CONFIRMATION OF SUBTYPE OF ALPHA-1 ADRENERGIC RECEPTOR PRODUCING RELAXATION IN GOAT ARTERY STRIP

A Dissertation submitted in partial fulfillment of the requirement for the Degree of Doctor of Medicine in Physiology (Branch – V) Of The Tamil Nadu Dr. M.G.R Medical University, Chennai -600 032

Department of Physiology

Christian Medical College

Vellore

May 2018

CHRISTIAN MEDICAL COLLEGE
Post Office: THORAPADI, VELLORE632 002.
India

Telephone : 2222102: 228+Extn.
Telegrams : MEDICOL
Telefax : India: 0416-2262788, 2262268
Telefax : Abroad: 0091-0416-2262788,2262268

Ref: ……Date:……

CERTIFICATE

This is to certify that the thesis entitled "**Confirmation of Subtype of Alpha-1 Adrenergic Receptor Producing Relaxation in Goat Artery Strip**" is a bonafide; original work carried out by Dr. Bhavithra Bharathi S, in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May- 2018.

Dr Sathya Subramani
Department of Physiology,
Christian Medical College,
Vellore – 632 002

CHRISTIAN MEDICAL COLLEGE
Post Office: THORAPADI, VELLORE 632 002.
India

Telephone : 2222102: 228+Extn.
Telegrams : MEDICOL
Telefax : India: 0416-2262788, 2262268
Telefax : Abroad: 0091-0416-2262788, 2262268

Ref: ……Date:……

CERTIFICATE

This is to certify that the thesis entitled "**Confirmation of Subtype of Alpha-1 Adrenergic Receptor Producing Relaxation in Goat Artery Strip**" is a bonafide; original work carried out by Dr. Bhavithra Bharathi S, in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May- 2018.

Dr Anna B Pulimood
Principal,
Christian Medical College,
Vellore – 632 002

DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**Confirmation of Subtype of Alpha-1 Adrenergic Receptor Producing Relaxation in Goat Artery Strip**" was carried out by me during my term as a post graduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

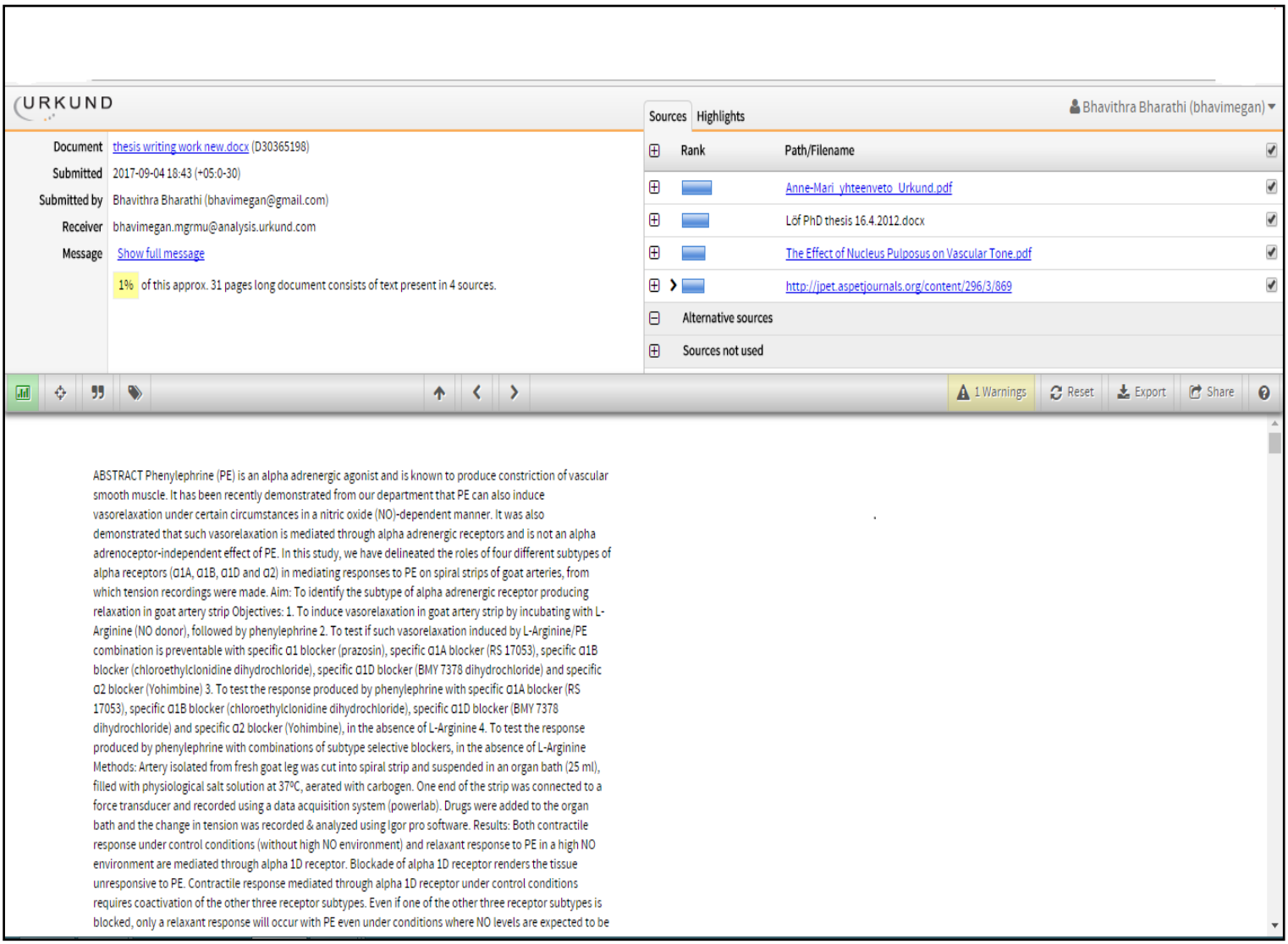
Dr Bhavithra Bharathi S,
Department of Physiology,
Christian Medical College,
Vellore – 632 002

ACKNOWLEDGEMENTS

I sincerely thank,

- ✓ Dr. Sathya Subramani, my guide and mentor, for her invaluable advice, guidance and encouragement throughout the study.
- ✓ Mr. Soosai Manickam, for his priceless help and support.
- ✓ Dr. Renu Tharion, for her advice and encouragement.
- ✓ Dr. Solomon Satishkumar for his advice and support.
- ✓ Dr. Silviya Rajakumari, Dr. Anand Bhaskar, Dr. Vinay Timothy Oomen and Dr. Elizabeth for their support and motivation.
- ✓ Dr. Neetu, Dr. Jesi, Dr. Upasana and Dr. Anandit for their kind support and encouragement.
- ✓ My friends, Renu Raj, Niranjini, Vasanth, Mahatabb, Farhan, Ankita and Alen for their wishes and cooperation throughout my study.
- ✓ Mrs. Geetha, Mr. Natarajan and Mr. Selvam for rendering timely help to complete my study.
- ✓ Mr. Henry, Mrs. Nalina and Mr. Vijay for arranging the laboratory to do the experiments.
- ✓ CMC Fluid Research Grant Committee, for funding the study.

I am greatly indebted to my parents, my in-laws and my husband Er. Megan David for all their love and support throughout the study. Above all I thank God Almighty for giving me strength to complete the thesis.



ABSTRACT Phenylephrine (PE) is an alpha adrenergic agonist and is known to produce constriction of vascular smooth muscle. It has been recently demonstrated from our department that PE can also induce vasorelaxation under certain circumstances in a nitric oxide (NO)-dependent manner. It was also demonstrated that such vasorelaxation is mediated through alpha adrenergic receptors and is not an alpha adrenoceptor-independent effect of PE. In this study, we have delineated the roles of four different subtypes of alpha receptors (α1A, α1B, α1D and α2) in mediating responses to PE on spiral strips of goat arteries, from which tension recordings were made. Aim: To identify the subtype of alpha adrenergic receptor producing relaxation in goat artery strip Objectives: 1. To induce vasorelaxation in goat artery strip by incubating with L-Arginine (NO donor), followed by phenylephrine 2. To test if such vasorelaxation induced by L-Arginine/PE combination is preventable with specific α1 blocker (prazosin), specific α1A blocker (RS 17053), specific α1B blocker (chloroethylclonidine dihydrochloride), specific α1D blocker (BMY 7378 dihydrochloride) and specific α2 blocker (Yohimbine) 3. To test the response produced by phenylephrine with specific α1A blocker (RS 17053), specific α1B blocker (chloroethylclonidine dihydrochloride), specific α1D blocker (BMY 7378 dihydrochloride) and specific α2 blocker (Yohimbine), in the absence of L-Arginine 4. To test the response produced by phenylephrine with combinations of subtype selective blockers, in the absence of L-Arginine Methods: Artery isolated from fresh goat leg was cut into spiral strip and suspended in an organ bath (25 ml), filled with physiological salt solution at 37ºC, aerated with carbogen. One end of the strip was connected to a force transducer and recorded using a data acquisition system (powerlab). Drugs were added to the organ bath and the change in tension was recorded & analyzed using Igor pro software. Results: Both contractile response under control conditions (without high NO environment) and relaxant response to PE in a high NO environment are mediated through alpha 1D receptor. Blockade of alpha 1D receptor renders the tissue unresponsive to PE. Contractile response mediated through alpha 1D receptor under control conditions requires coactivation of the other three receptor subtypes. Even if one of the other three receptor subtypes is blocked, only a relaxant response will occur with PE even under conditions where NO levels are expected to be

Table of contents

ABSTRACT

Phenylephrine (PE) is an alpha adrenergic agonist and is known to produce constriction of vascular smooth muscle. It has been recently demonstrated from our department that PE can also induce vasorelaxation under certain circumstances in a nitric oxide (NO)-dependent manner. It was also demonstrated that such vasorelaxation is mediated through alpha adrenergic receptors and is not an alpha adrenoceptor-independent effect of PE. In this study, we have delineated the roles of four different subtypes of alpha receptors (α_{1A}, α_{1B}, α_{1D} and α_2) in mediating responses to PE on spiral strips of goat arteries, from which tension recordings were made.

Aim:

To identify the subtype of alpha adrenergic receptor producing relaxation in goat artery strip

Objectives:

1. To induce vasorelaxation in goat artery strip by incubating with L-Arginine (NO donor), followed by phenylephrine
2. To test if such vasorelaxation induced by L-Arginine/PE combination is preventable with specific α_1 blocker (prazosin), specific α_{1A} blocker(RS 17053), specific α_{1B} blocker (chloroethylclonidine dihydrochloride), specific α_{1D} blocker (BMY 7378 dihydrochloride) and specific α_2 blocker (Yohimbine)
3. To test the response produced by phenylephrine with specific α_{1A} blocker(RS 17053), specific α_{1B} blocker (chloroethylclonidine dihydrochloride), specific α_{1D}

blocker (BMY 7378 dihydrochloride) and specific α_2 blocker (Yohimbine), in the absence of L-Arginine

4. To test the response produced by phenylephrine with combinations of subtype selective blockers, in the absence of L-Arginine

Methods:

Artery isolated from fresh goat leg was cut into spiral strip and suspended in an organ bath (25 ml), filled with physiological salt solution at 37ºC, aerated with carbogen. One end of the strip was connected to a force transducer and recorded using a data acquisition system (powerlab). Drugs were added to the organ bath and the change in tension was recorded & analyzed using Igor pro software.

Results:

Both contractile response under control conditions (without high NO environment) and relaxant response to PE in a high NO environment are mediated through alpha 1D receptor. Blockade of alpha 1D receptor renders the tissue unresponsive to PE. Contractile response mediated through alpha 1D receptor under control conditions requires coactivation of the other three receptor subtypes. Even if one of the other three receptor subtypes is blocked, only a relaxant response will occur with PE even under conditions where NO levels are expected to be normal.

Conclusion:

Alpha-1D receptor is required for the contractile or relaxant response with PE and coactivation of other three receptor subtypes is required for preventing relaxation and for contraction to occur under control conditions. The results suggest that each receptor subtype has a specific role to play in mediating the effects of PE and are not redundant mechanisms acting through the same pathway.

Keywords: NO-dependent vasorelaxation, Phenylephrine, Alpha adrenergic receptor, Vascular smooth muscle

INTRODUCTION

`Adrenergic receptors are classified into alpha and beta (1). The alpha adrenergic receptors are further classified into alpha 1 and alpha 2 (1). Beta adrenergic receptors are further classified into beta 1, beta 2 and beta 3 (2). The alpha adrenergic receptors are located in vascular smooth muscle (3). Beta 1 adrenergic receptors are predominantly located in heart, beta 2 in vascular & bronchial smooth muscle and beta 3 in adipose tissue (4). Alpha 1 adrenergic receptor is G_q protein coupled receptor and alpha 2 is G_i coupled receptor (5)(6). Beta adrenergic receptors are coupled to G_s protein (7). The well known action of alpha adrenergic activation is the contraction of vascular smooth muscle causing vasoconstriction and beta adrenergic activation leads to vasodilation (8). The vascular smooth muscle is predominantly innervated by sympathetic nervous system which releases nor-adrenaline and adrenaline as neurotransmitter. Thus the vascular smooth muscle tone is mainly regulated by the modulation of sympathetic discharge (9). Nor-adrenaline and adrenaline are non-specific agonists of adrenergic receptors (10).

Phenylephrine is a known vasoconstrictor which is a selective alpha adrenergic agonist (11). Phenylephrine after binding with alpha 1 adrenergic receptor causes activation of phospholipase C which converts phosphatidylinositol diphosphate (PIP_2) to Inositol triphosphate (IP_3) and Diacyglycerol (DAG). Inositol triphosphate acts on IP3 receptor located on sarcoplasmic reticulum. IP_3 receptor is a ligand-gated calcium channel, activated by IP3 which releases calcium. Calcium binds to calmodulin and this calcium-calmodulin complex causes activation of myosin light chain kinase (MLCK) which phosphorylates myosin protein and causes contraction of vascular smooth muscle.

The role of diacylglycerol is that it activates protein kinase C (PKC) which inhibits myosin light chain phosphatase, thereby preventing relaxation of vascular smooth muscle (12).

Beta adrenergic receptor is a G_s coupled receptor which is present in vascular smooth muscle. Stimulation of beta adrenergic receptor leads to increase in cyclic Adenosine monophosphate (cAMP) via activation of an enzyme Adenylyl cyclase and cAMP phosphorylates protein kinase A (PKA) which leads to decrease in intracellular calcium levels, thereby promoting vasodilation (7)(8). Nitric oxide which is one of the important second messenger signaling pathways in vascular smooth muscle also produce vasorelaxation via distinct pathway. Nitric oxide is synthesized from L-Arginine in vascular endothelium by endothelial nitric oxide synthase (eNOS) (13). Nitric oxide, then enters into the vascular smooth muscle and activates soluble Guanylyl cyclase (sGC) which converts Guanosine triphosphate (GTP) to cyclic Guanosine monophosphate (cGMP) (14). cGMP activates protein kinase G which then activates myosin light chain phosphatase leading to dephosphorylation of myosin producing vasorelaxation (15). cGMP is inactivated by converting it to 5' GMP, due to the action of phosphodiesterase (14). One of the conditions in which excess nitric oxide produced is septic shock which can cause serious complications like decompensated vasodilatory shock that is resistant to vasopressors like phenylephrine (16)(17) .

Thus, so far popularly known alpha adrenergic action on blood vessels is vasoconstriction and beta adrenergic action is vasodilation. However, the study published in plos one by Renu et al shows that phenylephrine in micromolar concentration

consistently produced vasorelaxation from the base-line tone under certain conditions in goat artery strip (18). Literature search shows that filippi et al also observed such vasorelaxation, but with nanomolar concentration of phenylephrine and that too from a pre-contracted rat mesenteric vessel (19). The study published by Renu et al shows three circumstances under which micromolar concentration of phenylephrine produce vasorelaxation in goat artery strip. One of these conditions involve increase in nitric oxide as with L-Arginine or sodium nitroprusside (while L-Arginine or sodium nitroprusside *per se* did not produce vasorelaxation). Among the other two circumstances, one involve decrease in cGMP as with soluble Guanylyl cyclase blocker like methylene blue and the other involve increase in cGMP as with sildenafil by decreasing the degradation of cGMP (due to inhibition of phosphodiesterase) while excess cGMP *per se* did not produce vasorelaxation. In the later two conditions nitric oxide is expected to be normal and it is being diverted to an unidentified vasorelaxant pathway. Vasorelaxation that is seen under the above three circumstances is said to be nitric oxide-dependent as the relaxant effect is blocked by L-NNA (Nω-Nitro-L-arginine), an eNOS blocker and the relaxant effect is independent of cGMP. The study shows that phentolamine, a non-selective alpha adrenergic receptor antagonist, abolished the vasorelaxation produced by phenylephrine in goat artery strip in the presence of excess nitric oxide like L-Arginine. This suggests that vasorelaxant effect of phenylephrine is mediated through alpha adrenergic receptor and the study also gives evidence that such vasorelaxation is not through beta adrenergic receptor as vasorelaxant effect is not abolished by beta blocker, propranolol (18).

Alpha adrenergic receptor has alpha 1 and alpha 2 subtypes, as already mentioned. Alpha 1 adrenergic receptor includes three subtypes as follows: alpha 1A, alpha 1B, alpha 1D (20). The known fact is that alpha adrenergic activation produce vasoconstriction. The receptor subtype through which phenylephrine acts to produce vasorelaxation and the downstream signaling pathways needs to be delineated. The study published by filippi et al has shown that in rat mesenteric vascular bed, alpha 1D adrenergic receptor is responsible for vasorelaxation effect induced by nanomolar concentration of phenylephrine whereas vasoconstriction by micromolar concentration of phenylephrine is through alpha 1A receptor. The study also shows that nitric oxide synthase activation which occurs due to stimulation of alpha 1D receptor is the mechanism for phenylephrine induced vasorelaxation in rat mesenteric vascular bed (19). Thus, each subtypes of adrenergic receptor have varying effects in mediating vascular tone and the different subtypes in vascular smooth muscle are not just redundant mechanisms, all producing the same effect, but each subtype has specific role in producing different actions of alpha adrenergic activation. Studies have also shown heterologous signaling pathways among alpha 1 adrenergic receptor subtypes (21).

Aim of the current study is to identify the alpha adrenergic receptor subtype through which phenylephrine acts to produce vasorelaxation in goat artery strip. Goat artery isolated from the goat leg is made into a spiral strip which is then mounted on organ bath and perfused with extracellular fluid solution with bicarbonate buffer at 37˚C and aerated with carbogen (95% oxygen and 5% carbondioxide). For recording tension, force transducer is connected to power lab data acquisition system. Initially a pre-load of

0.2gram is applied to the goat artery strip and the vascular tone is allowed to stabilize. L-Arginine, a NO donor, followed by 100 μmol/L phenylephrine is added (which is the control condition leading to vasorelaxation).

a) For the intervention group, L-Arginine is followed by the addition of alpha 1 selective antagonist prazosin, alpha 1A selective antagonist RS 17053, alpha 1B selective antagonist chloroethylclonidine dihydrochloride (CEC), alpha 1D selective antagonist BMY 7378 dihydrochloride, alpha 2 selective antagonist Yohimbine and subsequently phenylephrine is added, in each different set of experiments
b) In another set of experiments, addition of subtype selective blockers, RS 17053, CEC, BMY 7378 and Yohimbine is followed by the addition of phenylephrine in the absence of L-Arginine. If any of the subtype specific blockers in these set of experiments induce reduction in vascular tension with phenylephrine, then Nω-Nitro-L-arginine (L-NNA), an eNOS blocker was added to see if such relaxation is NO-dependent
c) Various combinations of subtype selective blockers is added in the absence of L-Arginine, followed by addition of phenylephrine

The change in tension is analyzed using Igor pro software and computed offline. The receptor subtype responsible for phenylephrine induced vasorelaxation is delineated based on changes in tension before and after phenylephrine, which is added subsequent to specific receptor blockers.

AIMS AND OBJECTIVES

Aim

To identify the subtype of alpha adrenergic receptor producing relaxation in goat artery strip

Objectives

1. To induce vasorelaxation in goat artery strip by incubating with L-Arginine followed by phenylephrine
2. To test if such vasorelaxation induced by L-Arginine/PE combination is preventable with specific α_1 blocker (prazosin), specific α_{1A} blocker(RS 17053), specific α_{1B} blocker (chloroethylclonidine dihydrochloride), specific α_{1D} blocker (BMY 7378 dihydrochloride) and specific α_2 blocker (Yohimbine)
3. To test the response produced by phenylephrine with specific α_{1A} blocker(RS 17053), specific α_{1B} blocker (chloroethylclonidine dihydrochloride), specific α_{1D} blocker (BMY 7378 dihydrochloride) and specific α_2 blocker (Yohimbine), in the absence of L-Arginine
4. To test the response produced by phenylephrine with combinations of subtype selective blockers, in the absence of L-Arginine

LITERATURE REVIEW

Vascular smooth muscle

Vascular smooth muscle is present on wall of the blood vessels. Contraction and relaxation of vascular smooth muscle alter the diameter of the blood vessel and hence regulate blood flow to various organs. Calcium is required for the contraction of vascular smooth muscle and the source of calcium is sarcoplasmic reticulum. Calcium concentration inside the sarcoplasmic reticulum is regulated by the cell membrane and at subcellular sites. Regulation of vascular smooth muscle tone is predominantly by the sympathetic nervous system. Various pharmacologic agents acts on specific receptors and modulate the contractile status of vascular smooth muscle by either increasing or decreasing the calcium concentration of sarcoplasmic reticulum (22)(9).

Vascular adrenoreceptors

The vascular smooth muscle has both alpha and beta adrenoreceptors. In 1933, W.B.Cannon described about the two chemical transmitters called sympathins, sympathin E for excitatory and sympathin I for inhibitory, during his research on the sympathetic nervous system. Subsequently in 1948, Raymond Ahlquist proposed the action of adrenaline on two distinct receptors, alpha and beta, which explains the excitatory and inhibitory actions of the same sympathetic mediator acting on two distinct receptors. Thereafter, the concept of receptor theory was accepted by the scientific community (23).

The alpha adrenergic receptors were thought to be a homogenous population of receptors until 1974. Later, it was proposed that alpha receptors can be classified into

alpha 1 and alpha 2 based on the differences in potency for phenoxybenzamine, an alpha adrenergic receptor antagonist. Then it was found in mid 1980s that the alpha 1 adrenoreceptors also show varying affinities for the agonist, oxymetazoline and the antagonist, WB4101 and phentolamine which lead to the concept of subtypes of alpha 1 receptor. Thus, alpha 1 adrenergic receptor is classified into alpha 1A, alpha 1B and alpha 1D and all the three receptors show high affinity to prazosin, an alpha 1 receptor antagonist. Another subtype of alpha 1 adrenergic receptor which has low affinity for prazosin is also discovered as alpha-1L. Alpha-1L may be the predominant receptor subtype that is responsible for the contractile response of prostatic smooth muscle to nor-adrenaline. Alpha 1 receptor antagonist is widely used in the treatment of benign prostatic hyperplasia (24).

Beta adrenergic receptors are further classified into beta 1, beta 2 and beta 3. Beta 1 adrenergic receptor is predominantly present in heart, beta 2 receptor in vascular & bronchial smooth muscle and beta 3 in adipose tissue (4). A normal human heart expresses 80% of beta 1 adrenergic receptor and 20% of beta 2 adrenergic receptor. Beta 1 adrenergic receptors are more selective to endogenous catecholamines like nor-epinephrine than beta 2 receptor. Downregulation of beta 1 receptor is seen in heart failure and the ratio of beta 1 and beta 2 receptor becomes approximately equal. Beta 3 receptor is mainly concerned with metabolic regulation (23).

Signaling pathways of vascular adrenoreceptors

The known action of alpha adrenergic receptor activation is vasoconstriction and beta adrenergic receptor activation is vasodilation (8). Adrenergic receptors are G protein coupled receptors (GPCR) which are seven helix transmembrane receptors. G proteins are heterotrimeric structure with α, β and γ subunits which are intracellular partners of GPCRs. They are activated after binding with GTP and its action is terminated by intrinsic GTPase activity. This cycle is regulated by the regulators of G-protein signaling proteins. Once the GTP is bound, Gα subunit gets separated from the βγ subunit and produces downstream effects. Gα subunit can be divided into $G\alpha_q$, $G\alpha_s$ and $G\alpha_i$ based on their action on various effectors. $G\alpha_q$ produce downstream effects by activating phospholipase C, $G\alpha_s$ by stimulating adenylyl cyclase activity and $G\alpha_i$ by inhibiting adenylyl cyclase activity. Gβγ subunit is involved in regulating kinases like mitogen activated kinases and small G proteins. Gβγ subunit re-combines with Gα subunit to form heterotrimeric structure once the G protein is inactivated by the intrinsic GTPase activity, replacing guanosine diphosphate (GDP) for GTP (25)

Alpha 1 adrenergic receptor is G_q coupled (5). Phenylephrine is a specific alpha 1 adrenergic agonist which is a known vasoconstrictor (11). Agonist such as phenylephrine after binding with G_q coupled receptor, activates phospholipase C which converts phosphatidylinositol diphosphate to IP_3 and DAG. IP_3 binds to IP_3 receptor located on the sarcoplasmic reticulum. IP_3 receptor is a ligand-gated calcium channel and on binding with IP_3, releases calcium into the cytosol. The cytosolic calcium increases both by the

intracellular calcium stores of sarcoplasmic reticulum and from the entry of extracellular calcium by receptor-operated calcium channels. Calcium binds to calmodulin and this complex activates myosin light chain (MLC) kinase which phosphorylates the 20-kDa light chain of myosin, allowing the interaction of myosin with actin. The actin-myosin interaction enable vascular smooth muscle contraction. The elevation in intracellular calcium concentration is transient and the vascular contractility is maintained by small G protein, Rhokinase. Guanine nucleotide exchange factor (RhoGEF) converts RhoA-GDP (inactive) to RhoA-GTP (active) form which inhibit myosin light chain phosphatase. MLC phosphatase under normal condition, dephosphorylates myosin light chain leading to smooth muscle relaxation. Inhibition of myosin light chain phosphatase by Rhokinase results in vascular smooth muscle contraction. This is the calcium sensitizing mechanism brought about by the activity of Rhokinase, which is activated at the same time when phospholipase C is activated. The role of DAG is that it activates protein kinase C along with calcium which inhibits MLC phosphatase activity, thereby promoting smooth muscle contraction. Decrease in intracellular calcium concentration and stimulation of MLC phosphatase will promote smooth muscle relaxation (12) (26).

Beta adrenergic receptor is coupled to G_s protein. Agonist such as nor-adrenaline after binding with G_s protein, stimulate adenylyl cyclase activity which convert ATP to cAMP. cAMP phosphorylates protein kinase A which produces various downstream effects by phosphorylating different proteins. One of the effects is decreasing the intracellular calcium concentration and thereby promoting vasodilation of blood vessel.

Other effects include modulation of myocardial contractility, mitogenic and proapoptotic actions of beta adrenoreceptor pathway. It was found that beta 2 receptor is coupled to G_i protein in addition to G_s/Adenylyl cyclase/PK-A whereas beta 1 is not coupled to G_i protein. The consequence of differences in coupling is that beta 1 receptor stimulation is found to be proapoptotic whereas beta 2 receptor stimulation is not proapoptotic in cardiomyocytes. It was found that overexpression of beta 2 receptor in transgenic mouse model has significantly improve myocardial performance (8) (23).

Alpha 2 adrenergic receptor is coupled to G_i proteins on vascular smooth muscle. The downstream effect is inhibition of adenylyl cyclase activity and hence decrease in the formation of cAMP (27). Decrease in level of cAMP was found to cause vasoconstriction (28). Thus activation of both alpha 1 and alpha 2 adrenoreceptors on vascular smooth muscle produce vasoconstriction (3). The alpha 2 receptor is further classified into three subtypes as alpha - 2A, 2B and 2C and all three subtypes acts via inhibition of cAMP. Alpha 2B receptor is predominantly present in peripheral vascular smooth muscle and mediate vasopressor effects. Alpha 2A and 2C subtypes are present in central nervous system and may produce sedation, analgesia and sympatholytic effects. The sympatholytic effect is due to the fact that pre-junctional alpha 2 receptors present in central nervous system inhibits further release of noradrenaline by negative feedback mechanisms. Reduction in cAMP prevents the calcium ion entry into the nerve terminal and hence produce feedback inhibition of noradrenaline release. Due to its sympatholytic action in the central nervous system, alpha 2 agonist like clonidine is used in the

treatment of hypertension to lower arterial blood pressure. Alpha 2 receptors also inhibit nociceptive neurons in spinal cord and hence alpha 2 agonist can be used in chronic pain disorders (29).

Studies have shown diversity in the signaling pathways, mediated by alpha 1 receptorsubtypes. Cloned subtypes, alpha 1A and 1B receptors, expressed in transfected NIH3T3 cells activates phosphatidylinositol 3-kinase (PI 3-kinase) and $P21^{ras}$, while alpha 1D receptor subtype did not activate this kinase. Norepinephrine activated PI 3-kinase in cells with alpha 1A and 1B receptor via pertussis toxin insensitive G proteins and did not activate this kinase in cells expressing alpha 1D receptor. Stimulation of both alpha 1A and 1B receptor activates $P21^{ras}$ and guanine nucleotide exchange on Ras protein. Studies have shown that alpha 1 receptor can activate phospholipase D in brain and causes release of arachidonic acid via phospholipase A2 activation through pertussis toxin sensitive G protein. There is also evidence suggesting the involvement of alpha 1 receptor in tyrosine kinase/Ras/mitogen-activated protein kinases signaling pathways with peptide growth factors that promote growth and proliferation of myocytes and vascular smooth muscle cells. PI 3-kinase mediates mitogen-mediated action with peptide growth receptors, cytokine receptors, G protein coupled receptors in different types of cells, that promotes cell growth. It also mediates various intracellular events such as protein kinase C independent serine phosphorylation and activation of ribosomal S6 kinase family, which induces mitogen-stimulated increase in protein synthesis and changes in cytosolic structure of proliferating cells. Gβγ-subunits are also found to activate PI 3-kinase in neutrophils

and platelets and also stimulates mitogen activated protein kinase (MAPK). Gβγ-subunit is found to be analogous to phosphorylated tyrosine kinases. Thus, the alpha 1 receptor subtypes was found to be involved in diverse signaling pathways by activation of PI 3-kinase, $P21^{ras}$,MAPK and mediates various biological processes (21).

Adenylyl cyclase, as a coincidence detector

Nine different forms of the mammalian enzyme, adenylyl cyclase has been found to exist in different types of cells and are regulated by various factors. Rather than having an unique regulatory function, each of the enzymes produce cell/tissue-specific changes which needs to be addressed in different types of cells. It was found that nitric oxide (NO) can inhibit adenylyl cyclase activity in N18TG2 neuroblastoma cells and the predominant isoform expressed in N18TG2 cells was found to be adenylyl cyclase type VI. NO was able to inhibit hormone-stimulated and Forskolin-stimulated increase in cAMP. Hormone-stimulated increase in cAMP was by secretin and prostaglandin E1. NO is involved in various regulatory function including smooth muscle relaxation on nonadrenergic noncholinergic neuronal stimulation and in synaptic plasticity. Recent studies have focussed on identifying the target molecules which is altered by NO to produce various biological effects. When the purified plasma membrane preparation of N18TG2 cells was incubated with NO donor such as sodium nitroprusside, forskolin-induced increase in cAMP production was inhibited. When such plasma membrane preparation was incubated with pertussis toxin, forskolin-induced cAMP production was not inhibited, showing that G_i is not involved in regulation of cAMP production through adenylyl cyclase activity.

Experiments also eliminated other factors such as phospho-diesterase, Gs, protein kinases, guanylyl cyclase and calmodulin as NO targets. Also it was found that NO mediated inhibition of cAMP is completely reversible. When NO-free media was provided for membrane preparations of N18TG2 cells, ability of forskolin-induced cAMP production was fully recovered after about 20 minutes. To see if peroxynitrite or hydrogen peroxide (H_2O_2) was involved in modulating cAMP production by NO, plasma membrane preparations was incubated with either superoxide dismutase (SOD) or catalase or combination of both in the presence of sodium nitroprusside (SNP). SOD coverts oxygen free radical to H_2O_2, thereby decreasing the possibility of converting NO to H_2O_2 and catalase converts H_2O_2 to $H_2O$and O_2. After 30 minutes incubation of plasma membrane preparation of N18TG2 cells with SOD or catalase or both, in the presence of SNP, inhibition of forskolin-stimulated cAMP production, by NO seems to be unaltered. It shows that NO will inhibit cAMP production by altering type VI adenylyl cyclase activity in N18TG2 cells, probably via s-nitrosylation and such regulation by NO depends on redox potential of the cell. Thus, the study gives a novel coincidence detection system of adenylyl cyclase, where NO will inhibit the G_s-stimulated effects and removal of the stimulus will reverse the situation, thus optimizing cell sensitivity to additional input signal. Studies have shown that expression of adenylyl cyclase type I in HEK-293 cells causes elevation of calcium, which switches on the reporter gene that mediates transcription through cAMP-mediated response element. Increase in calcium and stimulation of beta receptor by Isoproterenol leads to synergistic stimulation of transcription in HEK-293 cells and culture neurons as propranolol, a beta adrenergic

antagonist inhibited such synergistic stimulation. Thus, adenylyl cyclase type I is stimulated by calcium and neurotransmitters which play a role for synaptic plasticity by providing optimal cAMP level for regulation of transcription.

Adenylyl cyclases are proposed to be a 'coincidence detectors', which are affected by various factors and produces its effects via cAMP changes. Rsponses to two different signals could produce a synergistic response. For example, type II adenylyl cyclase could produce an effect via G_s-activators and the effect can be amplified by the simulataneous presence of betagamma subunit from G_i-activators or by protein kinase C stimulation via G_q-activators. 'Discordant coincidence detection' can occur where presence of one signal will be attenuated by simultaneous presence of other signal. For example, neuro-transmitters which increase calcium flux will stimulate type I adenylyl cyclase while it is inhibited by G_i-stimulation.

Nitric oxide as a key signaling molecule in cGMP-Dependent smooth muscle relaxation

Nitric oxide is one of the important signaling molecules in mediating various physiologic functions. It was called as 'molecule of the year' in 1992. Three scientists, Robert Furchgott, Ferid Murad, and Louis Ignarro were awarded noble prize in physiology or medicine in 1998 for their discovery on nitric oxide as a natural signaling molecule in mediating various cardiovascular functions. Nitric oxide produced by endothelium was initially called as "endothelium-derived relaxation factor" by Robert Furchgott and later

was studied extensively and characterized. Nitric oxide is synthesized by a family of enzymecalled nitric oxide synthases (NOS) which convert L-Arginine to L-citrulline and nitric oxide. NOS can be of three different types as endothelial nitric oxide synthases (eNOS), neuronal nitric oxide synthases (nNOS) and inducible nitric oxide synthases (iNOS). EventhougheNOS and nNOS are named after their discovery in endothelial and neuronal tissues respectively, they are expressed in various tissues. Nitric oxide produced by eNOS is mainly responsible for vascular smooth muscle relaxation and NO produced by nNOS acts as a neurotransmitter, particularly in non-adrenergic non-cholinergic neurons. iNOS is expressed mainly by inflammatory stimuli which produce NO that is involved in fighting against pathogens and shows cytotoxic effects. Both eNOS and nNOS are expressed constitutively, having a low basal activity. NOS is activated by calcium influx into the cells and by calcium/calmodulin complex. Nitric oxide synthases are regulated by several mechanisms including changes in transcription, phosphorylation, nitrosylation , etc.. (30) (14).

NO, produced in the endothelium enters into smooth muscle and activates soluble guanylyl cyclase 100-200 fold by tightly binding to the heme moiety in beta subunit of sGC. The activated enzyme, sGC will convert GTP to cGMP which activates protein kinase G-I (PKG I). PKG family includes PKG I and PKG II, of which PKG I is associated with sGC/cGMP signaling pathway. PKG then phosphorylates various proteins to bring about distinct physiologic effects. One such action of PKG is activation of myosin light chain phosphatases which dephosphorylates myosin, thus preventing actin-myosin

interaction leading to vasorelaxation. cGMP is inactivated by converting it to 5' GMP due to the action of phoshodiesterase-5 (PDE_5). Therapeutic drugs such as sildenafil which is used in the treatment of conditions such as erectile dysfunction, pulmonary hypertension acts by inhibiting PDE_5, thereby producing vasodilation by increase in cGMP and enhances blood flow or decreases vascular resistance. Glyceryl trinitrate, which is used in the treatment of angina pectoris also causes vasodilation and improves blood supply to the heart by releasing nitric oxide which acts via sGC/cGMP pathway. Thus, smooth muscle vasorelaxation produced by nitric oxide is stated to be a cGMP-dependent mechanism (14)(15).

cGMP-Independent smooth muscle relaxation by NO also requires alpha adrenergic receptor activation

Eventhough it is a known fact that alpha receptor activation produce vasoconstriction, the paper published by renu *et al* in plos one proposed that vasorelaxation produced in goat artery strip by alpha adrenergic agonist, phenylephrine, in the presence of excess or normal nitric oxide level is cGMP-independent but requires alpha adrenergic receptor activation and such relaxation was observed from the base-line tension with micromolar concentration of phenylephrine (18). Vasorelaxation produced by phenylephrine was also reported by filippi *et al* using nanomolar concentration of phenylephrine and that too from a pre-contracted rat mesenteric vessel. The vasorelaxation was claimed to be due to the activation of NOS by intracellular calcium mobilization, which was produced by alpha receptor activation (19). The paper published by renu et al gives three different

circumstances under which phenylephrine produced vasorelaxation. One of the circumstances was excess NO added in the presence of phenylephrine. Excess NO was created in the organ bath by adding NO donor, L-Arginine or sodium nitroprusside (while L-Arginine or sodium nitroprusside *per se* did not produced vasorelaxation). Out of the other two circumstances, one involve decrease in cGMP by adding sGC blocker, Methylene blue or ODQ (1H-[1,2,4] oxidiazolo [4,3-a]quinoxalin-1-one) and the other involve increase in cGMP by adding sildenafil (PDE_5 inhibitor) and NO levels are expected to be normal under these two conditions. Either Methylene blue or ODQ or sildenafil by itself did not produced vasorelaxation, but it occurred only with the presence of phenylephrine. These results suggest that vasorelaxation produced by phenylephrine is cGMP-independent (as the relaxant effect was observed in both situations where there is decrease or increase in cGMP) and also requires agonist-induced alpha receptor activation. The relaxant effect produced under various circumstances was prevented by L-NNA, an endothelial nitric oxide synthases blocker. These results summarizes the fact that vasorelaxation produced by phenylephrine is NO-dependent, cGMP-independent and requires alpha adrenergic receptor activation. The mechanism that was proposed is that NO was diverted to an putative pathway (in case of sildenafil, excess cGMP inhibit sGC by negative feedback mechanism and hence NO will be relieved from its action on sGC) and the point of interaction of NO and phenylephrine may be the inhibition of PKC. Initial activation of PKC by PMA (phorbol 12- myristate 13- acetate) or phenylephrine at the start of the experiment prevented further relaxation by the NO/PE combination (18). Endothelium dependent vasorelaxation by alpha adrenoreceptor activation was also

reported in other studies in rabbit bronchial artery and rat pulmonary artery where they suggest that alpha receptor activation induces nitric oxide release which was prevented by NOS inhibitor. Vascular tone is the balance between constriction and relaxation mechanisms which negatively modulate each other (31)(32).

Adrenergic receptor subtypes for relaxation in vascular smooth muscle

As already mentioned, alpha adrenergic receptor is divided into alpha 1 and 2. The alpha 1 receptor is further subdivided into alpha - 1A, 1B and 1D. It is known that contractility of vascular smooth muscle is mediated by activation of alpha 1 receptor. Studies have shown that alpha 1A is most potent while alpha-1B and 1D adrenergic receptor is less effective in producing contraction in rabbit abdominal aorta (33). The receptor subtype responsible for relaxation, induced by alpha receptor activation under high NO environment needs to be delineated. Literature search reveals that work done by renu *et al* shows phenylephrine induced vasorelaxation under high NO environment is through alpha adrenergic receptor, as such vasorelaxation is inhibited by phentolamine, a non-specific alpha receptor blocker. The paper also reiterated the fact that such vasorelaxation is not through beta adrenergic receptor as relaxant effect is not inhibited by propranolol, a beta receptor blocker (18). The work done by filippi *et al* gives evidence that alpha-1D receptor is involved in relaxant mechanisms caused by alpha receptor agonist, phenylephrine in nanomolar concentration, in rat mesenteric vessel. Activation of alpha-1D receptor results in inositol phosphate stimulation and calcium mobilization from IP_3 sensitive calcium stores in sarcoplasmic reticulum. Calcium stimulates NOS which lead to the production NO, which produce

vascular smooth muscle relaxation by sGC/cGMP pathway. However, micromolar concentration of phenylephrine produced vasoconstrictor response and is through alpha-1A receptor subtype. The vasorelaxant effect of phenylephrine is dependent on the ability of endothelium to produce NO, as such relaxation is prevented by NOS inhibitor, L-NAME and is not found in endothelium denuded vascular preparations. This endothelium dependent relaxation is inhibited by thapsigargin, an inhibitor of calcium-ATPase of the sarcoplasmic reticulum, suggest the involvement of IP_3 sensitive calcium stores of the sarcoplasmic reticulum in such relaxant effect (19)(34).

A study by Andrade *et al* also gives evidence for the existence of relaxant alpha-1 adrenoreceptor and shows alpha-1D subtype is responsible for relaxant

effect induced by phenylephrine in rat carotid artery. Such relaxant effect depends on NO production by endothelium and not by prostanoids. The physiological role of existence of relaxant effect induced by alpha-1D receptor is local control mechanism which modulates vasoconstrictor response to sympathomimetic amines. The study put forth the fact that there is impaired relaxation by alpha-1D receptor and hence there is an enhanced vasoconstrictor response to alpha-1 receptor agonist like phenylephrine in hyperhomocysteinemia, which is a known risk factor for cardiovascular diseases. Hyperhomocysteinemia model is created in rat by homocysteine diet and carotid artery is used for isolated vessel study. Such increased vasoconstrictor response is due to decreased NO bioavailabilty and impaired superoxide dismutase activity leading to generation of superoxide radicals. There is no morphological or pathological changes between control and hyperhomocysteinemic rat at optical microscopy. Endothelial dysfunction is one of the

earliest processes in the development of vascular diseases like atherosclerosis and the paper shows that alpha-1D induced relaxation is impaired during early stages of hyperhomocysteinemia and lead to enhanced vasoconstrictor response (35). Similar concept was also given by pernomian *et al*, where balloon catheter injury abolishes phenylephrine induced relaxant effect which lead to enhanced phenylephrine induced contractile responses in rat carotid artery. The mechanism that was proposed is that cyclooxygenase-2 (COX-2) generates superoxide anion which inactivates NO and thus impair NO-dependent vasorelaxation induced by phenylephrine (34).

Oscillatory vasomotion

Vasomotion is the spontaneous changes in tone or diameter of the vessel due to contraction and relaxation of vascular smooth muscle. Vasomotion is present in all vascular beds both in-vivo and in-vitro and it refer to oscillations of vascular tone with frequencies in the range of 1 - 20 per minute. The concept of vasomotion was described 150 years ago in bat wing. The mechanisms responsible for cellular oscillations are of three types. One of the mechanisms is based on an oscillatory release of calcium from intracellular stores of sarcoplasmic reticulum (cytosolic oscillator), second mechanism is based on oscillations due to ion channels in sarcolemma (membrane oscillator) and the third one is based on an oscillation of glycolysis (metabolic oscillator). There is less experimental evidence for the latter two mechanisms and cytosolic oscillator is the most important. To get a macroscopic oscillation in vascular tone, the oscillations in individual smooth muscle cells needs to be synchronized (36)(37).

The calcium waves are produced due to the intracellular release of calcium stores from the sarcoplasmic reticulum (SR) and absent if the SR calcium ATPase pump (SERCA) is blocked. Waves are present even in the absence of extracellular calcium but the waves disappear eventually because the SR calcium stores needs to be refilled by calcium channels on the cell membrane. The role of SERCA pump is to actively remove the cytosolic calcium and replenish the calcium stores for next contraction. Plasma membrane calcium ATPase (PMCA) and sodium/calcium exchanger (NCX) are also involved in removal of calcium from the cytosol. This oscillation in calcium waves due to SERCA pump is called cytosolic oscillator which contribute to vasomotion. In vascular smooth muscle, calcium is released from IP_3-sensitive channel via caffeine and ryanodine sensitive channel and IP3 is involved when agonist are used to induce calcium waves. The amplitude and frequency of calcium waves contain information that is decoded by various transcription factors and different oscillations leads to expression of different proteins. Even after the blockade of SERCA and calcium release from ryanodine sensitive channels, another type of oscillation is present in vascular smooth muscle, which is due to membrane oscillator. The membrane oscillator is due to oscillations in membrane potential caused by the interaction between large conductance calcium activated potassium channels and voltage-dependent calcium channels. Calcium oscillations in endothelial cells have also been reported (36).

SERCA pump is encoded by three homologous genes, SERCA 1 to 3. Its essential role is to refill the calcium stores in SR and is called housekeeping pump.

SERCA1 is mainly present in fast-twitch skeletal muscle. SERCA2a is present primarily in heart and slow-twitch skeletal muscle while SERCA2b is present ubiquitously and most importantly it is present in smooth muscle tissue. The role of SERCA pump in regulating smooth muscle contractility is studied using SERCA inhibitors, thapsigargin and cyclopiazonic acid. Phospholamban, which is a 52 amino acid phosphoprotein is an important modulator of SERCA pump. Phospholamban, in its unphosphorylated monomeric form will inhibit SERCA while phosphorylated pentameric form by calmodulin-dependent protein kinase II (CaMKII) will relieves the inhibition and increases the affinity of SERCA to calcium ions. Various studies have done in bladder, aorta, gastric antrum, portal vein using transgenic mice with phospholamban gene knockout and mutations in calcium clearance system leads to various smooth muscle pathology (38) .

PMCA is a calmodulin dependent calcium ATPase. There are four isoforms of PMCA as PMCA 1-4. PMCA 1 and PMCA 4 are ubiquitously present and these isoforms are reported to be present in smooth muscle. PMCA 2 and 3 express in the cell-specific pattern. PMCA helps in the extrusion of calcium across the sarcolemma. They play an important role in bladder and uterine smooth muscle contractility. Paul and colleagues, after utilizing *pmca1*$^{+/-}$, *pmca4*$^{-/-}$, and *pmca1*$^{+/-}$ × *pmca4*$^{-/-}$ mice, observed that half-time for force development to potassium chloride is prolonged in gene-targeted bladder. This indicates that loss of *pmca4* alleles limits depolarization induced calcium influx. This may be due to NCX present in the plasma membrane which extrudes calcium in-exchange for sodium. SERCA and PMCA each contribute to 20-25% of relaxation and the remaining

percent is by the NCX. NCX has three isoforms, of which NCX1.3 and NCX1.7 are predominantly expressed in vascular smooth muscle (38).

The oscillations produced by individual smooth muscle cells need to be synchronized which is possible by interaction between cytosolic and membrane oscillators. Calcium-activated chloride channel is stimulated by the calcium released from SR which produces membrane depolarization due to an inward current. The current generated in one cell is transferred to adjacent electrically coupled vascular smooth muscle cell via gap junctions. This synchronized depolarization lead to increased calcium release from SR. This occurs due to enhanced calcium influx, either via L-type calcium channels or due to membrane depolarization-induced potentiation of IP_3 production. All these electro-physiological events leading to synchronization are said to be cGMP-dependent. Initial sequential activation of smooth muscle is unsynchronized and synchronization predominantly refers to entrainment of active smooth muscle cells. Endothelium plays an important role in regulating vasomotion as removal of endothelium prevents vasomotion in certain arteries. Endothelium may provide certain levels of cGMP, for coordination of oscillators in smooth muscle cells and the calcium-activated chloride channel is also cGMP-dependent (39)(36).

Studies have shown oscillatory vasomotion induced by alpha-1 adrenergic agonist like phenylephrine in rat small mesenteric artery. This helps in local modulation of tissue perfusion in response to sympathetic activation. It is found to be mediated by endothelium derived hyperpolarizing factor which includes nitric oxide, as denudation of endothelium increased the contractile response of rat small mesenteric artery to sympathetic stimulation

and alpha 1 adrenergic agonist. Phenylephrine, which is an alpha-1 agonist, increases the intracellular calcium release in smooth muscle and the calcium diffuses into the endothelial cell from the smooth muscle via myoendothelial gap junctions. Rise in endothelial calcium will stimulate the opening of calcium-activated potassium channel which causes hyperpolarization and this change in membrane potential is conducted to smooth muscle via myoendothelial gap junctions, which is the endothelial derived hyperpolarizing factor-dependent component of oscillation. Thus, alpha-1 agonist can induce oscillatory vasomotion and helps to maintain intestinal perfusion, particularly if patients with circulatory shock are treated with alpha-1 agonists. Thus endothelium is an important modulator of phenylephrine induced vasoconstriction in rat small mesenteric artery and at higher concentrations produce oscillatory vasomotion, which is partly mediated by endothelium derived hyperpolarizing factor (40).

Therapeutic implications of alpha adrenergic agonist in septic shock

Sepsis which is the leading cause of death in critically ill patients represents an uncontrolled inflammatory response. The important pathophysiology in patients with sepsis is due to overproduction of one or many secondarily induced host mediators. Extensive animal and human studies shows that interleukin-1 and tumor necrosis factor α (TNF α) are the principal toxic secondarily induced host mediators but none of these anti-cytokine agents were successful in the treatment of sepsis in numerous clinical trials conducted. Recombinant activated protein C which has anti-thrombotic, anti-

inflammatory and pro-fibrinolytic properties reduces relative risk of death by 19.4% and absolute risk reduction of 6.1% but associated with increased risk of bleeding (41)(42).

Septic shock, which is a major complication of sepsis, characterized by hypo-tension and vascular collapse, is considered to be due to cytokine dependent induction of inducible nitric oxide synthases (iNOS) leading to excessive nitric oxide production causing pathological vasodilatation and tissue damage. It is most commonly due to endotoxin released by gram negative bacteria (endotoxic shock) but can also be caused by gram positive organisms, virus, fungi and parasites. The patients are in a hyperdynamic state with tachycardia and later causing progressive vasodilatation with compromised tissue perfusion and oxygenation. The bacterial endotoxin released is called lipopolysaccharide (LPS), a component in the outer membrane of gram negative bacteria, which is a major mediator of high morbidity and mortality in septic shock. The presence of bacterial products in blood causes the production of cytokines including TNF-α, IL-1β, IFN-γ, IL-6, which lead to the development of septic shock. Septic shock model can be created experimentally in animals by injecting LPS and cytokines are found to be elevated in such model. The relationship between excess production of NO and septic shock is demonstrated by the fact that administration of NOS inhibitors restore the blood pressure to normal range in patients and animal model with septic shock (42).

LPS binds with specific LPS binding protein (LBP) and this complex interacts with cell surface molecule CD14. Recent evidence suggests that transduction of signal occurs across the membrane via Toll-like receptors 2. TNF also binds with the receptor on cell

membrane, P^{55} for type I TNF, P^{75} for type II TNF and signals inflammatory response, apoptosis and proliferative actions. Although the precise mechanism of iNOS expression is not known, tyrosine kinase activation may be involved in secretion of cytokines and transduction of signal, once the cytokine binds with the receptor, as use of tyrosine kinase inhibitors have reported to prevent circulatory failure in mice. Sepsis can cause hepatocellular damage where liver enzymes like aspartate aminotransferase is found to be elevated and is the first sign of multiple organ dysfunction (MODS). MODS occur in the later stage of sepsis where there is hypotension and hyporeactivity to vasoconstrictor agents, leading to failure of major organs like kidney, lung, brain and liver, and eventually causing death (42).

Renu *et al* shows that when vasoconstrictor agents like phenylephrine was added after L-Arginine, which is a NO donor, the vascular tension reduces, in goat artery strip. Based on this observation, administration of vasoconstrictor agents for patients with septic shock, a condition with high NO levels in blood, have poor outcome as hypotensive situation may be worsened (18). The recommended first line vasopressor in the treatment of septic shock is norepinephrine and the other agents are dopamine, vasopressin, epinephrine and phenylephrine (43). Literature search shows that alpha-1 adrenergic receptor activation can increase the heart rate directly or decrease the heart rate indirectly through parasympathetic activation. Studies shows that in the presence of prazosin, an alpha-1 adrenergic antagonist, which inhibits the parasympathetic preganglionic alpha-1 receptors and then addition of phenylephrine exerts positive chronotropic effect by acting on alpha-1 receptor of heart which is also partly mediated

by β receptor (44). Studies have shown that alpha 1 blocker, prazosin as a useful adjunct to dopamine for the treatment of cardiogenic shock (45). Another study by bond *et al* shows that alpha 1 adrenergic receptor antagonist, tiodazosin, was able to prevent decompensatory vasodilation in hemorrhagic shock, created in rats using wiggers hemorrhagic shock protocol while alpha 2 receptor blockade accentuates the decompensation by 35%. Thus, usage of alpha 1 receptor blocker like prazosin helps in the treatment of septic shock (46). As the hypothesis is that alpha receptor activation in the presence of excess NO may worsen hypotension in septic shock, administration of adrenergic agonist after selectively blocking alpha receptor in patients with septic shock may improve the inotropic and chronotropic effect of heart and thus can ameliorate hypotension as pumping action of heart may be able to maintain the blood pressure.

Isolated tissue preparation to study smooth muscle function

An important tool for both physiologists and pharmacologists to study smooth muscle function is the isolated tissue bath assays. Although this method has been in use for more than 100 years, it is still considered to be the standard method for studying concentration-response curve of a particular drug and other functions of smooth muscle because of its simple, flexible and reproducible nature. Isolated tissue bath assays can be used to study tissues as small as murine mesenteric artery to as large as porcine ileum. The technique is also useful to study sequence of events such as receptor localization and interaction with drugs, signal transduction, second messenger system and response to the drugs. Studies which used isolated tissue bath assays has helped to understand the basic therapies and

drug discovery for various disorders such as asthma, hypertension, diabetes, heart failure, gastro-intestinal diseases etc, thus forming an important tool in basic research (47).

To start with the experiment, tissue of interest need to be isolated with minimal manipulation and mounted in an organ bath. One end of the tissue will be attached to bottom of organ bath using metal hook and the other end is connected to a force transducer, which is attached to a data acquisition system. The organ bath was preheated to 37°C, aerated with carbogen (95% O_2 and 5% CO_2) and filled with physiological salt solution (PSS). Each tissue has a particular length at which it produces optimum response, which is called passive tension. After setting up the tissue in organ bath, data acquisition system is switched on and stretched to its passive tension. The tissue will relax later to its resting tone. After equilibration period, desired drugs are added to the bath in appropriate concentration and response of the tissue can be recorded. Viability of the tissue can also be tested by adding drugs known to produce contractile response in smooth muscle tissue like potassium chloride. Data can be analyzed using appropriate software like Graph pad prism. The advantage of this system is that tissue is viable which functions as a whole and the physiological response (either contraction or relaxation) can be applied to the human body. Various pharmacological and physiological variables can be measured throughout the experiment which simulates the action of desired drug in the body as a whole. Multiple tissues can be isolated from one animal and the same animal can serve as its own control. One of the disadvantages is that if the tissue isolated is damaged during surgical removal or during mounting, then the recording may give varying and unreliable results, as for example, damaging endothelium during isolation of

vessel such as abdominal aorta and mesenteric artery. Hence, tissue needs to be isolated without injury, that may affect the results. Some drugs which are poorly soluble in water may precipitate in PSS which can also affect the results. Tissue viability can vary with time taken for completing the experiment and also varies with precautions taken during tissue preparation and mounting. This basic setup can be modified by incorporating electrodes for electrical field stimulation of innervating nerves and pH & temperature probe to study the effect of pH and temperature changes on tissue response (47).

Despite the traditional method of using isotonic/isometric force transducers to study dose-response relation of drugs on isolated tissue, studies have reported newer methods like using an image-sensor to study contractile responses of isolated tissue. The setup includes a high-resolution camera, a telecentric lens and an illumination system. The responses can be measured based on change in lumen diameter of isolated tissue such as, aortic ring. An image processing algorithm has been developed which measure the lumen area by calculating the number of pixels enclosed within the ring. A specific software computes an on-line dose-response curve at the same time. The advantage of this method over traditional technique is that it does not involve usage of tranducers, organ bath and the amount of drugs, buffers, gases and solutions consumed are less. Mechanical damage to the tissue is also minimal compared to traditional technique. Dose-response curve obtained for phenylephrine using an image-sensor method is equivalent to that obtained with classical force transducers in rat aortic ring. Thus, image-sensor method for measuring contractile responses in isolated tissue is claimed to be the fastest, cheaper, and requires less complicated equipment over classical force transducers (48).

Wire myograph and pressurized arterial myograph are other methods used to study vascular reactivity to various drugs and vascular function. Wire myograph is used to study resistance arteries with internal diameter of as small as 60μM. Wire myograph uses stainless steel wires for mounting the isolated ring preparations, which is secured to two supports. One of the supports is attached to micrometer for adjusting tension and other end is attached to an isometric transducer. After setting the wire-mounted artery to an appropriate internal diameter that gives maximal response, change in tension to desired drugs can be recorded. Pressurized myograph allows the study of artery more closely to its in-vivo condition and can study vascular reactivity in physiological and pathological states. It uses perfusing cannulae (glass pipette) that allows maintenance of intraluminal pressure close to physiological levels. One cannula is in a fixed position while other cannula can be moved to adjust arterial tension. Before connecting other end to movable cannula, artery is flushed to remove any blood or gas bubbles using perfusion solutions. Branches of the artery is sutured to prevent any leakage of solution. Pressure transducer is used to maintain desired pressure. Contractility of blood vessel is studied based on the changes in internal diameter of vessel by video camera attached to an imaging system. Vascular reactivity of both endothelium-intact and endothelium-denuded preparations can be studied using the above described methods. Removal of endothelium can be confirmed by loss of vasorelaxation to acetylcholine (49).

Factors affecting vascular smooth muscle contractility

Various factors can affect the vascular smooth muscle contractility including dissection skills, buffers, temperature of solutions, optimal resting tension, age of animal and isolation time. Tissue need to be handled with care and forceps is used for gentle removal of connective tissue surrounding the tissue without stretching the vessel. Appropriate buffer should be used for the experiment as both acidosis and alkalosis can affect tissue viability. If bicarbonate buffer is used, pH should be maintained by aeration with carbondioxide. HEPES buffer can also be used instead of bicarbonate buffer, where pH is neutralized to 7.4, with sodium hydroxide. Low temperature of dissecting media can slow down metabolic process and hence chilled media at around 4°C can be used for dissection. Optimal resting tension depends on type of artery and species of animal from which artery is isolated. The preload applied to an artery is an important factor to get maximal contractile response and hence need to be determined accurately. Age of the animal can also affect vascular function, as ageing can cause structural changes in blood vessel. Report has shown that endothelium-mediated vasodilator response to acetyl-choline progressively decreases with age in rat pulmonary artery. Hence, it is important to consider the age of animals used for experiment. Tissue isolation time need to be kept minimal as far as possible (49).

Classical method using isometric force transducer was used for all experiments in this study and recording was done using powerlab data acquisition system. Protocol followed for isolated vessel experiment was based on paper published by renu *et al* from our department. Isolated vessel from goat leg, instead of using as a whole strip or ring preparations as followed by most other studies, was cut into a spiral strip with the picture

that long axis of smooth muscle cells is parallel to spiral orientation, so that the vascular contraction can develop more fully. To achieve this, the orientation of spiral strip must be identical between the vessels (50)(49)(18).

In study reported by filippi et al, relaxant response of phenylephrine was seen with nanomolar concentration, and claimed that such relaxation occurs through alpha-1D receptor subtype, which is endothelium dependent, in a pre-contracted rat mesenteric artery with thrombaxane mimetic, U46619 (19). But the study published in plos one by our department gives evidence that phenylephrine also induce relaxation from basal tone in goat artery strip, in micromolar concentration and might have therapeutic implications in pathological states with high NO level like septic shock. Study also claimed that relaxation induced by micromolar concentration of phenylephrine was through alpha adrenergic receptor and not via beta receptor. The relaxant effect was NO-dependent and cGMP-independent (18). Thus, alpha agonist, decreasing vascular tension under certain circumstances is of great concern as they are used as a vasoconstrictor agent in various pathological states including septic shock. Hence, the receptor subtype responsible for such relaxation need to be delineated as it might contribute to therapeutic goals. Aim of current study is to find out the alpha adrenergic receptor subtype responsible for phenylephrine induced vasorelaxation by using micromolar concentration in goat artery strip. Objective mentioned in the study will be achieved by adding different receptor subtype-specific blockers into organ bath in which goat artery strip will be mounted and results will be interpreted based on responses to each receptor blockers.

MATERIALS AND METHODS

This study was given approval by the Institutional review board (IRB) of Christian medical college (IRB no: 9773, dated 10.11.2015). Study was done in the department of physiology, Christian medical college from a period of June, 2016 to June, 2017.

Materials required for isolated vessel experiment

- Double-jacketed organ bath
- Circulating water bath at 37°C
- Mammalian ECF solution
- White board
- Iris scissor – 1
- Toothed forces – 1
- Micro forceps straight - 1
- Surgical blade (22 size)
- Two petri dishes filled with mammalian ECF solution
- Thread
- Pipette tip (100ul) to make loop
- Micropipette with pipette tips (1000µl and 100µl) to add drugs
- Carbogen cylinder
- Force transducer
- Power lab data acquisition system
- Laptop

Fig 1: Powerlab data acquisition system

Stock solutions

Stock solution of 10mM (millimolar) concentration was prepared for each of the following drugs except potassium chloride (KCl), which was prepared as 1M (molar) concentration.

- NO donor, L-Arginine hydrochloride
- α receptor agonist, Phenylephrine hydrochloride
- α-1 blocker, prazosin hydrochloride
- α-1A blocker, RS-17053 hydrochloride
- α-1B blocker, chloroethylclonidine dihydrochloride (CEC)
- α-1D blocker, BMY 7378 dihydrochloride

- α-2 blocker, yohimbine hydrochloride

Stock solution for phenylephrine, L-arginine and KCl was prepared newly each time, on the day of experiment. Stock solution for other drugs was prepared and kept aliquoted, stored at -20°C, which was used as and when required.

Composition of normal extracellular solution

NaCl	100mmol/L
KCl	3mmol/L
$CaCl_2$	1.3mmol/L
NaH_2PO_4	0.5mmol/L
Na_2HPO_4	2mmol/L
$NaHCO_3$	25mmol/L
$MgCl_2$	2mmol/L
HEPES buffer	10mmol/L
Glucose	5mmol/L

pH was titrated to 7.4 by using 1M sodium hydroxide. 5 liters of solution was prepared at a time and stored at 4°C.

Fig 2: Force transducer

Methods

Isolated vessel preparation

Fresh goat leg was procured from a registered slaughter house on the day of experiment. Further procedure was started within 5 to 10 minutes of obtaining the leg. Additional legs, if available, which may be used for the second experiment on the same day, was stored at 4°C and it was used within 2 to 3 hours. Required solutions and drugs are prepared beforehand. Once the leg was obtained, cleaned under running tap water and kept on a white board for dissection. Two petri dishes filled with cold extracellular fluid (ECF) solution are kept ready before starting the dissection. Using surgical blade, skin was removed from the goat leg. Skin was dissected superficially, without damaging the underlying vessel. A medium-sized artery was identified and traced. The vessel was confirmed as artery by the patency of lumen, as a vein has collapsed lumen. Then the artery was dissected out with minimal manipulation and as lengthier as possible.

Immediately after dissection, the arterial strip was placed in a petri dish containing cold ECF solution. The metabolic rate of vascular smooth muscle cell will be decreased by chilled ECF solution and hence prolong the viability of tissue (49). Then, the extraneous connective tissue was removed with forceps and iris scissor, while the artery was still kept immersed in chilled ECF solution. Viability may be affected if the tissue was not kept immersed in ECF solution. Artery was completely cleaned off extraneous tissue as it may affect the results. After cleaning, artery was then transferred to the second petri dish which was filled with ECF solution. Isolated artery was then cut into small strips of 2 to 2.5cm in length, devoid of any side branches, as required for one experiment. Iris scissor was then introduced into the lumen of an arterial strip and spiral cut was made. Thus, the lumen was cut open in a spiral orientation, exposing the endothelium of vessel. The remaining arterial strips can be utilized for the subsequent experiments, if used within 2-3 hours of isolation and hence stored at 4°C. A pipette tip (100µl) was used to make a loop with thread at one end of the spiral strip while a knot was made at other end of strip, leaving a longer thread, enough to be connected with force transducer. The spiral strip was then mounted in organ bath of 25ml capacity. Loop was secured to a hook at the bottom of organ bath, while the other end was connected to a force transducer. The organ bath was filled with ECF solution, maintained at a temperature of 37°C by circulating water-bath and aerated with carbogen (95% oxygen and 5% carbondioxide). Force transducer was connected to the powerlab data acquisition system and recordings were made on a laptop.

Fig 3: Spiral strip of goat artery in ECF solution with a loop made at one end

Solutions required for experiment

All salts for the ECF solution was purchased from SIGMA. L-Arginine hydrochloride, prazosin hydrochloride, Phenylephrine hydrochloride, BMY 7378 dihydrochloride, Yohimbine hydrochloride, Chloroethylclonidine dihydrochloride, L-NNA, Forskolin and PMA was purchased from SIGMA. RS-17053 hydrochloride was purchased from TOCRIS BIOSCIENCE. 1000 times stock solution was prepared and appropriate concentration of the drug was added to organ bath to attain the final concentration. L-Arginine hydrochloride, BMY 7378 dihydrochloride, Phenylephrine hydrochloride, Yohimbine hydrochloride, Chloroethylclonidine dihydrochloride, PMA and KCl was dissolved in distilled water. RS-17053 hydrochloride and Indomethacin was dissolved in ethanol. prazosin hydrochloride was dissolved in methanol. L-NNA was dissolved in

1mol/L HCl and pH was adjusted to 7.4 with 1mol/L NaOH. Forskolin was dissolved in DMSO (Dimethyl sulfoxide).

L-Arginine hydrochloride

Molecular weight is 210.7 g/mol

0.02g was added to 10ml of distilled water to get 10mM stock solution

1000µl of 10mM stock solution was added to 25ml of ECF to get a final required concentration of 400µmol/L

Phenylephrine hydrochloride

Molecular weight is 203.67 g/mol

0.02g was added to 10ml of distilled water to get 10mM stock solution

250µl of 10mM stock solution was added to 25ml of ECF to get a final required concentration of 100µmol/L

Prazosin hydrochloride

Molecular weight is 419.86 g/mol

10mg was added to 2.4ml of methanol to get 10mM stock solution and was stored as aliquots, of 250µl each, at -20°C. One aliquot was added to 25ml of ECF to get a final

required concentration of 100µmol/L.

RS-17053 hydrochloride

Molecular weight is 449.42 g/mol

10mg was added to 2.2ml of ethanol to get 10mM stock solution and was stored as aliquots, of 250µl each, at -20°C. One aliquot was added to 25ml of ECF to get a final required concentration of 100µmol/L.

Chloroethylclonidine dihydrochloride (CEC)

Molecular weight is 408.58 g/mol

10mg was added to 2.5ml of distilled water to get 10mM stock solution and was stored as aliquots, of 250µl each, at -20°C. One aliquot was added to 25ml of ECF to get a final required concentration of 100µmol/L.

BMY 7378 dihydrochloride

Molecular weight is 458.42 g/mol

10mg was added to 2.18ml of distilled water to get 10mM stock solution and was stored as aliquots, of 250µl each, at -20°C. One aliquot was added to 25ml of ECF to get a final required concentration of 100µmol/L.

Yohimbine hydrochloride

Molecular weight is 390.90 g/mol

10mg was added to 2.6ml of distilled water to get 10mM stock solution and was stored as

aliquots, of 250µl each, at -20°C. One aliquot was added to 25ml of ECF to get a final required concentration of 100µmol/L.

L-NNA

Molecular weight is 219.2 g/mol

0.02g of L-NNA (dissolved in HCl and pH adjusted to 7.4 with NaOH) was added to 25ml of ECF to get a final required concentration of 1mmol/L.

Potassium chloride

Molecular weight is 74.55 g/mol

0.74g was added to 10ml of distilled water to get 1M stock solution

2000µl of 1M stock solution was added to 25ml of ECF to get a final required concentration of 80mmol/L.

Experiment protocol

After the spiral strip was mounted in an organ bath filled with ECF solution, power-lab data acquisition system and laptop was switched on. Force transducer, which senses changes in vascular tension, was connected to a powelab data acquisition system. Initial calibration was done with 10g weight, so as to get the recorded values in grams from millivolt. Appropriate maintenance of temperature and adequate supply of carbogen was ensured before starting the experiment. Initial preload applied was in the range of 0.2 to 0.3gram so that the thread becomes taut and then the arterial strip was allowed to stabilize

to its basal resting tone. Time taken for the arterial strip to stabilize was usually about 10 minutes. Once the tension was stabilized, drug of interest was added and waited for 5 minutes to notice the change in response as any change in tension can be appreciated within 5 minutes of adding the drugs. Twenty two different set of experiments are done which includes both control and intervention groups. Sample size for each of the experimental group was four to six. After equilibration period, 400μmol/L of L-Arginine was added into organ bath, followed by 100μmol/L phenylephrine and this was the control group. Any change, either vasoconstriction or vasorelaxation, was recorded as increase or decrease in vascular tension from the baseline respectively.

For the intervention group,

1. To test if such vasorelaxation induced by L-Arginine/PE combination is preventable with specific α_1 blocker (prazosin), specific α_{1A} blocker(RS 17053), specific α_{1B} blocker (chloroethylclonidine dihydrochloride), specific α_{1D} blocker (BMY 7378 dihydrochloride) and specific α_2 blocker (Yohimbine)
2. To test the response produced by phenylephrine with specific α_{1A} blocker(RS 17053), specific α_{1B} blocker (chloroethylclonidine dihydrochloride), specific α_{1D} blocker (BMY 7378 dihydrochloride) and specific α_2 blocker (Yohimbine), in the absence of L-Arginine. . If any of the subtype specific blockers in these set of experiments induce reduction in vascular tension with phenylephrine, then Nω-Nitro-L-arginine (L-NNA), an eNOS blocker was added to see if such relaxation is NO-dependent

3. To test the response produced by phenylephrine with combinations of subtype selective blockers in the absence of L-Arginine

After addition of each drug, comment was added immediately during the recording, which indicates the identity of drug added and its concentration. At the end of each experiment, ECF in organ bath, which contain various drugs added was drained off and refilled with new ECF solution, into which 80mmol/L potassium chloride was added to check the tissue viability. If the tissue was viable, increase in vascular tension was noted and results are included in the analysis. If KCl does not show increase in vascular tension, recordings obtained from such tissue was not included in analysis as the tissue was considered to be in-viable. Precaution was taken not to disturb the thread which was connected to force transducer, while drug was added or when the organ bath was re-filled with ECF solution for wash. There may be increase in noise if the thread is disturbed during recording.

Data was recorded at a frequency of 1000 hertz by powerlab data acquisition system. Text file was obtained from the data pad option in software. Text file was then exported to Igor pro software for further analysis. After the curve was smoothened to remove noise in Igor pro software using smoothing function, a graph was obtained with time (in hr:min) along X-axis and tension (in gram) along Y-axis. Values are noted down in an excel spreadsheet and any significance of results was further computed using statistical analysis.

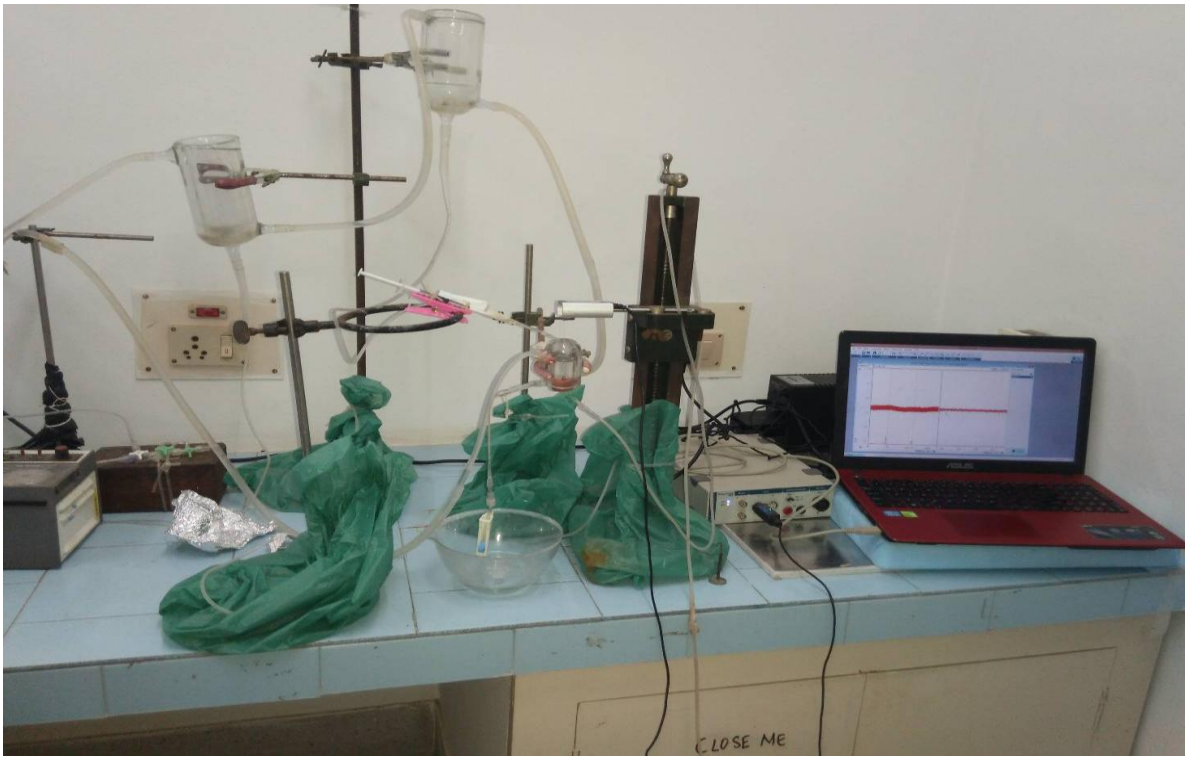


Fig 4: Experimental setup

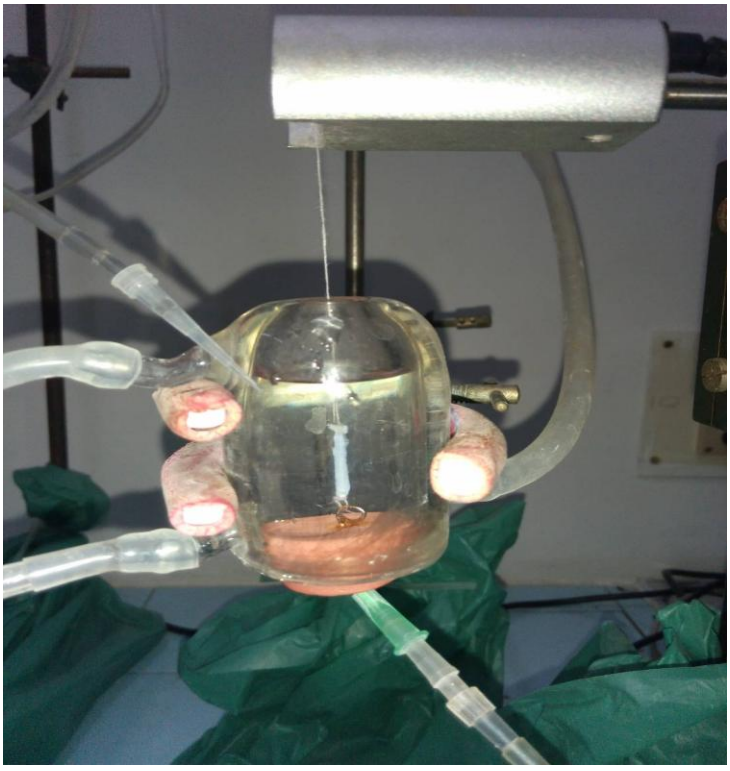
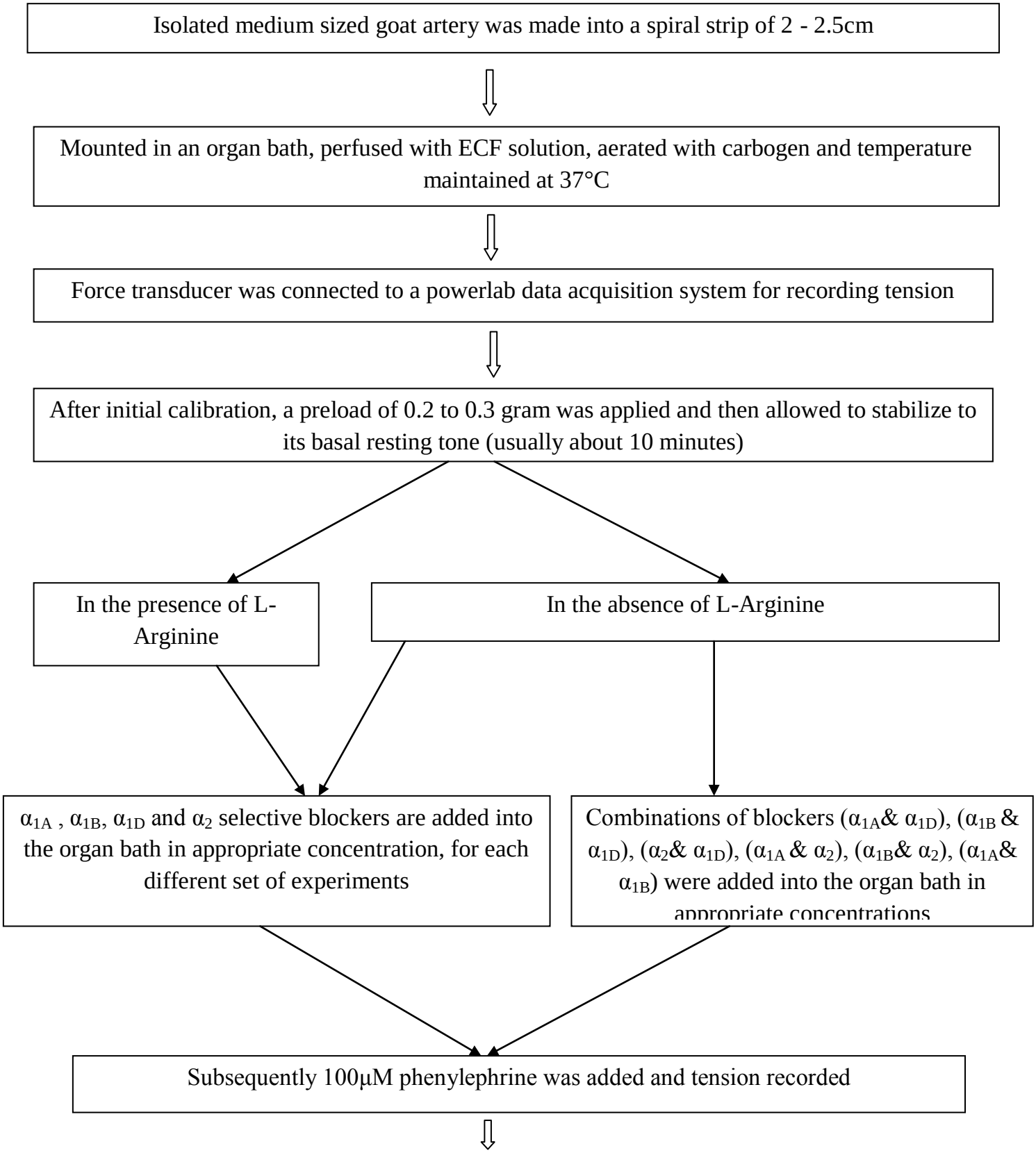


Fig 5: Spiral strip mounted in an organ bath filled with ECF

STATISTICAL ANALYSIS

The statistical analysis was done using SPSS software (version 16.0). The change in vascular tension before and after addition of drug within a group was compared using Wilcoxon's signed rank (WSR) test. The difference of percent change in vascular tension in the presence of specific blockers in intervention group was compared with the control group using Mann-Whitney U (MWU) test. P value of < 0.05 was considered as statistically significant. The results are expressed as scatter plots with median. Scatter plots are done using GraphPad prism software (version 6).


Isolated medium sized goat artery was made into a spiral strip of 2 - 2.5cm
Mounted in an organ bath, perfused with ECF solution, aerated with carbogen and temperature maintained at 37°C
Force transducer was connected to a powerlab data acquisition system for recording tension
After initial calibration, a preload of 0.2 to 0.3 gram was applied and then allowed to stabilize to its basal resting tone (usually about 10 minutes)
In the presence of L-Arginine
In the absence of L-Arginine
α1A , α1B, α1D and α2 selective blockers are added into the organ bath in appropriate concentration, for each different set of experiments
Combinations of blockers (α1A& α1D), (α1B & α1D), (α2& α1D), (α1A & α2), (α1B& α2), (α1A& α1B) were added into the organ bath in appropriate concentrations
Subsequently 100µM phenylephrine was added and tension recorded

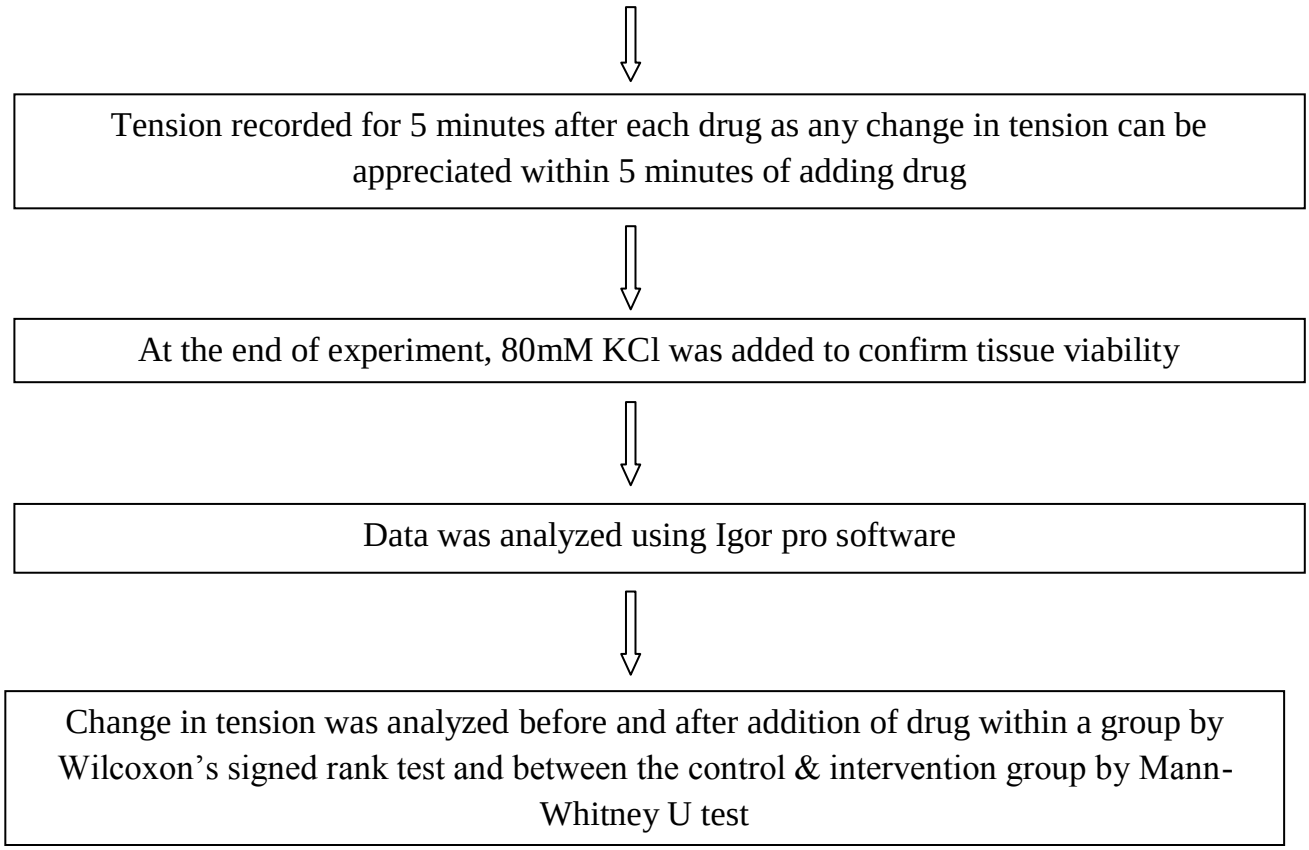


Fig 6: Detailed diagrammatic algorithm of the study

RESULTS

Change in tension was recorded by powerlab data acquisition system at a sampling rate of 1000Hz and data was analyzed using Igor pro software.

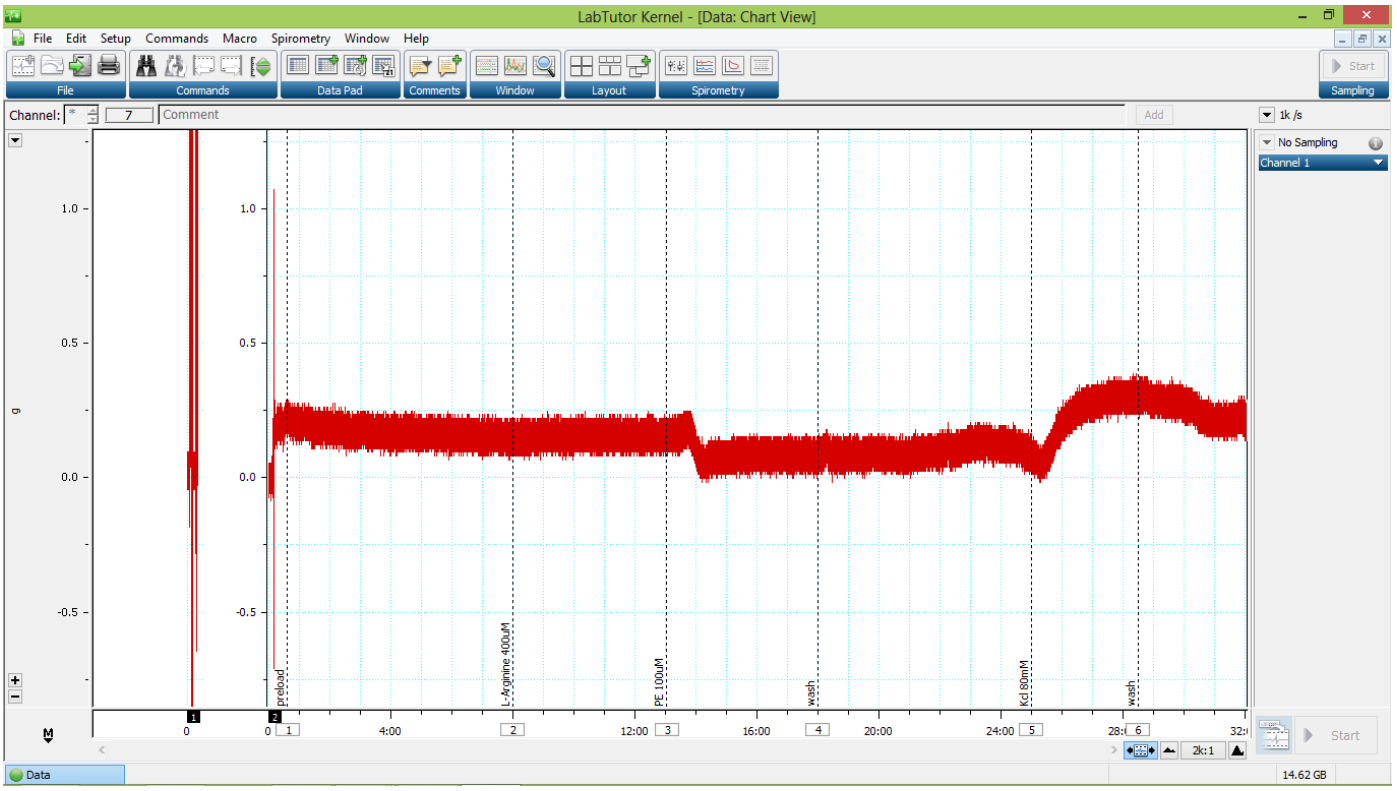
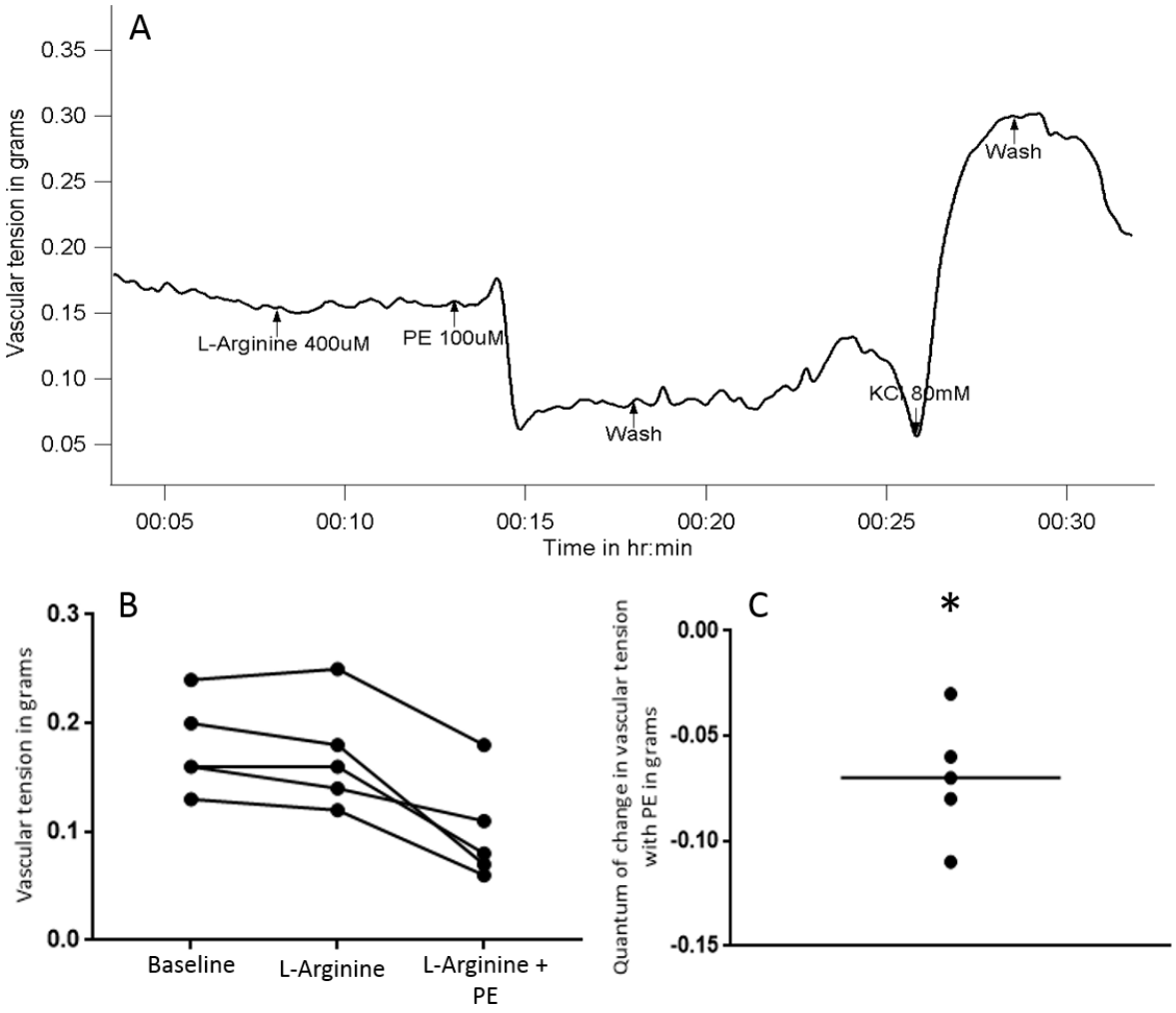


Fig 7: Powerlab data acquisition recording

Phenylephrine decreased vascular tension under high NO environment

While 400µmol/L L-Arginine, which is a NO donor *per se* did not decrease vascular tension, subsequent addition of 100µmol/L phenylephrine decreased vascular tension from 0.16 gram to 0.08 gram (median, n = 5).

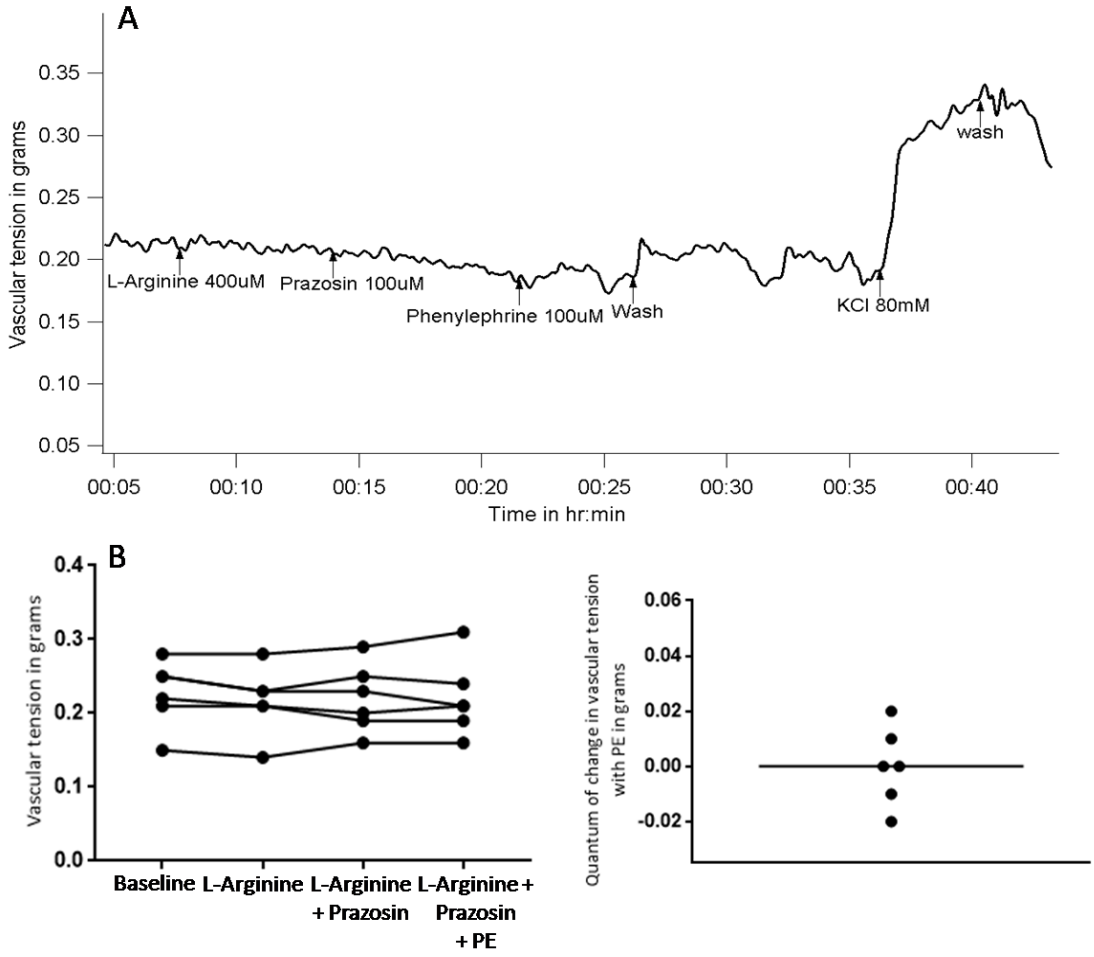
Tissue viability was confirmed by vasoconstriction, caused by the addition of 80mmol/L KCl. Exposure of smooth muscle to high potassium can cause membrane depolarization and thereby increases calcium influx through voltage-dependent calcium channels which produce contraction (49).



PE-induced vasorelaxation under high NO environment is through alpha 1 adrenergic receptor

L-Arginine/PE combination could not reduce vascular tension in the presence of 100μmol/L prazosin, an alpha 1 blocker. The vascular tension prior to the addition of PE (in the presence of L-Arginine & prazosin) was 0.22 gm and after addition of PE it was 0.21 gm (median, n=6, P= 1.0 with WSR test). (Fig 9)

There was a significant difference (P = 0.006 with MWU) when percentage changes in vascular tension due to L-Arginine/PE combination with and without prazosin were compared. In the absence of prazosin, L-Arginine/PE combination reduced tension to 50 % of base line (median, n=5) while in the presence of prazosin, tension remained at 100 % (median, n=6).



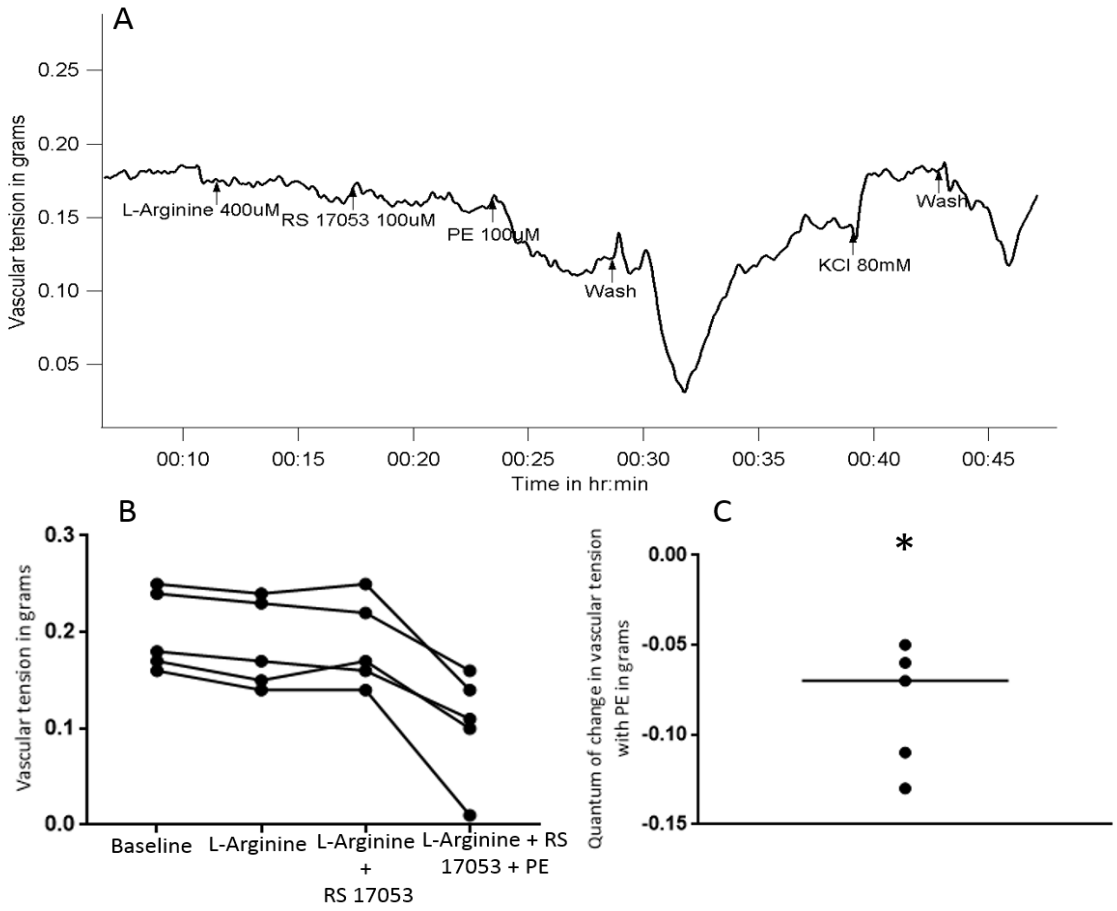
PE-induced vasorelaxation under high NO environment is through alpha-1D receptor subtype

Subtype of alpha 1 receptor responsible for PE-induced vasorelaxation under high NO environment was further delineated by adding subtype specific blockers. Reduction in

vascular tension with L-Arginine/PE combination was not prevented by 100μmol/L RS 17053, an alpha-1A blocker and 100 Chloroethylclonidine dihydrochloride (CEC), an alpha-1B blocker but was prevented by 100μmol/L alpha-1D blocker, BMY 7378 dihydrochloride.

The vascular tension prior to addition of PE (in the presence of L-Arginine & RS 17053) was 0.17 gm and after addition of PE it was 0.11 gm (median, n=5, p=0.043 with WSR test). (Fig 10)

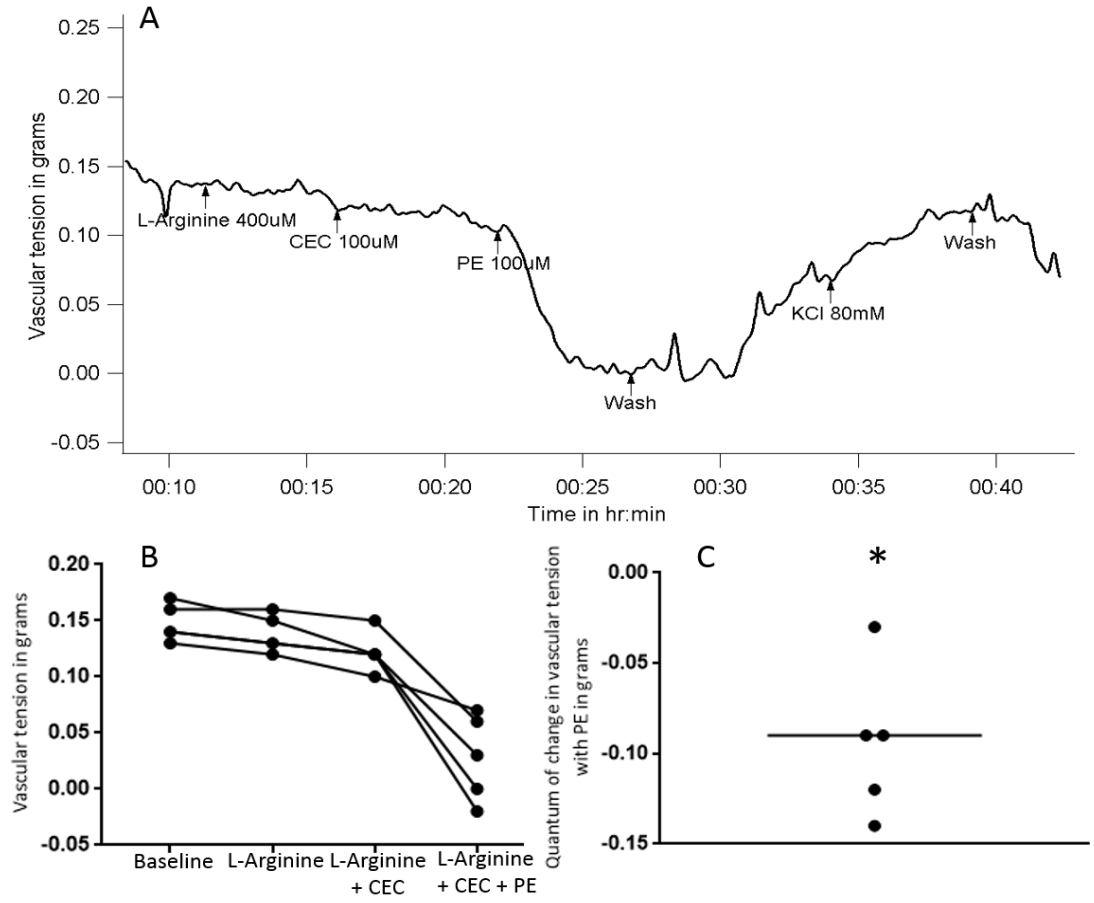
There was no significant difference (P = 0.917 with MWU test) when percentage changes in vascular tension due to L-Arginine/PE combination with and without RS 17053 were compared. In the absence of RS 17053, L-Arginine/PE combination reduced tension to 50 % of base line (median, n=5) while in the presence of RS 17053, tension was reduced to 58.82 % (median, n=5) of baseline.



Vascular tension prior to the addition of PE (in the presence of L-Arginine & CEC), was 0.12 gm and after addition of PE it was 0.03 gm (median, n=5, p=0.042 with WSR test). (Fig 11)

In the absence of CEC, L-Arginine/PE combination reduced tension to 50 % of base line (median, n=5) while in the presence of CEC, tension was 25% (median, n=5) of

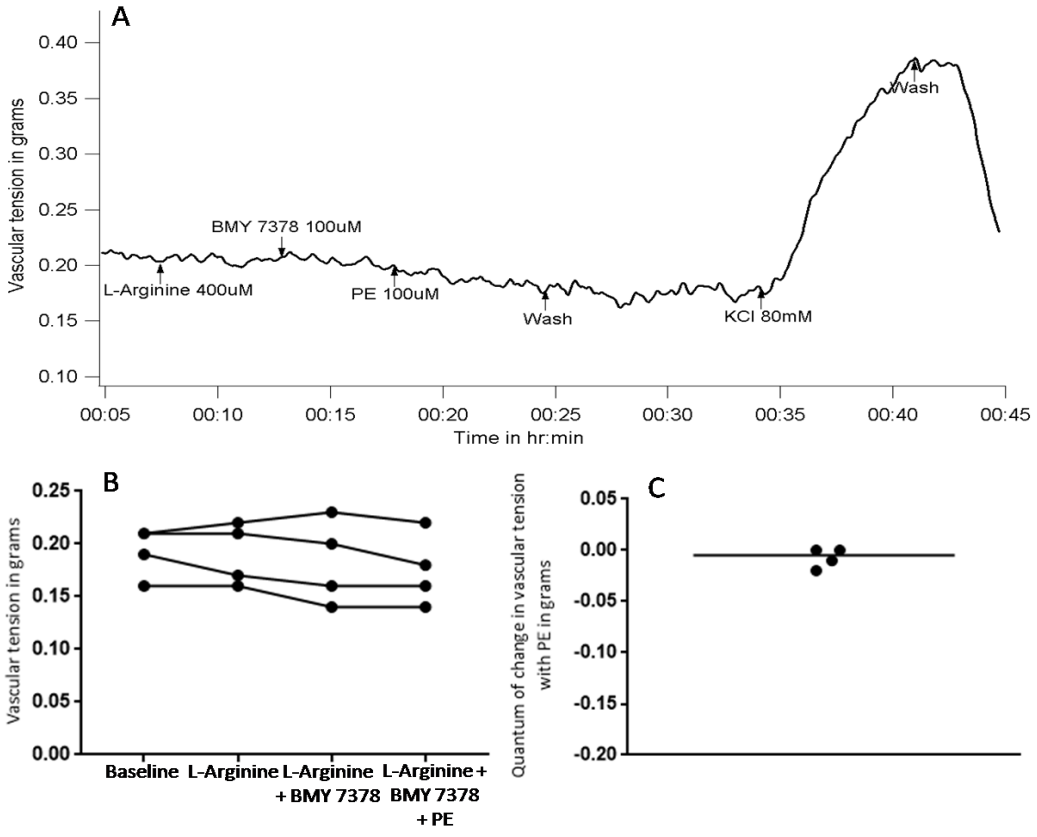
baseline. However, at this sample size, there was no significant difference when percentage changes in vascular tension due to L-Arginine/PE combination with and without CEC were compared (P=0.075 with MWU test).



Vascular tension prior to the addition of PE, in the presence of L-Arginine & BMY 7378 was 0.18 gm and after addition of PE it was 0.17 gm (median, n=4, p=0.180

WSR test). (Fig 12).

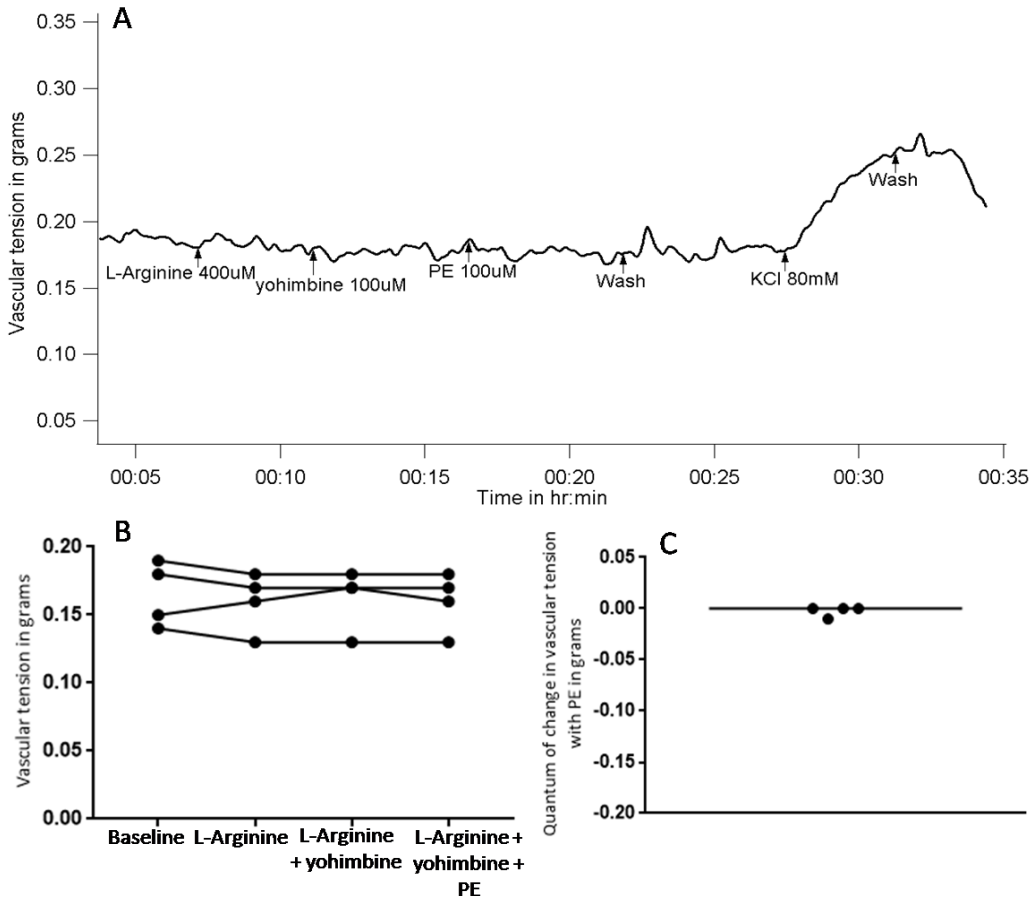
There was a significant difference (P = 0.014 with Mann-Whitney U test) when percentage changes in vascular tension due to L-Arginine/PE combination with and without BMY 7378 were compared. In the absence of BMY 7378, L-Arginine/PE combination reduced tension to 50% of base line (median, n=5) while in the presence of BMY 7378, tension remained at 97.83% (median, n=4) of baseline.



PE-induced vasorelaxation under high NO environment was also prevented by alpha 2 receptor blocker, Yohimbine

PE-induced vasorelaxation under high NO environment was prevented by 100μmol/L yohimbine, an alpha-2 blocker. Vascular tension prior to addition of PE (in the presence of L-Arginine & yohimbine) was 0.17 gm and after addition of PE it was still 0.17 gm (median, n=4, p=0.317 with WSR test). (Fig 13)

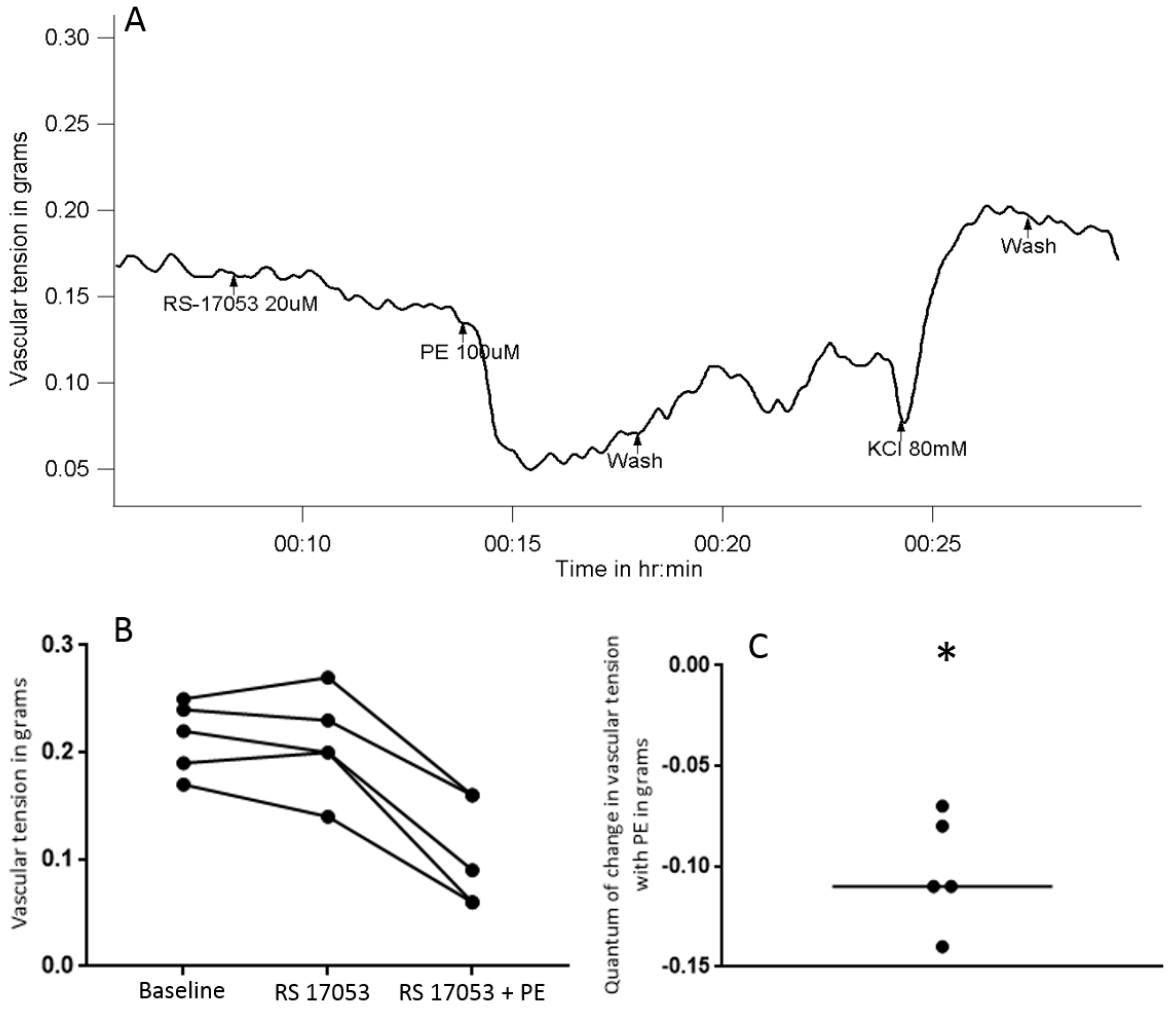
There was a significant difference (P = 0.012 with MWU test) when percentage changes in vascular tension due to L-Arginine/PE combination with and without yohimbine were compared. In the absence of yohimbine, L-Arginine/PE combination reduced tension to 50 % of base line (median, n=5) while in the presence of yohimbine, tension remained at 100 % (median, n=4).



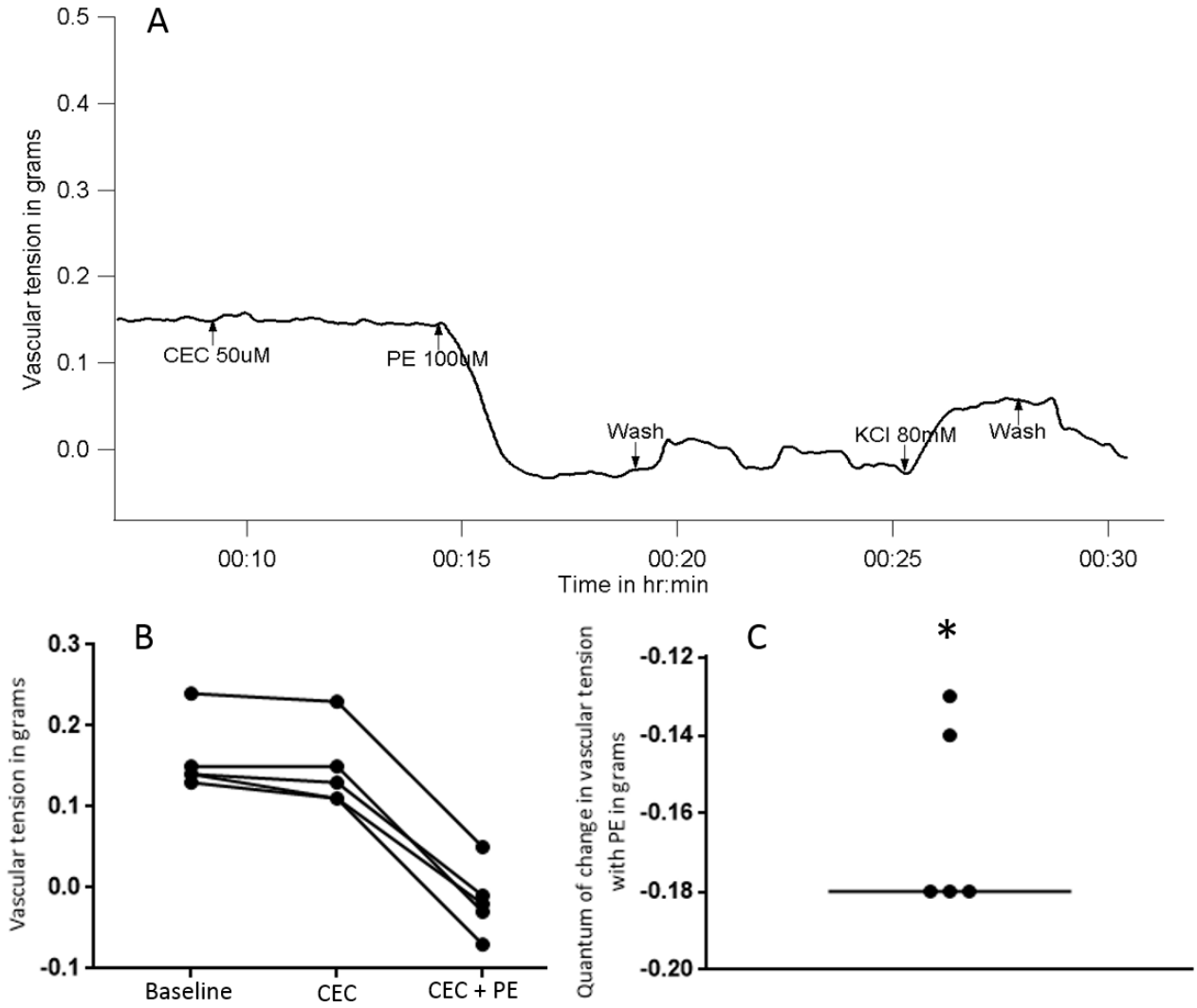
Even without a high NO environment, PE decreased vascular tension with blockade of alpha-1A & alpha-1B adrenergic receptors, but not with alpha 1D blockade

Even under conditions where high nitric oxide environment was not provided, PE decreased vascular tension when alpha-1A & alpha-1B were blocked. Alpha-1D blockade resulted in lack of response (either contraction or relaxation) to PE.

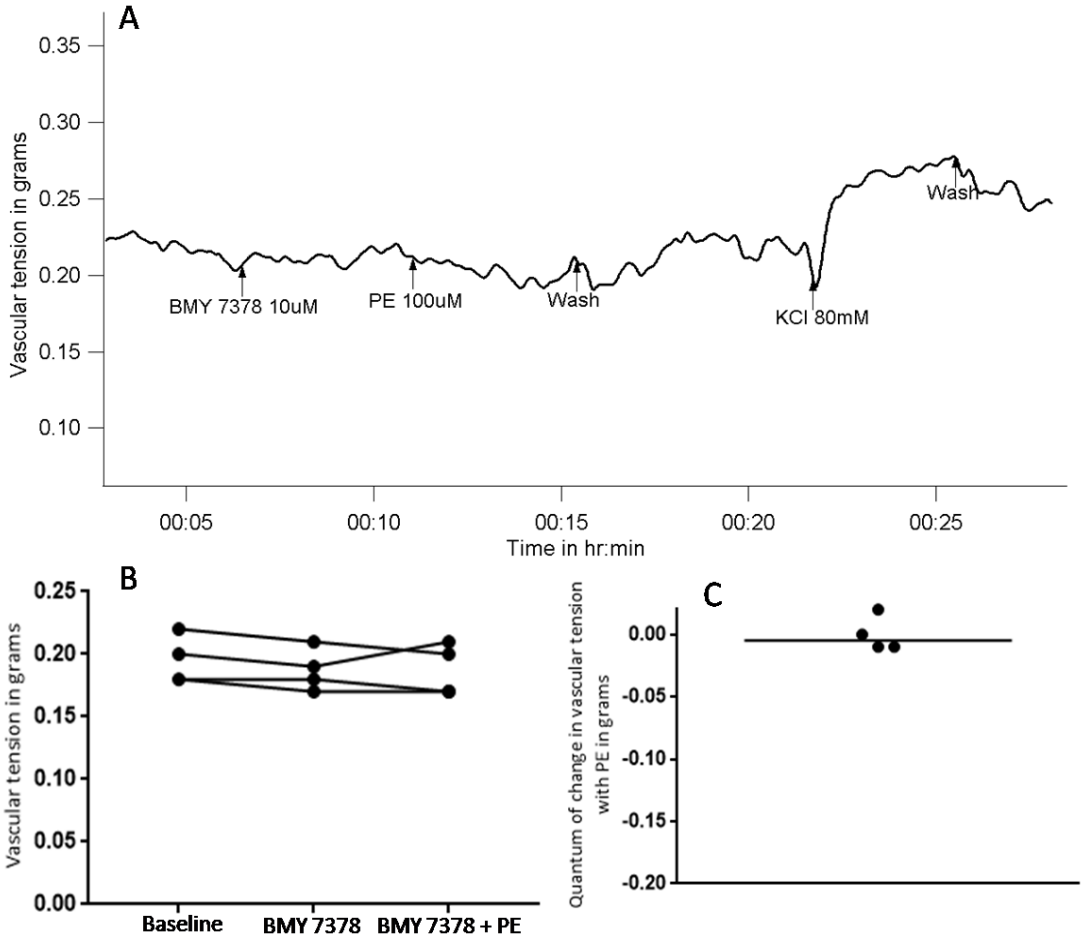
Vascular tension prior to the addition of PE (in the presence of 20µmol/L RS 17053) was 0.2 gm and after addition of PE it was 0.09 gm (median, n=5, p=0.042 with WSR test). (Fig 14)



Vascular tension prior to the addition of PE (in the presence of 50µmol/L CEC) was 0.13 gm and after addition of PE it was -0.02 gm (median, n=5, p=0.039 with WSR test). (Fig 15)

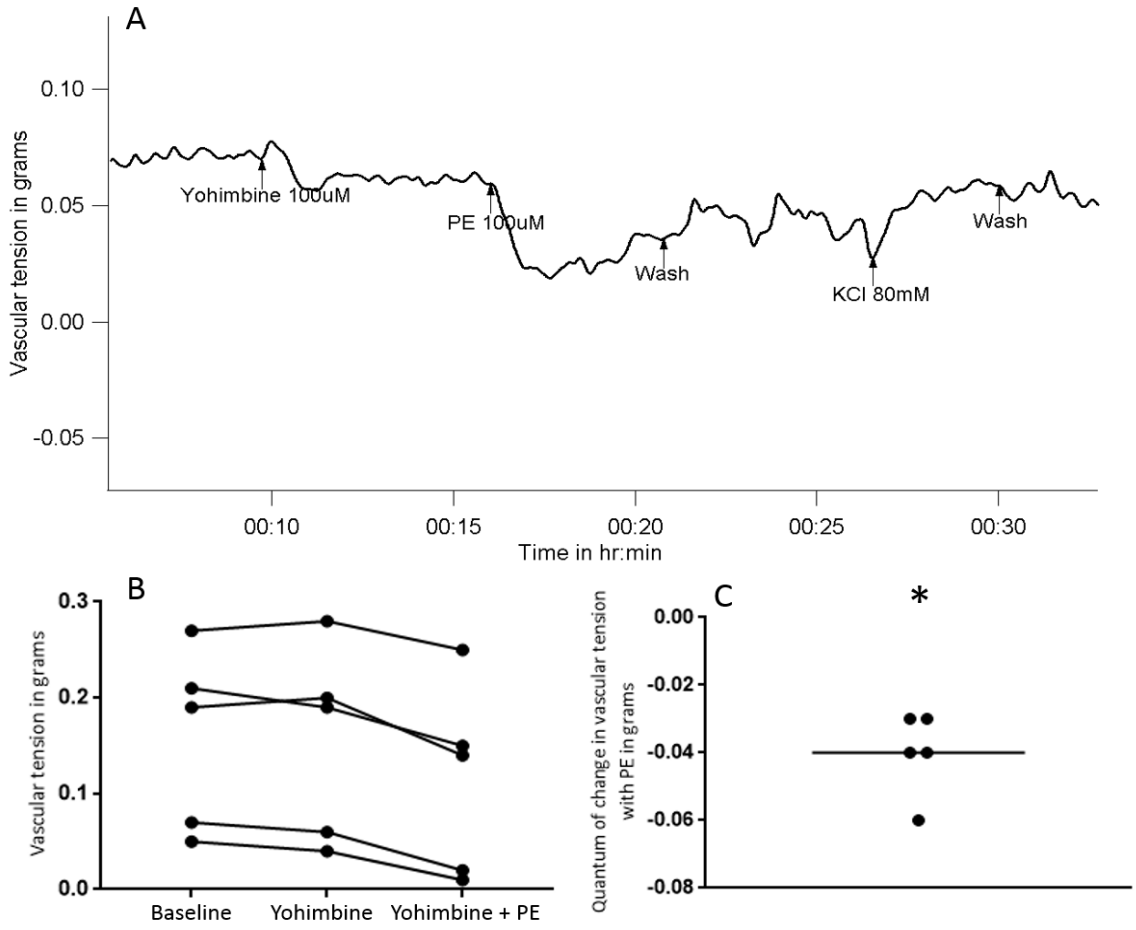


PE could not change tension in the presence of alpha-1D blocker, BMY 7378. Vascular tension prior to the addition of PE (in the presence of 10µmol/L BMY 7378) was 0.19 gm and after addition of PE, it remained at 0.19 gm (median, n=4, p=1.0 with WSR test). (Fig 16)



In the absence of a high NO environment, alpha 2 blockade favoured reduction in vascular tension with PE

Alpha-2 adrenoceptor blockade with yohimbine led to decrease in vascular tension with PE. Vascular tension prior to the addition of PE (in the presence of 100µmol/L yohimbine) was 0.19 gm and after addition of PE it was 0.14 gm (median, n=5, p=0.041 with WSR test). (Fig 17)

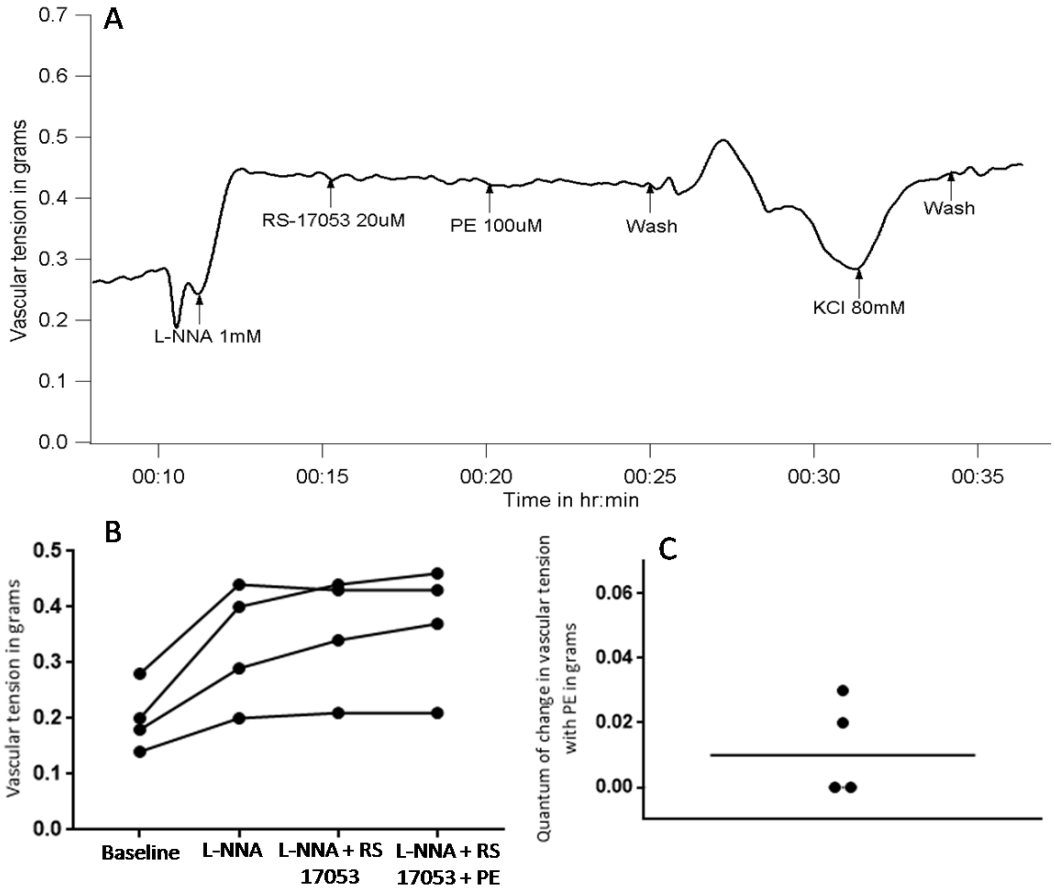


PE-induced vasorelaxation under normal NO environment in the presence of alpha-1A, 1B & alpha 2 receptor blockade was blocked by L-NNA, an inhibitor of eNOS

L-NNA is an inhibitor of endothelial nitric oxide synthase (eNOS). PE-induced reduction in vascular tension with RS 17053 (alpha-1A receptor blocker), CEC (alpha-1B receptor blocker) and Yohimbine (alpha-2 blocker) was prevented by 1mmol/L L-NNA, in each case.

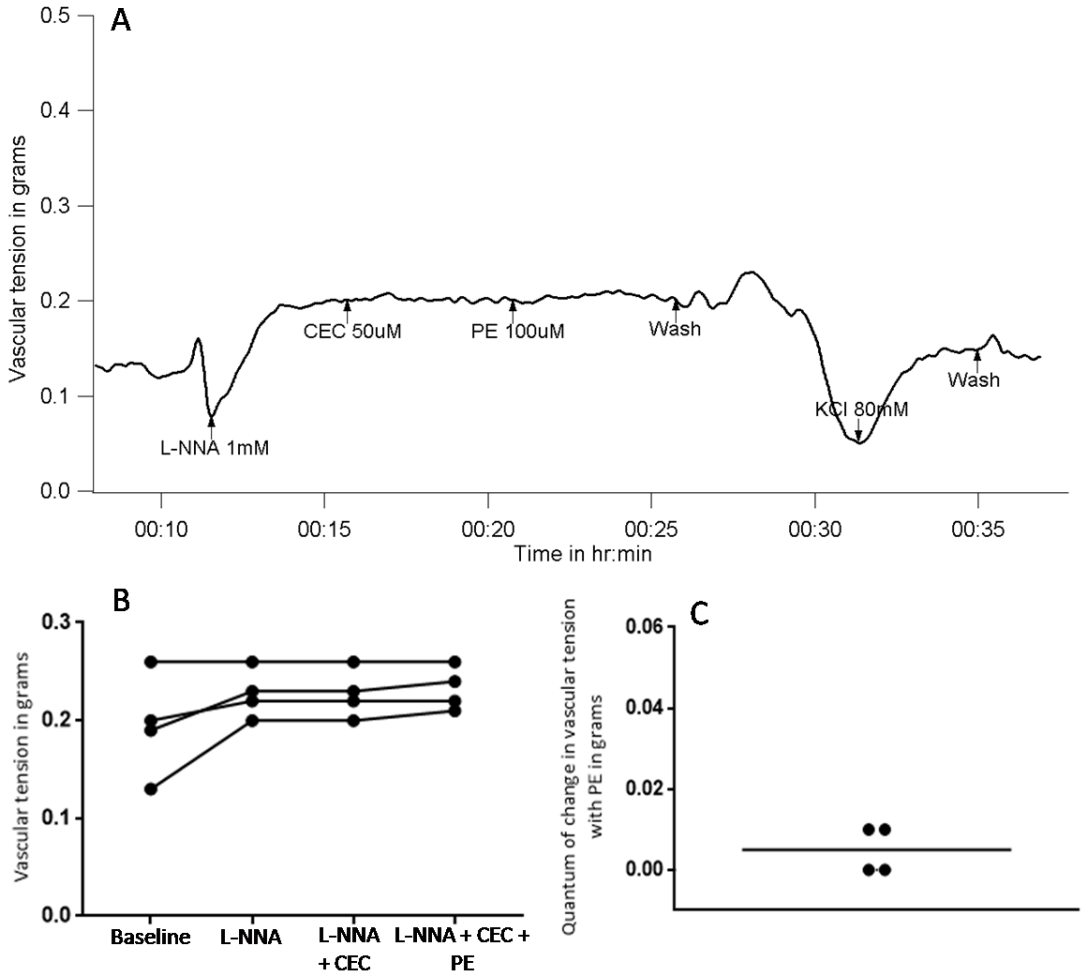
Addition of L-NNA increased tension from 0.19 gm to 0.35 gm (median, n=4). Subsequent addition of RS 17053 & PE did not change tension further. The vascular tension prior to the addition of PE (in the presence of L-NNA & RS 17053) was 0.39 gm and after addition of PE it remained at 0.4 gm (median, n=4, p=0.180 with WSR test). (Fig 18)

There was a significant difference (P = 0.014 with MWU test) when percentage changes in vascular tension due to RS 17053/PE combination with and without L-NNA were compared. In the absence of L-NNA, RS 17053/PE combination reduced tension to 45% of base line (median, n=5) while in the presence of L-NNA, tension remained at 102.27% (median, n=4).



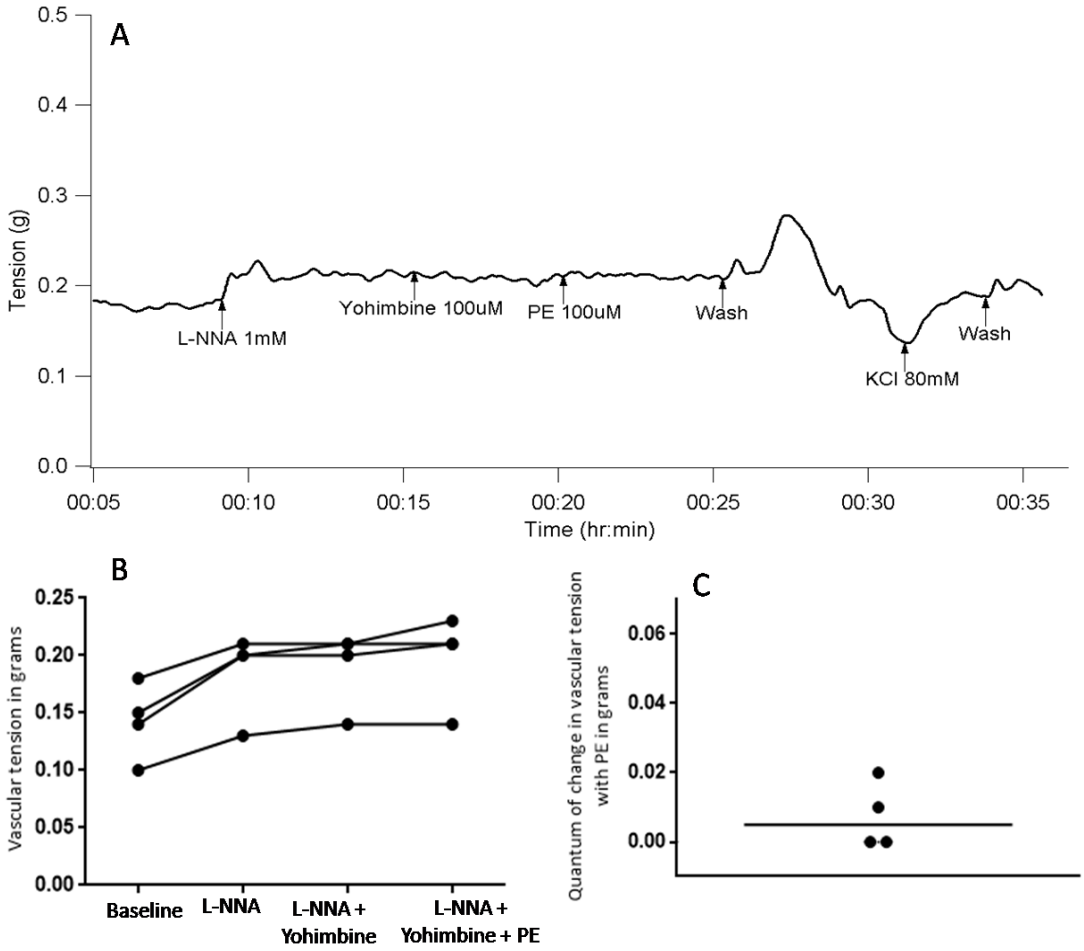
In another set of experiments, while L-NNA increased tension from 0.20 gm to 0.23 gm (median, n=4), subsequent addition of CEC & PE did not change tension any further. Vascular tension prior to addition of PE (in the presence of L-NNA & CEC) was 0.23 gm and after addition of PE it remained at 0.23 gm (median, n=4, p=0.157 with WSR test). (Fig 19)

There was a significant difference (P = 0.014 with MWU test) when percentage changes in vascular tension due to CEC/PE combination with and without L-NNA were compared. In the absence of L-NNA, CEC/PE combination reduced tension to -18.18 % of base line (median, n=5) while in the presence of L-NNA, tension remained at 102.17 % (median, n=4).



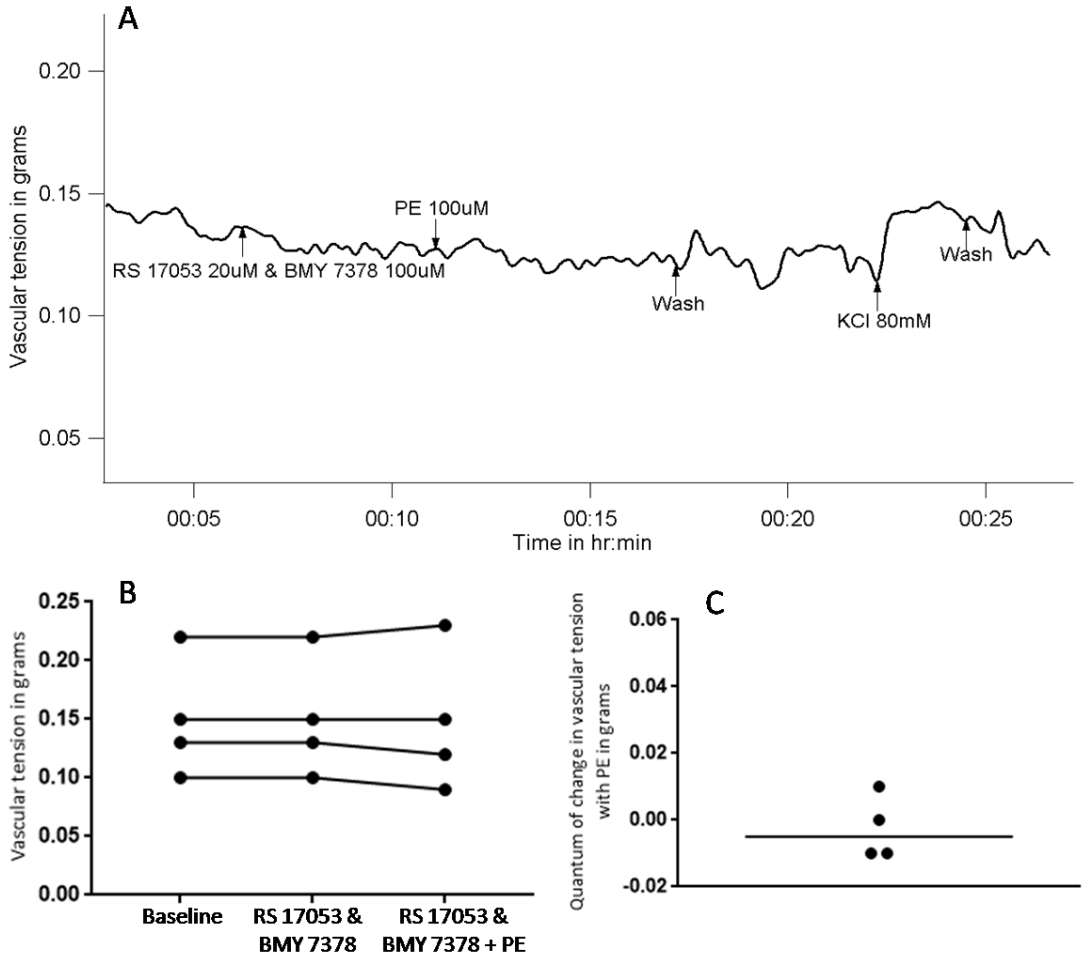
In another set of experiments, while L-NNA increased tension from 0.15 gm to 0.20 gm (median, n=4), subsequent addition of Yohimbine & PE did not change tension any further. Vascular tension prior to addition of PE (in the presence of L-NNA & Yohimbine) was 0.21 gm and after addition of PE it remained at 0.21 gm (median, n=4, p=0.180 with WSR test). (Fig 20)

There was a significant difference (P = 0.014 with MWU test) when percentage changes in vascular tension due to Yohimbine/PE combination with and without L-NNA were compared. In the absence of L-NNA, Yohimbine/PE combination reduced tension to 70 % of base line (median, n=5) while in the presence of L-NNA, tension remained at 102.50% (median, n=4).

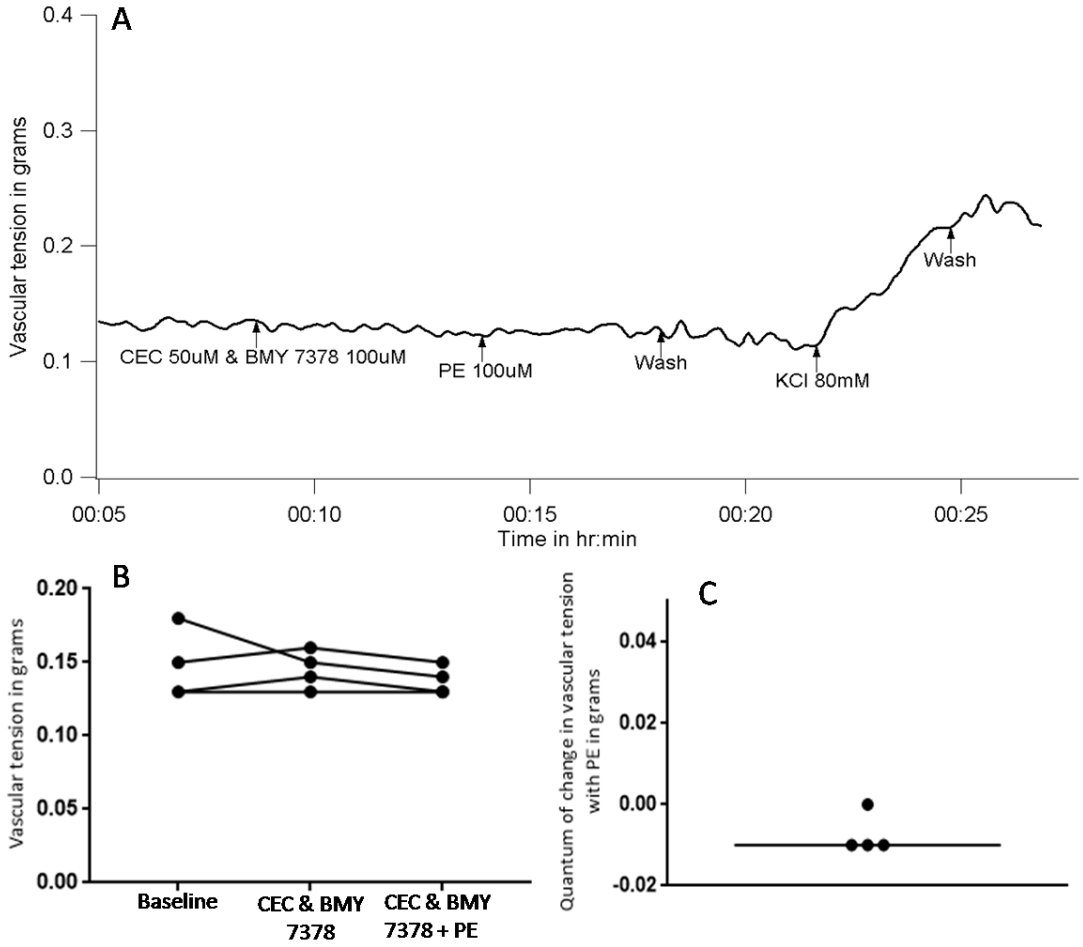


To emphasize the role of alpha-1D receptor in PE-induced vasorelaxation, various combinations of blockers were used.

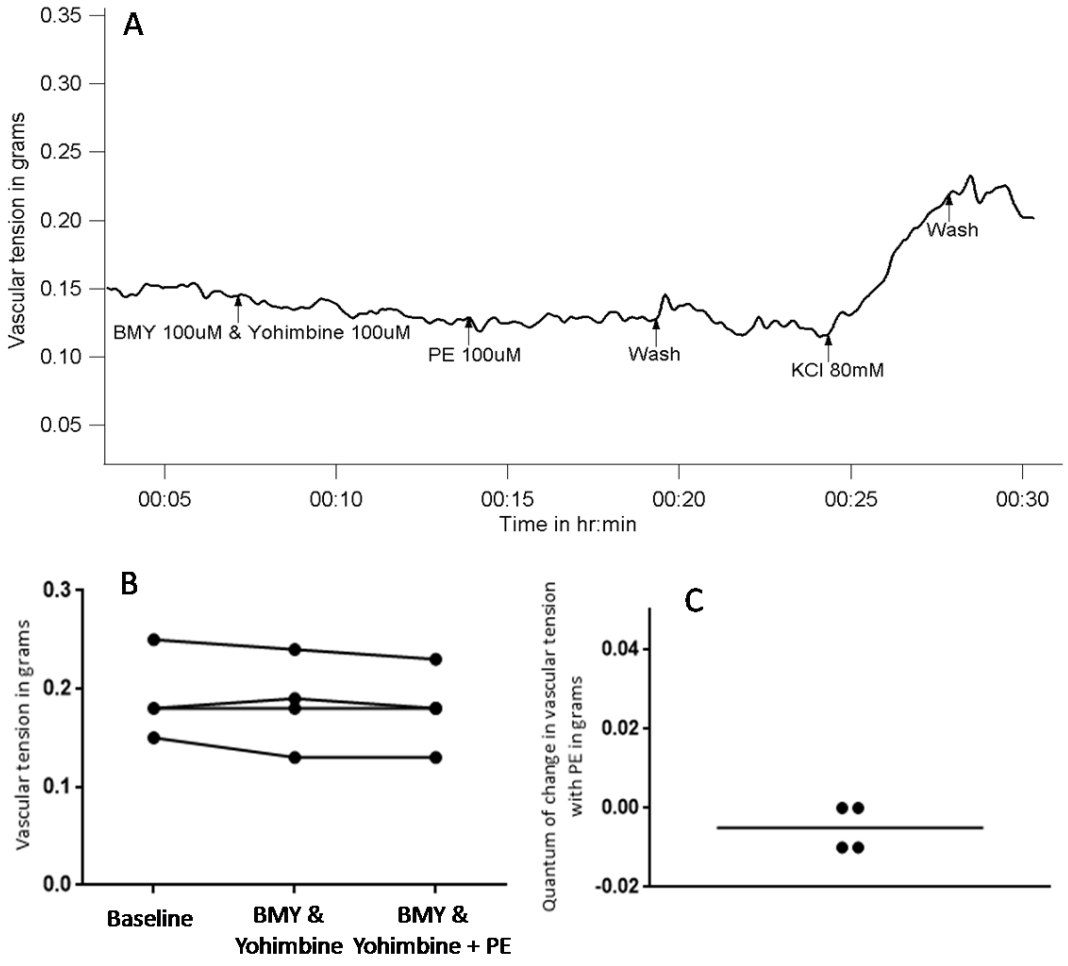
With simultaneous blockade of alpha-1A & alpha-1D receptors, PE was unable to induce reduction in vascular tension. Vascular tension prior to addition of PE (in the presence of RS 17053 & BMY 7378) was 0.14 gm and after addition of PE it remained at 0.14 gm (median, n=4, p=0.564 with WSR test). (Fig 21)



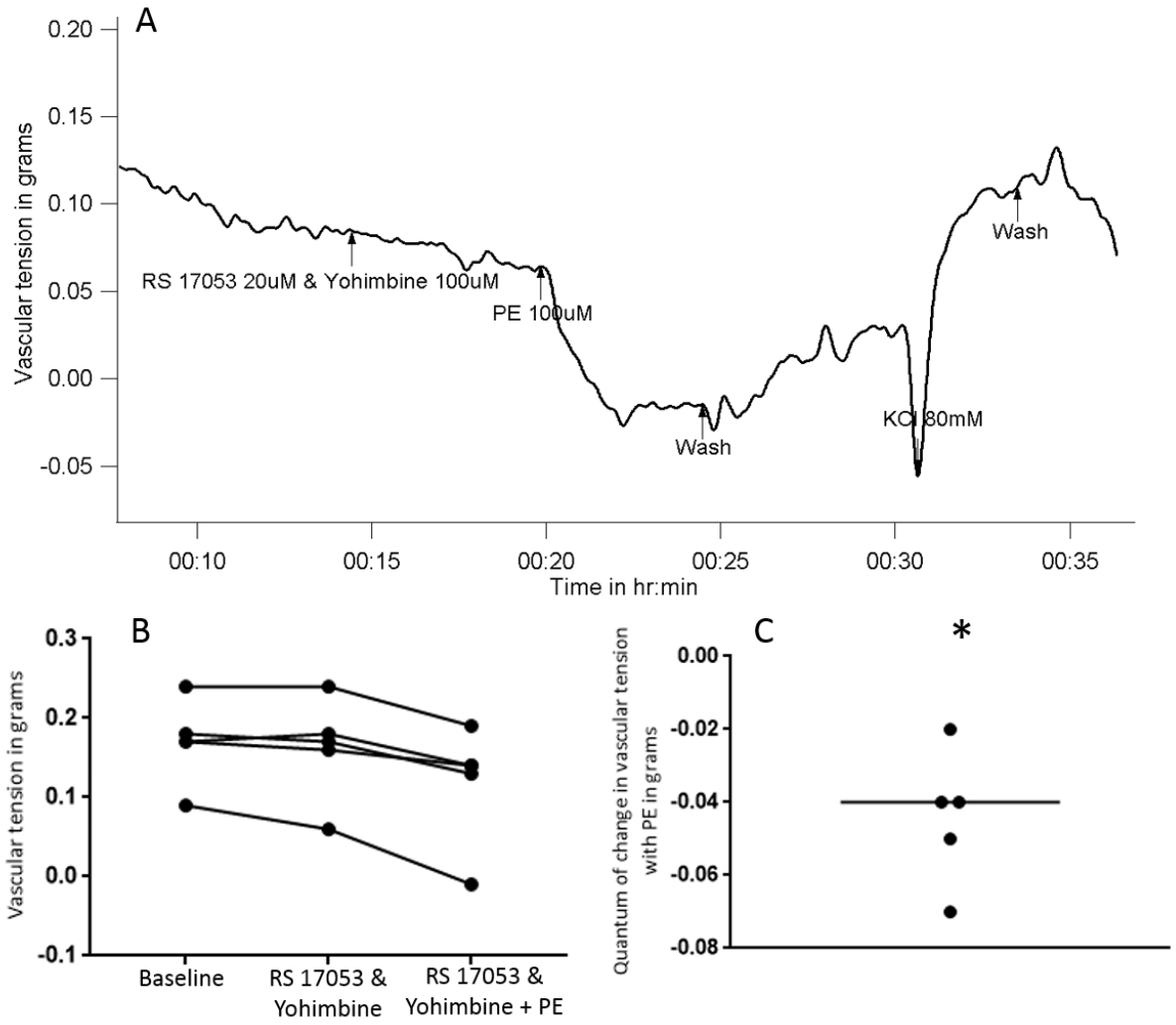
With simultaneous blockade of alpha-1B & alpha-1D receptors, PE was unable to induce reduction in vascular tension. Vascular tension prior to addition of PE (in the presence of CEC & BMY 7378) was 0.15 gm and after addition of PE it was 0.14 gm (median, n=4, p=0.083 with WSR test). (Fig 22)



With simultaneous blockade of alpha-2 & alpha-1D receptors, PE was unable to induce reduction in vascular tension. Vascular tension prior to addition of PE (in the presence of Yohimbine & BMY 7378) was 0.19 gm and after addition of PE it was 0.18 gm (median, n=4, p=0.157 with WSR test). (Fig 23)



Simultaneous blockade of alpha-1A & alpha-2 receptors caused PE to reduce vascular tension. Vascular tension prior to addition of PE (in the presence of RS 17053 & yohimbine) was 0.17 gm and after addition of PE it was 0.14 gm (median, n=5, p=0.042 with WSR test). (Fig 24)



Simultaneous blockade of alpha-1B & alpha-2 receptors caused PE to reduce vascular tension. Vascular tension prior to addition of PE (in the presence of CEC & yohimbine) was 0.15 gm and after addition of PE it was 0.11 gm (median, n=5, p=0.042 with WSR test). (Fig 25)

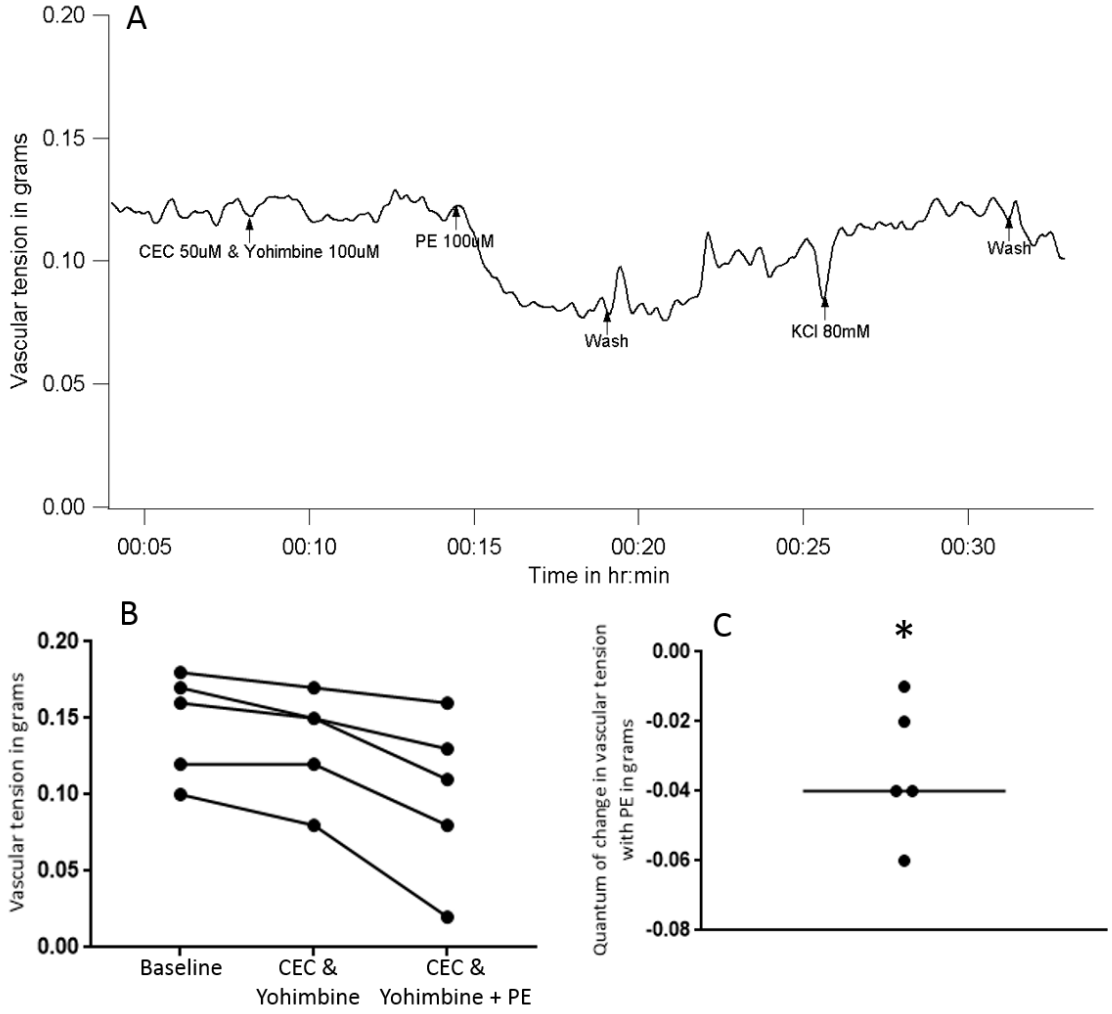


Fig 25(A) Representative raw tracing showing that simultaneous alpha-1B (CEC) & alpha-2 (Yohimbine) receptor blockade created conditions for reduction in tension with PE. (B) Vascular tension profiles due to addition of CEC & Yohimbine followed by PE. (C) Quantum of change in vascular tension with PE in the presence of CEC & Yohimbine (*P<0.05)

Simultaneous blockade of alpha-1A & alpha-1B receptors caused PE to reduce vascular tension. Vascular tension prior to addition of PE (in the presence of RS 17053 & CEC) was 0.17 gm and after addition of PE it was 0.08 gm (median, n=5, p=0.043 with WSR test). (Fig 26)

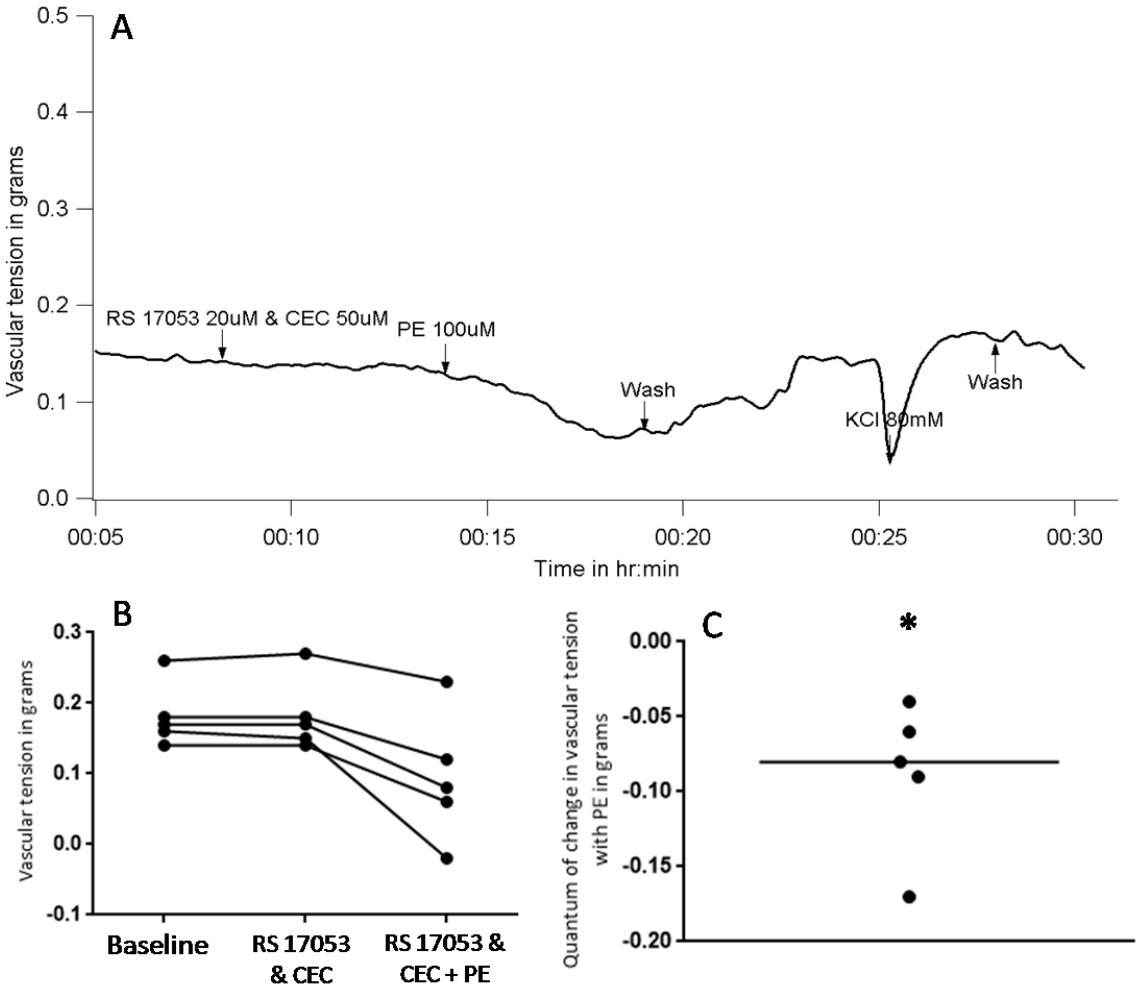
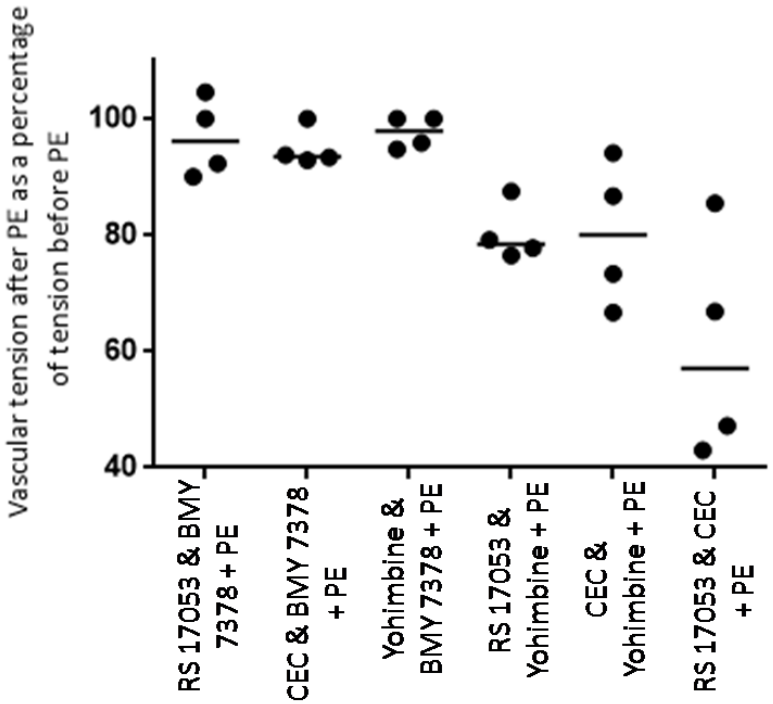


Fig 26(A) Representative raw tracing showing that simultaneous alpha-1A (RS 17053) & alpha-1B (CEC) receptor blockade created conditions for reduction in tension with PE. (B) Vascular tension profiles due to addition of RS 17053 & CEC followed by PE. (C) Quantum of change in vascular tension with PE in the presence of RS 17053 & CEC (*P<0.05)

Vascular tension after PE as a percentage of tension before PE in high NO environment
100
50
0
-50
L-Arginine + PE
L-Arginine + prazosin + PE
L-Arginine + RS 17053 + PE
L-Arginine + CEC + PE
L-Arginine + BMY 7378 + PE
L-Arginine + yohimbine + PE

Vascular tension after PE as a percentage of tension before PE
100
50
0
-50
-100
RS 17053 + PE
L-NNA + RS 17053 + PE
CEC + PE
L-NNA + CEC + PE
BMY 7378 + PE
Yohimbine + PE
L-NNA + Yohimbine + PE


Vascular tension after PE as a percentage of tension before PE
100
80
60
40
RS 17053 & BMY 7378 + PE
CEC & BMY 7378 + PE
Yohimbine & BMY 7378 + PE
RS 17053 & Yohimbine + PE
CEC & Yohimbine + PE
RS 17053 & CEC + PE

DISCUSSION

Phenylephrine, an alpha receptor agonist, is a known vasoconstrictor and it is been used as a vasopressor for hypotension due to various causes. The findings from our department shows that while phenylephrine increased vascular tension under control conditions, it led to a reduction in vascular tension under circumstances where NO levels were higher or even at normal NO levels, when NO may have been diverted to activate a vasorelaxant pathway (18). Such vasorelaxation by phenylephrine is of great concern while treating conditions like septic shock. Septic shock is a condition where there is excess production of nitric oxide and treatment with alpha agonist like phenylephrine might further worsen hypotension leading to death.

This study was conducted to delineate the alpha receptor subtype responsible for PE-induced vasorelaxation and to understand the pathways through which α-adrenergic stimulation of vascular smooth muscle leads either to a contractile response or a relaxant response depending on circumstances.

Here we have assessed the role of four alpha receptor subtypes (α_{1A}, α_{1B}, α_{1D} and α_2) in PE-induced reduction in vascular tension in a high NO environment. While α_{1A}& α_{1B} blockade did not prevent PE-induced reduction in vascular tension in the presence of L-Arginine, blockade of α_{1D} and α_2 receptors prevented reduction in vascular tension due to L-Arginine/PE combination. The inference that followed the experiments with L-Arginine was that reduction in vascular tension with PE required α_{1D} and α_2 receptor activity and did not require α_{1A}& α_{1B} activity.

However, when the blockers were used without L-Arginine, the results shows that independent blockade of α_{1A}, α_{1B} and α_2 receptors by itself caused PE to induce a reduction in vascular tension while with α_{1D} receptor blockade, PE was unable to induce any response – either contractile or relaxant.

From the observations made with receptor blockade, without L-Arginine, it may be concluded that co-activation of all four alpha receptor subtypes is essential for the contractile response to occur with PE. Even if one of them is blocked, the contractile response does not occur. In fact, with blockade of the three receptor subtypes other than α_{1D}, PE induces reduction in vascular tension. It may, therefore,be concluded that PE can activate either a contractile or a relaxant response through α_{1D} receptor activation and that co-activation of the other three receptor subtypes is mandatory to prevent relaxation and for contraction to occur.

It is clear therefore that these receptor subtypes are not just redundant mechanisms, all acting through a common pathway, but have specific roles in supporting the contractile response. The centrality of α_{1D} receptor activation for PE-induced reduction in vascular tension is further emphasized by the experiments where combinations of blockers are used. Whenever there is α_{1D} receptor blockade along with blockade of any other alpha adrenoceptor subtype, PE is unable to induce reduction in vascular tension and whenever any receptor combination other than α_{1D} is blocked, PE is able to reduce vascular tension.

It remains to be explained, why, when α_2 receptor blockade by itself led PE to cause reduction in vascular tension, there was no such response in the presence of L-Arginine. Raj & Subramani had demonstrated that NO-induced reduction in vascular tension is cGMP independent and alpha adrenergic receptor activation dependent (18). This theory is further substantiated by the results presented here and the alpha receptor subtype responsible for the reduction in vascular tension with PE is identified as α_{1D}. While interpreting the results from experiments with L-NNA as demonstrating a NO-dependence of PE-induced reduction in vascular tension, it is essential to emphasize that L-Arginine (or NO) by itself did not reduce vascular tension and that NO-induced reduction in vascular tension may always be dependent on alpha adrenergic receptor activation.

CONCLUSION

Co-activation of all four alpha adrenergic receptor subtypes is required for the contractile response to occur in vascular smooth muscle. Alpha-1D receptor subtype is essential for both contractile (under control condition) and relaxant responses (under high NO environment) to phenylephrine in vascular smooth muscle. It shows diversity in the signaling pathways of adrenergic receptor subtypes, with each having its own unique function in vascular smooth muscle. Second messenger signaling pathways responsible for the above described effect needs to be delineated in further studies.

REFERENCES

1. Strosberg AD. Structure, function, and regulation of adrenergic receptors. Protein Sci Publ Protein Soc. 1993 Aug;2(8):1198–209.

2. D'Allaire F, Atgié C, Mauriège P, Simard PM, Bukowiecki LJ. Characterization of beta 1- and beta 3-adrenoceptors in intact brown adipocytes of the rat. Br J Pharmacol. 1995 Jan;114(2):275–82.

3. van Brummelen P, Jie K, van Zwieten PA. α-Adrenergic receptors in human blood vessels. Br J Clin Pharmacol. 1986;21(Suppl 1):33S–39S.

4. Ladage D, Schwinger RHG, Brixius K. Cardio-Selective Beta-Blocker: Pharmacological Evidence and Their Influence on Exercise Capacity. Cardiovasc Ther. 2013 Apr 1;31(2):76–83.

5. Wu D, Katz A, Lee CH, Simon MI. Activation of phospholipase C by alpha 1-adrenergic receptors is mediated by the alpha subunits of Gq family. J Biol Chem. 1992 Dec 25;267(36):25798–802.

6. Remaury A, Larrouy D, Daviaud D, Rouot B, Paris H. Coupling of the α_2-adrenergic receptor to the inhibitory G-protein G_i and adenylate cyclase in HT29 cells. Biochem J. 1993 May 15;292(1):283–8.

7. Madamanchi A. β-Adrenergic receptor signaling in cardiac function and heart failure. McGill J Med MJM. 2007 Jul;10(2):99–104.

8. Silva AS, Zanesco A. Physical exercise, β-adrenergic receptors, and vascular response. J Vasc Bras. 2010 Jun;9(2):47–56.

9. McCorry LK. Physiology of the Autonomic Nervous System. Am J Pharm Educ [Internet]. 2007 Aug 15 [cited 2017 Jun 20];71(4). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1959222/

10. Lynch GS, Ryall JG. Role of β-Adrenoceptor Signaling in Skeletal Muscle: Implications for Muscle Wasting and Disease. Physiol Rev. 2008 Apr 1;88(2):729–67.

11. Siegl PKS, McNeill JH. Comparison of the alpha-adrenergic agonists, phenylephrine and methoxamine in rabbit papillary muscles. ResearchGate. 1980 Dec 1;30(2):221–31.

12. Webb RC. Smooth Muscle Contraction and Relaxation. Adv Physiol Educ. 2003 Dec 1;27(4):201–6.

13. Habib S, Ali A. Biochemistry of Nitric Oxide. Indian J Clin Biochem. 2011 Jan;26(1):3–17.

14. Francis SH, Busch JL, Corbin JD. cGMP-Dependent Protein Kinases and cGMP Phosphodiesterases in Nitric Oxide and cGMP Action. Pharmacol Rev. 2010 Sep;62(3):525–63.

15. Lee MR, Li L, Kitazawa T. Cyclic GMP Causes Ca2+ Desensitization in Vascular Smooth Muscle by Activating the Myosin Light Chain Phosphatase. J Biol Chem. 1997 Feb 21;272(8):5063–8.

16. Bonanno FG. Clinical pathology of the shock syndromes. J Emerg Trauma Shock. 2011;4(2):233–43.

17. Pollard S, Edwin SB, Alaniz C. Vasopressor and Inotropic Management Of Patients With Septic Shock. Pharm Ther. 2015 Jul;40(7):438–50.

18. Raj RR, Subramani S. Phenylephrine Decreases Vascular Tension in Goat Arteries in Specific Circumstances. PLoS ONE [Internet]. 2016 Jun 30 [cited 2017 Jul 5];11(6). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4928870/

19. Filippi S, Parenti A, Donnini S, Granger HJ, Fazzini A, Ledda F. alpha(1D)-adrenoceptors cause endothelium-dependent vasodilatation in the rat mesenteric vascular bed. J Pharmacol Exp Ther. 2001 Mar;296(3):869–75.

20. Piascik MT, Perez DM. Alpha1-adrenergic receptors: new insights and directions. J Pharmacol Exp Ther. 2001 Aug;298(2):403–10.

21. Hu Z-W, Shi X-Y, Lin RZ, Hoffman BB. Contrasting Signaling Pathways of α1A- and α1B-Adrenergic Receptor Subtype Activation of Phosphatidylinositol 3-Kinase and Ras in Transfected NIH3T3 Cells. Mol Endocrinol. 1999 Jan 1;13(1):3–14.

22. Bohr DF, Webb RC. Vascular Smooth Muscle Function and Its Changes in Hypertension. Am J Med. 1984 Oct 5;77(4):3–16.

23. Wachter SB, Gilbert EM. Beta-Adrenergic Receptors, from Their Discovery and Characterization through Their Manipulation to Beneficial Clinical Application. Cardiology. 2012;122(2):104–12.

24. Langer SZ. History and nomenclature of alpha1-adrenoceptors. Eur Urol. 1999;36 Suppl 1:2–6.

25. McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signaling: back to the future. Cell Mol Life Sci CMLS. 2005 Mar;62(5):551–77.

26. Kaneko-Kawano T, Takasu F, Naoki H, Sakumura Y, Ishii S, Ueba T, et al. Dynamic Regulation of Myosin Light Chain Phosphorylation by Rho-kinase. PLOS ONE. 2012 Jun 19;7(6):e39269.

27. Remaury A, Larrouy D, Daviaud D, Rouot B, Paris H. Coupling of the α2-adrenergic receptor to the inhibitory G-protein Gi and adenylate cyclase in HT29 cells. Biochem J. 1993 May 15;292(1):283–8.

28. Volicer L, Hynie S. Effect of catecholamines and angiotensin on cyclic AMP in rat aorta and tail artery. Eur J Pharmacol. 1971 Jul 1;15(2):214–20.

29. Giovannitti JA, Thoms SM, Crawford JJ. Alpha-2 Adrenergic Receptor Agonists: A Review of Current Clinical Applications. Anesth Prog. 2015;62(1):31–8.

30. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012 Apr;33(7):829–37.

31. Zschauer AO, Sielczak MW, Smith DA, Wanner A. Norepinephrine-induced contraction of isolated rabbit bronchial artery: role of alpha 1- and alpha 2-adrenoceptor activation. J Appl Physiol Bethesda Md 1985. 1997 Jun;82(6):1918–25.

32. Boer C, Scheffer G, Lange J de, Westerhof N, Sipkema P. Alpha-1-Adrenoceptor Stimulation Induces Nitric Oxide Release in Rat Pulmonary Arteries. J Vasc Res. 1999;36(1):79–81.

33. Gnus J, Czerski A, Ferenc S, Zawadzki W, Witkiewicz W, Rusiecka A, et al. Role of α_1-adrenergic receptor subtypes in contractility of the rabbit abdominal aorta *in vitro*. Acta Vet Brno. 2013 Nov 17;82(3):331–6.

34. Pernomian L, Gomes M, de Oliveira A. Balloon catheter injury abolishes phenylephrine-induced relaxation in the rat contralateral carotid. Br J Pharmacol. 2011 Jun;163(4):770–81.

35. de Andrade CR, Fukada SY, Olivon VC, de Godoy MAF, Haddad R, Eberlin MN, et al. α1D-adrenoceptor-induced relaxation on rat carotid artery is impaired during the endothelial dysfunction evoked in the early stages of hyperhomocysteinemia. Eur J Pharmacol. 2006 Aug 14;543(1):83–91.

36. Aalkjær C, Nilsson H. Vasomotion: cellular background for the oscillator and for the synchronization of smooth muscle cells. Br J Pharmacol. 2005 Mar 1;144(5):605–16.

37. Koenigsberger M, Sauser R, Bény J-L, Meister J-J. Effects of Arterial Wall Stress on Vasomotion. Biophys J. 2006 Sep 1;91(5):1663–74.

38. Oloizia B, Paul RJ. Ca2+ clearance and contractility in vascular smooth muscle: evidence from gene-altered murine models. J Mol Cell Cardiol. 2008 Sep;45(3):347–62.

39. Peng H, Matchkov V, Ivarsen A, Aalkjaer C, Nilsson H. Hypothesis for the initiation of vasomotion. Circ Res. 2001 Apr 27;88(8):810–5.

40. Okazaki K, Seki S, Kanaya N, Hattori J, Tohse N, Namiki A. Role of Endothelium-derived Hyperpolarizing Factor in Phenylephrine-induced Oscillatory Vasomotion in Rat Small Mesenteric Artery. Anesthesiol J Am Soc Anesthesiol. 2003 May 1;98(5):1164–71.

41. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001 Mar 8;344(10):699–709.

42. Titheradge MA. Nitric oxide in septic shock. Biochim Biophys Acta. 1999 May 5;1411(2–3):437–55.

43. Avni T, Lador A, Lev S, Leibovici L, Paul M, Grossman A. Vasopressors for the Treatment of Septic Shock: Systematic Review and Meta-Analysis. PLoS ONE [Internet]. 2015 Aug 3 [cited 2017 Jul 19];10(8). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4523170/

44. Flavahan NA, McGrath JC. alpha 1-adrenoceptor activation can increase heart rate directly or decrease it indirectly through parasympathetic activation. Br J Pharmacol. 1982 Oct;77(2):319–28.

45. Oliver LE, Horowitz JD, Dynon MK, Jarrott B, Brennan JB, Goble AJ, et al. Use of dopamine and prazosin combined in the treatment of cardiogenic shock. Med J Aust. 1980 Jul 26;2 Suppl 1:42–5.

46. Bond RF, Johnson G. Cardiovascular adrenoreceptor function during compensatory and decompensatory hemorrhagic shock. Circ Shock. 1984;12(1):9–24.

47. Jespersen B, Tykocki NR, Watts SW, Cobbett PJ. Measurement of smooth muscle function in the isolated tissue bath-applications to pharmacology research. J Vis Exp JoVE. 2015 Jan 19;(95):52324.

48. Díaz-Martín D, Hernández-Jiménez JG, Rodríguez-Valido M, Borges R. Measuring the contractile response of isolated tissue using an image sensor. Sensors. 2015 Apr 20;15(4):9179–88.

49. Ko EA, Song MY, Donthamsetty R, Makino A, Yuan JX-J. Tension Measurement in Isolated Rat and Mouse Pulmonary Artery. Drug Discov Today Dis Models. 2010;7(3–4):123–30.

50. Herlihy JT. Helically cut vascular strip preparation: geometrical considerations. Am J Physiol. 1980 Jan;238(1):H107-109.

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

December 18, 2015

Dr Bhavithra Bharathi S,
PG Registrar,
Department of Physiology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research grant project NEW PROPOSAL: (Animal Study)**
Confirmation of subtype of alpha-1 adrenergic receptor producing relaxation in goat artery strip.
Dr Bhavithra Bharathi S (Employment Number: 21237), Physiology, Dr Sathya Subramani, Employment Number: 14123, Physiology, Dr Renu Raj R,Employment number: 21093, Physiology,

Ref: IRB Min No: 9773 [OTHER] dated 10.11.2015

Dear Dr Bhavithra Bharathi S,
The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Confirmation of subtype of alpha-1 adrenergic receptor producing relaxation in goat artery strip" on December 03rd 2015.

The Committee reviewed the following documents:

1. IRB Application format
2. Cvs of Drs Bhavithra Bharathi, Sathya Subramani, Dr Renu Raj R
3. No. of documents 1 – 2

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 03rd 2015 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson(Research Chairperson), Member Secretary (Ethics Committee), IRB. CMC, Vellore	Internal, Clinician
Dr. RV. Shaji		Professor, Heamatology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS, MCh	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Comm IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, V	External, Scientist &Epidem
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgica Nursing, CMC, Vellore	Internal, Nurse
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Comn Health, CMC, Vellore	Internal, Clinician

We approve the project to be conducted in its presented form.

This proposal will also need to be submitted to the Institutional Animal Ethics Committee (IAEC) for approval.

The animal requirements and budget will have to be discussed with the Animal House Staff prior to submission of the proposal to the Institutional Animal Experimentation Committee.

Fluid Grant Allocation:

A sum of Rs.74,500/- INR (Rupees Seventy Four Thousand five hundred Only) will be granted for 2 years and out of which a maximum of Rs.5000/- can be spent for stationery, printing, Xeroxing and computer charges(if computers used are within the institution)

Yours sincerely

Chairperson (Research Committee) &
Principal
Christian Medical College
Vellore - 632 002, Tamil Nadu, India

Dr. Alfred Job Daniel
Principal & Chairperson (Research Committee)
Institutional Review Board

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
CHRISTIAN MEDICAL COLLEGE, VELLORE

Dr. Alfred Job Daniel
Principal and Chairman
email: princi@cmcvellore.ac.in

Dr. Vinay Timothy Oommen
Secretary
email: vinayoommen@cmcvellore.ac.in

25th February 2016

To
Dr. S Bhavithra Bharathi
PG Registrar
Dept. of Physiology
CMC Vellore

Dear Dr. Bhavithra

Re: Animal Ethics approval for Project " Confirmation of subtype of alpha-1 adenergic receptor producing relaxation in goat artery strip", IRB min no 9773

The above proposal need not be taken up by the Institutional Animal Ethics Committee as long as tissue is being utilized from the slaughter house and no animal is being slaughtered for the purpose of this study.

With best wishes,
Yours sincerely,

Dr. Alfred Job Daniel,
Principal & Chairperson
Institutional Animal Ethics Committee

Cc:
Dr. Vinay Timothy Oommen
Secretary, IAEC