induced depressor response when compared to LNAME and Pen A alone (**Fig 9C**).

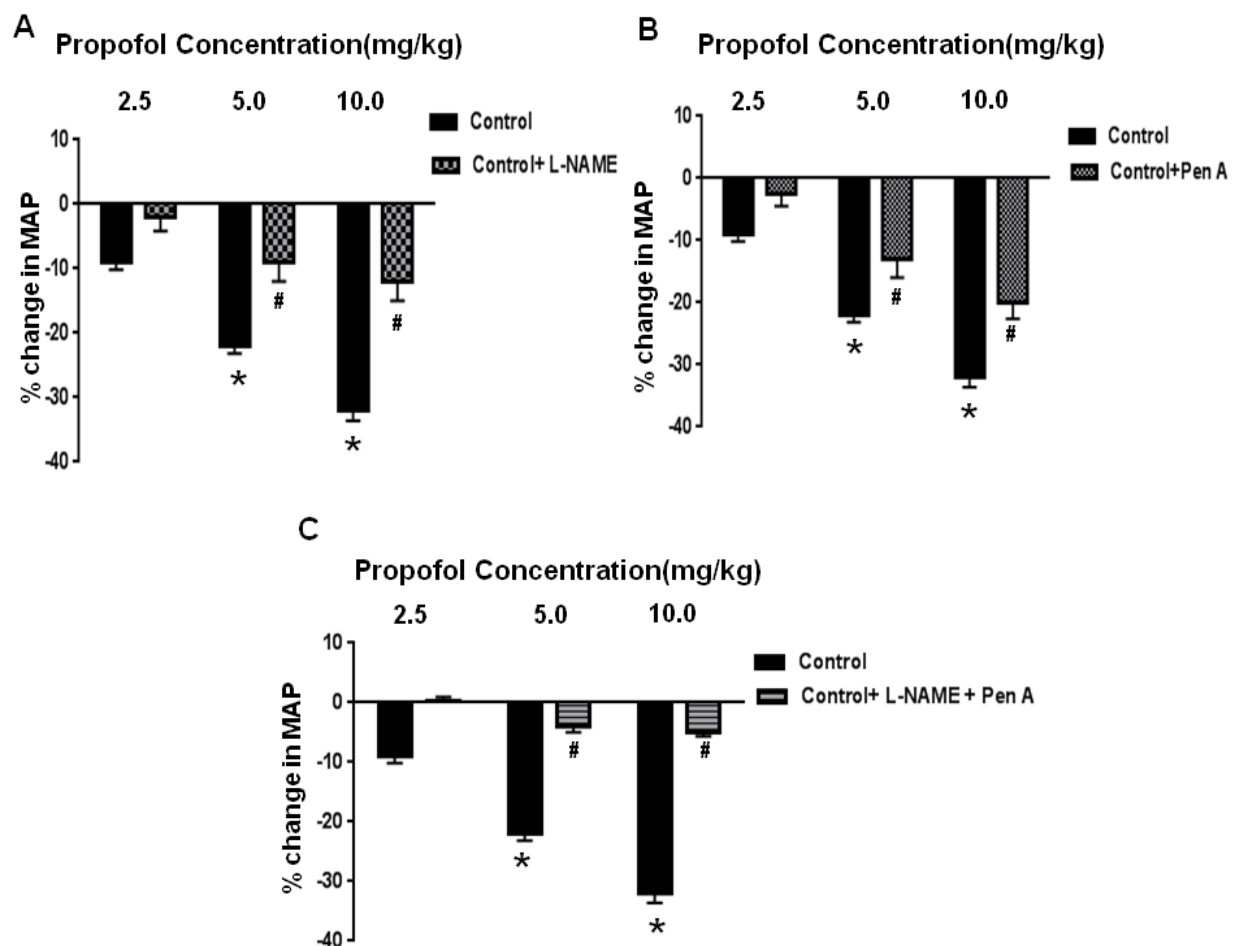


Fig 9: Propofol induced changes in mean arterial pressure (MAP) in control mice in presence of a specific endothelial nitric oxide synthase inhibitor L-NAME and a specific BK_{Ca} channel inhibitor Pen A and combination of both. *A* and *B*. Summary data illustrating the attenuation of propofol-induced depressor responses in control mice in presence of L-NAME and in presence of Pen A (n=6 in each group). *C*. Summary data demonstrating the propofol induced depressor response in control animals (n=6) in presence of a combination of LNAME and Pen A. * $P < 0.05$ vs. baseline. # $P < 0.05$ vs. control at that concentration.

1.4: Propofol induced depressor response was attenuated in TRPA1^{-/-} mice: Role of NO and BK channels:

To further delineate the downstream regulators of the TRP channels in propofol induced depressor response L-NAME (difference from base-line: 11.4 ± 2.5 mmHg) and Pen A (difference from base-line: 1.2 ± 3.13 mmHg) were used to inhibit endothelial nitric oxide synthase and BK_{Ca} channel. Propofol induced depressor response was not significantly attenuated in TRPA1^{-/-} mice in presence of L-NAME (**Fig 10A**) suggesting NO as a possible downstream target of TRPA1 channel. On the other hand administration of Pen A also did not significantly attenuate propofol induced depressor response in TRPA1^{-/-} mice suggesting that NO and BK channel pathways are severely compromised in TRPA1^{-/-} mice (**Fig 10B**). Furthermore a combination of L-NAME and Pen A (difference from base-line: 17.5 ± 3.8) also did not further attenuate propofol induced depressor response in TRPA1^{-/-} mice (**Fig 10C**).

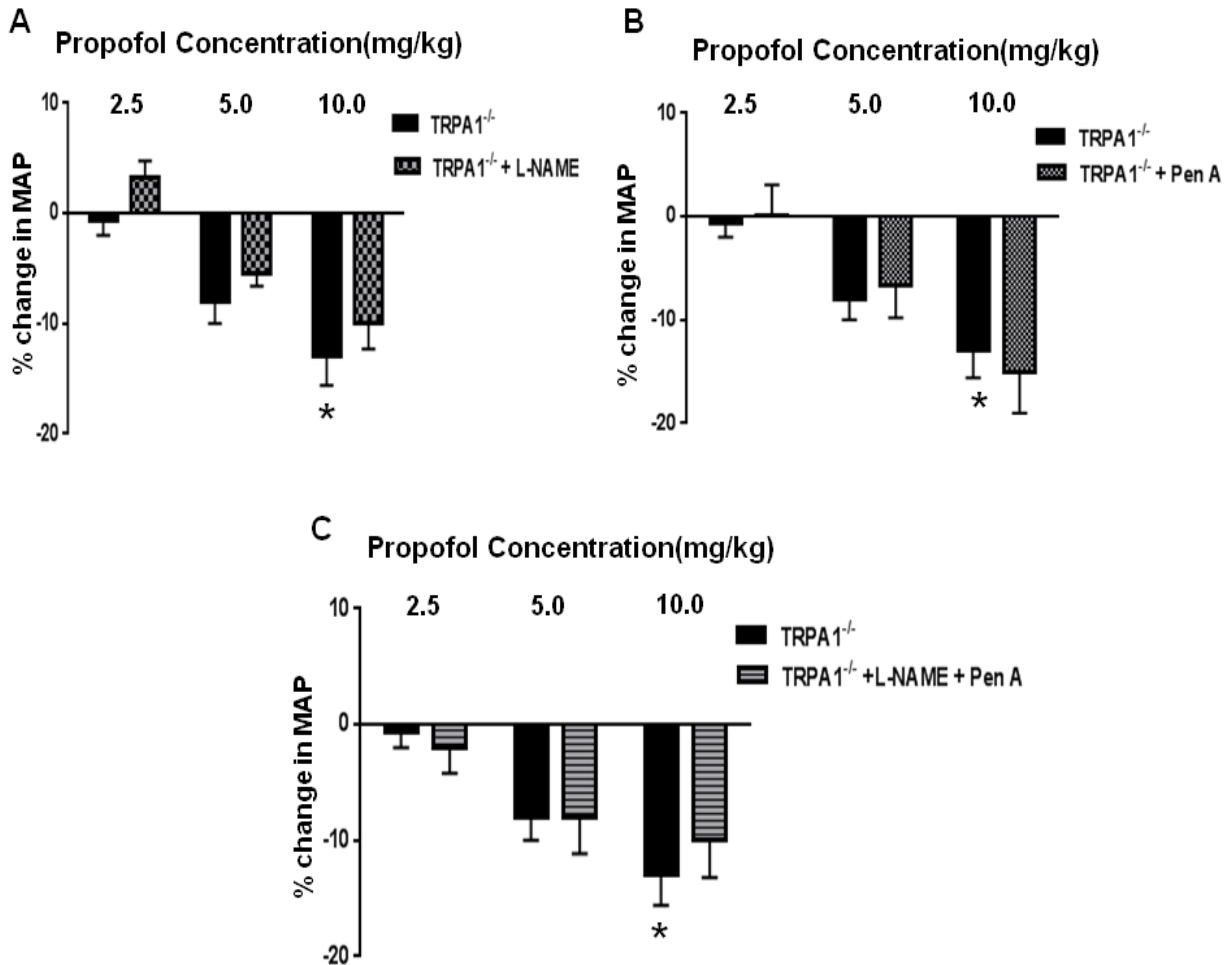


Fig 10: Propofol induced change in mean arterial pressure (MAP) in TRPA1^{-/-} mice in presence of L-NAME, Pen A and combination of L-NAME and Pen A. A: Summary data depicting attenuation of propofol induced depressor responses in presence of LNAME (n=6). B: Summary data depicting propofol induced depressor response in TRPA1^{-/-} mice in presence of Pen A. C: Summary data depicting propofol induced depressor response in TRPA1^{-/-} mice in presence of both L-NAME and Pen A (n=6). * $P < 0.05$ vs. baseline.

1.5: Propofol induced depressor response involves NO and BK channel in TRPV1^{-/-} mice:

To further investigate the role of NO and BK_{Ca} channel in TRPV1^{-/-} mice, the same protocol was repeated in TRPV1^{-/-} mice. L-NAME and Pen A was used to block NO and BK_{Ca} channel respectively. Propofol induced depressor response was attenuated in the presence of LNAME (**Fig 11A**) and Pen A (**Fig 11B**) in TRPV1^{-/-} mice. Further attenuation was achieved when both L-NAME and PenA (**Fig 11C**) was used suggesting that propofol induced depressor response is not mediated via TRPV1 channel and NO and BK_{Ca} channels are acting independently and not downstream of TRPV1 channels.

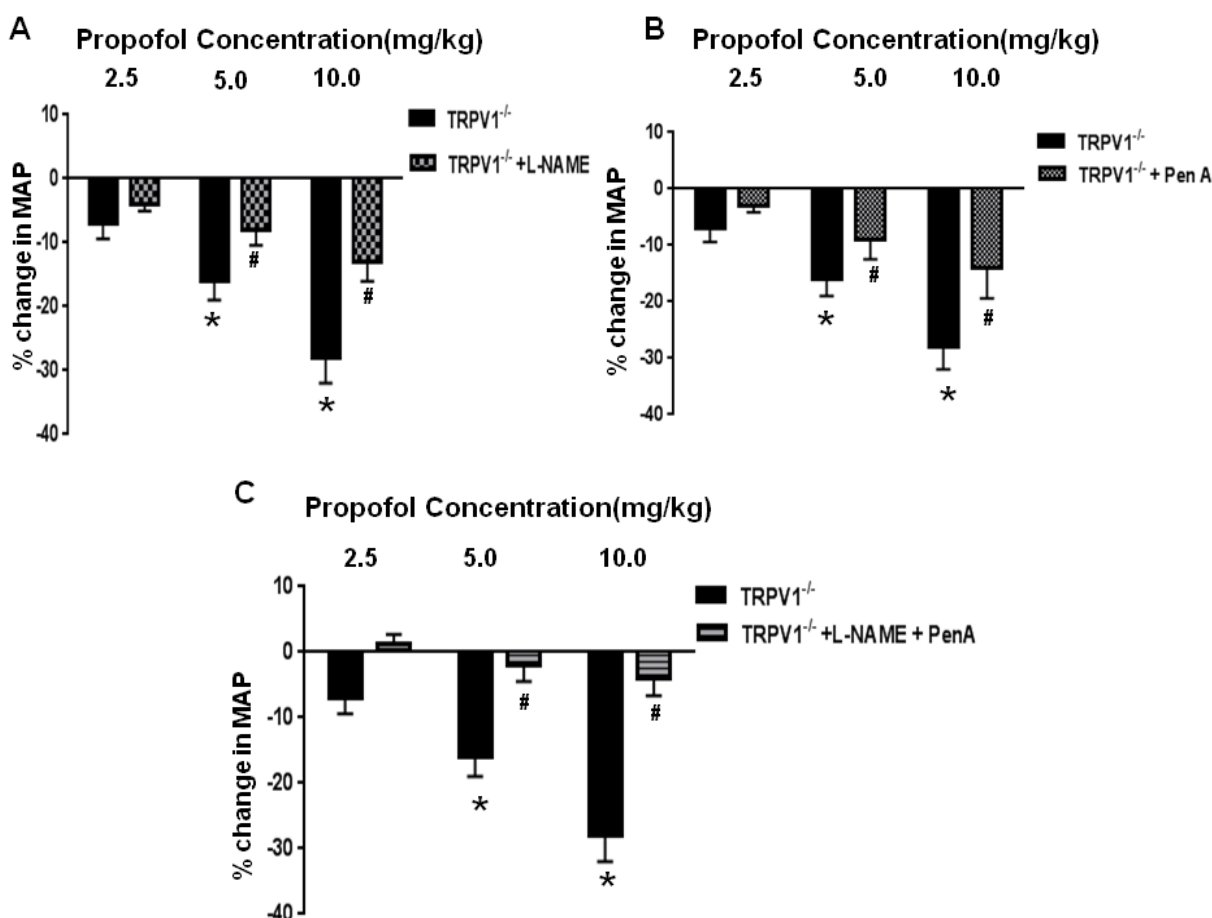


Fig 11: Propofol induced change in mean arterial pressure (MAP) in TRPV1^{-/-} mice in presence of L-NAME, PenA and combination of L-NAME and Pen A. A: Summary data

depicting attenuation of propofol induced depressor responses in presence of L-NAME (n=6). *B*: Summary data depicting propofol induced depressor response in TRPV1^{-/-} mice in presence of Pen A. *C*: Summary data depicting propofol induced depressor response in TRPV1^{-/-} mice in presence of both LNAME and Pen A (n=6). * $P < 0.05$ vs. baseline. # $P < 0.05$ vs. TRPV1^{-/-} at that concentration.

1.6: Propofol induced depressor response involves NO and BK channel in TRPAV^{-/-} mice:

To further investigate the role of nitric oxide and BK channel in TRPAV^{-/-} mice, the same protocol was repeated in TRPAV^{-/-} mice. L-NAME and Pen A were used to block the NO and BK channel respectively. Propofol induced depressor response was not attenuated in the presence of LNAME (**Fig 12A**) and Pen A (**Fig 12B**) and with the combination of L-NAME and Pen A in TRPAV^{-/-} mice (**Fig 12C**). The data further confirms the notion that NO and BK_{Ca} channel pathways are impaired in the absence of TRPA1 channel and propofol induced depressor response is solely dependent on TRPA1-NO-BK channel pathway and TRPV1 has no role.

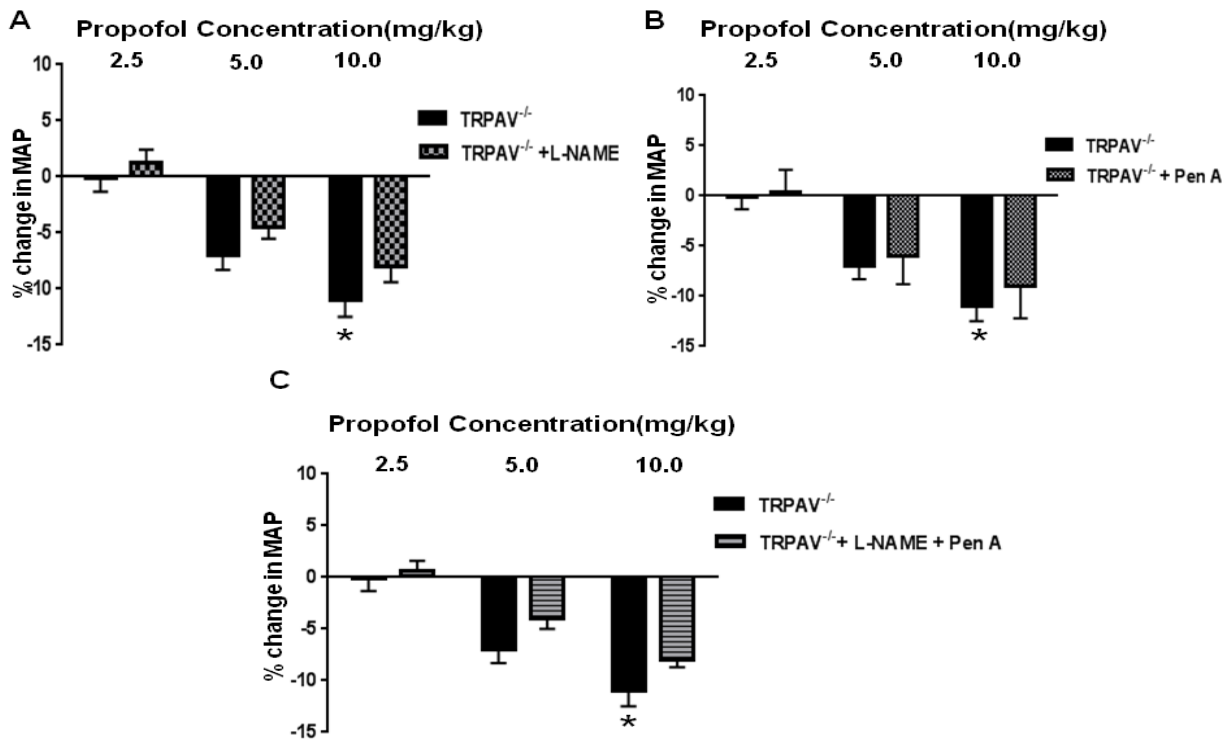


Fig 12: Propofol induced change in mean arterial pressure (MAP) in TRPAV^{-/-} mice in presence of L-NAME, Pen A and combination of L-NAME and Pen A. *A*: Summary data depicting attenuation of propofol induced depressor responses in presence of L-NAME (n=6). *B*: Summary data depicting propofol induced depressor response in TRPAV^{-/-} mice in presence of Pen A. *C*: Summary data depicting propofol induced depressor response in TRPAV^{-/-} mice in presence of both L-NAME and Pen A (n=6). * $P < 0.05$ vs. baseline.

To summarize Aim 1:

- Propofol induced depressor responses involves the role of TRPA1
- There is no significant role of TRPV1
- NO and BK channels are downstream mediators of this process.

CHAPTER 3:

AIM-2: To determine the role of TRPA1 and TRPV1 in propofol-induced vasodilation *in-vitro*

Background and Rationale:

Several studies have demonstrated the vasodilatory effects of propofol in coronary arteries. Klockgether *et al.* 2000 illustrated that propofol and thiopental caused a dose-dependent vasorelaxation that is independent of endothelium-derived factors. Ouattara *et al.* 2001 also investigated the coronary effects of propofol in rabbit hearts and concluded that propofol stimulated an increase in coronary blood flow that was not compromised during cardiac hypertrophy. Interestingly, Yamanoue *et al.* 1994 proposed that propofol-induced vasodilation in porcine coronary artery is dependent on the antagonistic properties of calcium channels. Park *et al.* 1995 have successfully demonstrated that propofol-induced vasodilation in rat coronary arteries is mediated by NO. Studies by Gacar *et al.* 1995 have also demonstrated that propofol-induced relaxation in bovine coronary arterial rings is dependent on the presence of an intact endothelium. Klockgether *et al.* 2004 have also illustrated a role for a high conductance potassium channel, BK_{Ca} in propofol-induced hypotension in porcine coronary arterial rings. Lam *et al.* 2010 demonstrated a role for K_{ATP} channels in propofol-induced vasorelaxation. However, no studies have looked at the role of TRP channels in propofol-induced vasodilation in isolated coronary arterioles obtained from mouse hearts. For instance Gazzieri *et al.* 2006 showed that TRPV1 increases coronary blood flow and hence dilates coronary arteries that are mediated by CGRP. Bratz *et al.* 2008 have shown that capsaicin induced relaxation is dependent on the production of NO/BK channels via TRPV1 in porcine coronary artery. His group for the first time has identified the location of TRPV1 in porcine coronary artery endothelial cells. Guarini *et al.* 2012 reported that TRPV1-mediated vasorelaxation in murine coronary arteries is dependent

on TRPV1-mediated production of NO and BK channel activation. Surprisingly, other studies have also depicted that capsaicin-induced vasorelaxation is independent of TRPV1-CGRP pathway in porcine and human distal coronary arteries (Gupta *et al.* 2007). Hopps *et al.* 2012 have also delineated that capsaicin-induced vasorelaxation in porcine coronary arteries and rat aorta is dependent on the antagonistic behavior of L-type calcium channels. However no studies have investigated the role of TRPV1 in mediating propofol-induced vasodilation in the coronary circulation. Moreover, only a few studies have demonstrated a role for TRPA1 in mediating endothelium-dependent relaxation (Earley *et al.* 2009).

Though studies have shown cross-talk between these two channels in sensory neurons and heterologous expression systems none of the studies have implicated the role of these channels in vascular bed, specifically to the coronary bed. Therefore, our primary goal was to identify whether propofol-induced coronary vasodilation involves TRPA1 and TRPV1 channels. In addition, we investigated the extent to which endothelium-dependent NO production and BK_{Ca} activation play a role in the propofol-induced vasodilatory effect in isolated coronary arterioles. As demonstrated in our *in vivo* studies TRPA1 appears to play a prominent role in mediating propofol-induced depressor responses. However, because the systemic responses observed *in vivo* are dependent on a multitude of factors, we utilized an *in vitro* approach to directly examine the role of the endothelium, TRPA1 and TRPV1 crosstalk and the cellular signaling pathways involved in propofol-induced depressor responses observed *in vivo*. Our next hypothesis was to investigate the role of nitric oxide in isolated cells. Since *in-vivo* data demonstrated that there is a predominant role of nitric oxide in propofol induced

vasodilation, our next goal was to measure nitric oxide release in cells isolated from control, TRPA1^{-/-}, TRPV1^{-/-} and TRPAV^{-/-} animals. Previous studies have illustrated that nitric oxide activates TRPA1 and TRPV1 in primary neurons and also in heterologous expression system (Miyamoto *et al.* 2009). Studies have also illustrated that nitric oxide activates recombinant TRP protein via cysteine S-nitrosylation (Yoshida *et al.* 2006). But none of these studies have investigated the role of nitric oxide as downstream regulators of these channels. We have isolated cells from mouse aorta of four different groups of animals and measured nitric oxide using a specific fluorometric dye. Our goal was to investigate the role of nitric oxide as a downstream signaling target of TRP pathway. None of the studies before have measured nitric oxide synthesis in cultured cells isolated from TRPA1^{-/-}, TRPV1^{-/-} and TRPAV^{-/-} animals. To support our *in-vivo* studies we have isolated cells from mouse aorta using specific CD31 beads so that we can discriminate the endothelial cells from the smooth muscle cells. Several studies have investigated the role of propofol in nitric oxide production. Petros *et al.* 1993 proposed that propofol results in nitric oxide generation in porcine aortic endothelial cells. In this study cGMP was measured to evaluate nitric oxide production as cGMP is a downstream effector of the nitric oxide pathway, so this group concluded that nitric oxide production in these cells have resulted in the increase of cGMP in these cells. Interestingly Yamamoto *et al.* 1999 have also demonstrated that in cultured rat ventricular myocytes propofol resulted in the activation of acetylcholine-nitric oxide-cGMP pathway. Other reports by Luo *et al.* 2005 have also illustrated that propofol results in a dose-dependent increase in nitric oxide production in human umbilical vein endothelial cells. On the other hand Wang *et al.* 2007 have depicted that propofol can

upregulate endothelial nitric oxide synthase in human umbilical vein endothelial cells. But none of these studies were linked to the TRP pathways. Our main hypothesis was to investigate the role of nitric oxide as a downstream mediator of the TRP pathway in relation to propofol. Finally using immunocytochemistry we have investigated the co-localization of TRPA1 and TRPV1 in endothelial cells isolated from mouse aorta.

Materials and Methods:

Mice:

Mice were housed in a room with a 12:12-h light-dark cycle and maintained at constant room temperature with continuous access to food and water. Experiments were performed on 8-12 week old males of C57Bl6, TRPA1^{-/-}, TRPV1^{-/-} mice and in the double knockout of both TRPV1 and TRPA1 (TRPAV^{-/-}) mice. The generation of TRPAV^{-/-} mice was previously described in the Chapter 1.

Coronary Vessel Cannulation and Pressure Myography:

Hearts were excised from anesthetized mice and placed in ice-cold physiological salt solution (HEPES). Mouse coronary arterioles were dissected free from ventricular wall tissue in buffer containing the following (in mM): 145 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.17 MgSO₄, 25.0 NaHCO₃, and 10 glucose, (pH 7.4). Isolated microvessels were cannulated with glass pipettes and secured with silk suture in a temperature- controlled chamber (Danish Myotech, DMT, Atlanta, GA). The chamber was then transferred to the stage of an inverted microscope outfitted with a video camera, and edge detection analyzing software. Coronary arterioles were pressurized to 60 mmHg and warmed to

37°C and allowed to equilibrate for 1 hr. Vessel viability was examined using 60 mM KCl to induce contraction. Vasoreactivity studies were performed in endothelium intact and endothelium denuded coronary microvessels. The endothelium of microvessels was disrupted by passing ~ 1 ml of air through the lumen. The denuded endothelium was then assessed in the presence of acetylcholine (1 μ M). Experiments were performed in presence of L-NAME (a specific endothelial NOS inhibitor) and Penitrem A (a BK_{Ca} channel inhibitor) to investigate the downstream mediators of this channel.

Culture of isolated primary endothelial cells from mouse aorta:

Mice were placed on surgical chamber and a ventral incision was made. The aorta was isolated, placed in ice-cold HEPES solution, cleaned immediately and cut into 2-3 mm segments. Aortic segments were pooled into 1 ml of dissociation solution containing 1 mg/ml papain and 1 mg/ml dithioerythritol (DTT). After 30 mins incubation at 37°C, segments were transferred into 1 ml of dissociation solution containing 1.5 mg/ml collagenase and 1 mg/ml soybean trypsin inhibitor, and further incubated for ~15 min at 37°C. Afterwards, this solution was replaced with 2 ml of ice-cold HEPES solution and incubated for 10 min at room temperature. The supernatant was then replaced with 1 ml HEPES solution, before trituration of vessel segments to release the cells. The cells were centrifuged at 500g for 5 mins. The supernatant was aspirated completely. The cells were re-suspended in 60 μ l of endothelial medium (DMEM Low glucose + 1% Antibiotic-Antimycotic + 10% Nu-Serum + 10% FBS + Endothelial cell growth serum (ECGS) + heparin), centrifuged and again re-suspended in media. After re-suspension the cells were magnetically separated using LS Column and MidiMacs Separator

(CD31-Microbead Endothelial Isolation kit – Miltenyl Biotec, Auburn, CA). The column was first rinsed with 3 ml of media and then the cell suspension was passed through the LS Column. The cells were collected and the column was again washed with 2 ml of medium. The washing step was repeated for three times. Now the column was removed from the separator and was placed in a suitable collection tube. The 2 ml of media was added and immediately the magnetically labeled cells were flushed out by firmly pushing the plunger into the column. Cells were then seeded onto a 5 mm dish and the media was changed daily until the cells reached 70% confluence.

Measurement of Nitric Oxide from Primary Endothelial Cells:

The endothelial cells isolated from four different animals were seeded onto polystyrene tissue culture plates to ensure 30-40% confluence on the day of the experiment. Before 48hrs of the experiment, the cells were provided with a special media which has no L-arginine. On the day of the experiment the cells were washed twice with PBS to remove the media. The cells were then loaded with NO detection reagent and incubated under normal tissue culture conditions for 2 hours. After 2 hrs the cells were washed twice with wash buffer and then treated with experimental test agents such as Propofol (10-100 μ M) and L-NAME(100 μ M). After the treatment, cells were rinsed with wash buffer and observed under a fluorescence/confocal microscope using the filter set Cyanine 5 (650/670nm).

Immunocytochemistry of mouse aortic endothelial cells:

Mouse aortic endothelial cells were grown on coverslips pre-coated with gelatin/fibronectin and was fixed in 4% formaldehyde for 30 minutes. Fixed cells were

blocked and permeabilized in phosphate-buffered saline (PBS) containing 3% normal donkey serum and 0.1% Triton X-100, and was incubated overnight at 4°C with primary antibodies. Omission of the TRPA1 and TRPV1 primary antibodies provided as a negative control. Coverslips were then washed for 3 times both before and after incubation with fluorescently-labeled secondary antibodies. Coverslips was then mounted to microscope slides in Vectashield anti-fade reagent with DAPI, a nuclear stain (Vector Laboratories), and was sealed with nail polish before viewing it under the confocal microscope.

Drugs and Chemicals:

All drugs were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise stated. Drugs were dissolved in distilled water as concentrated stock solutions unless otherwise stated. Propofol stock solution was diluted in saline. L-Nitro-Arginine Methyl Ester (L-NAME) stock solution was prepared in saline. U46619 and Penitrem A (Pen A) stock solutions were prepared in DMSO. Primary TRPA1 rabbit polyclonal antibody was purchased from Novus Biologicals. Mouse monoclonal TRPV1 antibody was purchased from Millipore, USA. Secondary antibodies were purchased from Invitrogen, USA.

Data analysis and statistics:

Summary data are expressed as means \pm SE. Statistical comparisons were performed using One-way Analysis of Variance (Simple ANOVA)/ Kruskal-Wallis One way Analysis of Variance on ranks followed by pairwise multiple comparison procedures such as Tukey/Dunn test as appropriate. For statistical analyses, Sigma Plot 11.0 software for Windows XP (Systat Software, San Jose, CA) was utilized. Calculation of percent

change in relaxation was performed using Excel 2007 (Microsoft, Redmond, WA). A value of $p < 0.05$ was considered statistically significant. For nitric oxide assay, treated endothelial cells were analyzed and compared with non-treated endothelial cells using ImageJ software (NIH, Bethesda, MD).

Results:

2.1 Propofol induced relaxation in coronary microvessels involves the role of

TRPA1 and TRPV1 channels:

To further evaluate propofol-induced vasorelaxation in isolated coronary arterioles, coronary microvessels were dissected from control, TRPA1^{-/-}, TRPV1^{-/-} and TRPAV^{-/-} mouse hearts. **Figures 13A** and **13B** are the representative raw trace of control and TRPA1^{-/-} animals depicting the effect of propofol on luminal diameter of endothelium-intact coronary microvessels following exposure to the thromboxane mimetic, U46619. In endothelium-intact microvessels obtained from TRPA1^{-/-} and TRPV1^{-/-} mice, propofol-induced relaxation was partially attenuated. In contrast, propofol-induced vasodilation was completely lost in coronary microvessels obtained from TRPAV^{-/-} mice (**Fig 13C**). These data implicate role of TRPV1 in regulating propofol-induced vasodilation.

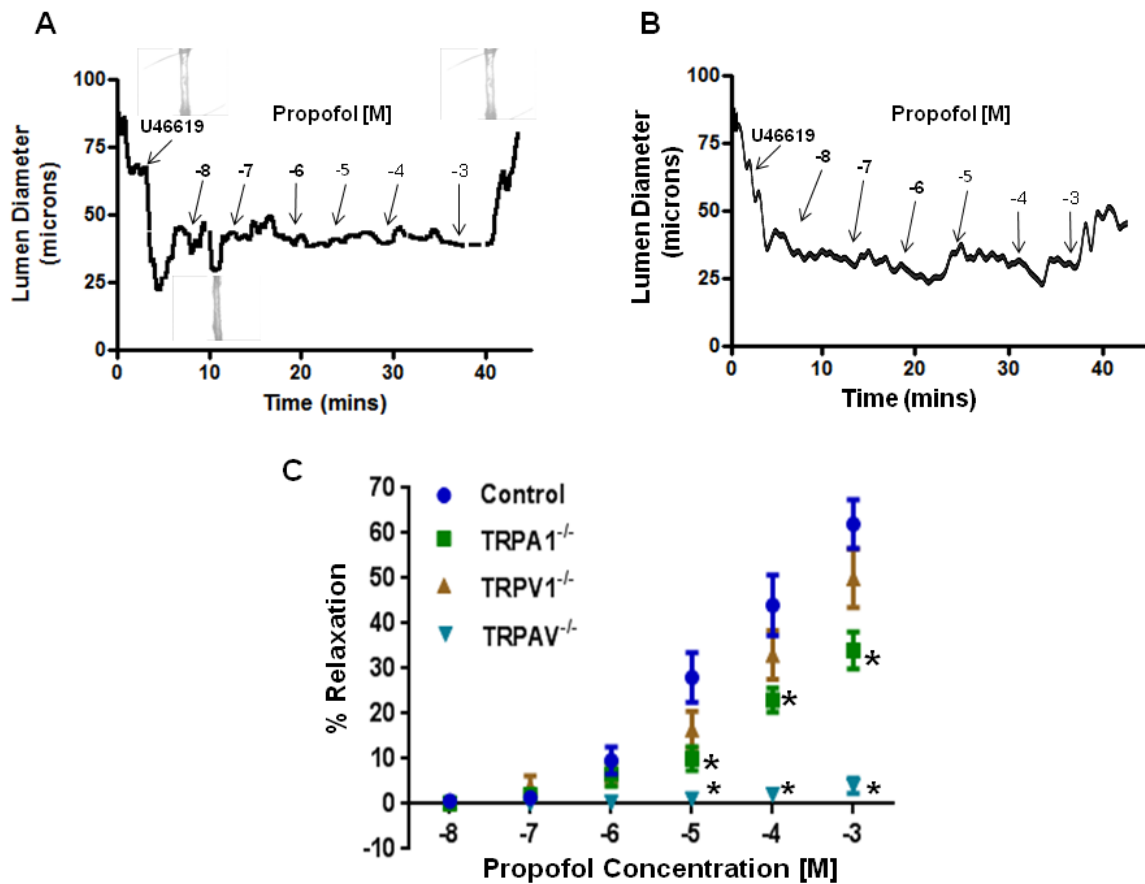


Fig 13: Propofol relaxes isolated mouse coronary microvessels: *A* and *B*: representative trace of diameter changes in coronary arteriole from control animal and TRPA1^{-/-} mice. *Insets*: images of artery diameters under baseline conditions and in response to U46619 (1 μ M) and propofol (10^{-7} and 10^{-3} M). *C*: Summary data showing propofol-induced relaxation from control mice (n=16), TRPA1^{-/-} mice (n=20), TRPV1^{-/-} mice (n=15) and TRPAV^{-/-} mice (n=8). *P< 0.05 vs. control.

2.2: Propofol induced relaxation in coronary microvessels involves the role of TRPA1 channels:

To further evaluate propofol induced vasorelaxation in isolated arteries, coronary microvessels were dissected from hearts from control mice. Propofol dose-dependently relaxed vessels which were significantly attenuated in the presence of a specific TRPA1 antagonist HC-030031 (50 μ M); (**Fig 14**). This data suggest that there is a prominent role of TRPA1 in propofol induced vasodilation.

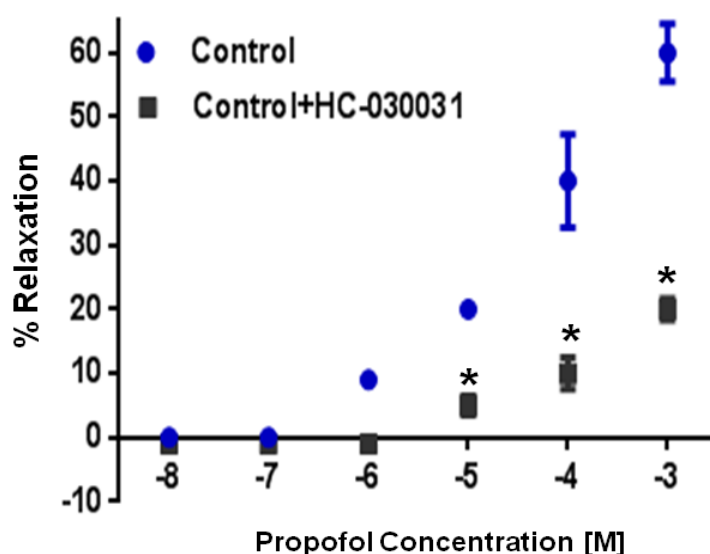


Fig 14: Attenuation of propofol induced vasorelaxation in presence of HC-030031: Summary data representing propofol induced vasorelaxation in control mice (n=4) which was attenuated in presence of a specific TRPA1 antagonist HC-030031 (n=4). *P< 0.05 vs its respective control.

2.3: Propofol induced relaxation in coronary microvessels involves the role of TRPA1 and TRPV1 channels:

To further evaluate propofol induced vasorelaxation in isolated arteries, coronary microvessels were dissected from hearts from TRPA1^{-/-} and TRPV1^{-/-} mice. Propofol dose-dependently relaxed vessels which were significantly attenuated in the presence of a specific TRPA1 antagonist HC-030031 (50 μ M) in TRPV1^{-/-} mice (**Fig 15A**) and a specific TRPV1 antagonist SB-366791(50 μ M) in TRPA1^{-/-} mice (**Fig 15B**). Our data suggest that there is a predominant role of TRPA1 and TRPV1 in propofol induced vasodilation.

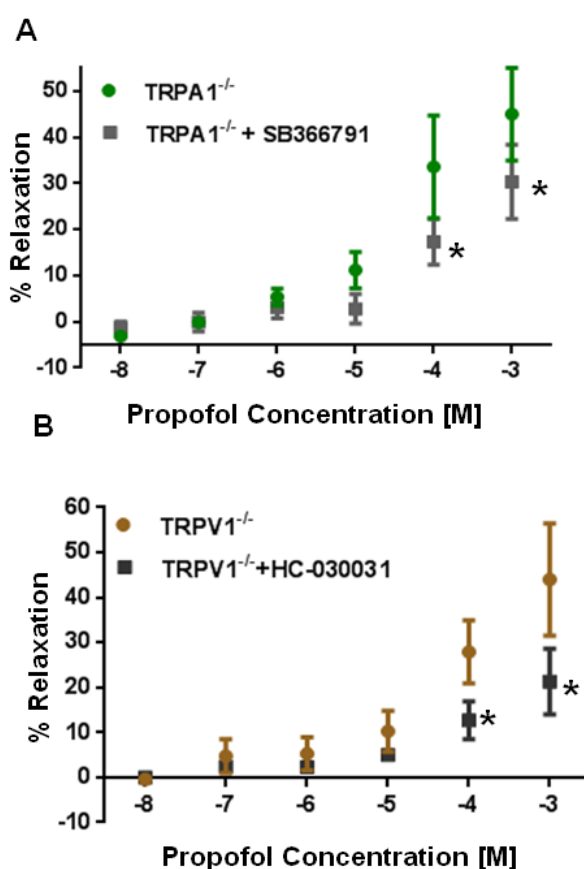


Fig 15: Attenuation of propofol induced vasorelaxation in presence of SB-366791 and HC-030031. A. Summary data representing propofol induced vasorelaxation in TRPV1^{-/-} mice (n=4) which was attenuated in presence of a specific TRPA1 antagonist HC-

030031 (n=4). *B.* Summary data representing propofol induced vasorelaxation in TRPA1^{-/-} mice (n=4) which was attenuated in presence of a specific TRPV1 antagonist SB-366791 (n=4). *P< 0.05 vs control.

2.4: Propofol induced relaxation in coronary microvessels involves the role of BK channels:

To further evaluate propofol induced vasorelaxation in isolated arteries, coronary microvessels were dissected from hearts from TRPA1^{-/-} mice and TRPV1^{-/-} mice. Propofol dose-dependently relaxed vessels which were significantly attenuated in the presence of a specific TRPV1 antagonist SB-366791(50μM) and BK_{Ca} channel inhibitor Penitrem A (50μM) in TRPA1^{-/-} mice (**Fig 16A**). On the other hand in TRPV1^{-/-} mice propofol induced dose-dependent vasorelaxation was attenuated in the presence of TRPA1 antagonist HC-030031(50μM) and BK_{Ca} channel inhibitor (50μM) (**Fig16B**). Our data suggest that BK_{Ca} channel plays a prominent role in mediating propofol induced vasorelaxation.

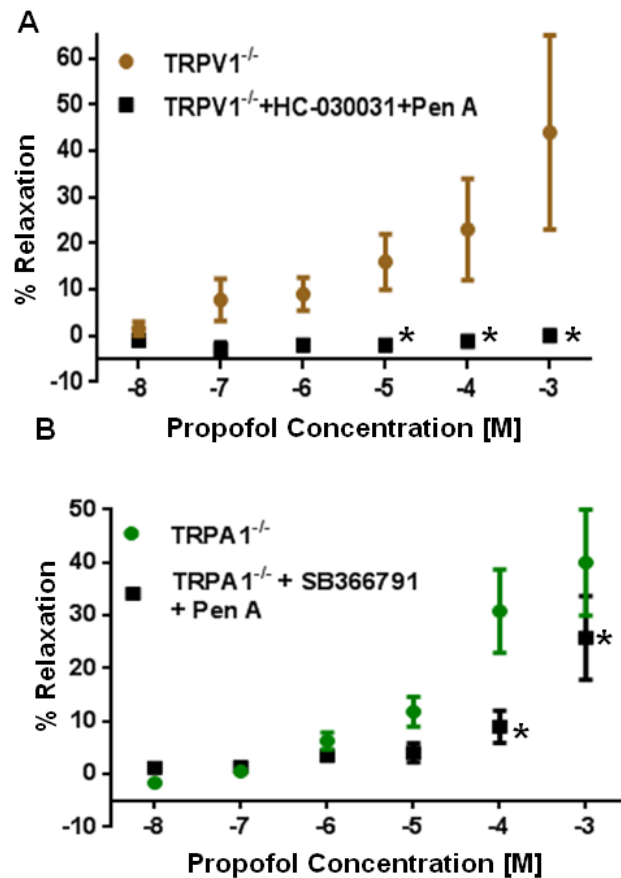


Fig 16: A: Summary data representing propofol induced vasorelaxation in TRPV1^{-/-} mice (n=6) which was attenuated in presence of a specific TRPA1 antagonist HC-030031 and BK_{Ca} channel inhibitor Pen A (n=6). B: Summary data representing propofol induced vasorelaxation in TRPA1^{-/-} mice (n=4) which was attenuated in presence of a specific TRPV1 antagonist SB-366791 and BK_{Ca} channel inhibitor Pen A (n=4). *P < 0.05 vs control.

2.5: Role of NO in propofol-induced vasodilation:

To further investigate the role of NO in propofol-induced relaxation in coronary microvessels, L-NAME was used to block NO production by nitric oxide synthase. At the

same time endothelial layer of the microvessels were denuded. Propofol-induced vasodilation was completely blunted in the presence of L-NAME (200 μ M) in all groups (**Fig.17 A-D**). To further validate the role of NO in propofol-induced relaxation, parallel experiments were performed in endothelium-denuded vessels (**Figures 17 A-D**).

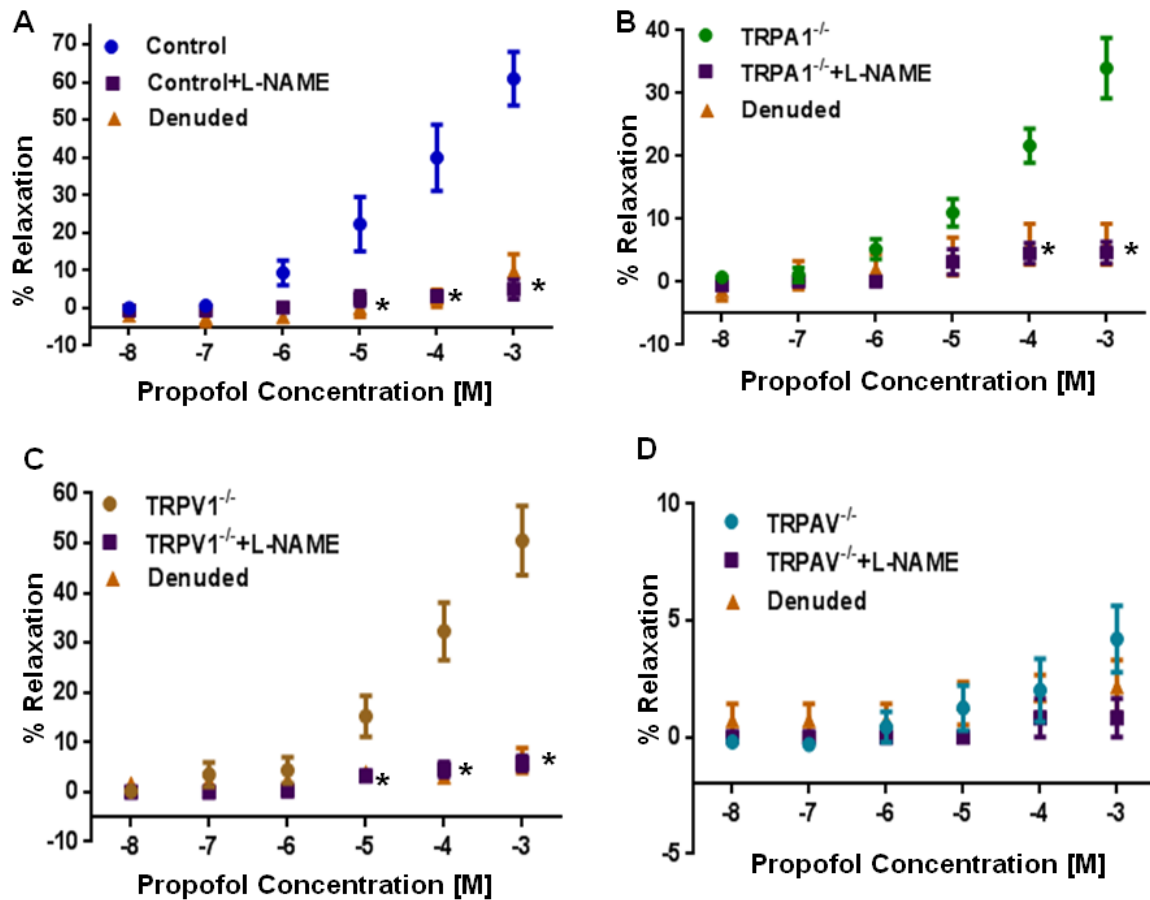


Fig 17: Role of NO in propofol induced vasorelaxation: Summarized data representing propofol induced vasorelaxation in the presence, absence of L-NAME and denuded vessels isolated from control mice (n=6), TRPA1^{-/-} mice (n=6), TRPV1^{-/-} mice (n=6) and TRPAV^{-/-} mice (n=6) (A-D). *P< 0.05 vs. its respective control.

2.6: Propofol induced relaxation in coronary microvessels involves BK channels:

To further investigate the role of the BK_{Ca} channel in propofol-induced relaxation of isolated coronary microvessels, Pen A was used to inhibit the BK channel. Propofol-induced vasorelaxation was markedly attenuated (about 50%) in presence of Pen A (50μM) in control mice (**Fig 18A**). In contrast, the presence of Pen A (50μM) had no effect on propofol-induced vasorelaxation (**Fig 18B**) in TRPA1^{-/-} mice. Similar to control mice, Pen A markedly attenuated propofol-induced vasorelaxation in TRPV1^{-/-} mice (**Fig 18C**). In contrast to TRPV1^{-/-} and TRPA1^{-/-} mice, propofol-induced vasodilation was completely blocked in TRPAV^{-/-} mice (**Fig 18D**). These data further suggest the notion that BK channel is a downstream mediator of TRPA1 pathway. Similar experiments were also performed in presence of L-NAME and Pen A in control, TRPA1^{-/-}, TRPV1^{-/-} and TRPAV^{-/-} mice (**Fig 18A-D**).

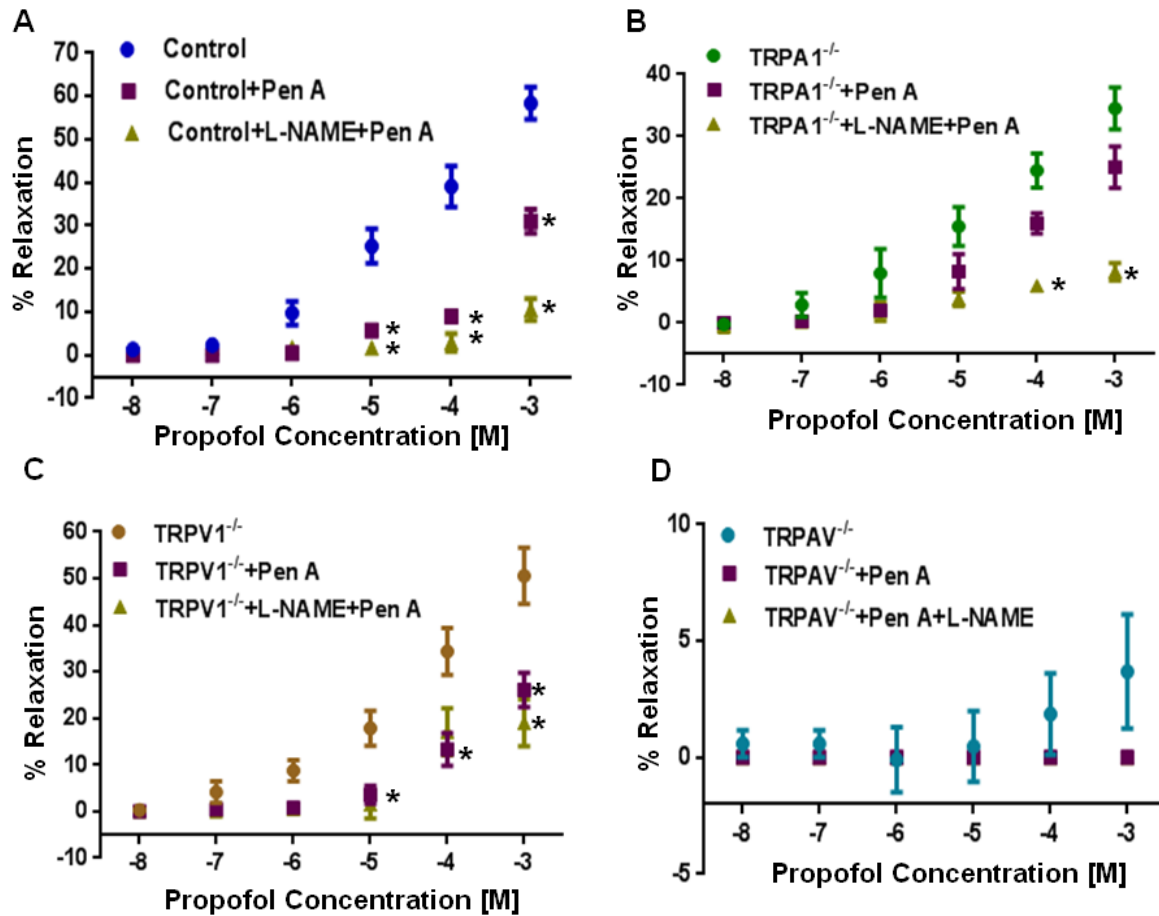


Fig 18: Propofol induced relaxation in coronary microvessels involves the role of BK channels: Summarized data representing propofol induced vasorelaxation in the presence, absence of Pen A and a combination of L-NAME and Pen A isolated from control mice (n=6) TRPA1^{-/-} mice (n=6), TRPV1^{-/-} mice (n=6) and TRPAV^{-/-} mice (n=6); (A-D). *P< 0.05 vs its respective control.

2.7: Endothelial cells were screened based on morphology:

After isolation of cells using a CD31 microbead isolation kit, cells were further categorized based on their morphology under the microscope. The cells were seeded on cover-slips and were visualized under the confocal microscope. Based on the

morphology the cells were screened and NO fluorescence values were taken from those cells.



Fig 19: Morphology of isolated endothelial cells from mouse aorta

2.8: Propofol-induced NO production in endothelial cells:

Propofol dose-dependently resulted in an increase in NO production in endothelial cells isolated from control, TRPA1^{-/-}, TRPV1^{-/-} and TRPAV^{-/-} mice which was attenuated in the presence of a specific nitric oxide synthase inhibitor, L-NAME. Interestingly propofol-induced NO production was less in TRPAV^{-/-} mice compared to control, TRPA1^{-/-} and TRPV1^{-/-} mice (**Fig 20**).

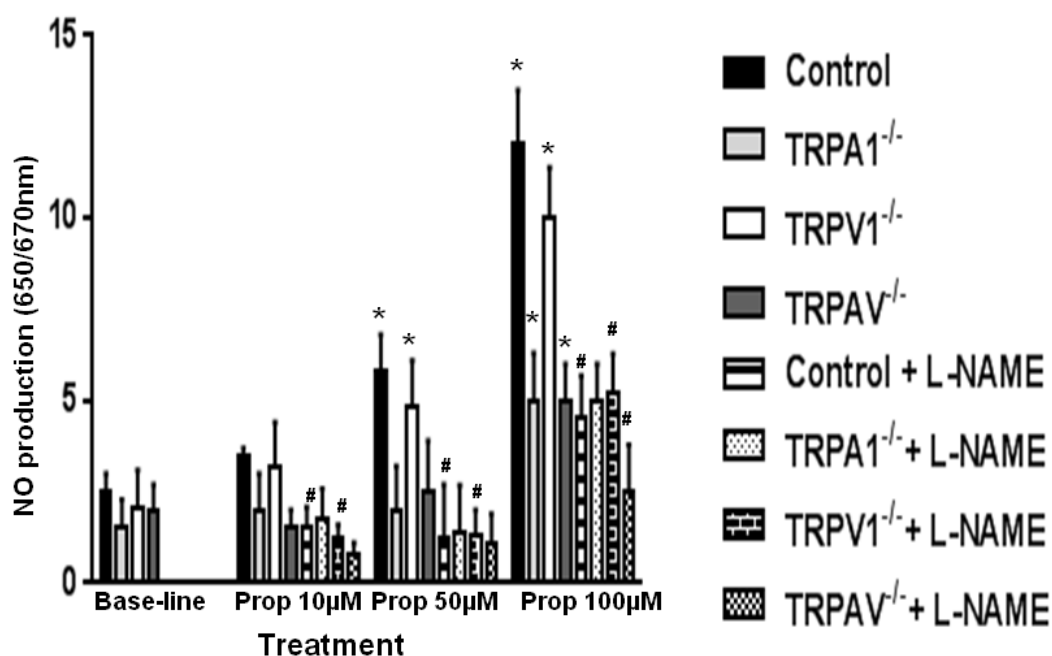


Fig 20: Schematic graph showing the production of NO in 4 different groups of animals in the presence and absence of L-NAME (*= $p < 0.05$ vs. base-line; # $p < 0.05$ vs. control).

2.9: Immunocytochemical studies show the co-localization of TRPA1 and TRPV1 in mouse aortic endothelial cells:

Figures 21A and 22A represent the nuclear staining by DAPI. Immunocytochemical TRPV1 expression using a monoclonal TRPV1 antibody conjugated with a Texas-red secondary antibody is shown in **21B and 22B** which indicates dense TRPV1 staining along the perimeter of the cell. The TRPA1 staining using a polyclonal antibody conjugated with FITC secondary antibody is shown in **21C and 22C** which signifies the

location of TRPA1 in the cell-membrane as well as in the cytoplasm and perinuclear regions. Furthermore the colocalization of these channels is shown in **21D and 22D**. Finally the bright-field images of the cells are shown in **Figures 21E and 22E**. The negative controls are shown in **Figure 23 A-D**.

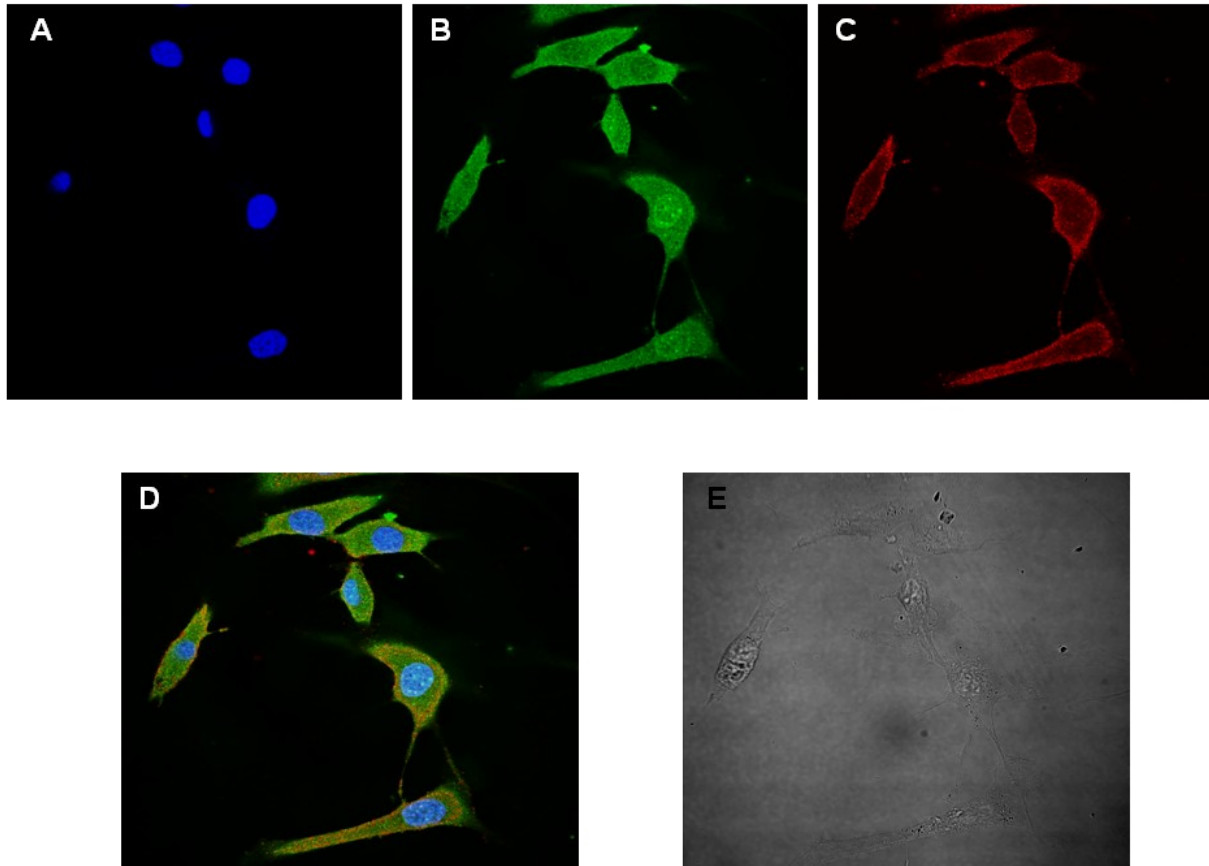


Figure 21: Immunocytochemical studies shows TRPA1 and TRPV1 expression in mouse aortic endothelial cells: A: Blue represents DAPI staining, B: green shows TRPA1 and C: red shows TRPV1staining. The panel D shows the merge of the three channels and panel E is the bright-field image.

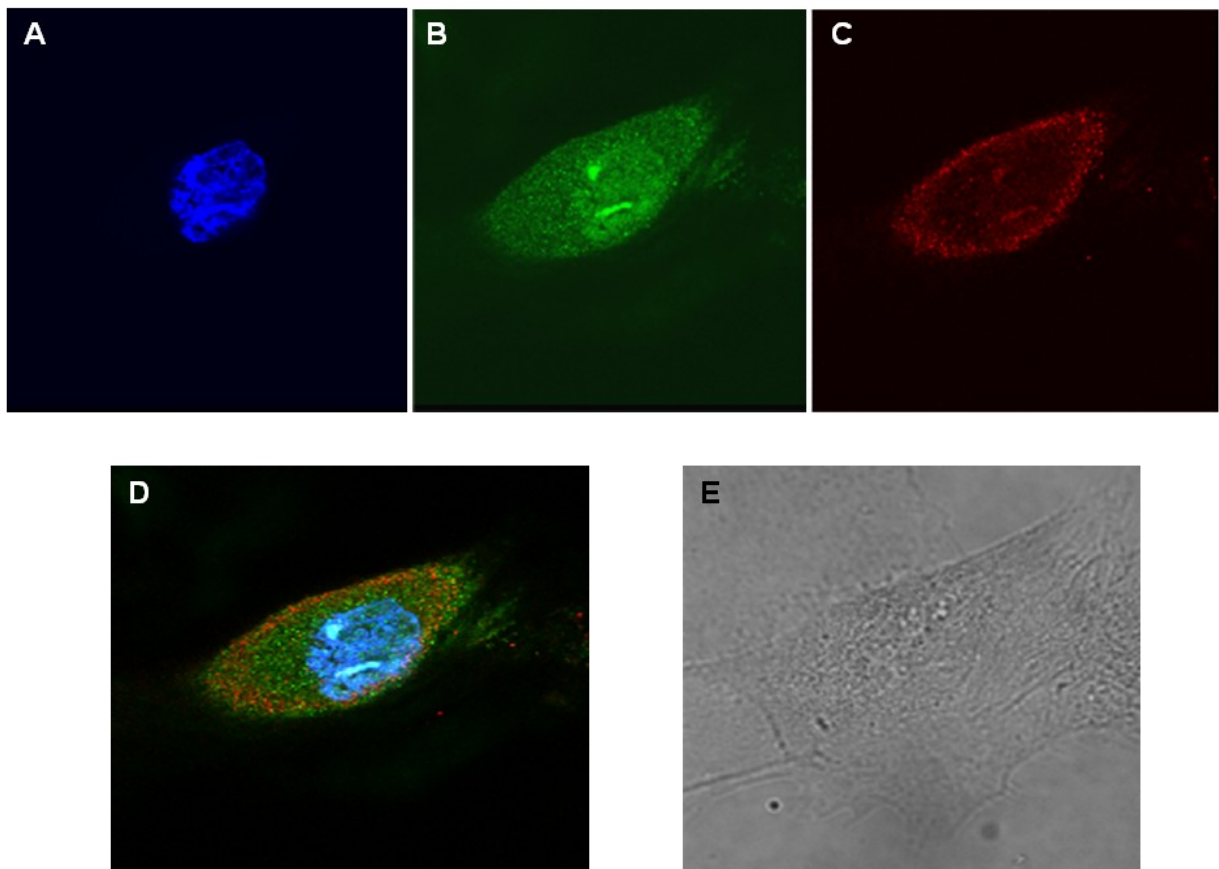


Figure 22: Confocal Z-stacking shows TRPA1 and TRPV1 expression in a single representative cell: *A*: Blue represents DAPI staining, *B*: green shows TRPA1 and *C*: red shows TRPV1 staining. The panel *D* shows the merge of the three channels and panel *E* is the bright-field image.

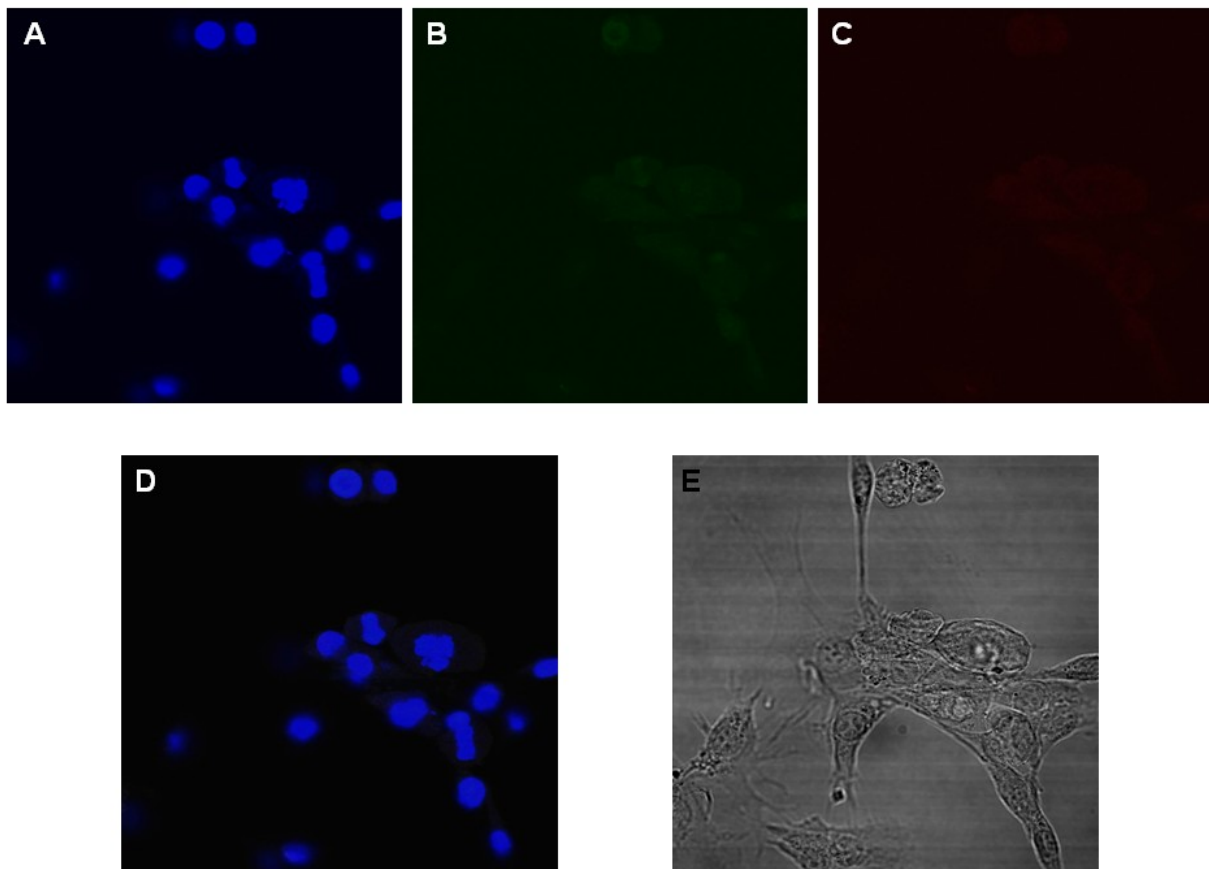


Figure 23: Negative control representing the selectivity of TRPA1 and TRPV1 antibodies: A: Blue represents DAPI staining, B: green shows TRPA1 and C: red shows TRPV1 staining. The panel D shows the merge of the three channels and panel E is the bright-field image.

To summarize the results of Aim 2:

- Propofol induced vasodilation is dependent on TRPA1 and TRPV1
- Propofol may activate TRPA1 directly.
- Propofol can activate nitric oxide pathway and hence can indirectly turn on TRPV1.
- Nitric oxide can be a downstream regulator of TRP channels.

- BK channel is a downstream regulator of TRPA1
- *In-vitro* data implicates a possibility of cross-talk between TRPA1 and TRPV1 channels

CHAPTER 4:

OVERALL CONCLUSIONS AND CLINICAL SIGNIFICANCE

Propofol induced depressor response is mediated by TRPA1 *in-vivo*: This is the first study to demonstrate the role of propofol in regulation of hypotension via TRPA1 *in vivo*. Several studies have demonstrated before that TRPA1 and TRPV1 cross-regulate with each other in neurons and in heterologous expression systems (Ruparel *et al.* 2008; Akopian *et al.* 2008; Salas *et al.* 2009). Studies have also revealed a functional interaction between these channels (Akopian *et al.* 2010). Parallel studies from our lab have also investigated the cross-talk mechanisms between these channels in sensory neurons and heterologous expression systems (Wickley *et al.* 2010; Zhang *et al.* 2011). Interestingly recent findings have demonstrated the role of anesthetics such as lidocaine, desflurane in activation and modulation of TRP channels (Eilers *et al.* 2010; Satoh *et al.* 2009; Leffler *et al.* 2008; Leffler *et al.* 2011). Moreover recent reports have also shown that propofol activates TRPA1 (Matta *et al.* 2008, Fisher *et al.* 2010). But most of these studies were performed in neurons and transfected cells, moreover none of these studies were done to delineate the signaling mechanisms by which anesthetics causes vasodilation. The primary objective of the first part of this study was to delineate the signaling cascade by which propofol causes depressor responses *in vivo*. The *in-vivo* data have shown for the first time that propofol induced depressor response is completely regulated by TRPA1 and there is no role of TRPV1. Previous studies have shown that a known TRPA1 agonist, cinnamaldehide increases paw blood flow in WT mice which was decreased in TRPA1^{-/-} mice. Pozsgai *et al.* 2010 have also demonstrated that activation of TRPA1 leads to reflex changes in blood pressure and heart rate which is more likely dependent on vasovegal responses. These results also concluded that substance P and CGRP do not contribute to the lowering of blood

pressure *in-vivo*. So according to their perspective there is no role of CGRP and substance P when used under physiological conditions. Here authors have seen a biphasic role of the TRPA1 agonist cinnamyldehydro, where interperitoneal injection of this drug at lower concentration causes depressor response whereas at higher concentration it leads to increase in blood pressure. None of these effects were seen in our study as we have seen a dose-dependent profound decrease in blood pressure when propofol was used in our study. This group have seen a very negligible role of TRPV1 in this whole process. They have seen no difference between WT and TRPV1^{-/-} mice in the depressor phase but have only seen a difference between WT and TRPV1^{-/-} mice in the pressor phase where a very high dose of cinnamyldehydro was used (320µmol/kg). Grant *et al.* 2005 have also demonstrated before that topical application of mustard oil, another TRPA1 agonist results in increase in blood flow in mouse ear which is mediated by CGRP and Substance P though using cinnamyldehydro *in vivo* this group have not seen any role of CGRP and substance P. Parallel studies by Graepel *et al.* 2011 have also shown that intraplantar injections of 4-ONE, a compound which is a metabolite of arachidonic acid results in vasodilation in hindpaw of mice which is also mediated by CGRP and Substance P. Other studies have also demonstrated the role of TRPA1 agonists in bradycardia and bradypnoea (Harada *et al.* 1975, Brimblecombe *et al.* 1972). Other reports have also demonstrated that activation of TRPA1 also results in meningeal vasodilation which is also mediated by CGRP (Kunkler *et al.* 2011). But interestingly our data has shown for the first time that propofol induced depressor response is mediated via a TRPA1 dependent-NO-BK channel pathway.

Implication of cross-talk mechanisms in the coronary vascular bed:

Previous studies have illustrated TRPA1 expression in cardiac myocytes (Stokes *et al.* 2006). Our *in-vivo* data shows that there is a promising role of TRPA1 in propofol induced depressor responses and no significant role of TRPV1. As in an *in-vivo* setting there are multitude of factors involved so to narrow down our *in-vivo* findings we have performed vasoreactivity studies in coronary isolated rings. Our coronary data suggest for the first time that TRPA1 and TRPV1 cross-talk with each other in the coronary vascular bed. This is the first study to prove the notion that these channels play an important role in regulating vasomotor tone in the heart. On the other hand this is the also the first study to show that propofol regulates the functionality of these channels in mouse coronary bed thereby causing vasorelaxation. In the first set of experiments we have seen that propofol induced vasodilation is significantly attenuated in TRPA1^{-/-} mice and completely blunted in TRPAV^{-/-} mice proving our hypothesis that propofol induced vasorelaxation is mediated by both TRPA1 and TRPV1. Moreover our findings propose that propofol activates TRPV1 but to a much lesser extent compared to TRPA1 which supports the previous findings (Fisher *et al.* 2010; Tsutsumi *et al.* 2001). Our data illustrates that propofol induced vasodilation is partially attenuated in TRPV1^{-/-} mice compared to controls though not significant which suggest that there is an indirect/partial role of TRPV1 in mediating propofol induced vasodilation in coronary vascular bed. As unpublished patch-clamping and calcium-imaging studies from our lab have shown that propofol do not activate TRPV1 directly, so there is a possibility of an indirect activation of TRPV1 via propofol. As report exists that NO activating TRP channels via S-nitrosylation (Yoshida *et al.* 2006) and propofol induced vasodilation is

also dependent on NO in rat distal coronary arteries (Park *et al.* 1995); so there is a possibility of propofol activating NO via some different mechanisms which is then activating TRPV1.

Role of Nitric Oxide in propofol induced depressor responses *in-vivo* and in isolated coronary rings:

Our next goal was to determine the role of the downstream regulators of TRPA1 and TRPV1 in propofol induced vasodilation. Earlier studies have shown that propofol upregulates eNOS, stimulates NO release from porcine aortic endothelial cells (Petros *et al.* 1993; Park *et al.* 1995; Wang *et al.* 2007) but none of these studies have determined that how propofol turns on nitric oxide. Our major hypothesis was to determine that whether NO pathway is independent or dependent on TRP pathway. On the other hand none of these previous studies were performed *in-vivo*. Most of these studies were performed in isolated rings/arteries. This is the first *in-vivo* study where the depressor mechanisms were investigated using propofol as an anesthetic. Interestingly our results suggested that propofol induced depressor responses is completely dependent on NO. Our *in-vivo* data suggest that NO is a downstream modulator of TRPA1 pathway which further supports our coronary data suggesting that NO is a downstream target of TRP channel. In presence of L-NAME (a specific endothelial nitric oxide synthase inhibitor) or denuded arteries in TRPAV^{-/-} mice our results have shown that there is no further attenuation of propofol induced vasodilation. These results suggest that in the absence of TRPA1 and TRPV1 channels in coronary bed, the nitric oxide pathway is severely compromised.

Functionality of BK channel is impaired in TRPA1^{-/-} mice:

Our next hypothesis was to investigate the role of BK channel in propofol induced vasodilation. Our results illustrates that propofol induced vasodilation is attenuated in control mice in the presence of a specific BK_{Ca} channel inhibitor Pen A. Interestingly in presence of Pen A propofol induced vasodilation is attenuated in TRPA1^{-/-} mice but not in TRPV1^{-/-} mice which suggest that BK_{Ca} channel is a downstream regulator of TRPA1 both *in-vivo* and in isolated coronary microvessels. Previous finding have shown that propofol induced vasodilation in porcine coronary arteries is dependent on big conductance calcium activated K⁺ channels (BK_{Ca} channel)(Klockgether *et al.* 2004). Earley *et al.* 2009 have shown that AITC, a known TRPA1 agonist activates TRPA1 present in the myoendothelial junctions located at the cerebral arteries thereby activating intermediate and small conductance K⁺ channels and hence causing vasodilation. Our data suggest that propofol induced depressor response is dependent on BK channel which is acting downstream of TRPA1.

Possible roles of other mediators in propofol induced depressor responses:

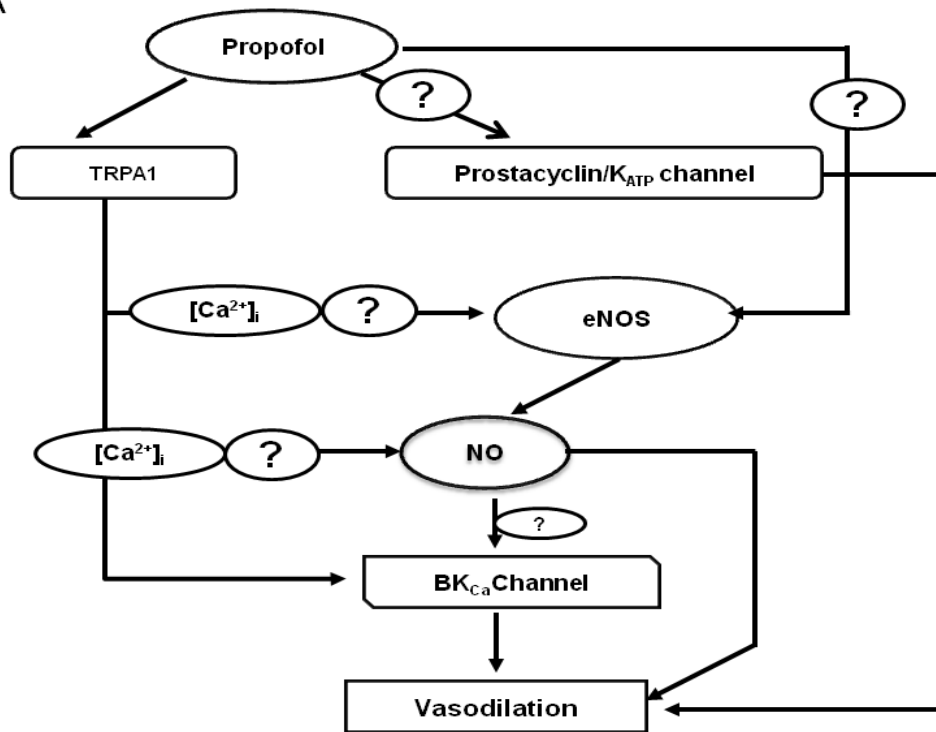
Though our data have shown that there is a prominent role of TRPA1 in propofol induced depressor response and nitric oxide and BK channel are the downstream mediators of this channel, but there may be roles of other mediators in this process. As propofol induced depressor response is not completely attenuated in TRPA1^{-/-} mice *in-vivo* it may be possible that there is a role of other channels or pathways involved in this process. Previous reports have demonstrated the role of ATP-gated K⁺ channels and the role of prostacyclins, cyclooxygenases in propofol induced vasodilation (Lam *et al.* 2010; Liu *et al.* 2009). So it is possible that these mediators have a prominent role in

regulating propofol-induced depressor response which can be independent of TRPA1 pathway. Furthermore there may be a role of substance P, CGRP and vasovagal reflexes in TRPA1 mediated vasodepressor responses. In conclusion this is the first study to prove the notion that propofol induced depressor responses is dependent on TRPA1 *in-vivo*. There is also an implication of cross-talk between TRPA1 and TRPV1 channels in heart. Moreover there is a prominent role of NO and BK channel in this process which are downstream regulators of these channels.

Finally we have also looked at the expression of these channels in the endothelial cells isolated from mouse aorta which have proved the expression of these channels in the vascular bed. Our immunocytochemistry data shows a prominent expression of TRPV1 in the periphery of the cell whereas TRPA1 is expressed both in the periphery and in the perinuclear regions. Our data have also signified co-localization of these channels at the periphery of these cells.

The following flow-diagrams delineate the signaling cascade by which propofol induced depressor responses *in-vivo* and in coronary vessels.

A



B

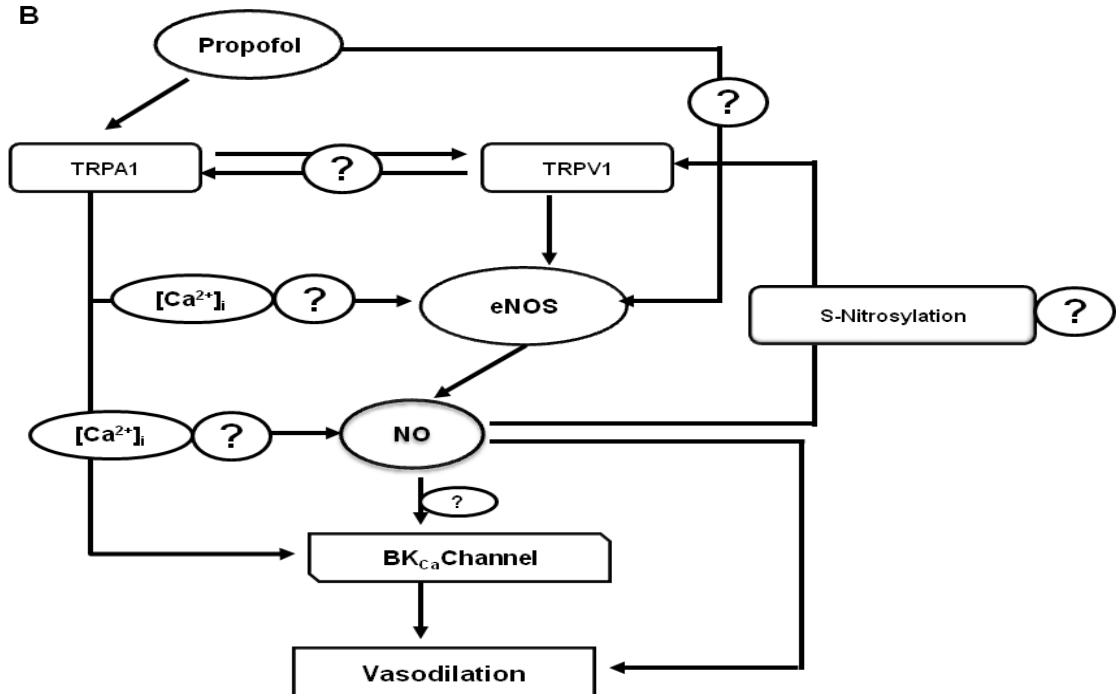


Figure 24: Schematic flow-diagram depicting the signaling cascade by which propofol induced vaso-depressor responses *in-vivo* (A) and in isolated coronary arterial rings (B)

Unpublished findings from our lab have shown that propofol activates TRPA1 intracellularly. Though research is ongoing and we are still in the process of performing mutagenesis to investigate the amino acids which are involved in the process. Future experiments will further implicate the binding site of propofol and will help us to modify this anesthetic. These modifications can hence help to mitigate the potential harmful side-effects such as pain on injection and hypotension. This will in turn ensure a better and faster post-operative recovery of patients that will be especially beneficial to “at risk” patient groups such as those suffering from diabetes and other cardiovascular disorders.

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