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A study of α-Adrenoceptor Agonists on Porcine Nasal and Extra-Nasal Vasculature: Insight into Decongestant Activity

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Abstract

Background and purposes: Sympathomimetics have long been used as medications to relieve the symptoms associated with nasal congestion, but in the last 15 years, the worldwide market has moved from largely topical formulation to oral consumption. Many of the existing drugs are thought to cause vasoconstriction by activating α-adrenoceptors present on nasal mucosa and reduce local blood flow and swelling. This thesis aims to re-evaluate the activity of various sympathomimetic drugs on the nasal and extra-nasal blood vessels and better characterise the pharmacological characteristics of α-adrenoceptors on these vessels. In addition to that, I have explored the potential for lithium ions to modify the action of directly-acting sympathomimetics on both nasal and extra-nasal blood vessels and vasculature.

Results: Isometric tension recordings of the porcine isolated splenic and nasal arteries showed that both noradrenaline and phenylephrine caused concentration-dependent contractions, but surprisingly were less potent (3-4-fold, respectively) in the latter vessel. Concentrations of phenylephrine known to occur *in vivo* following oral consumption (10-30nM) failed to either induce direct vasoconstriction or alter the magnitude and duration of electrically evoked contractions of the porcine vasculature. While pseudoephedrine failed to contract either nasal or extra-nasal vessels directly, it significantly enhanced electrically evoked noradrenergic contractions and increased the duration of responses. This effect was more marked in the nasal artery. Pharmacological characteristics of contractile responses to noradrenaline in porcine arteries, using a variety of selective and non-selective antagonists, revealed unsourmountable inhibition (Schild plot < 1) and evidence for the presence of two subtypes of α_1-adrenoceptors. Attempts to reveal a constrictor role for α_2-

adrenoceptors in the splenic artery were only successfully provided by experimental conditions included both elevations of intracellular calcium ions and cyclic AMP.

Comparison of the vasoconstrictor effect of phenethylamine derivatives, e.g. phenylephrine or metaraminol, in splenic and nasal arteries revealed that responses to high concentrations were not sustained. However, the presence of 1mM lithium increased the magnitude and duration of constrictor responses. This effect was not observed when α_1-adrenoceptors were stimulated by imidazoline derivatives. Using the perfused porcine nasal snout revealed that various sympathomimetics were also able to increase perfusion pressure and the inclusion of 1mM lithium in the perfusate significantly increased the maximum response and duration of pressor responses to phenylephrine.

Conclusion and implication: In summary, these data show that while the decongestant activity of topically applied phenylephrine could be accounted by a localised constrictor effect on nasal blood vessels, no mechanistic justification was uncovered for a naso-selective effect of orally consumed phenylephrine (10mg). In contrast, pseudoephedrine selectively enhanced noradrenergic contractions in the nasal vasculature. The major mediators of vasoconstriction in porcine blood vessels appear to involve two subtypes of α_1-adrenoceptors (mainly α_{1A}-adrenoceptors and probably α_{1B}-adrenoceptors). For arterial vessels, α_2-adrenoceptors could not be detected *in vitro* study without simultaneous elevation of cellular calcium ions and cyclic AMP. Finally, my findings raise the possibility that the nasal decongestant activity of topically applied phenylephrine may be enhanced by the presence of lithium ions, which may reduce the frequency of administration.

Thesis related abstract publications

1. A. Abdulrahman, H. Denfria, J. Heer, R. Mahajan, V. Wilson. Comparison of the direct and indirect vascular actions phenylephrine and pseudoephedrine, as orally-active nasal decongestants, with reference to plasma concentrations found in man. *Pharmacology 2016* Queen Elizabeth II Conference Centre London. www.pA2online.org, 199P.

2. A. Abdulrahman, H. Denfria, R. Mahajan, S. Chan, V. Wilson. Neither phentolamine nor prazosin behave as classical competitive antagonists against noradrenaline-induced contractions in the porcine isolated splenic artery. *Pharmacology 2016* Queen Elizabeth II Conference Centre London. www.pA2online.org, 151P.

3. A. Abdulrahman, S. Chan, V. Wilson. Pharmacological examination of decongestant activity of phenylephrine in the porcine isolated nasal artery: homeopathy hidden in plain sight? *Pharmacology 2017* Queen Elizabeth II Conference Centre London. www.pA2online.org, 075P.

Award

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Abbreviations

ACh	Acetyl choline
AD	Adrenaline
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BK_{ca}	large conductance calcium sensitive potassium channels
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CaM	Calmodulin
CEC	Chloroethylclonidine
CI	Confidence Interval
CR	Concentration ratio
CRCs	Concentration-response curves
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DPX	Resinous mounting medium for histology; a mixture of distyrene, a plasticizer, and xylene
EC_{50}	Agonist concentration that causes 50% of observed action in absence of the antagonist
EC_{50}'	Agonist concentration causing 50% of observed action in presence of the antagonist
EDHF	Endothelial derived hyperpolarising factor
EDTA	Ethylenediaminetetraacetic acid disodium salt
EFS	Electrical field stimulation
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
ET-1	Endothelin

FDA	Food and Drug Administration
GPCR	G-protein coupled receptors
IK_{ca}	Intermediate conductance calcium sensitive potassium
IMPase	Inositol Monophosphatase enzyme
iNOS	Inducible nitric oxide synthase
IP_3	Inositol-1,4,5-trisphosphate
L-NAME	*N*-nitro-L-arginine methyl ester
MLC	Myosin light chain
MLCK	Myosin light chain kinase
n	Number of observations
NA	Noradrenaline
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
OD	Optical density
OCT	Optimum Cutting Temperature embedding medium
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
OTC	Over-the-counter
PBS	Phosphate buffered saline
pD_2	The base -10 logarithm of EC_{50}
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G

R_{-max}	Maximum response to cumulative addition of an agonist
S.E.M	Standard error of mean
SK_{ca}	Small conductance calcium-sensitive potassium channels
SR	Sarcoplasmic reticulum
TC	Time course
TNF-α	Tumour necrosis factor-α
U46619	9,11-dideoxy-9α,11α -epoxymethanoprostaglandin $F_{2\alpha}$

Chapter One

General Introduction

1 General Introduction

General outline of the thesis

Nasal congestion is one of the main uncomfortable and significant symptoms of the common cold and nasal obstruction, which can be treated by using OTC nasal decongestants. Nasal decongestant medications are sympathomimetic amines that mediate their vasoconstriction action in nasal blood vessels by activating α-adrenoceptors either directly or indirectly leading to a net reduction in oedema and nasal secretions (Vaidyanathan *et al*., 2012).

Clinically, nasal congestion describes a non-critical condition. However, nasal congestion severely affects quality of life by its impact on daily life, especially sleep, social life and time lost from work and school, for example, the common cold is associated with 22 million and 20 million absences from school and work annually, respectively (Pappas *et al*., 2008). As well as the amount of money spent on OTC medicines and based on total economic impact, cold remedies possess a role in the industrial pharmacy that was expected to reach $2 billion dollars annually in 1996 (Ho *et al.,* 1997). Based on this development, the sales of intranasal drug products including nasal decongestants were expected to reach approximately $5.2 billion by 2017, in the United States market alone (Touitou and Illum, 2013), but remarkably the growing sales of cold remedies, particularly on decongestants, were unexpected and even extended to approximately 40 billion dollars on an annual basis in the United State (Ta'i *et al*., 2012). While in England, a growing estimation, and an increase in demand for OTC medicines, which include decongestants, extended to approximately £569 million in the year previous to June 2017 (NHS England and NHS Clinical Commissioners, 2018).

Historically, phenylpropanolamine, ephedrine and pseudoephedrine were the first effective decongestants to be used in the 1930s (Empey *et al*., 1980: Kernan *et al*., 2000; Flavahan, 2005) and formed the largest market over the past 70 years ago. Even though phenylpropanolamine was effective as a decongestant, in October 2000, the Food and Drug Administration Nonprescription Drugs Advisory Committee announced that phenylpropanolamine should be removed because of safety concerns about hemorrhagic strokes (Kernan *et al*., 2000; Meadows, 2001; Flavahan, 2005; Eccles, 2006; Hendeles and Hatton, 2006). Also, the important issue of extensive use of pseudoephedrine to make methamphetamine illegally caused for it be placed "behind the counter" (Eccles, 2006). This removal of pseudoephedrine-containing products from in front of the counter lead to a dramatic increase in the manufacturing and use of phenylephrine as a nasal decongestant after 2004. Phenylephrine was approved to be safe and effective according to final monograph of OTC nasal decongestant drug products at 1994 and was first introduced as a nasal spray then the formulation was changed to an oral form, but questions should be raised about its efficiency as a decongestant. However, in the last 20 years, prescribing OTC decongestants is changing and it is now phenylephrine incorporated into oral medicines either as an oral decongestants tablet/capsule or as part of cough remedies.

To date, many people use oral OTC medications that contain antihistamines, decongestants, analgesics or a combination to self treat the symptoms of the common cold. As the main objective of this study is to understand more clearly the action and efficacy of various α-adrenoceptor agonists as nasal decongestants, therefore, this chapter has been organized in the following way. The first section of the general introduction will cover information related to adrenoceptors, their history, types and

mechanism of action and finally the therapeutic role of adrenoceptor agonists and antagonists.

This will be followed by a description of the structure of the vascular system, particularly the blood vessels and basic biochemical pathways involved in the regulation of the vascular tone. Then to improve our therapeutic modalities, we need to further our understanding of the structural anatomy of the nose, moving to the nasal congestion and its definition, mechanism, epidemiology, effects of it in our health and life and to finish with the treatment of nasal congestion. Finally, I will highlight the aims of this thesis.

1.1 Adrenoceptors

Adrenoceptors have a crucial role in the vascular smooth muscle, as it is important in the regulation of the vascular tone reactivity.

1.1.1 Story of adrenoceptors

The origins of the receptor concept start in 1896 when Oliver and Schafer found that injection of adrenal gland extracts caused an elevation of blood pressure. The approval of the drug-receptor theory was delayed because of conflicting ideas about drug action. Uncertainties and/or hesitations about the drug receptor idea continued until the work started by the German bacteriologist and immunologist Paul Ehrlich and of the British physiologist John Newport Langley at the end of the nineteenth and the beginning of the twentieth century (Quirke, 2006). Langley (1905) studied the influence of nicotine, curare and the breakdown of motor nerves in striated muscles of the fowl and he assumed that there was a new idea concerning with direct relationship between nerve and affected organ from one side and the direct actions of the different agonist on the nerve endings from another side.

In his study Langley, (1905) was the first to assume that there were substances receiving stimuli and transmitting them to the affected tissue in order to start the required function and those substances were called receptive substance (Langley, 1906). Later on, Dale (1906) in his study developed the idea of receptive substance then applied it on the sympathetic nervous system involving the sympathetic receptors and stated that there were various effects of adrenaline either sympathelin I (inhibitory) or sympathelin E (excitatory), however, this influence differed with various muscles. In the course of his work, Dale was able to present the evidence of different sympathetic receptors, yet the first scientist who used the term receptor was Ehrlich (Piascik *et al*., 1996). Other researchers developed further the idea of the receptive substance. It was concluded that, in addition to adrenaline, which was the main sympathetic neurotransmitters released; there were other neurotransmitters (Gaddum and Kwiatkowski, 1938). Supplementary studies concluded the availability and action of noradrenaline, which was also released from sympathetic nerve endings as an active neurotransmitter, in addition to the presence of other neurotransmitters such as sympathin I (inhibitory) and sympathin E (excitatory) (Von Euler, 1946; West, 1947).

1n 1948, a pharmacological work done by Ahlquist developed the context of Langley's concept to describe the idea of adrenoceptors using a quantitative technique as the "hypothetical structures or systems located in, or near the muscle or gland cells affected by adrenaline". Ahlquist (1948) suggested the presence of two adrenoceptor subtypes to which different endogenous catecholamines can react and those two adrenoceptors were α and β depending on various rank orders of potency, within groups of structurally related natural and synthetic adrenoceptors.

During Ahlquist's work, a comparison between the action and potencies of noradrenaline, adrenaline and isoprenaline using various tissues was conducted and he assumed that the rank order was adrenaline > noradrenaline > isoprenaline for vasoconstriction and isoprenaline > adrenaline > noradrenaline for vasodilation. The next main confirmation of the adrenoceptor subclassification came when dichloroisoprenaline was used, initially as a selective antagonist of the β-adrenoceptor (Powell and Slater, 1958).

The development and acceptance of the receptor concept and rational drug design was largely affected in 1965 by Sir James Black who was awarded the Nobel Prize in 1988 for the introduction and use of propranolol as ''the first clinically useful β-receptor blocker'' and its revolutionary use in solving problems with cardiovascular diseases, from angina pectoris to hypertension (Quirke, 2006). The impact of introducing propanolol on receptor theory was that it became a key agent in terms of the subdivision of adrenoceptor into α- and β-subtypes. Subsequent studies considering the optimal conditions for classifying receptors in isolated tissue and a further subclassification of adrenoceptors was clarified by Furchgott, who used the rank order of potency and potency ratios to characterise receptor subtypes in a range of responses (Furchgott, 1972). In 1972, Furchgott stated rank orders for the catecholamines in several tissues indicated that there was more than one type of adrenoceptor and the rank order was:

Adrenaline > *noradrenaline* > *phenylephrine* > *isoprenaline* (indicated α-adrenoceptor mediated response), while rank order of:

Isoprenaline > *adrenaline* > *noradrenaline* > *phenylephrine* (indicated β-adrenoceptor mediated response).

Later on, studies with agonists and antagonists confirmed Ahlquist's theory of two receptors and the use of receptor theory as a tool for pharmacological innovation in the classification of receptor types (Delbarre and Schmitt, 1973; Lefkowitz, 1976; Molinoff, 1984; Bylund, 1992). Consequently, pharmacology and molecular studies found that there is a sub-classification of α-adrenoceptors depending on the anatomical subdivision, pre and post-junctional α-adrenoceptors which were assumed to be α_2- and α_1-adrenoceptor, respectively (Langer, 1980). Further studies changed the sub-classification of α-adrenoceptor according to functional subdivision depending on the function of each receptor outside its anatomical location in which the inhibitory response was caused by α_2-adrenoceptors while the excitatory response was caused by α_1-adrenoceptors (Berthelsen and Pettinger, 1977). Finally, after the development and characterisation of potent and highly selective α_1- and α_2-adrenoceptor agonists and antagonists, in addition to radioligand binding studies, the researchers and studies recommended that pharmacological sub-classification be used to subdivide α_1- and α_2-adrenoceptors (Bylund *et al*., 1994).

1.1.2 Types and mode of action of adrenoceptors

Adrenoceptors are a common receptor spread throughout the body and are considered as cell membrane receptors of the seven transmembrane domain spanning G protein-linked family of receptors, stimulated by the physiological agonists, adrenaline and noradrenaline and this occurs by eliciting a response in the cell (Docherty, 2019). Generally, G protein-coupled receptor family members couple via a G protein which constitutes three subunits (α, β, γ) and these receptors are classified into at least three main types, which have a characteristic mode of action (Figure 1.1). Each adrenoceptor

subtype is attached selectively to certain types of G protein, such as $G_{q/11}$ is the kind of G protein-coupled receptors to which α_1-adrenoceptors subtypes related, while α_2-adrenoceptor linked to G_i kind G protein linked receptors and Gs to β-subtype (Williams *et al.*, 1998; Foord *et al.*, 2005; Westfall and Westfall, 2006b; Alexander *et al*., 2017). Then G-protein-coupled effects, by various secondary messengers, which include one or more of the following pathways: the adenylyl cyclase/cAMP pathway, the pathway of inositol trisphosphate (IP_3) and diacylglycerol (DAG) discharge, etc. changing the vascular tone of the smooth muscle. In general, adrenoceptors are considered potent receptors in the regulation of several systems such as the cardiovascular system, reproductive system, nervous system, urinary system and gastrointestinal tract system (Civantos Calzada and Aleixandre de Artinano, 2001). Therefore, agents acting on adrenoceptors, such as adrenaline and noradrenaline, have a vital role in the regulation of most organ systems and control the blood pressure, the blood flow, myocardial contractile rate and force, airway reactivity and different types of metabolic functions.

Adrenoceptors are classified into two main types: α–and β-adrenoceptors. α-Adrenoceptors mediate most excitatory functions and are important for the control of the vascular tone (Starke, 1981). In the 1970s, α-adrenoceptors were distinguished into α_1- and α_2-adrenoceptors. The action of the α_1-adrenoceptor type were commonly seen in the effector organ, while the α_2-adrenoceptor type was found presynaptically and controlled the release of the neurotransmitter but it was also located postsynaptically (Berthelsen and Pettinger, 1977). β-Adrenoceptors are classified into (β_1-, β_2-, and β_3-) subtypes and their action is related to excitatory functions (cardiac stimulation including increased heart rate, contractility and conduction velocity) and

inhibitory function, for example, vasodilation, uterine muscle relaxation and bronchodilation (Coleman and Somerville, 1977; Lefkowitz *et al*., 1983) (Figure 1.1).

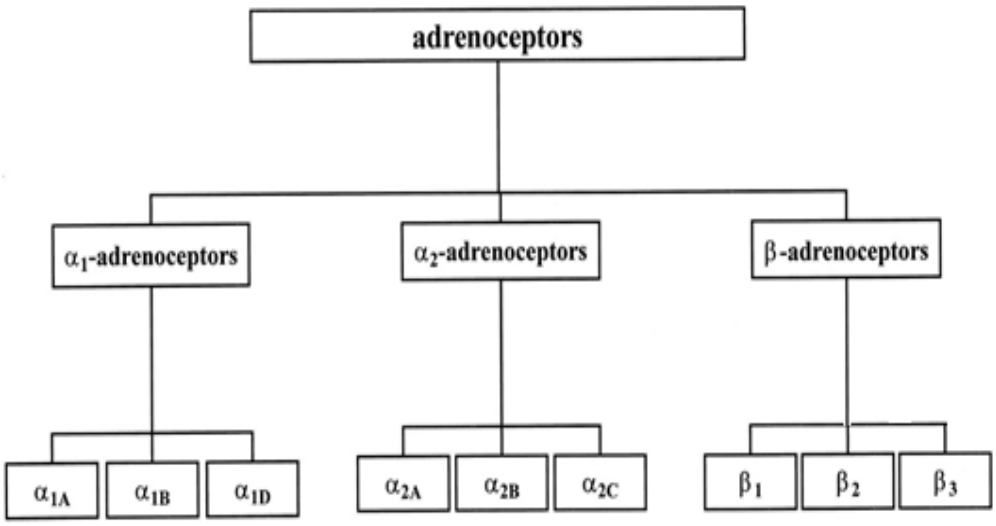


Figure 1.1 Schematic diagrame of adrenoceptors classification.

Further molecular, functional and pharmacological (agonist/antagonist) studies are used to characterise the α-adrenoceptor subtypes. In relation to pharmacological studies using α-adrenoceptor antagonists, prazosin is the main selective α_1-adrenoceptor antagonists, while phenoxybenzamine and phentolamine are non-selective α-adrenoceptor antagonists. In animal studies, prazosin is used to characterise α-adrenoceptor subtypes as it has a 10,000-fold greater affinity for α_1-adrenoceptors than α_2-adrenoceptors (Hoffman *et al.*, 1979; Hoffman and Lefkowitz, 1980). Phentolamine is the antagonist of choice to help distinguish between α- and β-adrenoceptors for responses mediated by either noradrenaline or adrenaline. Furthermore, it has become clear from radioligand binding, molecular biology, isolated tissue experiments and functional studies that phentolamine does not distinguish between the two major subtypes of α-adrenoceptors (Starke, 1981; Bylund, 2005). From another aspect, yohimbine is a selective α_2-adrenoceptor antagonist, with about 500-fold greater affinity for α_2-adrenoceptors than α_1-adrenoceptors (Hoffman *et al.*, 1979).

Functional and autoradiographic studies reported that α_2/α_1 affinity ratio for yohimbine was 45, whereas those of idazoxan and rauwolscine (α_2-adrenoceptor antagonists) were 245 and 3, respectively (Doxey *et al*., 1984; Mallard *et al*., 1992). The highly selective α_2-adrenoceptor antagonists include the 2-alkoxy substituted analogues of idazoxan, RX-821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) and RX-811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline) (Harris and Clarke, 1993; Alexander *et al.*, 2017). In summary, the affinity and the selectivity of the antagonists depends on the type and concentration of agonist used, the blood vessels tested and the condition of the experiments and studies, for example, rauwolscine showed the binding profile with 5-HT_{1A} receptors and α_2-adrenoceptors in rat cortex (Broadhurst and Wyllie, 1986), rabbit and human cerebral cortex (Convents *et al*., 1989). While the autoradiography study showed that rauwolscine associated with dopamine receptors in its primary distribution within the rat brain (Boyajian *et al*., 1987) (Figure 1.2).

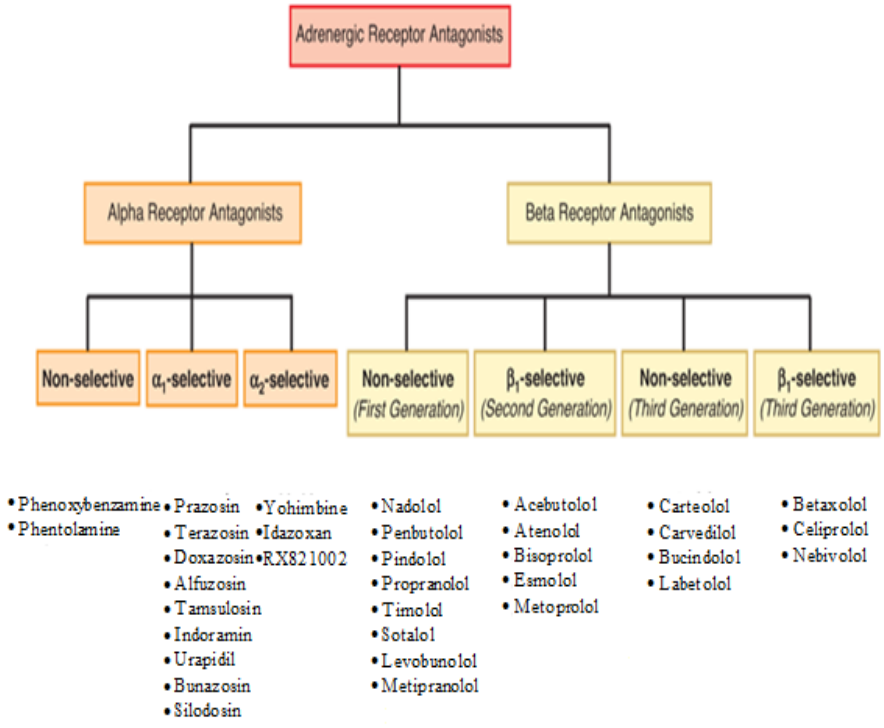
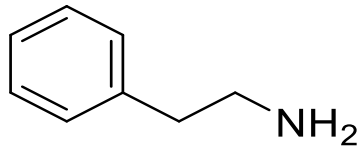


Figure 1.2 Classification of adrenoceptor antagonists. Figure modified from Westfall and Westfall, (2006a).

α-Adrenoceptors are usually divided into two main molecular existences according to their structure, phenethylamine and imidazoline agents, both of which bind to α-adrenoceptors (Raulli and Crewst, 1991). It has been found that phenethylamines are structurally related to endogenous catecholamines (adrenaline and noradrenaline) and showed tight coupling of receptor occupancy and response (Insel, 1989). By contrast, imidazolines are structurally unrelated to endogenous catecholamines (Insel, 1989).

Generally, imidazoline and phenethylamine ligands are potent agonists in cell-based or isolated organ systems and demonstrate a broad range of efficacies (Raulli and Crewst, 1991; Kukkonen *et al*., 2001) such as phenethylamine agents (noradrenaline and guanfacine) had a relative efficacy of 1.0 and 0.13 ± 0.02 and a potency of 6.54 ± 0.06 and 6.82 ± 0.06, respectively. Imidazoline agents, such as clonidine and oxymetazoline, had a relative efficacy of 0.20 ± 0.05 and 0.13 ± 0.02 and potency of 7.36 ± 0.13 and 7.66 ± 0.13, respectively, in dog saphenous vein (Maclennan *et al*., 1997). However, the therapeutic activity of phenethylamine and imidazoline agents and the α-adrenoceptors subtype through which they act is still unclear. Table 1.1A and 1.1B illustrates the chemical structure of some phenethylamine and imidazoline agents.

Table 1.1A Phenethylamine and structurally related α-adrenoceptor agents.



phenethylamine

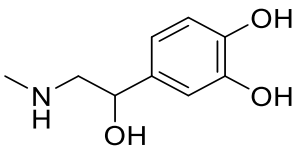
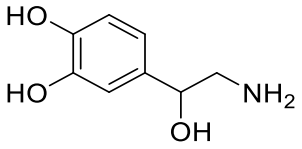
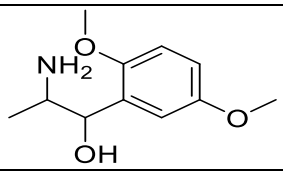
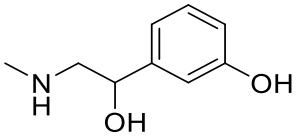
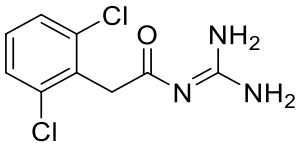
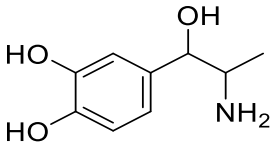
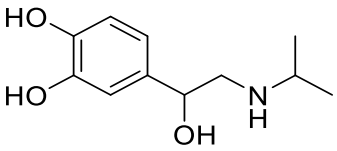
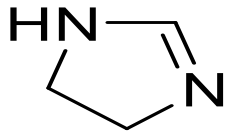
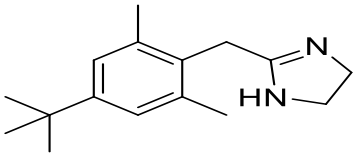
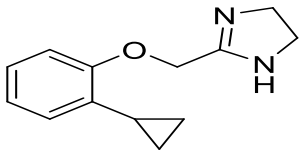
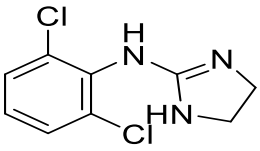
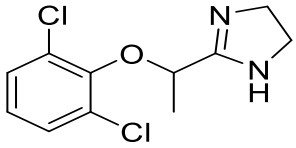
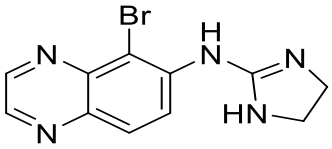
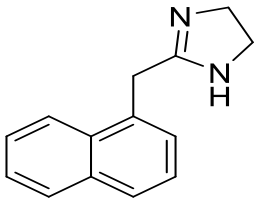
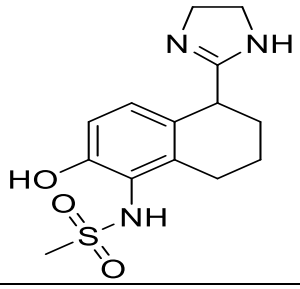
	**Adrenaline**
	**Noradrenaline**
	**Methoxamine**
	**Phenylephrine**
	**Guanfacine**
	**α-methyl noradrenaline**
	**Isoprenaline**

Table 1.1B Imidazoline and structurally related α-adrenoceptor agents.



Imidazoline

	**Xylometazoline**
	**Cirazoline**
	**Clonidine**
	**Lofexidine**
	**Brimonidine**
	**Naphazoline**
	**A61603**

1.1.2.1 α-Adrenoceptors

Ahlquist classified adrenoceptors as alpha (α)- and beta (β)-adrenoceptors based on their pharmacological responses (Ahlquist, 1948).

1.1.2.1.1 α_1-Adrenoceptors

α_1-Adrenoceptors are abundant in different tissues and organs including vascular smooth muscle, intestinal smooth muscle, hepatic cell, salivary gland, radial muscle of the iris, sweat gland, uterus, kidney, ureter, bladder sphincter, vas deferens, pilomotor muscle of skin and in the nervous system. α_1-Adrenoceptors are distributed predominantly in postsynaptic smooth muscles and have a vital role in pharmacological manipulation of vascular tone through activation of $G_{q/11}$, and thus leads to the production of inositol-1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), as second messengers. IP_3 activates receptors on the endoplasmic/sarcoplasmic reticulum resulting in relaese of calcium into the cytosol, while DAG activates protein kinase-C (PKC) by phospholipase C activation dependent mechanism and this finally results in vasocontraction (Yu *et al*., 1998).

Lithium ions inhibit inositol monophosphatase and elevate IP_3 in smooth muscle (Fox *et al*., 1985), therefore, it has been hypothesised that lithium ions may lead to drug repurposing or finding a new formulation of existing α_1-adrenoceptor medications. Additional signaling mechanisms related to α_1-adrenoceptors involve phospholipase A_2 activation (arachidonic acid release), and phospholipase D activation (phosphatidic acid release). Phosphatidic acid is a second messenger prompting the intracellular release of calcium, and is metabolised to DAG by phosphatidic acid phosphatase (Westfall and Westfall, 2006b).

Contractile activity of the sympathetic nervous system following post-junctional activation of α_1-adrenoceptors has been clarified in numerous blood vessels and species. For example, electrically stimulated cntraction of the rabbit hindlimd (Madjar *et al*., 1980), rabbit isolated pulmonary artery (MacLean *et al*., 1993) and horse penile resistance arteries (Simonsen *et al*., 1997) responded to α_1-adrenoceptor blockade.

A previous study has established that the contractile responses of small arteries were related extensively to the stimulation of α_1-adrenoceptors (Angus *et al*., 1988; McGrath *et al*., 1989). This finding was supported by Wright *et al*., (1995a) who examined the existence of α-adrenoceptors using both functional and radioligand binding techniques and reported that only post-junctional α_1-adrenoceptors were functionally active and responsible for mediating most of the vasoconstrictor action in the porcine splenic artery. On the other hand, *in vivo* studies with human vasculature, the roles of post-junctional α_1 and α_2-adrenoceptors can be elucidated on the forearm blood flow via intra-arterial infusions of selective α_1 and α_2-adrenoceptor agents and blockers (Jie *et al*., 1984). Additionally, Fukui *et al*., (2005) demonstrated the presence of post-junctional α_1 and α_2-adrenoceptors in human gastroepiploic arteries, which mediate the electrically stimulated vasoconstriction activity. I hope that this *in vitro* study will try to confirm these findings and clarify the action of α-adrenoceptors in porcine blood vessels.

McGrath in 1982 demonstrated the presence of more than one α_1-adrenoceptor subtypes in the rabbit basilar artery, rat anococcygeus and vas deferens depending on the variable potency series for different agonists (McGrath, 1982). McGrath noted that in the rat anococcygeus, the response to low concentrations of phenylethenylamines and non-phenylethenylamines was mediated by α_{1A}-

adrenoceptor while the response to high concentrations of the same agents was caused by α_{1B}-adrenoceptors. In addition to this, McGraths, (1982) study compared the effects and potencies of various antagonists in the rat vas deferens and the functional study carried out by Ruffolo, (1985) provided evidence for the availability of more than one type of α_1-adrenoceptor. Subsequently, developed studies with ligand binding, molecular cloning, and finally, functional assays using selective antagonists elucidated that α_1-adrenoceptors could be sub-classified into $\alpha_{1A,}$ α_{1B}, α_{1D} subtypes (Bylund *et al*., 1994; Bylund, 2005; Alexander *et al*., 2017).

The sub-classification schemes for α_1-adrenoceptors were based on their affinities for antagonists (WB4101, phentolamine, 5-methyl-urapidil and (+)-niguldipine) and agonists (phenylephrine and oxymetazoline), which reported that WB4101 and phentolamine blockers were approximately 40- and 30-fold more potent at the α_{1A}-adrenoceptor than the α_{1B}-adrenoceptor binding site, respectively (Morrow and Creese, 1986). Later on, Johnson and Minneman, (1987) found that the hydrophilic alkylating analogue of clonidine (chloroethylclonidine, CEC) inactivation has been used as one criterion to subtype α_1-adrenoceptors. As the α_{1A}-adrenoceptor showed a lower sensitivity to CEC opposed to the α_{1B}-adrenoceptor (90% of the total receptor population irreversibly inactivated), while the α_1-adrenoceptor that was 70% inactivated by CEC was designated as a α_{1D}-adrenoceptor (Perez *et al*., 1991; Bylund *et al*., 1994).

Extensive investigations have shown that all three subtypes of α_1-adrenoceptors are present across vascular adventitial, medial smooth muscle and endothelial cells, and are capable of functional impact (McGrath, 2015). Of the three subtypes, α_{1A}-adrenoceptor is the major receptor responsible for contractions in both arteries (e.g.

splenic, mesenteric, renal, mammary), and veins (e.g. vena cava, pulmonary, saphenous).

Knepper *et al*., (1995) found that A61603 is a potent α_{1A}-adrenoceptor agonist in rat vas deferens, (200 to 300)-fold more potent than norepinephrine or phenylephrine, respectively and in isolated canine prostate strips, (130 to 165)-fold more potent than norepinephrine or phenylephrine, respectively. Silodosin (KMD-3213) and 5-methyl-urapidil show high affinity for α_{1A}-adrenoceptor ligand binding sites and act as selective α_{1A}-adrenoceptor antagonists (Perez *et al*., 1991; Murata *et al*., 1999; Murata *et al*., 2000).

While, α_{1B}-adrenoceptor is the most abundant subtype in heart, and spleen and α_{1B}-adrenoceptor exists structurally in combination with α_{1A}-adrenoceptors (Docherty, 2019). However, until now no validated, selective competitive antagonist for α_{1B}-adrenoceptors has been found, as their potency *in vivo* is very low (Barbieri *et al*., 1998; Yoshiki *et al*., 2014; Docherty, 2019). α_{1D}-Adrenoceptor is the main vasoconstrictor in the aorta and coronary arteries involved in the control of the blood pressure and BMY 7378 (8-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride) is considered as a selective α_{1D}-adrenoceptor antagonist (Nash, 1990; Perez *et al*., 1991; Goetz *et al*., 1995; Westfall and Westfall, 2006b; Docherty, 2019).

Previous functional studies performed by (Holck *et al*., 1983; Medgett and Langer, 1984; Agrawal *et al*., 1985; Drew, 1985), which reflect heterogeneity in the affinity of the population of postsynaptic α_1-adrenoceptors to prazosin and yohimbine antagonists in different type of isolated tissues. Therefore, Flavahan and Vanhoutte, (1986) divided the α_1-adrenoceptors into two subtypes: one with high affinity or sensitivity to prazosin and yohimbine (α_{1H}-adrenoceptors) (pA_2 values > 9 for prazosin and > 6.4 for

yohimbine and the other (α_{1L}-adrenoceptors) with low affinity or insensitive to these two blockers (pA_2 values < 9 and 6.2, respectively).

α_1-Adrenoceptors in blood vessels were further subdivided into three subtypes, α_{1H}, α_{1L} and α_{1N}, (N: neither α_{1H} nor α_{1L}) based on their affinities for prazosin, WB4101, HV723 and yohimbine antagonists in the following order: α_{1H}-adrenoceptor is more sensitive to prazosin than HV723 and WB4101 pA_2 value for prazosin > 9.5, while α_{1L}-adrenoceptor has a pA_2 values for prazosin, HV723 and WB4101 ranging between 8-9 and finally α_{1N}-adrenoceptor is more sensitive to HV723 and WB4101 than prazosin with pA_2 values for HV723, WB4101 and prazosin > 9, > 8.4 and < 8.3, respectively (Muramatsu *et al*., 1990; Muramatsu, 1991). Ford and colleagues (1994) anticipated four subtypes of α_1 adrenoceptors: three showed high affinity to prazosin (α_{1A}, α_{1B} and α_{1D}) and one showed low affinity to prazosin and was designated as α_{1L}-adrenoceptor. To date, due to development in functional and cloning studies with α-adrenoceptors, the α_{1L}-adrenoceptors are regarded as a subclass of α_{1A}-adrenoceptors while the α_{1H} is assumed to belong to all of the three subtypes of α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) (Docherty, 2019).

1.1.2.1.2 α_2-Adrenoceptors

Generally, α_2-adrenoceptors are widely distributed throughout the body. For example, presynaptic α_2-adrenoceptors are present on sympathetic nerve endings and noradrenergic neurons in the autonomic nervous system (autoreceptors) where they inhibit the release of noradrenaline, therefore α_2-adrenoceptors acts as a negative feedback mechanism for modulating the release of noradrenaline (Langer, 1980; Jie *et al*., 1987; Zeng *et al*., 1989; Piascik *et al*., 1996). Postsynaptic α_2-adrenoceptors exist

in a number of tissues (heart, liver, pancreas, platelets, kidney, eye and adipose tissue) where they have a distinct physiological function (Figure 1.3).

For example, activation of the α_2-adrenoceptor subtype causes contraction of the sphincter of the gastrointestinal tract and has a role in activation of platelet aggregation, and inhibition of insulin and glucagon release from the pancreas (Westfall and Westfall, 2006b). It was observed that the action of α_2-adrenoceptors in various organs and tissues differed according to the type of blood vessels being affected, type of receptor subtype being activated and the location. For example, the α_{2A}-adrenoceptor subtype appeared to couple in an inhibitory fashion to L-type voltage-gated calcium channels in the locus ceruleus of the brain-stem, while, in the vasculature, the α_{2B}-adrenoceptor subtype coupled in an excitatory manner to the same effector mechanism (Kamibayashi and Maze, 2000).

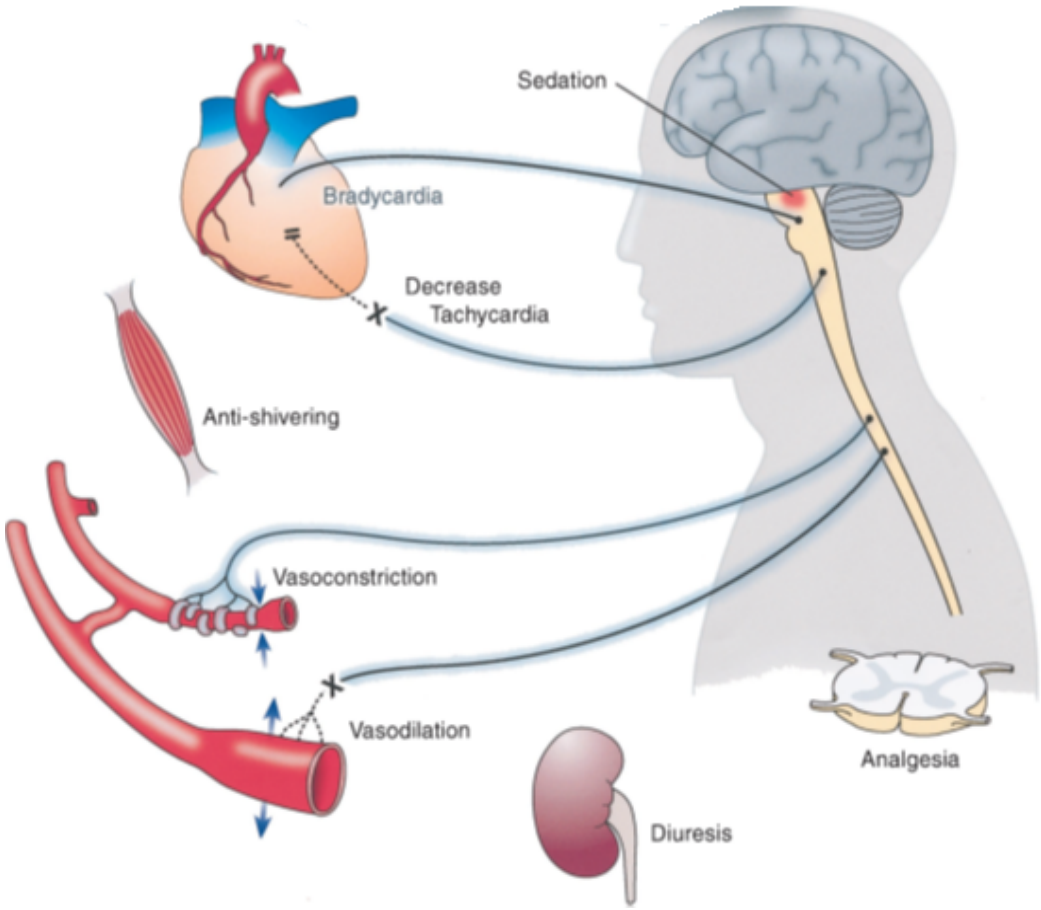


Figure 1.3 Different responses caused by α_2-adrenoceptors. The location for a sedative action is in the locus ceruleus of the brain-stem, while the main location for the analgesic action is possibly in the spinal cord; although, there is clear indications for both a peripheral and a supraspinal location of action. In the heart, the dominant action of α_2-agonists is a reduction in tachycardia (through block of the cardioaccelerator nerve) and bradycardia (through a vagomimetic action). In the peripheral vasculature, there are both vasodilatory actions via sympatholysis and vasoconstriction mediated through the receptors in the smooth muscle cells. The mechanism for the antishivering and diuretic actions have yet to be established firmly. Figure adopted from Kamibayashi and Maze, (2000).

In vascular smooth muscle, α_2-adrenoceptors play a critical role in the regulation of vascular reactivity. For example, $\alpha_{2A/D}$- and α_{2B}-adrenoceptor subtypes facilitate contraction of arterial blood vessels, while α_{2C}-adrenoceptor subtype mediates contraction of the venous side (Gavin *et al.*, 1997; Civantos Calzada and Aleixandre de Artinano, 2001). It is quite difficult to demonstrate the involvement of α_2-adrenoceptors in the constrictor action of isolated blood arteries. However, a response of post-junctional α_2-adrenoceptors has been identified to the α_2-adrenoceptors agonist brimonidine in small blood vessels such as human subcutaneous resistance vessels (Nielsen *et al*., 1989).

From another perspective, Barbieri *et al*., (1998) found no evidence for α_2-adrenoceptors in the vasoconstrictor activity during the pharmacological characterisation of responses to noradrenaline and only α_1-adrenoceptors were functionally relevant in a porcine splenic artery. This study suggested by using phenylephrine and various α_1-adrenoceptor subtype selective antagonists that only α_{1A}-adrenoceptors were expressed on the porcine splenic artery, while evidence of α_{1B}-adrenoceptors was elusive and no evidence was found for α_{1D}-adrenoceptors.

Additional studies found that α_2-adrenoceptors alone could not evoke sufficient responses and functional effects of α_2-adrenoceptors in blood vessels required a previous methodological manipulation like enhancing the pressure perfusion system by arginine vasopressin in the isolated vascular bed of the rat tail (Templeton *et al*., 1989). Another manipulation method included raising the vascular tone either by incubation with a non-adrenoceptor vasoconstrictor like angiotensin II in rabbit isolated distal saphenous artery (Dunn *et al*., 1989) or the presence of a combination of unrelated vasoconstrictor (e.g. the thromboxane-mimetic U46619) and forskolin (to elevate the

cellular cyclic AMP) in the palmar lateral vein (Wright *et al*., 1995b), the ear artery (Roberts *et al*., 1999) and the rectal artery (Minyan *et al*., 2001). These manipulations imply that post-junctional α_2-adrenoceptors are more widely distributed than generally recognised from standard *in vitro* studies.

α_2-Adrenoceptors mediate vasoconstriction by activation of G_i -protein which inhibits adenylyl cyclase, thus reducing the generation of cAMP. As myosin light chain kinase, an essential enzyme in regulation of contractility, is inactivated by cAMP-dependent protein kinase, the reduction of cAMP formation is associated with vascular smooth muscle contraction. Also, α_2-adrenoceptor functions were sensitive to pertussis toxin, which suggests the activation of G_i protein (Docherty, 2019). Roberts and co-workers (1999) concluded during their studies that α_2-adrenoceptors-evoked contraction was not mediated only by a reduction in cAMP, but by using agents that elevate adenylyl cyclase and thus stimulating the production of cAMP. α_2-Adrenoceptors may work to decrease forskolin-stimulated cAMP production.

In a study of the guinea pig cauda epididymis, α_2-adrenoceptors agonists were found to stimulate the contractions to through inhibition of forskolin-stimulated cAMP accumulation (Haynes and Hill, 1996). There were also studies showing that α_2-adrenoceptors caused activation of other signal transduction pathways such as activation of potassium channels, phospholipase A_2 and Na^+/H^+ exchange (Bylund *et al*., 1994).

Somlyo and Somlyo (1998) concluded that α_2-adrenoceptors also mediated action by calcium sensitisation, like α_1-adrenoceptors. More recent evidence, suggests that there are also subclassification schemes of α_2-adrenoceptors into: α_{2A}, α_{2B} and $\alpha_{2C,}$ which

were based initially on their affinity to prazosin (Bylund *et al*., 1994).

Early studies found that the receptors with low affinity to prazosin were considered α_{2A} subtypes, while the α_2-adrenoceptors, which had high affinity to prazosin, were designated as α_{2B}-adrenoceptors (Bylund, 1985). Later on, the pharmacological subclassification of α_2-adrenoceptors was determined by the ability of various antagonists like ARC239, BAM1303, WB4101, yohimbine and rauwolscine to compete for α_2-adrenoceptor antagonist binding sites. α_{2C}-Adrenoceptor possess higher affinity to prazosin similar to α_{2B}-adrenoceptors, but distinguished from it by its higher affinity to BAM1303, WB4101 and rauwolscine antagonists (Bylund *et al*., 1994).

Based on agonist affinity to sub-classify the α_2-adrenoceptors, only the partial agonist oxymetazoline was approximately (50-100)-fold more potent at the α_{2A}-adrenoceptor relative to the α_{2C}- and α_{2B}-adrenoceptors, respectively (Bylund, 1992; Alexander *et al*., 2017). Furthermore, α_{2D}-adrenoceptors as a fourth type of α_2-adrenoceptors were identified with a characteristic lower affinity for yohimbine and rauwolscine antagonists than the other three subtypes (Bylund, 1992; Piascik *et al*., 1996). α_{2D}-Adrenoceptors were first discovered in zebrafish then also found in other tissues such as bovine pineal gland, submaxillary gland and islet cell tumor-derived cell line of the rat (Piascik *et al*., 1996). Yet, this type may be not relevant in human and there is a controversy related to its characterisation as a fourth subtype of α_2-adrenoceptor for example, the rat α_{2D}-adrenoceptor is considered as a species orthologue of the human α_{2A}-adrenoceptor (Bylund *et al*., 1994; Docherty, 2019).

Functional α_2-adrenoceptors in the rat submandibular gland, rat vas deferens (Smith and Docherty, 1992), rat cerebral cortex (Trendelenberg *et al*., 1993), pithed rat heart

(Smith *et al*., 1995) and mouse atria resemble the α_{2D}-adrenoceptor ligand binding site, whereas those in rabbit cerebral cortex (Trendelenberg *et al*., 1993), dog mesenteric artery (Daniel *et al*., 1995) and human saphenous vein (Molderings and Gothert, 1995; Rizzo *et al*., 2001) resembles the α_{2A}/α_{2C}-adrenoceptor. As α_{2D}-adrenoceptors share many similarities with α_{2A}-adrenoceptors, most of the studies mention only three types (α_{2A}, α_{2B} and α_{2C}) (Civantos Calzada and Aleixandre de Artinano, 2001; Bylund, 2005; Alexander *et al*., 2017). $\alpha_{2A}/_{B}$-Subtype of adrenoceptor is the predominant functional post-junctional α_2-adrenoceptor subtype (Docherty, 2019).

Brimonidine is an agonist acting at α_{2A}-adrenoceptors (Jansson *et al*., 1998; Alexander *et al*., 2017) and can only produce obvious contractions by α_2-adrenoceptors in isolated blood vessels with a high receptor density (> 300 fmol mg^{-1} protein) (Wright *et al*., 1995a). Oxymetazoline and guanfacine are considered as a selective for the $\alpha_{2A/D}$-adrenoceptor agonists; however, there is no selective agonist available for the other α_2-adrenoceptor subtypes (Civantos Calzada and Aleixandre de Artinano, 2001; Alexander *et al*., 2017). In relation to α_2-adrenoceptor antagonists, Young *et al*., (1989) have confirmed the presence of selective antagonists for α_2-adrenoceptors for example, BRL 44408 is a selective α_{2A}-adrenoceptor, imiloxan is a selective α_{2B}-adrenoceptor, (Michel *et al*., 1990) and JP1302 is a selective blocker at α_{2C}-adrenoceptor subtype (Sallinen *et al*., 2007).

1.1.2.2 β-adrenoceptors

There are two main subtypes of β-adrenoceptors depending on their pharmacological response and the degree of potency to the agonists (Lands *et al.*, 1967). These β-adrenoceptors are β_1-adrenoceptors, which have been found in the heart and intestinal smooth muscles and have similar sensitivity to catecholamines (noradrenaline and

adrenaline) and their dominance. Activation of β_1-adrenoceptors results in an increase in heart rate and contractility while activation in the intestine results in smooth muscles relaxation (Lands *et al.*, 1967; Frielle *et al*., 1987).

On the other hand, β_2-adrenoceptors are most common in the liver, striated muscle, the smooth muscle of gastrointestinal tract, genitourinary tract, bronchial tree, and blood vessels feeding skeletal muscle and have less sensitivity to noradrenaline. Activation of β_2-adrenoceptors is responsible for hepatic glycogenolysis, muscle tremor, bronchodilatation, relaxation of visceral smooth muscle, and vasodilatation (Westfall and Westfall, 2006a, Westfall and Westfall, 2006b; Barisione *et al*., 2010). At the end of the 20th century, studies showed a third type of β-adrenoceptors, not responding to non-selective agonists and antagonists (propranolol). This third type is β_3-adrenoceptors which are present in the adipose tissue and bladder of the human and are responsible for lipolysis and thermogenesis (Guimaraes and Moura, 2001, Alexander *et al*., 2017; Docherty, 2019). Generally, the β-adrenoceptor signal transduction pathway starts via activation of Gs protein subunits that leads to adenylate cyclase stimulation, thus increasing the production of cAMP (Strosberg and Pietri-Rouxel, 1996).

1.1.3 Therapeutic use of α-adrenoceptor agonists and antagonists

Commonly, both α_1 and α_2-adrenoceptors mediate vasoconstriction, yet the degree of contribution of each receptor in the contraction responses is still controversial. In general, α-adrenoceptors have been used as a target for therapeutic agents and play an important physiological role in the cardiovascular system as the parenteral use of α_1-adrenoceptors to elevate blood pressure in patients during anesthesia (Dyer *et al*., 2009;

Foss *et al*., 2014), septic shock (Morelli *et al*., 2008) and hemorrhagic shock (Hoen *et al*., 2005). Distribution of α-adrenoceptors in the central nervous system, pre-junctional autonomic fibers and post-junctional on smooth muscle cells have found clinical utility in drugs that activate these receptors. Agonists are used to treat hypertension (Sica, 2007), migraine (Rapoport and Bigal, 2004), opiate withdrawal (Yu *et al*., 2008), nasal congestion (Valdyanathan *et al*., 2010), preoperative sedation (Blaudszun *et al*., 2012), acute hemorrhoidal disease (Hernandez-Bernal *et al*., 2014), muscle spasm (Vakhapova *et al*., 2010) and glaucoma (David, 2001).

A considerable amount of literature has been published to describe the clinical application of α-adrenoceptosr agonists as nasal decongestants, to reduce nasal airway resistance and thus facilitate nose breathing. For example, a controlled study by Dressler and colleagues (1977) showed that the interior rhinomanometric measurements were improved with decongestants (0.25% phenylephrine nasal spray, ephedrine inhaler and phenylpropanolamine 25mg oral syrup). In their case study, Dressler *et al*., (1977) concluded the greatest decongestant action was seen with 0.25% phenylephrine nasal spray which reduced the baseline congestion index, especially after 15 minutes post-treatment time from 24.4 to 5.6 (P <0.5, n=10).

A number of studies have investigated the therapeutic useful action of the α_2-adrenoceptor agent (BHT-920) in reducing vascular nasal mucosa dilation and therefore inhibiting mucosal swelling (Corboz *et al*., 2005; Corboz *et al*., 2007; Corboz *et al*., 2008). For example, selective α_2-adrenoceptor agonists produced significant contractions in nasal strips of variable species including dog, Yucatan mini-pig, Rhesus monkey and Cynomolgus monkey with a potency range of 6.2-7 for BHT-920 and 6.1-7.4 for PGE-6201204 agonists. Corboz and colleagues performed a similar series of

histological experiments on nasal human mucosa and pig nasal veins and found an effective role for BHT-920-activates α_2-adrenoceptors in reducing the engorgement of blood vessels in those tissues (Corboz *et al*., 2005; Corboz *et al*., 2007) (Figure 1.4).

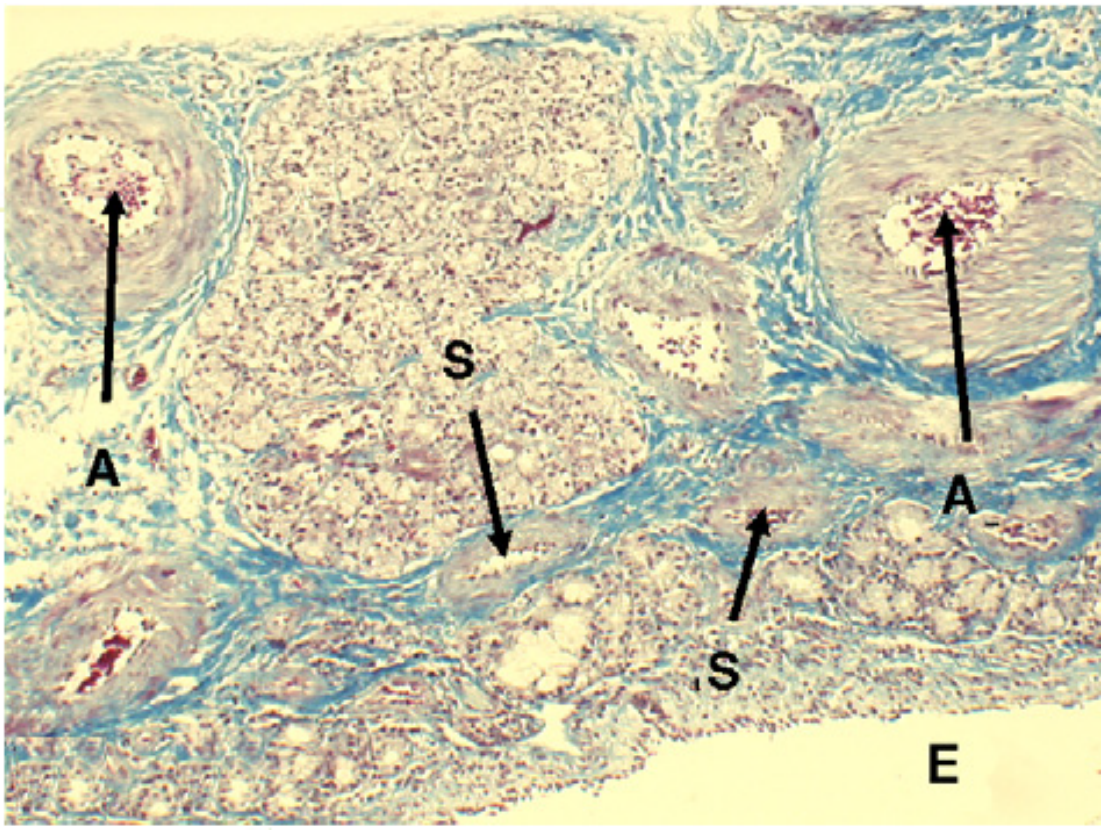


Figure 1.4 Light microscopy of the pig nasal mucosa showing a section of lamina propria following treatment with the α_2-adrenoceptor agonist BHT-920 (10μM) illustrating collapse (contraction) of thin-walled venous sinusoids (S) and minimal to no effect on small arteries (A) using hematoxylin and eosin stain; 10x magnification (Corboz *et al*., 2007).

Later on, the same authors concluded that the use of BHT-920 α_2-adrenoceptor agents improved patency of the nasal cavity by evaluating the real-time contractility assay in isolated pig nasal explants and *in vivo* cat model of congestion (Corboz *et al*., 2008).

Akerlund *et al*., (1989) approved the use of different concentrations 0.25, 0.5 and 0.1mg/ml oxymetazoline nose drops for more than four decades as a decongestant by

measuring the effect of it on the nasal airway resistance and the symptoms of nasal congestion in 106 patients. Akerlund and co-workers (1989), concluded there was a dose-response relationship between the nasal airway resistance and the nasal congestion symptoms, especially by using 0.5mg/ml and volume 0.1ml in each nostril of oxymetazoline, which appeared to be effective and caused significant healing of nasal blockage up to 7 hours.

Additionally, in a randomized controlled study done in thirty patients requiring nasendoscopy, after two weeks administration of five puffs of co-phenylephrine (0.5% phenylephrine and 5% lignocaine) nasal spray, the ability of endoscopists to insert nasendoscopy and the quality of visualization of the upper airway were increased by 84 (95% CI: 80-89) compared with the use of 5% lignocaine alone 77 (95% CI: 73-81) ($P < 0.01$) according to visual analogue score and co-phenylephrine spray also reduced the pain experienced by the patients (Douglas *et al*., 2005).

A relationship exists between the topical applications of α_1-adrenoceptor agents and controlling of bleeding time. Kratz and Danon, 2004 reported that the topical spray of α_1-adrenoceptor agents contributed to the decline of bleeding time from superficial injuries and spraying 0.25% phenylephrine caused a shorter mean bleeding time compared with 0.9% saline (1.9 ± 0.14 minutes versus 4.8 ± 0.43 minutes, respectively, $P < 0.001$) compared to 0.05 and 0.25% oxymetazoline (4.46 ± 0.54 and 5.5 ± 0.58 minutes, respectively, $P < 0.001$). A recent study by Christensen *et al*., (2017) indicated that 17 from 25 patients administered 0.5% of phenylephrine intranasally before nasotracheal intubation were connected with increased ease of insertion of nasotracheal tube and a decrease in their blood loss.

Clonidine was established as a peripheral vasoconstrictor agent for the treatment of nasal blockage and congestion (Stahle, 2000). But, remarkably, a few cases argued a major hypotensive effect, in addition to other systemic side effects including bradycardia, and dryness of the mouth that limits clonidine usage as a nasal decongestant (Frye and Vance, 2000; Johnson *et al*., 2011; Perruchoud *et al*., 2012). However, the clinical use of clonidine as an antihypertensive also has been limited by its sedative effects, but compromises benefits in anaesthetic field due to their ability to regulate the sympathetic tone, which gave a desirable haemodynamic profile and sustained myocardial oxygen demand during surgery (Engelman *et al*., 1987; Khan *et al*., 1999). De Kock *et al*., (1995) described the 350 patients undergoing major abdominal surgery who administered intravenous 4 $mg.kg^{-1}$ clonidine at induction followed by 2 $mg.kg^{-1}.h^{-1}$ infusion. Fewer adverse hemodynamic responses were recorded compared with the 52 controls and only two cases of severe hypotension and bradycardia were detected in the clonidine group.

Turning now to the therapeutic use of α-adrenoceptor blockers. Phentolamine which was discovered by Urech *et al*., (1950) as a non-selective α-adrenoceptor antagonist and was used in clinical situations including the diagnosis of pheochromocytoma and the treatment of hypertensive crises in emergency due to pheochromocytoma, epinephrine and norepinephrine-related dermal necrosis, cocaine-associated acute coronary syndrome, erectile dysfunction, and also a diagnostic and therapeutic action in complex regional pain syndrome (Hoffman and Lefkowitz, 1980; Tuncel and Ram, 2003; Hong *et al*., 2005; Rowbotham, 2006; Bavadekar *et al*., 2008). Additionally, recent developments in anticancer drug therapies have highlighted the reintroduction of phentolamine as an effective new chemotherapy for castration-resistant prostate cancer (Ho *et al*., 2015).

This present study reported that phentolamine-induced anti-proliferative effect and activation of related apoptotic signaling pathways in castration-resistant prostate cancer cells, and thus lead to mitotic arrest of the cell cycle and further mitochondrial damage. Authors of this study also found that a combination of phentolamine with another chemotherapeutic agent (paclitaxel) caused a synergistic effect on apoptotic cell death (Ho *et al*., 2015). This outcome hypothesised the reintroduction and/or repurposing of phentolamine, which can be used for lithium cations along with α_1-adrenoceptor agents as a new topical decongestant medication to enhance their action or increase their duration.

1.2 Structure of the vascular system

Generally, the vascular system is composed of a complicated network of vessels, which function to circulate the blood and nutrients throughout the body. As this dissertation has investigated the porcine splenic artery, vein and nasal vasculature. Therefore, this study will focus on those blood vessels, which represents both arteries and veins and are composed of three layers: intima, media and adventitia. The thinnest layer is the intima; one layer of endothelial cells and the basal lamina. The media exhibits a greater amount of smooth muscle, inserted in a matrix of collagen, a small amount of elastin and various glycoproteins separated from the adventitia by an external elastic lamina. The third layer (adventitia) is composed of collagen, fibroblasts, vaso vasorum, lymphatics and nerves (Pugsley and Tabrizchi, 2000; Jain, 2003; Rhodin, 2011; Klabunde, 2012).

Smooth muscle cells are the major constituent of the blood vessel wall and are classically 5-10 μm in diameter and ranges from 50-300 μm in length. Small invaginations (caveolae) are present in the cell membrane, which are thought to increase the surface area of the cell. Contraction of the smooth muscle cells is the main regulator of the vascular tone as they are composed of the contractile proteins (actin and myosin) with the bands of actin filaments merged to each other and attached by dense bodies or dense bands like Z-lines. Every myosin filament is bounded by numerous actin filaments. Smooth muscle cells are linked electrically to each other by the connection between smooth muscle and endothelial cells using gap junctions, promoting cell-to-cell propagation of depolarising currents (Yamamoto *et al.*, 2001; Figueroa and Duling, 2009; Klabunde, 2012).

1.2.1 Vascular smooth muscle contraction

Contraction of the smooth muscle cells is regulated by neuronal or humoral stimuli and this can vary in various vessels. Vascular smooth muscles have the ability to produce slowly and highly sustained forces of contraction. Generally, smooth muscle contractile activity is promoted by a variety of calcium-mediated signaling pathways. Basically, a surge in vascular tone is initiated by an elevation in the cytosolic calcium ion concentration, by either stimulation of voltage-gated or receptor-operated calcium channels or stimulation of intracellular second messengers that start a sequence of events ending with the discharge of calcium ions from their internal stores (sarcoplasmic reticulum) (Streefkerk *et al.*, 2002).

1.2.1.1 Activation of smooth muscle myosin

Activation of L-type calcium channels by G protein-coupled receptor (GPCR) agonists, cell membrane depolarisation and numerous signal transduction pathways result in an elevation in intracellular free calcium ion concentration and this process ends with vascular smooth muscle contraction (Webb, 2003). A combination of free calcium and calmodulin (a specific calcium binding protein) (Rembold, 1992) produces a calcium-calmodulin complex. This complex, in turn, activates a catalytic subunit of myosin light chain kinase enzyme (MLCK) in the presence of adenosine triphosphate (ATP) and this subunit phosphorylates serine at position 19 on the regulatory light chain of myosin (MLC). This phosphorylation leads to cross-bridge cycling of myosin heads along the actin filaments and is considered as a key episode in smooth muscle contraction (Gao *et al*., 2013) (Figure 1.5).

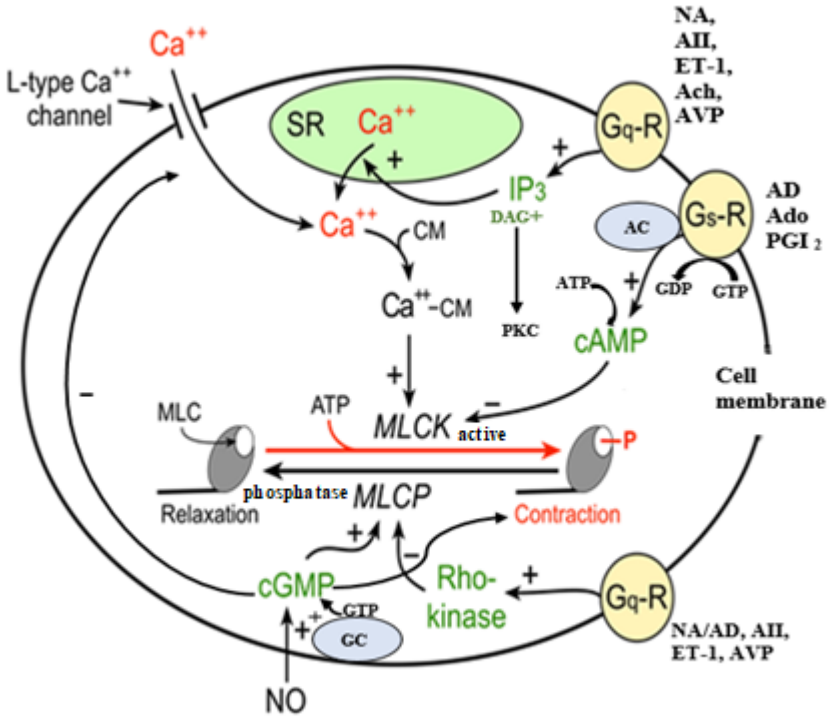


Figure 1.5 Diagram of smooth muscle contraction. Noradrenaline (NA); angiotensin II (AII); arginine vasopressin (AVP); acetylcholine (ACh); and endothelin I (ET-1) activate phospholipase through the Gq protein that leads to: either IP_3 formation from phosphatidylinositol (PIP_2). IP_3 causes the release of calcium from the sarcoplasmic reticulum. The formation of diacylglycerol (DAG) from PIP_2 leads to activation of protein kinase C, or activate the Rho kinase, which inhibits the activity of MLC phosphatase and promotes contraction. Adrenaline (AD); adenosine (Ado); and prostacyclin (PGI_2) activate adenylyl cyclase through activation of Gs protein, which catalyses the formation of cyclic adenosine monophosphate (cAMP). cAMP causes inhibition of MLCK that decreases phosphorylation of myosin light chain heads and inhibits the interaction between actin and myosin. NO passes from the endothelial cells to the smooth muscle cells and activates soluble guanylyl cyclase that leads to an increase in cyclic guanosine monophosphate (cGMP) and results in relaxation. R, receptor; Gs, stimulatory G-protein; Gq, phospholipase C-coupled G-protein; AC, adenylyl cyclase; PL-C, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; SR, sarcoplasmic reticulum; GC, guanylyl cyclase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; ATP, adenosine triphosphate; Ca^{2+}, calcium. Figure modified from (Klabunde, 2012; Audrey *et al.*, 2016).

1.2.1.2 Mechanism of Inositol trisphosphate (IP3) and diacylglycerol (DAG) discharge

Different agonists, hormones and neurotransmitters such as noradrenaline, adrenaline, angiotensin II (AT_1 receptor), vasopressin (V_1 receptor), acetylcholine (ACh) (M_3 receptor), and endothelin I (ETA), α_1-adrenoceptor agonists, etc. act on cell membrane receptors. Once activated, the receptor binds to Gq protein, which then stimulates phospholipase C enzyme (a membrane-bound enzyme). This enzyme then activates the phosphatidylinositol cascade by the formation of two second messengers: Inositol-1,4,5-trisphosphate generation (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). In turn, IP_3 enters and activates receptors on the sarcoplasmic/endoplasmic reticulum resulting in release of calcium into the cytosol. In addition, DAG with calcium activates protein kinase C (PKC) that can cause vascular smooth muscle contraction (Webb, 2003; Klabunde, 2012) (Figure 1.5). PKC has numerous isoforms in smooth muscle. The α, β, and γ isoforms of PKC are calcium-dependent, while δ and ε are calcium-independent (Brozovich *et al.*, 2016).

PKC can cause vascular smooth muscle contraction by protein phosphorylation of L-type calcium channels or cross bridge proteins (Webb, 2003). PKC activation causes indirectly PKC-dependent activation of double specificity mitogen-activated protein kinase kinase, which causes an increase in extracellular signal-regulated kinases (ERK) phosphorylation. This phosphorylation leads to phosphorylation of caldesmon. Caldesmon (an actin-binding protein) inhibits the myosin entrance to actin, and thus stops cross bridge formation. The C-terminal of caldesmon inhibits directly myosin ATPase activity (Bryan, 1990; Wang *et al.*, 1991).

1.2.1.3 Calcium Sensitisation pathway

Numerous agents enhance the tone of vascular smooth muscle via increasing intracellular calcium levels temporarily (Bradley and Morgan, 1985). The relationship between calcium levels and contraction events can be described by the phosphorylation of the myosin light chain, which is the major determinant of smooth muscle tone. MLC phosphatase has three subunits; catalytic region, myosin-binding region, and variable region. The most vital subunit is the myosin-binding subunit, which inhibits MLC phosphatase activity, causing persistence of myosin light chain phosphorylation, and thus supporting the cross-bridge formation between myosin heads and actin filaments (Somlyo and Somlyo, 2003). Activation of the ERK pathway by agonists prompts the continuation of smooth muscle contraction at lower calcium concentrations, which phosphorylates caldesmon, thereby supporting cross-bridge formation between actin and myosin (Kordowska *et al.*, 2006).

Another major component of the physiological calcium sensitization pathway is carried out by Rho kinase. Rho-kinase (a serine/threonine kinase) plays a significant role in the phosphorylation of myosin-binding subunits of the MLC phosphatase. *In vitro* studies with many agonists, which activated Rho kinase by G protein RhoA signaling pathway through guanine nucleotide factors, caused an inhibition of MLC phosphatase and in prolongation of smooth muscle contraction (Pearson and Vanhoutte, 1993; Chitaley *et al.*, 2001). Uehata *et al.*, (1997) demonstrated a pyridine derivative (Y-27632), which can selectively suppress smooth muscle contraction by suppressing Ca^{2+} sensitization. Y-27632 acts as a Rho-associated protein kinase inhibitor and consequently stops RhoA-signaling pathway and thus vasocontraction results in practical correction of hypertension in several hypertensive rat models.

1.2.2 Vascular smooth muscle relaxation

Relaxation of vascular smooth muscle occurs by two routes. Firstly, removal of the vasoconstrictor effects, secondly by diminishing of vasoconstrictor mechanisms. The first effects happened when there is inhibition of the intracellular calcium concentrations or myosin dephosphorylation. Thus, this inhibition suppresses the interaction between actin and myosin and causes smooth muscle relaxation.

The pathway of intracellular calcium diminishing occurs either by pumping calcium from the cytosol to the extracellular space through the plasma membrane calcium ATPase or pumping calcium from the cytosol to the sarcoplasmic reticulum, which depends on ATP hydrolysis (Poburko *et al*., 2009). The phosphorylation of sarcoplasmic/endoplasmic reticulum Ca^{2+}, Mg^{2+}-ATPase causes transport of two calcium ions which are then stored in the lumen of the SR. The phosphorylation process requires magnesium to bind at the catalytic position of ATPase to promote enzyme activity. Additionally, calmodulin has an auto inhibitory effects in which it binds to Ca^{2+}, Mg^{2+}-ATPase to decline the intracellular calcium concentrations. In fact, there is also another mechanism for decreasing intracellular calcium concentrations by sodium/calcium exchange, receptor-operated, and voltage-gated channels through the plasma membrane (Campbell and Paul, 1992; Marin *et al*., 1999; Barron *et al*., 2000; Webb, 2003).

1.2.2.1 Regulation of smooth muscle relaxation by the endothelium

Generally, the endothelium is actively involved in vascular relaxation by releasing potent vasoactive agents that elicit contraction or relaxation of vascular smooth muscle. The most pivotal endothelial-derived vasodilator substances are nitric oxide (NO),

prostacyclin and endothelial-derived hyperpolarising factor (Villar *et al.*, 2006; Mitchell *et al*., 2008).

1.2.2.2 The nitric oxide–cGMP system

NO is a key endothelial-vasodilator agent that is formed from the cationic amino L-arginine during the conversion to L-citrulline. This conversion is catalysed by one of three nitric oxide synthase enzymes (NOS); endothelial (eNOS, NOS-3), neuronal (nNOS, NOS-1) and inducible (iNOS, NOS-2) (Guterbaum *et al.*, 2013; Tsutsui *et al.*, 2014).

Nitric oxide is activated by binding endothelial-dependent vasodilator substances (substance P, Acetylcholine and bradykinin) to receptors located on the endothelium. Released NO diffuses from the endothelial cells to the adjacent vascular smooth muscle cells and activates soluble guanylyl cyclase resulting in augmentation in the cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP, in turn, activates protein kinase G (PKG) which has in the same polypeptide chain, regulatory (R) and catalytic (C) domains. Consequently, the active form of PKG inhibits cytosolic calcium by both reduction of calcium influx and intracellular calcium release and ends with smooth muscle relaxation. However, the activated PKG can enhance directly a number of pathways including plasma membrane calcium ATPase, calcium-dependent potassium channels and this can cause hyperpolarisation. Additionally, it stimulates MLC phosphatases and decreases IP_3 levels (Robertson *et al.*, 1993; Archer *et al.*, 1994; Stasch *et al.*, 2002; Evgenov *et al.*, 2006).

1.2.2.3 Endothelium-dependent hyperpolarising factor

Vascular endothelium mediates hyperpolarisation of vascular smooth muscle and vasodilation by the production of endothelial-derived hyperpolarising factor (EDHF) (Bolton *et al*., 1984). Acetylcholine, bradykinin, serotonin and catecholamines activated the release of EDHF which causes an enhancement in intracellular calcium levels in the endothelium, that actually stimulate small conductance calcium sensitive potassium channels (SK_{ca}), intermediate conductance calcium sensitive potassium channels (IK_{ca}) and large conductance calcium sensitive potassium channels (BK_{ca}). (Busse *et al.*, 2002; Gluais *et al.*, 2005; Edwards *et al.*, 2010; Garland *et al.*, 2011).

The possible pathways for vasodilatory effects of EDHF include potassium release through endothelial SK_{ca} and IK_{ca} to stimulate vascular smooth muscle inwardly rectifying potassium channels (K_{ir}) and the ouabain sensitive Na^+/K^+ ATPase pump, respectively and end with hyperpolarisation and dilation of the smooth muscle in rat arteries (Edwards *et al.*, 1998; Edwards *et al.*, 2010) and/or transfer of the endothelium hyperpolarising current by gap junctionas in myoendothelial cells (Chaytor *et al.*, 1998; Chaytor *et al.*, 2003; Chadha *et al.*, 2011; Kerr *et al.*, 2012).

1.2.2.4 Prostacyclin and cAMP-dependent vasodilation system

In addition to nitric oxide and EDHF that are produced by the vascular endothelium, prostacyclin (PGI_2) is also a potent endothelium-dependent vasodilator that promotes vasodilation by stimulation of IP prostanoid receptors and hence Gs protein, which stimulates the formation of cyclic adenosine monophosphate (cAMP) (Klabunde, 2012) (Figure 1.5).

The G protein is a heterotrimer consisting of Gα (includes G_s and G_i), Gβ and Gγ subunits. Activation and inhibition of adenylyl cyclase, respectively are regulated by G_s and G_i. The generation of cAMP, in turn, activates protein kinase A (PKA) that consists of two regulatory domains (R) that maintain its two catalytic (C) domains inactive, at a low level of cAMP. Thus, activation of PKA activates target proteins by mobilising a phosphate group from ATP to serine or threonine residues (Sassone-Corsi, 2012; Taylor *et al.*, 2012). Actually, activation of cAMP and PKA inhibits MLCK affinity for calcium-calmodulin complex and thereby decreases phosphorylation of myosin light chain heads, and the cross-bridge interaction between actin filaments and myosin heads resulting in vasodilation (Sanders *et al.*, 2007).

1.2.3 Regulation of vascular tone by mitochondria

Mitochondria play a pivotal role in the synthesis of ATP, which is used as an energy source for many metabolic cellular processes (Buttgereit and Brand, 1995). Mitochondria releasing energy (ATP) by an electron efflux process in a series of organised events under control of enzymes happened and identified as the mitochondrial respiratory chain, resulting in hyperpolarisation of the mitochondrial inner membrane and energy (Yang *et al.*, 1997; Freed *et al.*, 2014).

The key enzymatic sources of reactive oxygen species that include superoxide and hydrogen peroxide within the vascular wall are mitochondrial electron transport chains. Even though, reactive oxygen species are considered to be a toxic byproduct of metabolic cellular activities, as it contributes in a wide range of cellular processes like cell signaling, cell migration and growth (Felty *et al.*, 2005; Gutterman, 2005). Reactive oxygen species seem to be vital for stimulating endothelium-dependent vascular relaxation during activation of eNOS by changing intracellular calcium

concentrations (Cai *et al.*, 2002; Waypa *et al.*, 2002) and activation of transcription factors such as NF-κB and extracellular signal-regulated kinase including ERK, p38 MAPK and Akt (Pahl and Baeuerle, 1994; Wang *et al.*, 2004; Connor *et al.*, 2005).

1.2.4 Certain proteins can regulate vascular tone

The first group of protein substances that contributes to the vascular tone regulation include the pannexin family that includes three members: pannexin-1, pannexin-2 and pannexin-3. Pannexin-1 is present in vascular smooth muscle, particularly resistance arteries and arterioles as well as the vascular endothelial cells of large arteries, resistance arteries, arterioles and capillaries (Billaud *et al*., 2011; Burns *et al.*, 2012; Lohman *et al.*, 2012; Billaud *et al*., 2015; Kaneko *et al.*, 2015; Lohman *et al.*, 2015).

The second group includes transient receptor potential (TRP) channel superfamily, which are expressed in the endothelium and smooth muscle. These proteins have a pivotal role in mediating NO and EDH vasodilation response via stimulation of TRPV4 channels (members of the vanilloid TRP subfamily), and TRPC3 channels (members of the canonical TRP subfamily). Furthermore, these channels activate hyperpolarisation and vasodilation of the vascular smooth muscle by enhancing calcium influx and transient calcium-sensitive potassium channel activity (Huang *et al.*, 2011; Bagher *et al.*, 2012; Bubolz *et al.*, 2012; Sukumaran *et al.*, 2013; Zheng *et al.*, 2013).

1.3 Anatomical structure of the nose

The nose is a complicated cartilaginous structure that has two parts, the external nose and the nasal cavities; the nose has been clarified by a septum located in the mid line (Snell, 2004). The right and left nasal cavities are located in the middle of the craniofacial skeleton. Each nasal cavity has lower, upper, lateral and medial walls. The roof of the hard palate, which separates it from the oral cavity, forms the lower wall. The structure of the upper wall consists of a bony body of the sphenoid and cribriform plate that defines the cavity from the anterior cranial fossa. While the lateral walls consist of three bony extensions called the inferior, middle and superior turbinate. These turbinate structures are an important part of the nasal cavity structure because they provide a hole-like cavity and thus increases the surface area of the nasal mucosa to catch any soluble substance entering the nasal cavity. In addition, the turbinate provides humidity and controls the temperature of the inspired air (AlSuleimani and Walker, 2007) (Figure 1.6).

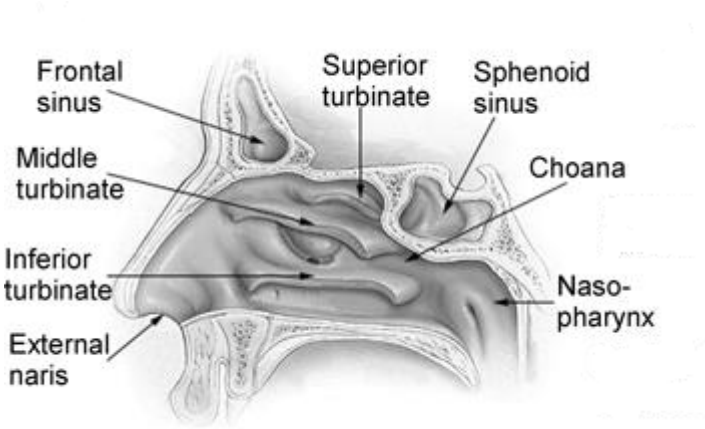


Figure 1.6 Anatomy of the nasal cavity (Nagarajan *et al*., 2004).

There is an inner part located under each turbinate, called the meatus, and its name belongs to the turbinate above it (inferior meatus, middle meatus and superior meatus). The dorsal portions of the nasal cavity connect to the nasopharynx. The nasal sinuses consist of four groups of double sinuses, which are named according to their location in the maxillary, frontal, ethmoid and sphenoid bones. As a result, the sinuses are called maxillary sinuses, frontal sinuses, ethmoidal air cells and sphenoid sinuses. However, these sinuses are covered with the respiratory epithelium and draw off into the nasal cavity, which is the same site at which the lacrimal gland also draws off (Snell, 2004; Monkhouse, 2007; Gosling *et al*., 2008).

In addition to being considered as the first part of the airways, the nose traps dust particles and foreign bodies in the nasal vestibule just after the nostril that is covered with non-keratinized stratified squamous epithelium with many nasal hairs. Therefore, the main functions of the nose are olfaction, sensation, immunology, mucociliary clearance and finally, maintaining the humidity, cleaning and heating of inspired air (AlSuleimani and Walker, 2007; Hall, 2011; Tate, 2011; Young *et al*., 2014). The blood supply to the nose comes from the internal carotid artery (ethmoidal branches of the ophthalmic) and external carotid artery (branches of the maxillary artery and facial artery) which feed the nasal mucosa with a blood flow greater than the liver or the brain (Figure 1.7). This blood supply passes throughout the capillary bed by arteriovenous anastomoses with a function related to temperature and water regulation. The blood vessels and glands in the nasal mucosa are supplied by afferent and efferent neurons.

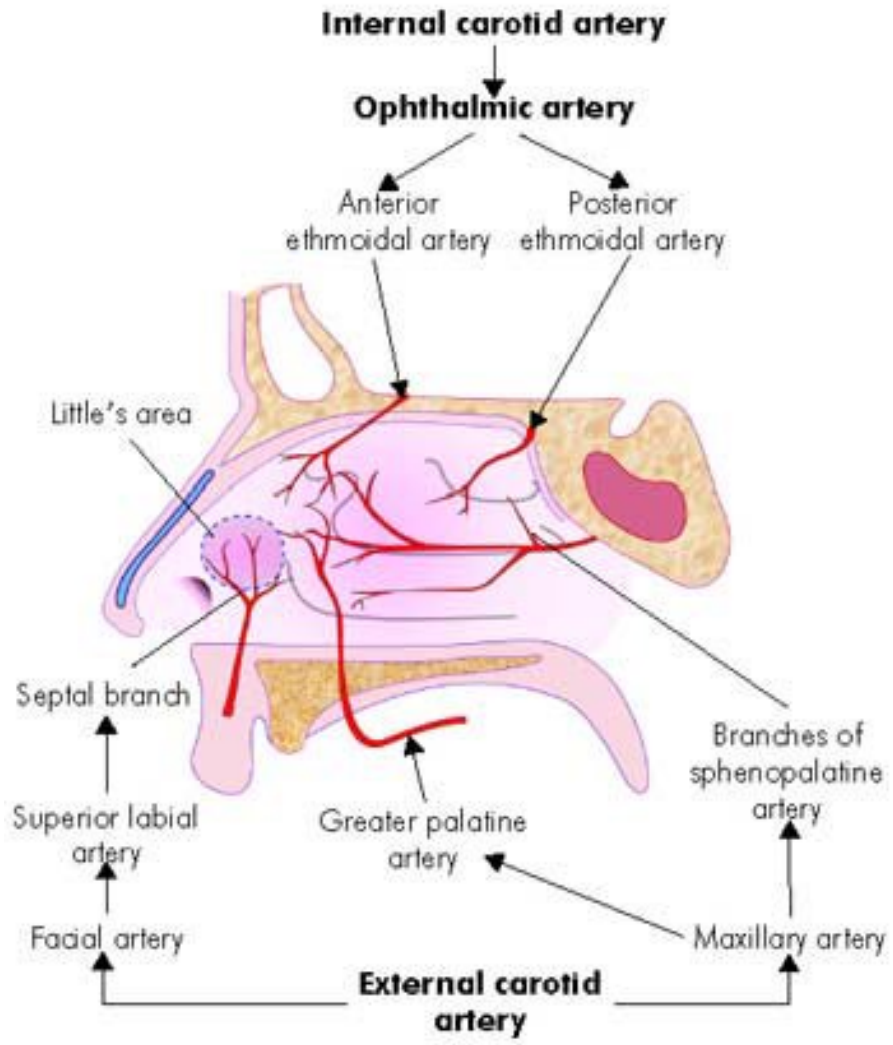


Figure 1.7 Blood supply to the nasal septum (Hall, 2011)

The afferent nerve is divided into two portions. First, the olfactory nerve from the cranial nerve I and discharge into olfactory mucosa and its role related to the sensation of smell. Secondly, the trigeminal nerve from cranial nerve V and discharge into the epithelium and its role related to awareness of airflow. In the nose, the blood vessels are supplied by both sympathetic and parasympathetic nerve supply, however, the regulation is chiefly by the sympathetic nerve (Jones, 2001; AlSueimani and Walker, 2007).

The anterior one-third of the nasal cavity is covered by a squamous and transitional epithelium. The top portion of it is lined with an olfactory epithelium and the rest by a ciliated, pseudostratified and columnar epithelium, which consists of four cell types: basal cells, ciliated columnar cells, non-ciliated columnar cells and goblet cells. The ciliated and non-ciliated columnar cells are lined by approximately 300 microvilli distributed over the surface, to assist the exchange process and provide moisture for ciliary function. A dense net of blood vessels (arterioles and venous plexuses) and extension of seromucous glands are located under the epithelium layer, which is attached to the surface by a small duct (Ovalle *et al*., 2008; Young *et al*., 2014) (Figure 1.8).

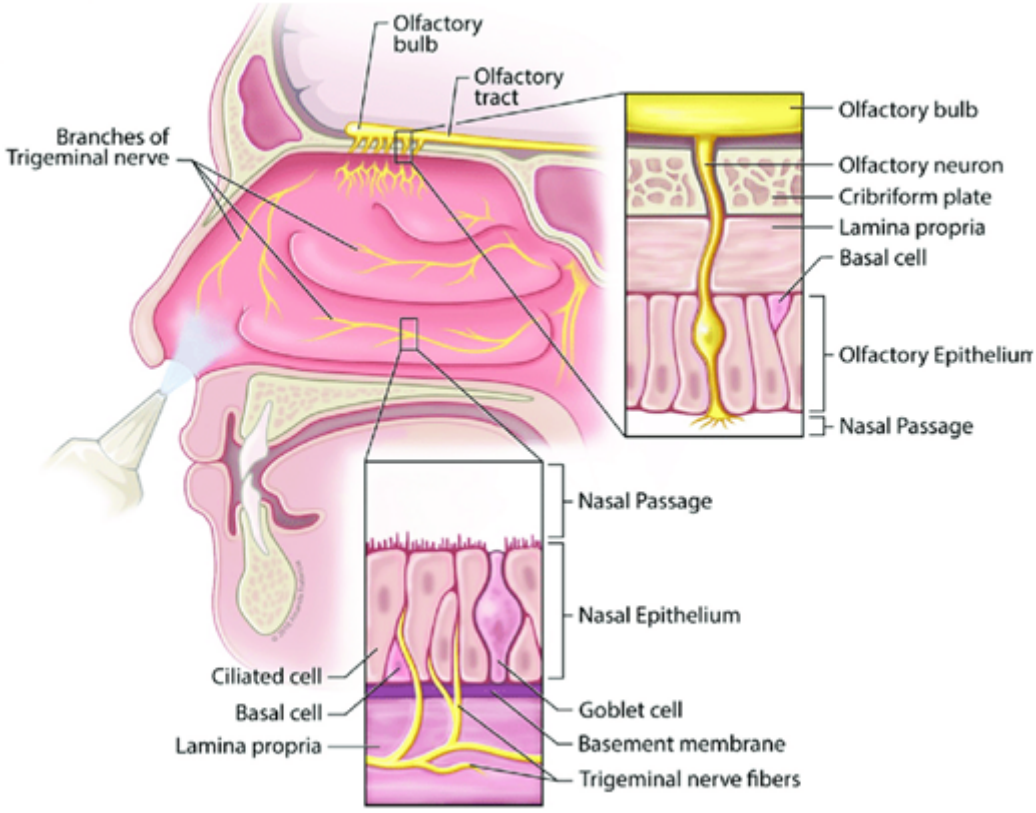


Figure 1.8 Lateral section of the nose shown the key anatomical structures, location of olfactory and trigeminal nerves in yellow. Details of olfactory and respiratory epithelia in enlarged sections (Chen *et al*., 2018)

1.4 Nasal congestion

1.4.1 Nasal Congestion Definition

In general, nasal congestion can be defined as a displeasing feeling, which usually occurs with other symptoms like a sore throat, headache, inability to taste, and smell, rhinorrhoea and sneezing, however, all these symptoms are frequently present with the common cold or with an allergic condition to several types of allergic substances. Commonly those symptoms suggest that the airway passage of the nose is not working normally and could be described as nasal stuffiness, nasal congestion, nasal obstruction and nasal blockage. Because nasal congestion could occur with other medical conditions as one of the symptoms of another disease such as allergic rhinitis, nonallergic rhinitis, rhinosinusitis, otitis media, asthma and nasal polyposis.

Congestion can also be caused by physical obstruction of nasal passages and/or modulation of sensory perception. Therefore, it is necessary to give an exact definition of nasal congestion as the diagnosis and treatment of many clinical conditions and epidemiology of many diseases depend on giving a definite definition of the clinical condition (Baumann, 2013). Mosby's Dictionary of Medicine, Nursing and Health Professions (2016) describes nasal congestion in a complicated definition as "a narrowing of the nasal cavity, thereby reducing the breathing capacity, caused by an irregular or deviant septum, nasal polyps, foreign bodies or enlarged turbinates", however, this complicated definition gives different options of care and treatment according to the causal factor of nasal congestion. Nathan, (2008) describes nasal congestion as "a subjective sensation of nasal blockage, accompanied by varying degrees of reversible objective airflow limitation".

This broad definition should differentiate between nasal congestion and nasal airflow obstruction because sometimes those two situations could not happen simultaneously. As a result, congestion is defined according to a medical dictionary (Dorland, 2012) as "an excessive or abnormal accumulation of fluid, as of blood, in part of the body". Therefore, the enormous and unnatural collection of fluid and/or blood in the nose refers to nasal congestion, which will lead to nasal obstruction. Yet, not all nasal obstructions result from nasal congestion. Van Spronsen *et al*., (2008) in their evidence-based guidelines for nasal congestion, defined the most complete definition of nasal congestion as: "an important symptom in nasal pathology and can be defined as an objective restriction of nasal cavity airflow because of mucosal pathology and/or increased mucus secretion (excluding anatomical variants) "(Figure 1.9).

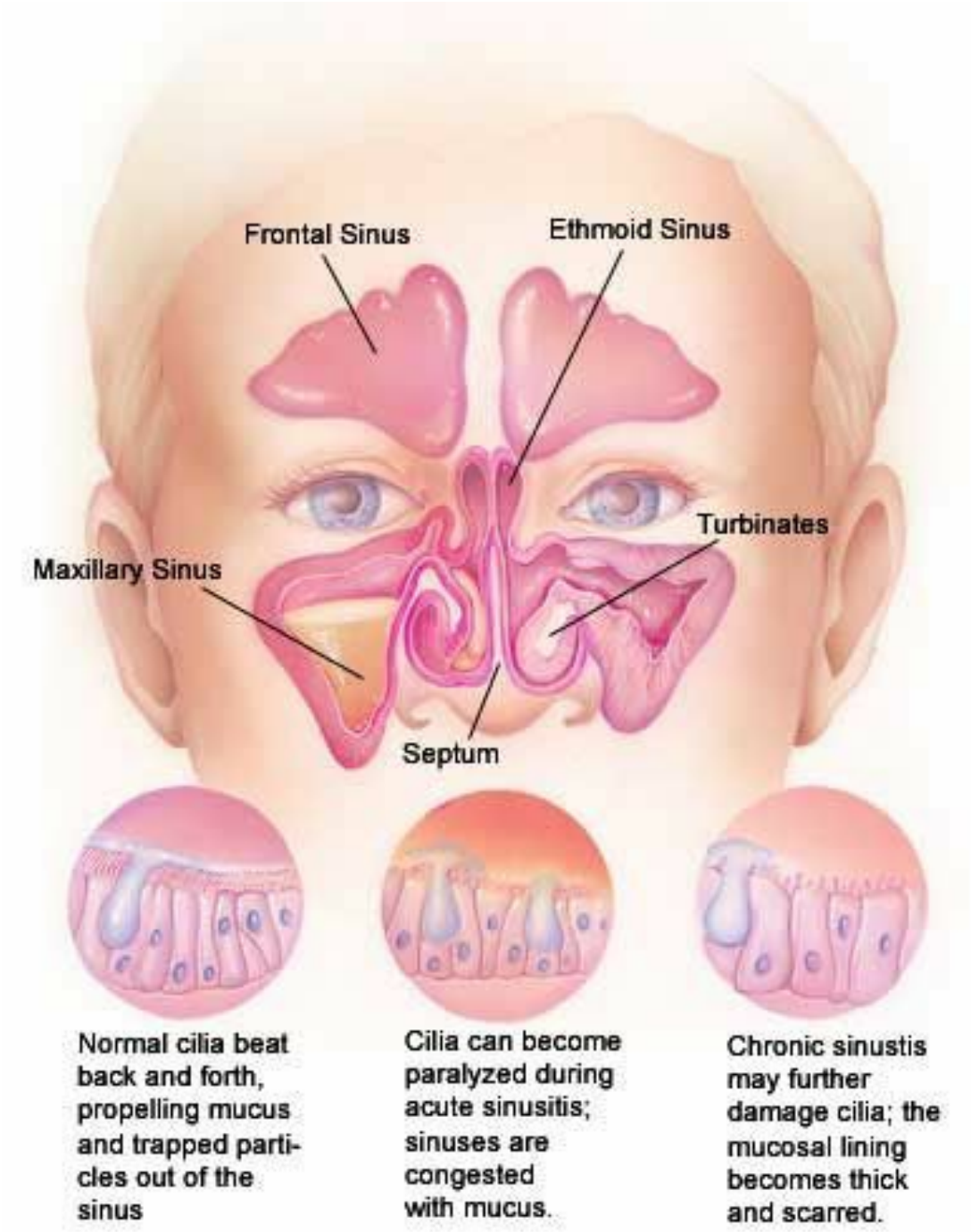


Figure 1.9 Figure illustrated the nasal congestion

1.4.2 Nasal congestion mechanism

Nasal congestion principally results from mucosal inflammation, which is the main vascular reactions that are associated with all the mechanisms involved in nasal obstruction such as intensification of nasal secretion, and vascular permeability, both of which result in engorgement of venous sinusoids, increased nasal secretion and tissue swelling or odema. Increased understanding of the mechanisms underlying inflammation can ease better-quality treatment choice and the improvement of new therapies for congestion.

The nasal mucosal inflammation starts from the increase in the response of the immune system in the respiratory tract, especially degranulation of the mast cells, inflammatory cells and granules, which causes release of histamine and protease in addition to formation and release of inflammatory mediators (leukotrienes, prostaglandins, thromboxane, tumor necrosis factor TNF-α and others) which stimulate the nose (Naclerio *et al*., 2010). Leukotrienes attract eosinophils and this results in more growth of eosinophil precursors, the facility of eosinophil adhesion and inhibition of eosinophil apoptosis. Other major inflammatory mediators include prostaglandins, which connect with the nose hypertrophic inflammation and recruitment of eosinophils. The principal inflammatory mediator is tumor necrosis factor-alpha (TNF-α) which causes activation of T cells, endothelial cells, fibroblasts and macrophages. Also, TNF-α increases the release of inflammatory cytokines, the expression of cell adhesion molecules and vascular cell adhesion molecules (Baraniuk, 1997; Baraniuk, 2001; Pawankar, 2007; Pawankar *et al*., 2011).

In the nasal cavity, all these inflammatory mediators cause vasodilation of the sinusoids and mucus hypersecretion, which results finally in nasal congestion that could be a chronic inflammatory condition known as allergic rhinitis (Togias, 2000).

Another vital contributing factor that may be involved in causing nasal obstruction includes structural problems (for example septal deviation, choanal atresia, choncha bullosa, cleft palate, adenoid hyperatrophy and neoplasia). Additionally, change and abnormality in sympathetic tone that regulate blood flow in the nasal cavity. For example, endogenous biochemical products, physical and chemical stimuli can cause nasal mucosal afferent sensory neuron stimulation and then carry stimuli to the central nervous system to end with nasal obstruction (Sarin *et al*., 2006; Corboz *et al*., 2007; Corboz *et al*., 2008; Naclerio *et al*., 2010). This pathological mechanism, which includes a vascular reaction, hyperstimulation of exocrine glands and release of immune cells to the inflammation site, requires long-term treatment and healing, especially in chronic conditions (Rogers, 2003).

1.4.3 Nasal congestion epidemiology

Nasal congestion is one of the most prominent characteristic symptoms of allergic rhinitis, which is regarded as an important factor in the occurrence of congestion. According to Naclerio *et al*., (2010) allergic rhinitis refers to "an abnormal inflammation of the membrane lining the nose, which is characterised by nasal congestion, rhinorrhoea, sneezing, itching of the nose and/or post nasal drainage". This definition means that nasal congestion is considered as a dependant clinical problem that occurs in association with allergic rhinitis. Generally, allergic rhinitis has a high prevalence rate reaching to approximately 20-50% of all populations in certain countries. Also, at the same time allergic rhinitis has different prevalence rate according to age such as younger patients with 17 years or less have 32% prevalence rate, while 18-44 years of age reaches 43% prevalence rate (Bauchau *et al.*, 2004; Bauchau *et al.*, 2005; Nathan, 2008). As a result, it is distributed most commonly in younger age groups.

The total costs of treating allergic rhinitis include the direct costs of the therapeutic and care management, and the indirect costs of decreased productivity and negative influence on daily functioning which leads to absenteeism at work and school (Nathan, 2008). The number of absences from work for example, in the United States is approximately 3.5 million a year (Shedden, 2005). Due to high distribution rates and unpleasant influences of nasal congestion and the complexity of its cause which could include neural, vascular and inflammatory elements, it is difficult to find studies investigating nasal congestion alone without combination with other medical conditions such as asthma, upper respiratory tract infections, allergic rhinitis and obstructive sleep apnoea and nasal septal deviation (Corey *et al*., 2000).

1.4.4 Nasal congestion influence

The effects of nasal congestion differ according to several factors such as the degree of severity and age, for example, nasal congestions could be only uncomfortable and embarrassing for adults, yet it might be critical for newborn babies and neonates, who may suffer from difficulties in breathing and later become dehydrated that require a number of therapeutic treatments. Other persistent conditions of nasal congestion might be a failure to thrive and in most serious conditions of nasal obstruction; the infants suffer from life-threatening respiratory disorders (Prescott, 1995).

Generally, nasal obstruction might have critical effects on human health and life. One of the most important factors in the aetiology of snoring is a persistent nocturnal obstruction, which might be connected with other serious cases, for example, obstructive sleep apnoea (Young *et al*., 2001). The severity of obstructive sleep apnoea appears in its influence in human daily activity and functioning because it affects normal sleeping and thus causes negative individual outcomes. In addition, it is

correlated with other clinical conditions such as ischemic heart disease and hypertension.

In addition, the influence of obstructive sleep apnoea could be seen obviously in certain type of tests used to examine the quality of life in patients with sino nasal difficulties such as the Sino-Nasal Outcome Test (SNOT-20) or it could be named as the Quality of Life 20-item test (QOL-20) (Browne *et al*., 2007). To decrease the adverse influence of nasal obstruction in human life, a number of studies have been developed to assess the delivery of therapeutic treatment in the nose. For example, Wadell *et al*., (1999) and Wadell *et al*., (2003) developed an *in vitro* model using a chamber diffusion system with three various types of porcine nasal mucosa (cavity mucosa, natural septum mucosa and dermatomed septum mucosa). These authors found that the viability and integrity of the tissues remained for several hours and the cavity mucosa was the appropriate one to examine, which survived for more than 8 hours after dissection. However, those studies that developed to examine the nasal cavity as a route of drug delivery, faced a lot of problems and difficulties because of the presence of nasal congestion and in order to overcome these difficulties septoplasty and nasal decongestants develop the effectiveness of nasal sprays into the nasal mucosa (Mi *et al*., 2011).

1.4.5 Nasal congestion treatment

Therapeutic management of nasal congestion necessitates a mixture of medications used for treatment obstruction, in addition to medications used for management of the cause. Generally, treatment of nasal congestion combines between drug therapies for diminishing the inflammatory reactions and at the same time declining the influence of inflammatory mediators on the nasal mucosa, such as allergic rhinitis. Allergic rhinitis is usually treated by using a combination of medications and because of the poor

efficiency of antihistamine and corticosteroids when used alone in comparing with the nasal decongestants. Therefore, a combination of the following medications is used: systemic and topical first- and second- generation antihistamines, systemic and topical glucocorticoids and furthermore, mast cell stabilizers, leukotriene modifier (montelukast) and finally oral and topical nasal decongestants (Meltzer *et al*., 2015). Additionally, the first management target in the control of any clinical condition is the prevention as it considered one of the most vital rules in medicine "prevention is better than cure" thus prevention is done by identification and avoidance of the causal allergens (Van Cauwenberge *et al*., 2000). Because of the limited scope of this study, the treatment of nasal congestion will be centering on nasal decongestants only.

Nasal decongestants are considered the first-line drug therapy existing for nasal congestion either in topical or oral form. In general, there are five different nasal decongestants available, ephedrine (oral/topical), phenylephrine (oral/topical), pseudoephedrine (oral), xylometazoline (topical) and oxymetazoline (topical) (Kumarnsit *et al*., 1999; Lehman and Blaiss, 2010). The nasal decongestants (or vasoconstrictors) are sympathomimetic compounds acting on α-adrenoceptors and thus, influence the sympathetic tone controlling blood vessels resulting in vasoconstriction (Davis and Eccles, 2004; Van Cauwenberge *et al*., 2000). α_1-Adrenoceptor agonists (e.g. phenylephrine) act by activating directly the α_1-adrenoceptors, while ephedrine and pseudoephedrine act indirectly by causing a release of noradrenaline from the nerve endings and thereby activating non-selectively α-adrenoceptors and β-adrenoceptors (Kobayashi *et al*., 2003). Topical decongestants include oxymetazoline and xylometazoline, both of which have vital α_2-adrenoceptor agonistic activity (Haenisch *et al.*, 2010).

All these decongestants are effective in the treatment of nasal congestions, however, topical decongestants are considered more effective than oral decongestants. Using topical nasal decongestants for more than 5 days is linked with the progress of rhinitis medicamentosa, also known as "rebound rhinitis," which can cause user dependency (Pray and Pray, 2004). Therefore, it is recommended that decongestants, especially topical should be used for a short duration, fewer than ten days as long-term use leads to adverse effect profiles in addition to the known rhinitis medicamentosa syndrome. Rhinitis medicamentosa identified by serious nasal mucosal swelling, congestion, hyper-reactivity and finally becomes resistant to nasal decongestant treatments (Van Cauwenberge *et al*., 2000; Nathan, 2008). This syndrome can be treated by slowly stopping the topical decongestants and all the patients with nasal congestion should be advised that their congestion might extend in duration from 2-4 weeks after their nasal decongestant agents are stopped (Corey *et al*., 2000).

The oldest molecules introduced for the treatment of nasal congestion in the 1930s include phenylpropanolamine, ephedrine and pseudoephedrine. These medications approved to be safe and effective for nonprescription relief of congestion according to food and drug administration review panel in 1976 (Empey *et al*., 1980; Flavahan, 2005; Hendeles and Hatton, 2006; Laccourreye *et al*., 2015) and they have the same stimulant action but pseudoephedrine has less vasopressor response. However, in 2000 phenylpropanolamine was withdrawn from most countries due to its connection with cardiovascular episodes of hemorrhagic stroke in addition to its abuse effect as a diet (Kernan *et al*., 2000; Meadows, 2001; Flavahan, 2005; Eccles, 2006; Hendeles and Hatton, 2006). Moreover, due to similarity of chemical structure of pseudoephedrine and ephedrine to the addictive psychostimulant amphetamine which was initially used for the treatment of cold symptoms. Safety and tolerability concerns were issued about

their illogical potential to be used as precursors for the manufacturing of methamphetamine (Kumarnsti *et al*., 1999; Eccles, 2006; Hatton *et al*., 2007; Laccourreye *et al*., 2015).

Based on these facts, accessibility to these medications have been limited and restricted to be provided by the pharmacist or behind the counter. As a result, the pharmaceutical industry such as Pfizer and Inc (Morris Plains, NJ) replaced the original decongestants with phenylephrine (Kumarnsti *et al*., 1999; Horak *et al*., 2009; Freeman *et al*., 2010), which has become as the main decongestant available in over-the-counter supply for relief symptoms of congestion. Phenylephrine intranasal was developed to diminish the potential of systemic side effects promoted by the wide distribution of α-adrenoceptors all over the body and it was designed to deliver maximally the drug particles straight to the nasal mucosa while the oral form of decongestants is more liable to cause systemic unwanted effects.

Interestingly, now in the United Kingdom market phenylephrine as a decongestant exists only in the oral form (capsule, tablet, syrup, power for dissolution) (Figure 1.10). This can be explained either by the finding of Aviado *et al*., (1959) who postulated that the oral form of phenylephrine was supposed to act selectively on the nasal mucosa. However, no initial and even current evidences were found related to the oral use of phenylephrine as a decongestant in relieving symptoms of congestion compared to placebo (McLaurin *et al*., 1961; Meltzer *et al.,* 2015; Meltzer *et al.,* 2016). This outcome requires further studies to assess and compare the exact efficacy of phenylephrine in nasal and extra-nasal vasculature or can be clarified by the association of rebound medicamentosa with the topical form, which raised a key question regarding the therapeutic availability of other topical decongestants.

Figure 1.10 Products in the United Kingdom that contain phenylephrine as an oral form.

1.5 Aims of the study

This study set out to provide a better understanding of the decongestant actions of the most common products used to diminish the symptoms of congestion. Using isometric tension recordings, I will compare the effect of decongestants and related products on nasal and extra-nasal blood vessels from the pig, both in terms of their direct vasoconstrictor effects and indirect effects on sympathomimetic neurotransmission.

Additionally, the main query of the present study was to investigate, in more detail, the α-adrenoceptor subtype(s) mediating vasoconstriction of the isolated porcine splenic artery and nasal artery using selective and non-selective antagonists. From another perspective, α_2-adrenoceptors are implicated to varying extents in the control of porcine blood vessels. Therefore, I will carry out a number of experiments to uncover the action of α_2-adrenoceptors using splenic artery of the pig with different pharmacological interventions using variable approaches and contractile agents. To this end, I will examine the contraction to a number of α-adrenoceptor agonists in the porcine vascular smooth muscle and porcine snouts in the presence and absence of lithium ions. Generally, I am interested to find whether if it is possible either for α_1-adrenoceptor or α_2-adrenoceptor agonists to incorporate topically with some substances like the contractile agents or the lithium cation as a new decongestant topical formulation to improve the decongestant action of the already available products.

Chapter Two

General Methods, Protocols and Materials

2 Materials, General Methods and Protocols

2.1 Contractility studies

2.1.1 Collection of material

In my experiments, I used porcine blood vessels, which provide an excellent model to conduct research about the functional effects of α-adrenoceptor agonists and antagonists. Human and pig are similar anatomically, physiologically, histologically and biochemically, especially in the nasal mucosa, in which they share the same morphology such as the ciliated and non-ciliated cells, basal cells, goblet cells and serous glands (Corboz *et al.,* 2003; Kobayashi *et al.*, 2003). Porcine snouts and spleens were collected from a local abattoir and immediately placed in ice-cold Krebs-Henseleit buffer (see table 2.1) that had been gassed with 95% O_2/ 5% CO_2 for 15 minutes and the tissue samples were transported within three hours to the laboratory. Isolation of about 6 cm lengths of the dorsal branch of the splenic artery and an associated vein was carried out by coarse dissection (Figure 2.1).

In contrast, dissection of a consistent sample of the major blood vessels supporting the nasal mucosa is difficult as they go through the nasal cavity of the cranium and the snout. Therefore, collecting of the subcutaneous nasal arteries was made according to a preceding thesis (Denfria, 2016). Dissection of the subcutaneous nasal artery was carried out by following the infra-orbital artery that ends distally, then goes together to the anterolateral aspect of the nasal bone, and bifurcates at the proximal part of the nasal cartilage. The infra-orbital artery branch goes medially and runs through a small foramen covered with a fibrous membrane to pass in the nasal cavity.

A)

B)

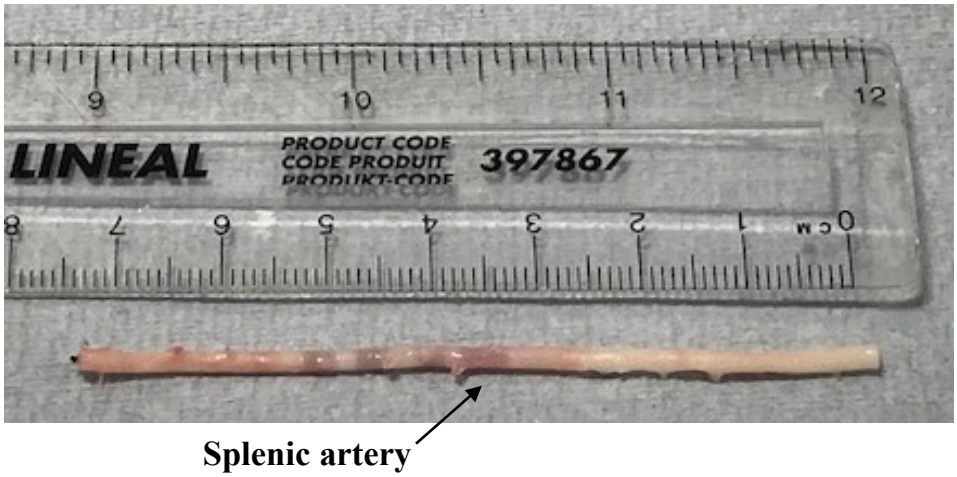


Figure 2.1 Images of the pig spleen showing the splenic artery and vein with its anatomical positions. (A) the dorsal branch of splenic artery with its corresponding vein (B) portion of the splenic artery (dorsal branch) finely dissected and is ready for use.

The proximal part of the artery was dissected cautiously by making two full-thickness incisions in the skin of the snout using a surgical scalpel. A longitudinal incision, which runs postero-anteriorly in a median plane starting from the snout's cut edge towards the tip of the snout, was the first incision. The second incision was a transverse incision which started just above one side of the upper lip and it ended just above the contralateral side of that lip taking a semi-circular course parallel to anterior margin of the snout and approximately half a centimeter posterior to it. The two incisions met each other at the dorsum of the snout forming a 'T' shape (Figure 2.2A). Excision of the skin and the soft structures from the skeleton of the snout were made using a scalpel illuminating the required artery, which goes adjacent to the periosteum (Figure 2.2B). The isolated segment with the surrounding connective tissue and fat was refrigerated overnight at 4°C in Krebs-Henseleit solution.

The following day, after samples were allowed to reach room temperature over 20 minutes, fine dissection of the isolated splenic artery, splenic vein and nasal artery was conducted by removing excess connective tissue and fat. The tissue was divided into rings of about 4-5 mm in length (approximately 2 mm internal diameter at rest). Each ring segment was then immersed between two stainless steel metal wires holders (0.2 - 0.4 mm thick) in 20ml organ baths. The upper wire was attached to a force transducer (World Precision Instruments, Sarasota, Florida, USA) using a cotton line (0.4 mm in diameter), which in turn was linked to an AD Instruments Quad Bridge pre-amplifier. The lower wire was connected to a fixed glass rod (Figure 2.3). Data were displayed using either Mac-Lab Chart v3.5 program software running on a Macintosh LC IV computers or Mac-Lab Chart v7 program software running on a Window XP computers. Each organ bath contained Krebs-Henseleit solution, maintained at 37°C by a thermo-circulator pump and continually gassed with 95% O_2/ 5% CO_2 to maintain pH at 7.4.

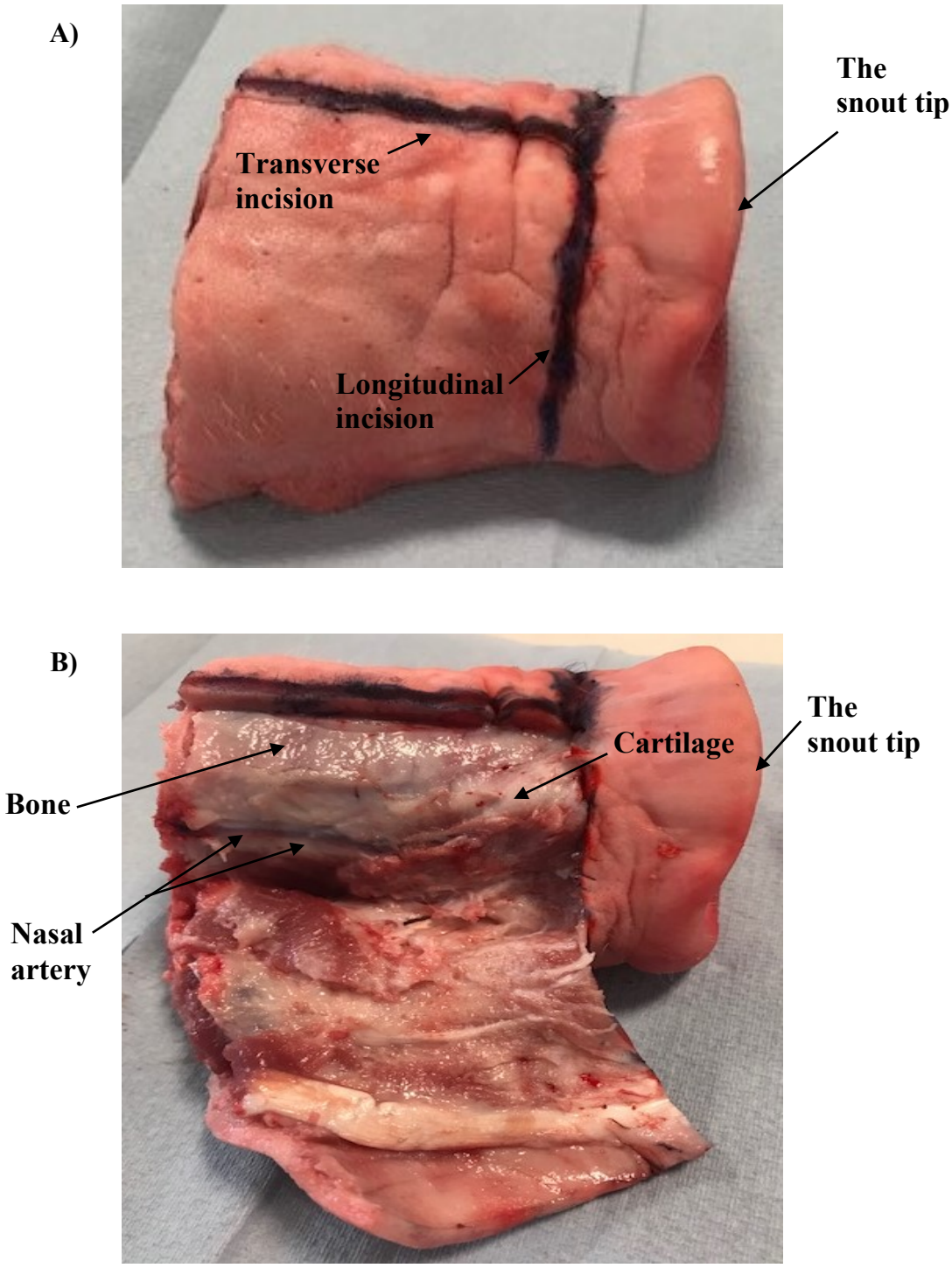


Figure 2.2 Images of the pig snout showing (A) the tip of the snout and the marks of the transverse and longitudinal incision (B) the nasal artery tracks on the horizontal side of the snout and split at the bone-cartilage junction.

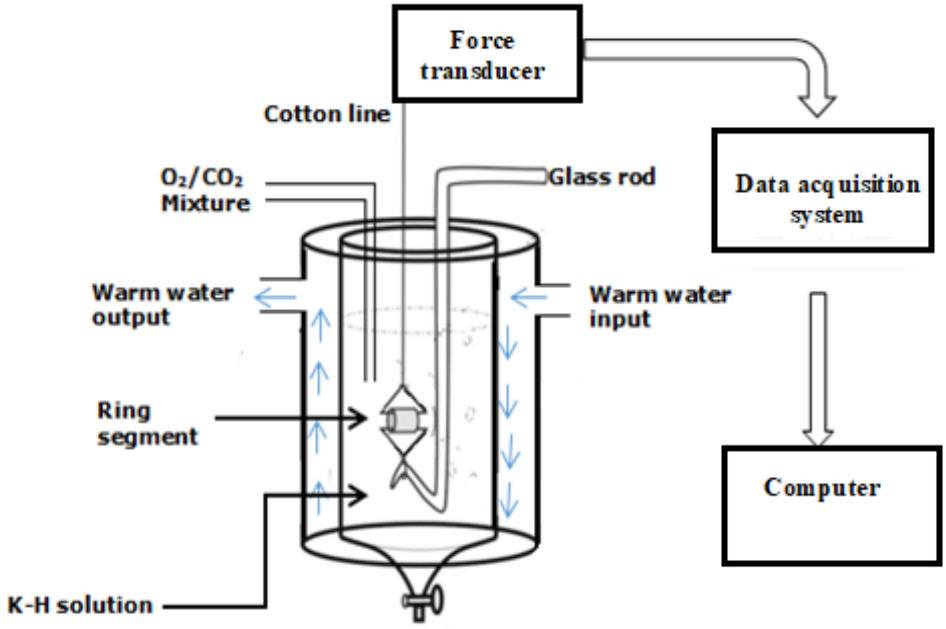


Figure 2.3 Schematic diagram of the organ bath set-up for porcine blood vessels contractility studies. Investigated drugs were added into a 20ml organ bath containing warmed (37^0C) Krebs-Henseleit buffer solution.

2.1.2 Experimental protocol

Studying nasal decongestants were carried out using the most common method, which is isometric tension recording usually with the pig (Corboz *et al.*, 2003; Varty *et al.*, 2004; Chu *et al.*, 2006; Corboz *et al.*, 2013).

After a 40-50 minute equilibration period, approximately 100mN tension was slowly applied to the arterial segments over a 2 minutes period, while 50mN was used for the venous segments. Each segment was allowed to slowly relax over 40-50 minutes to a final resting tension of between 30-40mN for arteries or 10-20mN for veins. Segments were then exposed to 60mM KCl to assess viability. After 15-20 minutes or when KCl responses had plateaued, each bath was washed out with Krebs-Henseleit twice (at 5-minute intervals). The tissue was re-tensioned to approximately half the initial resting tension and allowed for a further 30 minutes equilibration. Two additional contractions to 60mM KCl were elicited 40 minutes apart until reproducible responses were obtained so that the final two responses differed by less than 10%. Between each response, tissues were washed three times with Krebs-Henseleit buffer and allowed to recover for 20 minutes.

Cumulatively increasing concentrations of the agonists were used to generate concentration-response curves, with additional increments being made at 5-minute intervals in the absence of a contraction or until the response was reached equilibrium usually (5-10 minutes). Various concentrations of an antagonist, or a combination of the antagonists and drugs, were incubated for a minimum of 45-60 minutes in the bathing medium before commencing the agonist concentration-response.

Cocaine (10μM) to inhibit $uptake_1$, corticosterone (30μM) to inhibit $uptake_2$ and propranolol (1μM) to inhibit β-adrenoceptors were also included the bathing solution 40 minutes before the start of experiments when either noradrenaline or adrenaline agents were used. Table 2.1 lists the drugs and chemicals that were used for contractility studies.

Table 2.1 List of reagents and chemicals used for contractility studies. Details on the concentrations used will be identified in the following results chapters.

Reagents	Solvent	Source/Manufacture
Krebs-Henseleit buffer, pH 7.4, gassed for 15 minutes with 95% oxygen, 5% carbon dioxide		
Sodium chloride (120 mM)	Distilled water	Fisher Scientific, Loughborough, UK
Potassium chloride (4.8 mM)	Distilled water	
Sodium hydrogen carbonate (25 mM)	Distilled water	
Calcium chloride (1.25 mM)	Distilled water	
Magnesium sulfate (1.1 mM)	Distilled water	
Glucose (11 mM)	Distilled water	
Potassium dihydrogen orthophosphate (1.2 mM)	Distilled water	BDH, Laboratory Supplies, Poole, UK
Chemicals		
U46619 (9,11-didexoy-9α,11α-methanoepoxy prostaglandin F2α methyl acetate)	Ethanol	Tocris Bioscience
A61603 hydrobromide (N-[4,5-dihyro-1H-imidazole-2-hydroxy-5,6,7,8tetrahydronaphthalen-1-yl]- methane sulphonamide)	Distilled water	
Prazosin hydrochloride	DMSO	
Ethylene diamine tetra-acetic acid disodium salt (EDTA)	Distilled water	BDH, Laboratory Supplies, Poole, UK
Cocaine hydrochloride	Distilled water	Nottingham University Hospital Pharmacy, UK
Phentolamine mesylate	Distilled water	Alliance, UK
RX-811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline)	Distilled water	Imidazoline hydrochloride, Reckitt and Coleman, Hull, UK
Brimonidine tartrate	DMSO	TEVA UK Limited, Eastbourne, UK

Sudafed decongestant syrup contain pseudoephedrine hydrochloride 30mg/5ml	Distilled water	McNeil products, Maidenhead, Berkshire, UK
Ethanol	Used as solvent	Honeywell International, Manchester, UK.
DMSO (dimethylsulphoxide)	Used as solvent	Sigma Aldrich, Poole, UK
Tyramine hydrochloride	Distilled water	
ODQ (1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one)	Distilled water	
L-NAME (*N*-nitro-L-arginine methyl ester)	Distilled water	
Noradrenaline or (±)-norepinephrine (+)-bitartrate salt bitartrate	EDTA	
Adrenaline bitartrate	EDTA	
Corticosterone	DMSO	
Propranolol hydrochloride	Distilled water	
Phenylephrine or (R) – (-)-phenylephrine hydrochloride	Distilled water	
Forskolin	Ethanol, DMSO	
Xylometazoline hydrochloride	Distilled water	
Guanfacine hydrochloride	Distilled water	
Metaraminol(+)-biatartrate	Distilled water	
Corynanthine hydrochloride	Distilled water	
Lithium sulfate 1mM	Distilled water	
S(-)-BayK8644 (4S)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester.	Distilled water	
Raulwoscine hydrochloride	Distilled water	

2.1.3 Pharmacological manipulation

In some experiments, the addition of the α-adrenoceptor agent to the organ bath was made in the presence of other vasoactive agents (pharmacological manipulation) (Roberts *et al*., 1999). Subsequently, each preparation was pre-contracted with the thromboxane A_2 analogue U46619 (0.005-0.03μM) to produce a change in tone equivalent to 40-50% contraction of the response to 60mM KCl. The segments were then exposed to increasing concentrations of forskolin (30nM-0.1μM) and allowed to relax to their baseline (< 5-10% of the response to 60mM KCl) before being exposed to the cumulative concentrations of the agonist.

Additional methods of manipulations were performed by raising the vascular tone using different vasoconstrictor agents including the following compounds 1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ. The vasoconstrictors were applied to the organ bath and incubated for 40-45 minutes, which caused an approximately 5-10% elevation in the vascular tone then the tissue segments were exposed to the cumulative concentrations of the agonist. All additives or antagonists were added to the preparation after the beginning of the pharmacological manipulation process and at least 40 minutes before starting the agonist cumulative concentration-response curves (Roberts *et al.*, 1999).

2.1.4 Electrical field stimulation

Each porcine splenic and nasal artery were prepared for isometric tension measurement. However, for electrical field stimulation, the glass rods that hold the lower stainless steel wire hooks were replaced by plastic rods. The plastic rods hold the hooks and carrying two platinum electrodes parallel to the long axis of the hook and these electrodes were

connected to electrical stimulation where electrical pulses were generated with a Digitimer (Ltd) (UK) (SYNC+GATE MULTISTIM SYSTEM D330) pulse stimulator (Welwyn Garden city, Hertfordshire, United Kingdom) (Figure 2.4).

After relaxation and stabilisation from 60mM KCl induced-contraction, ring segments were exposed to trains of electrical pulses (2-64 Hz, 0.3ms pulse width, 200mA, for either 5 or 30s duration) at 10-minute intervals. To determine the effect of sympathomimetic on electrically evoked contractions, the nasal artery (4Hz, 0.3ms pulse width, 200mA for 30s) and the splenic artery (8Hz, 0.3ms pulse width, 200mA for 30s) were stimulated at 10-minute intervals. After three consistent responses, the preparations were exposed to the examined drugs.

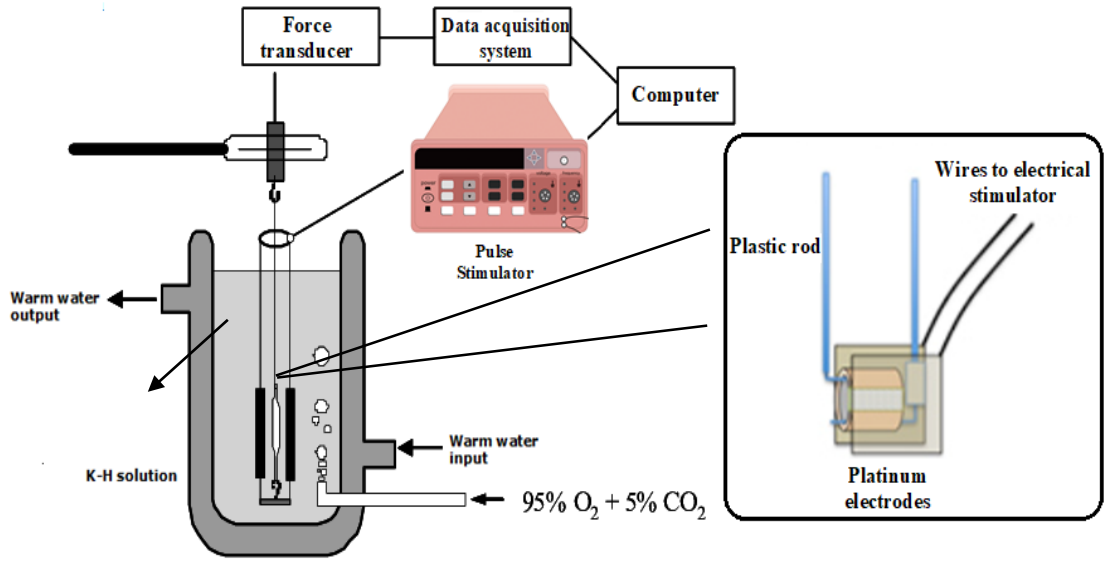


Figure 2.4 Schematic diagram of the electrical field stimulation set-up for electrically evoked contraction of the porcine blood vessels. Investigated drugs were added into a 20ml organ bath containing warmed (37^0C) Krebs-Henseleit buffer solution.

2.2 Histological examination of the porcine splenic and nasal arteries

Samples were collected and performed according to the methods mentioned in section 2.1.1. Fine dissection as previously described in section 2.1.2 was performed on the same day without storing the samples overnights in Krebs-Henseleit buffer.

0.5-1 μm thickness samples from the nasal artery and splenic artery were collected to be examined for their histological structure. They were stored for 48 hours at 4°C in 4% formaldehyde for initial fixation. Afterward, individual samples were washed three times with PBS buffer and placed in numbered cassettes and stored at 4°C using a 30% sucrose in 1X phosphate-buffered saline (PBS) until tissues sink, then embedded in OCT to form moulds. Table 2.2 lists the compunds and materials that were used for histological examination.

Embedded samples prepared for H&E staining were sectioned into 10 μm sections using a Leica-RM2245 microtome. Sections were floated on the water surface in a warm water bath, then placed on gelatin-coated slides and allowed to dry over these slides by placing them on a slide hotplate. Samples were dehydrated with a series of ethanol concentrations, dewaxed using xylene, stained with haematoxylene and eosine, then rehydrated before being finished, covered with appropriate coverslip and sealed with DPX resin mountant. Finally, samples were examined using a light microscope.

Table 2.2 List of compounds and materials used in histological examination.

Compounds/ Reagents	Source/Manufacture
Dulbecco's phosphate buffered saline (PBS)	Sigma Aldrich, Poole, UK
Sucrose (30%), 30 g of sucrose dissolved in 100m distilled water.	BDH, Poole, UK
Optimum cutting temperature embedding medium (OCT)	VWR International, UK
Paraformaldehyde solution (4%)	
Paraformaldehyde powder. 4 g of paraformaldehyde dissolved in distilled water, which is 2/3final volume (100ml). Heating up the temperature to 60°C.	Sigma Aldrich, Poole, UK
Sodium hydroxide solution (2 M) (NaOH). The pH of paraformaldehyde solution was adjusted to 9 by NaOH solution. After the solid dissolve, solution allowed to be cooled to room temperature and 1/3 volume of PBS was added.	
HCl (1M). The pH of paraformaldehyde solution was adjusted to 7.2 - 7.4 and stored at 4°C until needed.	
Haematoxyline and Eosine stain-H08 (H&E stain):	
Harris Haematoxyline reagents	Fisher Scientific, Loughborough, UK
Xylene	
Varying grades of ethanol	Honeywell International, Manchester, UK.
Acid alcohol (1m of concentrated hydrochloric acid mixed with 99ml of 70% ethanol)	Sigma Aldrich, Poole, UK
Lithium carbonate (Lithium carbonate 0.5 g in Distilled water 1000ml) / Scott's tap water (Magnesium sulfate ($MgSO_4$)30g, Sodium bicarbonate 2.0 g dissolved in tap water 3000ml)	
1% Eosin	RAL diagnostics, artillac, France
Resinous mounting medium for histology examination (a mixture of distyrene a plasticizer, and xylene) (**DPX**)	Thermo Fisher Scientific, Rockford, USA

2.3 Cannulation and tracking of the nasal artery

In some experiments, the pig snout was sectioned and used for nasal mucosa perfusion experiments. Dissection of the pig snout was initiated by careful dissection of the area around the nasal artery to clearly visualize the vessel according to the approach described in section 2.1.2. A narrow nasogastric tube of 5mm internal diameter was used to cannulate the artery and the tube secured in place by fixing the artery to the periosteum with a few stitches. Supplementary stitches were made along the course of the tube to prevent the backward flow of the perfusate (Figure 2.5A).

In preliminary experiments Evans blue dye was infused through the nasogastric tube at a rate of 0.75ml/minute using an ECOline peristaltic pump VC-MS/CA4-12 microprocessor controlled tubing peristaltic pump (Four Channels and 12 rulers) connected to ISMATEC rubber tubes of 5mm internal diameter and 0.86mm wall thickness (IDEX Health Science, Wertheim, Germany) (Figure 2.5B). The back pressure in the system was measured using MLT0380/D reusable blood pressure transducer (AD Instruments Ltd, UK) connected to Windows XP operated PC computer via AD Instruments Quad Brigde pre-amplifer unit and recorded on Lab Chart v7 program software (Figure 2.6). The blue color of the dye started to appear on the perfused side of the nasal mucosa in the nasal cavity after one minute of perfusion. This indicates that the selected artery is linked to the nasal mucosa either as a major blood supplier or just as an anastomosing artery (Figure 2.7A). To have a better observation on the distal part of the mucosa, the nasal cartilage was then removed to examine the presence of the blue dye inside the nasal cavity (Figure 2.7B).

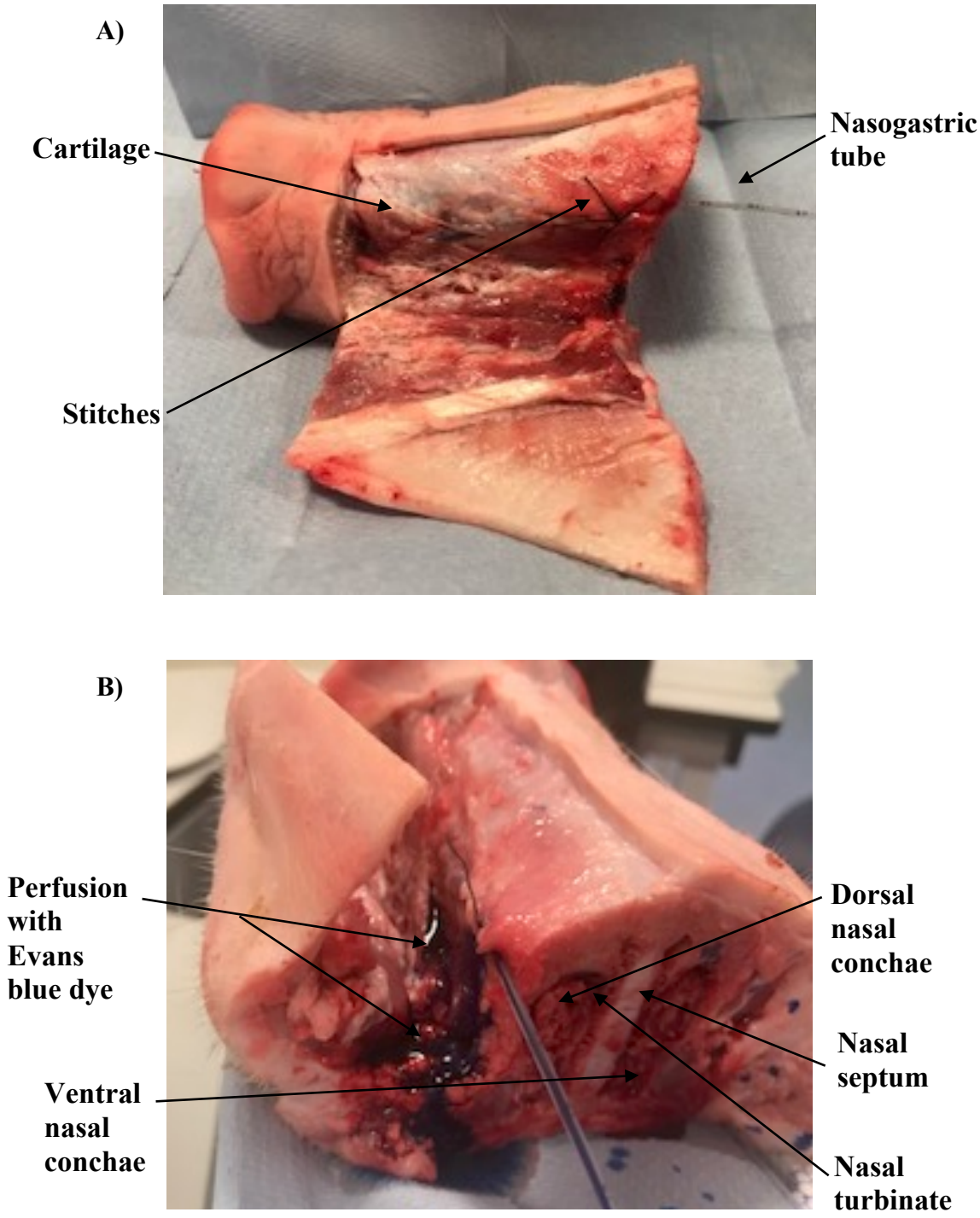


Figure 2.5 Images of a dissected pig snout showing (A) the nasal cartilage and the nasal artery is being cannulated and the cannula is fixed with stitches (B) posterior image of the same snout displaying the nasal septum, dorsal nasal conchae, ventral nasal conchae, the turbinate and the nasogastric tube, which is implanted in the nasal artery and perfusate with an Evans blue dye.

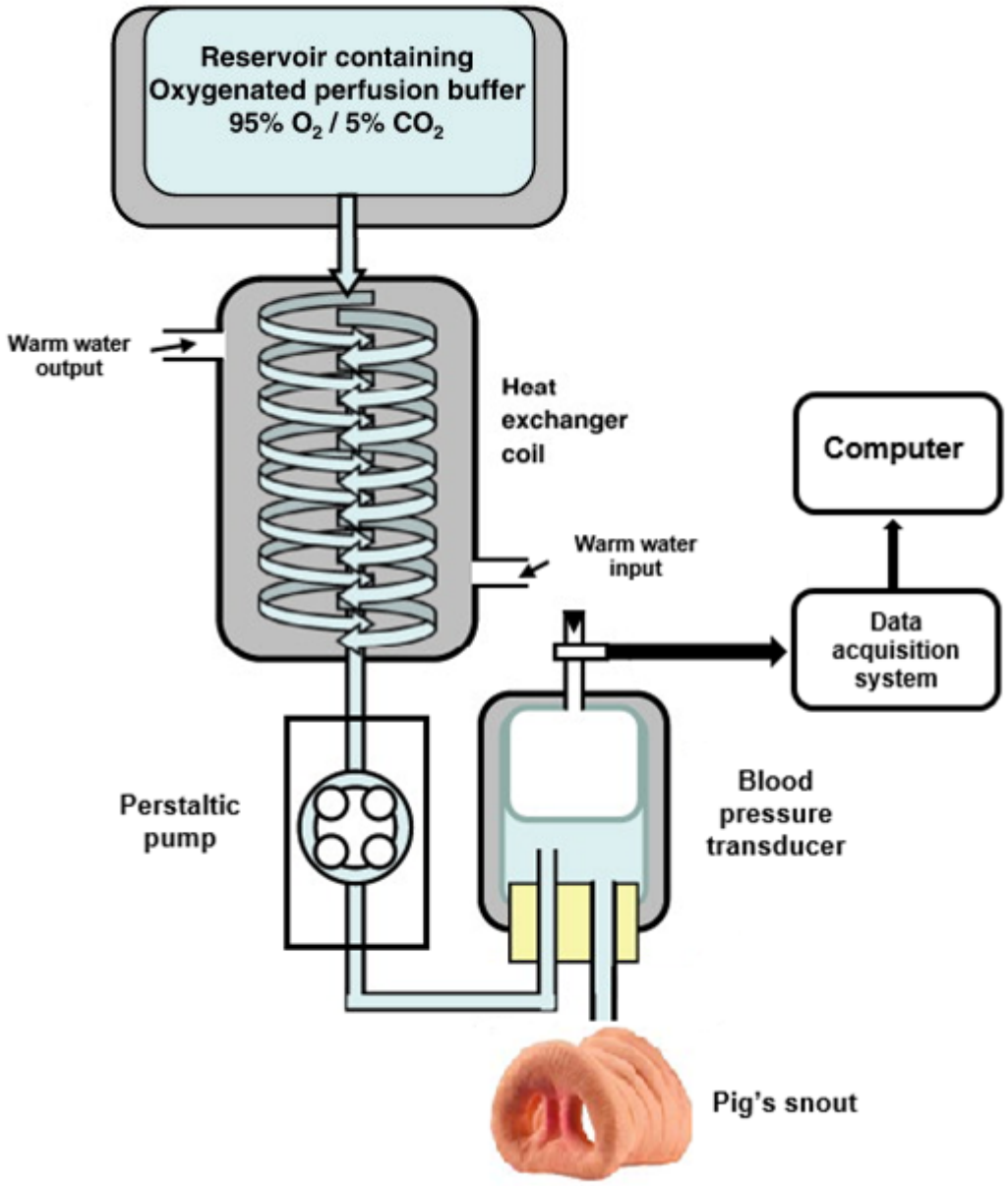


Figure 2.6 Schematic diagram of the nasal mucosa perfusion set-up. Investigated drugs were added into a 20ml reservoir tube containing pre-gassed Krebs-Henseleit buffer solution. Figure modified from Bell *et al*., (2011).

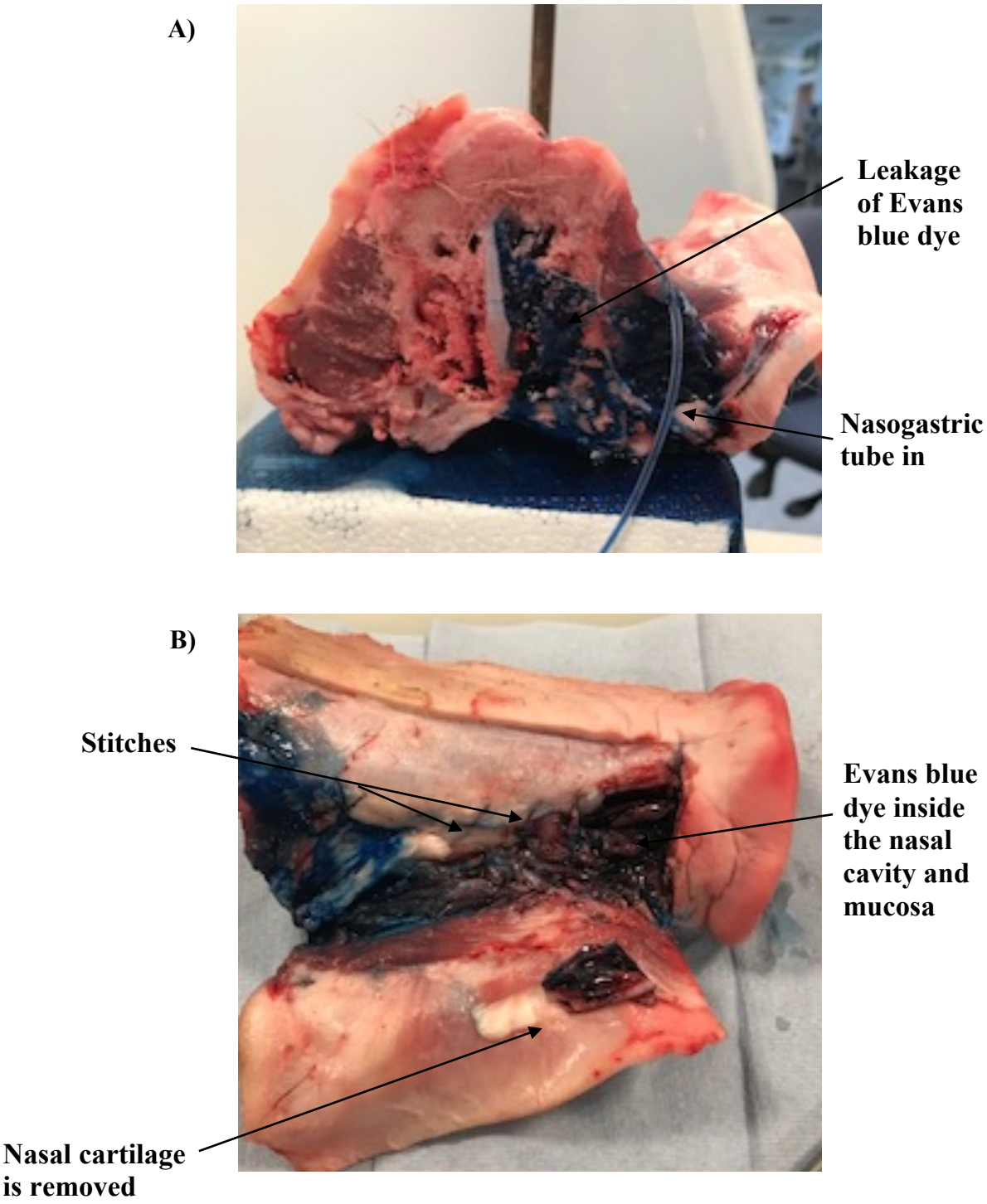


Figure 2.7 Image of perfusate pig snout with Evans blue dye showing (A) posterior image of the snout and the dye is leaking through the nasal mucosa (B) side image of the snout and the perfusion of the blue dye inside the nasal cavity when the nasal cartilage is removed.

2.3.1 Perfusion of the porcine nasal mucosa

After cannulation of the nasal artery as mentioned above, Krebs-Henseleit solution gassed with 95% O_2/ 5% CO_2 mixture and maintained at 37°C was injected through the cannula to run through the artery and reach the inside of the nasal mucosa. Krebs-Henseleit solution gradually pumped with a low rate of 0.5ml/minute to be increased every one or two minutes (when pressure stabilises) by a rate of 0.5ml/minute at a time. The flow rate was gradually increased over the next hour to 2.5ml/minute, thereby allowing the mucosa to accommodate to the flow and temperature of the perfused Krebs-Henseleit buffer and any collapsed mucosal vessels to reopen and adapt to the new conditions of the artificial circulation. When the perfusion rate reached 2.5ml/minute, it was continued until the end of the experiment.

After a stable baseline pressure was maintained with Krebs-Henseleit buffer within an hour, the nasal mucosa was warm enough and exposed to KCl (60mM) to assess viability. After 10 minutes or when KCl responses had plateaued, the nasal mucosa was washed out with Krebs-Henseleit several times and the perfused snout left for a further 30-60 minutes or when a stable baseline pressure was maintained. Later, α-adrenoceptor agents were added to the perfusion fluid in half log unit cumulatively increasing concentrations, each concentration taking approximately 5-10 minutes to reach equilibrium and additions were made only after pressure was stable for 3 minutes. Unless otherwise stated, all drugs used to influence the effect of α-adrenoceptor agonists were added and incubated to the perfusate fluid for a minimum of 40-45 minutes before agonists were applied. At the end of the snout perfusion experiments, the perfused snout exposed to KCl (60mM) to check that the nasal mucosa responded in a reproducible manner. After 10 minutes or when KCl responses had plateaued, the nasal mucosa was washed out with Krebs-Henseleit several times.

2.4 General statistical analysis methods

Contractions produced by the agonists were expressed as milliNewtons (mN) and the response of the blood vessels to each agonist concentration (sympathomimetics drugs) and electrically evoked contractions were expressed as a percentage of the contraction to 60mM KCl. All calculations and statistical tests were performed using Microsoft Office Excel and graphs were produced using the Kaleidagraph software (4.5.2, Synergy Software). The logistic equation (Kaleidagraph version 4.5.2 Synergy software, PA, USA) was used to draw the best-fit curve for the data from which the following parameters were obtained: R_{max} (maximum response to cumulative addition of an agonist), EC_{50} (The molar agonist concentration (sympathomimetics drugs) producing 50% of the maximum response). The latter was used to determine the negative logarithm of the concentration required to produce 50% of the maximum response (pD_2).

The agonist concentration ratio (CR) was determined from the concentration of the agonist causing 50% of the observed activity in presence of the antagonist (EC_{50}'), compared to agonist causing 50% of the observed action in absence of the antagonist or it is the ratio of EC_{50} of curve with the presence of antagonist over the EC_{50} of the control curve ($CR=EC_{50}'/EC_{50}$). In experiments with pharmacological inhibition produced by a series of antagonist concentrations, seven antagonist concentrations (3-fold increments up to 1000-fold range) were used. Schild plot analysis was produced by plotting log (CR-1) against the negative logarithm of antagonist concentration and the line of best fit for each tissue determined by regression analysis based on data obtained from tissues from each animal (Arunlakshana and Schild 1959).

In some instances, a CR value for the highest concentrations of the antagonist could not be accurately estimated because the agonist concentration used did not produce a

response greater than 75% of the estimated maximum. These values were not included in the Schild analysis.

The intersection of the Schild plot with log (CR-1) = 0 with the log [Antagonist] x-axis is the pA_2 value. For the calculation of pK_B (the potency of a competitive antagonist) values, the calculation was done manually, by using the Gaddum equation as the formula given below where B is the concentration of antagonists used where $CR=EC_{50}'/EC_{50}$

$$\textit{Log } K_B = \textit{Log [B] - Log (CR-1)}$$

Statistically significant differences between the mean values were assessed using one-way ANOVA test for experiments on different antagonist incubation time and their controls. The results were considered statistically significant at a value of $P < 0.05$, where the null hypothesis was rejected. For RX-811059, the dissociation constant against noradrenaline contractions was estimated from the agonist-concentration ratio in the absence and presence of 1μM of the antagonist.

For experiments related to electrical evoked contractions of the nasal artery (4Hz, 30s) and splenic artery (8Hz, 30s) the average of three responses were measured before and 40 minutes after exposure to the sympathomimetic. The peak response was measured and the time required for the response to declining by 50% after cessation of stimulation (t_{50}). The percentage change in the response after exposure to the sympathomimetic was calculated and used for statistical analysis (Figure 2.8).

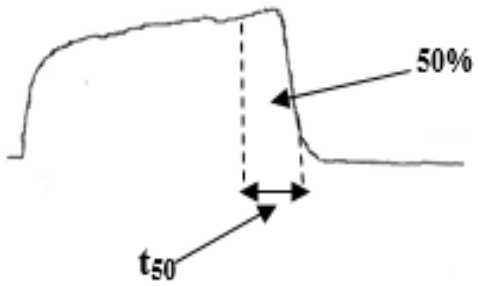


Figure 2.8 Diagram representing the calculation of t_{50}

All results of perfusion experiments were expressed as the change in the perfusion system pressure using millimeters of mercury (mmHg) units. Since the pulse pressure recorded by the MLT0380/D reusable blood pressure transducer could vary from 5-10 mmHg, I estimated the mean pressure over 15-20s before the addition of the drug and the mean pressure at the peak response. The difference was determined by LabChart software (AD Instruments Ltd, UK). The responses are expressed as the mean of the pressure difference of the number of observation included ± standard error of the mean (S.E.M) and were plotted against the base -10 logarithm of the concentration of the agonist. Also to compare the response of perfusate snout one to each other, a 2-tailed, Student's unpaired *t*-test was used to compare differences between two unrelated animal groups. In general, all values were expressed as the mean ± standard error of the mean (S.E.M) of observations (n). The number of observations in different animals for each experiment was expressed as (n). In the majority of experiments, a 2-tailed, Student's paired *t*-test was used to compare differences between two groups, and one-way ANOVA followed by *post hoc* Dunnett's test if more than two groups were needed to be compared. The *P*-value less than 0.05% was considered statistically significant.

Chapter Three

Contractility studies of porcine blood vessels

3 Contractility studies of porcine blood vessels

3.1 Introduction

As previously mentioned in the first chapter (general introduction), sympathomimetics are commonly and highly consumed throughout the world either in an oral or in topical forms as nonprescribed nasal decongestants. The topical route of administration was developed to offer the advantage of reducing the potential of systemic adverse effects by delivering the drug particles directly to the nasal mucosa. The oral form of decongestants is more liable to cause systemic undesirable effects due to distribution of α-adrenoceptors all over the human body. However, the oral form of phenylephrine (a selective α_1-adrenoceptor agonist) as pointed out by Aviado *et al*., (1958) and Aviado *et al*., (1959) acts selectively on the nasal mucosa and has high sensitivity at the nasal blood vessels. Additionally, from the market perspective, phenylephrine and pseudoephedrine form a large part for cold remedies that, from the sales of drug products including nasal decongestants, were estimated to be worth £569 million pounds in the year 2016-2017 (NHS England and NHS Clinical Commissioners, 2018).

Depending on these facts and as there are little studies to clarify and compare the action of nasal decongestants on nasal and extra-nasal circulation, I carried out a number of experiments to understand the pharmacology of nasal decongestants and related compounds on nasal and extra-nasal blood vessels from the pig, taking into consideration that there are a number of factors which have a vital role in the blood vessel responses.

The conventional view of phenylephrine and pseudoephedrine is that they lead to activation of α_1-adrenoceptors associated with the vasculature of the nasal mucosa, either

directly or indirectly, causing a reduction in the calibre of the associated blood vessels. The subsequent reduction in local blood flow reduces the overall volume of the tissue enclosed in the nasal cavity and leads to an improvement in the cardinal symptoms of nasal congestion (Eccles *et al*., 2005; Koller *et al*., 2007). While phenylephrine is known to have a direct action on nasal post-junctional α_1-adrenoceptors (Lacroix and Lundberg, 1989b; Johannssen *et al*., 1997), the action of pseudoephedrine is generally attributed to the release of noradrenaline from sympathetic neurones, subsequent to selective uptake into the cytosol via uptake$_1$ (Kobayashi *et al*., 2003; Rothman *et al*., 2003). For pseudoephedrine, there is abundant convincing evidence of efficacy in terms of patient-reported symptoms and also when nasal airflow is monitored (Jawad and Eccles 1998; Taverner and Latte, 2007; Horak *et al*., 2009). For oral phenylephrine, however, neither early (McLaurin *et al*., 1961) nor recent clinical trials (Meltzer *et al.,* 2015; Meltzer *et al.,* 2016) revealed evidence of a significant improvement in symptoms of congestion, compared to placebo.

α_1-Adrenoceptors are widely distributed throughout the cardiovascular system and the direct vasoconstrictor action of parenterally administered phenylephrine has been associated with an increase in both blood pressure (Keys and Violante, 1942) and systemic vascular resistance. For example, in septic shock intravenous infusion of phenylephrine caused a 20% increase in mean arterial pressure and 30% increase in total peripheral resistance in patients (Morelli *et al*., 2008), while in spinal anaesthesia induced hypotension in a qualitatively similar pressor effect was noted in women undergoing caesarean section (Dyer *et al.,* 2009; Foss *et al.,* 2014).

Moreover, α_1-adrenoceptors are widely available on other parts of the body like the vasoconstrictor action of phenylephrine to constrict the radial dilator muscle of the eye,

which was adopted effectively for topical ophthalmic examination. Also, phenylephrine vasoconstrictor response reduces the anal cushion blood flow and haemorrhoidal volume (Hernandez-Bernal *et al*., 2014). Similarly, oral pseudoephedrine has been reported as being a valuable adjunct to replace the use of intravenous vasopressor agents in patients with spinal cord injury and hypotension (Wood *et al*., 2014).

From a therapeutic viewpoint, there is a pharmacological paradox at play here. While the efficacy of parenteral phenylephrine and pseudoephedrine in clinical settings associated with hypotension requires widespread vasoconstriction via α_1-adrenoceptors and results in predictable haemodynamic changes, the oral use of both agents as decongestants relies upon the notion of selective activation of nasal α_1-adrenoceptors without general haemodynamic changes. The former necessitates the use of the drugs in a controlled clinical environment, while the latter generally allows for widespread, largely unsupervised consumption by the public. If the nasal blood vessel has a higher sensitivity to sympathomimetics, compared to the rest of the circulation (Aviado *et al*., 1958; Aviado *et al*., 1959), this could account for favourable vasoconstrictor effects for both pseudoephedrine and phenylephrine without major haemodynamic changes. However, the available evidence on precapillary resistance vessels from human nasal mucosa is that pseudoephedrine (10μM) is inactive (Wang *et al*., 2006), while phenylephrine-induced contractions were only noted at a concentration greater than 1μM (Johannssen *et al*., 1997), approximately 100-fold greater than the known plasma concentration following 12mg oral dosing (Gelotte and Zimmerman, 2015).

Furthermore, as far as we are aware, a direct comparison of the potency of phenylephrine on nasal and extra-nasal blood vessels have not previously been reported. However, a summary related to the effect of phenylephrine as a vasoconstrictor agent in

human isolated blood vessels (Table 3.1 and figure 3.1) reveals that in general extra-nasal conduit arteries, veins and resistance arteries are between (2-3)-fold more sensitive to this agonist than the comparable nasal artery, which could challenge and disagree with the standard opinion that the nasal vasculature possesses a greater sensitivity to phenylephrine and pseudoephedrine, compared to the rest of the circulation conducted by Aviado *et al*., (1958) and Aviado *et al*., (1959). Therefore, in the present study we have examined and compared the actions of phenylephrine and pseudoephedrine on isolated nasal and extra-nasal vessels from the pig, both in terms of direct vasoconstriction and also against responses arising from activation of sympathetic nerves, to establish whether these agents exert a selective action on the nasal vasculature. Additionally, these examinations provide a better understanding of the systemic action of these widely consumed drugs and develop approaches to direct specific therapies.

Table 3.1 Published data on the potency (pD_2) of phenylephrine in human isolated nasal and extra-nasal (systemic) blood vessels

Human Vessel	Phenylephrine pD_2
Extra-nasal vessels	
Mammary Artery [a]	6.93 ± 0.17
Mammary Artery [b] Saphenous Vein	5.70 5.70
Mammary Artery [c]	6.00 ± 0.09
Mesenteric Artery [d]	5.60 ± 0.1
Omental Artery [e] Omental Vein	5.62 ± 0.51 5.65 ± 0.36
Hand Vein [f]	5.59 ± 0.15
Resistance Arteries [g] Skeletal Muscle	6.10 ± 0.1
Resistance Arteries [h] Mesenteric Skeletal Muscle	 6.40 ± 0.2 6.50 ± 0.1
Resistance Arteries [i] Subcutaneous Skeletal Muscle	 5.85 6.30
Nasal vessels	
Resistance Artery [J]	5.30 ± 0.31

a Vidal *et al*., (2014).
b Weinstein *et al*., (1989).
c Xiaowen *et al*., (1999).
d Owaki *et al*., (2015).
e Steen *et al*., (1984).
f Arner and Hogestatt, (1986).
g Jarajapu *et al*., (2001).
h Hui *et al*., (2015).
i Coats and Hillier, (2000).
j Johannssen *et al*., (1997).

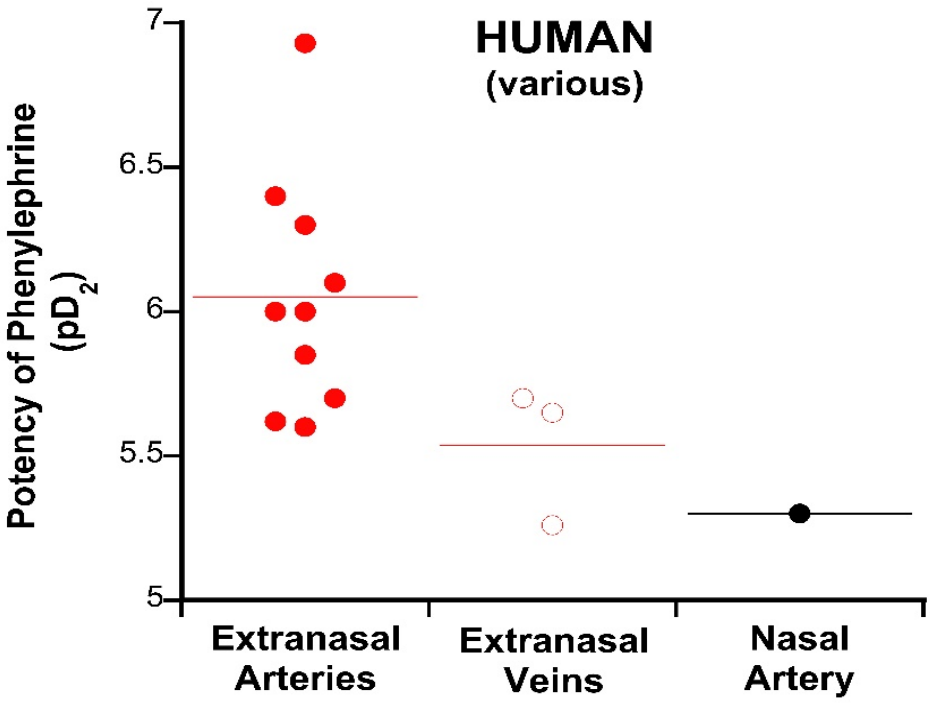


Figure 3.1 Comparison of the potency of phenylephrine (pD_2) in human isolated nasal and extra-nasal (systemic) blood vessels.

3.1.1 Aims

The aims of this study were

1. To determine whether phenylephrine and pseudoephedrine possess selectivity for the nasal vasculature by comparing the decongestant actions of both medications either directly or indirectly in the electrically evoked contraction of the porcine nasal artery and porcine splenic artery.
2. To compare the response of porcine nasal artery and porcine splenic artery to electrically evoked stimulation (2-64 Hz, 0.3ms) using two different durations (5 and 30s) of electrical stimulation.
3. To determine the vascular effects of electrically stimulated porcine nasal artery segments to cocaine and an indirect sympathomimetic agent (tyramine).
4. To determine the vascular effects of pseudoephedrine and cocaine in the porcine nasal artery response to noradrenaline.

3.2 Materials and Methods

3.2.1 Materials

Materials used for the experiments of this chapter were listed in table 2.1.

3.2.2 Methods

The collection of material and tissue preparation for contractility studies were described in chapter 2, section 2.1.1. Isometric tension measurement experiments were carried out in the same apparatus using the same buffer and the general protocol mentioned in chapter 2, section 2.1.2. However, all experiments in this chapter related to the electrical field stimulation were mentioned in detail in section 2.1.4 and were used the same apparatus of isometric tension measurement experiments, but were considerably adjusted in which electrical field pulses were generated with a Digitimer (Ltd) (UK) (SYNC+GATE MULTISTIM SYSTEM D330) pulse stimulator (Welwyn Garden city, Hertfordshire, United Kingdom).

3.2.3 Data analysis

Contractions were measured as a change in milliNewtons force (mN). The response of the porcine blood vessels to sympathomimetics and electrically evoked contractions have been expressed as a percentage of the contraction to 60mM KCl. All calculations and statistical tests were performed using Microsoft Office Excel software and analysed with Kaleidagraph version 4.5.2 Synergy software, PA, USA as already mentioned in chapter 2 (section 2.4).

The potency of the sympathomimetic drugs was estimated as the negative logarithm of the concentration producing 50% of the maximum response (pD_2). For electrically evoked contractions of the nasal artery (4Hz, 30s) and splenic artery (8Hz, 30s) the average of three responses were measured before and 40 minutes after exposure to the sympathomimetic. The peak response was measured and the time required for the response to declining by 50% after cessation of stimulation (t_{50}). The percentage change in the response after exposure to the sympathomimetic was calculated and used for statistical analysis. Comparisons between two groups (baseline and variation) were carried out using two-tailed Student's *t*-test for paired observations, and one-way ANOVA followed by *post hoc* Dunnett's test if more than two groups needed to be compared with *P*-value < 0.05% was considered to be statistically significant. All values were expressed as the mean ± standard error of the mean (S.E.M) of observation in tissues from different animals (n).

3.3 Results

3.3.1 The effects of various sympathomimetics on contractions of porcine blood vessels

Exposure of the porcine isolated splenic artery and nasal artery to 60mM KCl was associated with a contraction equivalent to 12.3 ± 0.3 g wt. (n=21) and 11.6 ± 0.8 g wt. (n=21), respectively. The present study sought to identify whether phenylephrine and pseudoephedrine possess selectivity for the nasal vasculature. Therefore, the responses of the porcine splenic artery and nasal artery segments to the cumulative concentration of noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) and phenylephrine starting from 0.01 to 100μM and pseudoephedrine starting from 1 to 100μM were carried out.

Figure 3.2 shows noradrenaline and phenylephrine caused concentration-dependent contractions of the porcine isolated splenic and nasal arteries and the contraction responses induced by phenylephrine are not sustained in porcine arteries. The rank order of potency of the agonists in the splenic artery was noradrenaline (pD_2 6.18 ± 0.09, n=32) > phenylephrine (pD_2 5.87 ± 0.07, n=20). Similarly, in the nasal artery the rank order of potency was noradrenaline (pD_2 5.71 ± 0.15, n=6) > phenylephrine (pD_2 5.33 ± 0.13, n=7). In the splenic and nasal arteries, noradrenaline was more potent than phenylephrine. However, pseudoephedrine failed to produce a contraction in either blood vessel (up to 100μM) (Figure 3.2). Phenylephrine elicited threshold contractions at 0.1 and 0.3μM in the splenic and nasal artery, respectively (Figure 3.3A). The comparison of the maximum response showed that the magnitude of phenylephrine maximum response in the isolated splenic artery was 140 ± 12% of the response to 60mM KCl, which was greater than the magnitude of the phenylephrine maximum response in the nasal artery (60 ± 8% of the response to 60mM KCl).

The intriguing results from this experiment are that phenylephrine was approximately 4-fold more potent in the splenic artery than in the nasal artery. A similar sort of action was seen with noradrenaline which was also approximately 3-fold more potent in the isolated splenic artery than in the nasal artery. As a result, the nasal vasculature in the pig is less sensitive to phenylephrine and noradrenaline than the rest of the extra-nasal circulation. Moreover, other vascular beds would be similarly affected and the systemic extra-nasal blood vessels have a higher sensitivity to phenylephrine than the nasal vascular bed. Yet, 1-100μM pseudoephedrine failed to elicit a contraction of either of the blood vessels (Figure 3.3B).

Based on the above observations, phenylephrine acted by direct activation of α_1-adrenoceptors in both nasal and splenic arteries and the plasma concentrations of (10-30nM) achieved following oral ingestion of phenylephrine was not able to produce appreciable constriction of the nasal vasculature by α_1-adrenoceptors and even in the splenic artery.

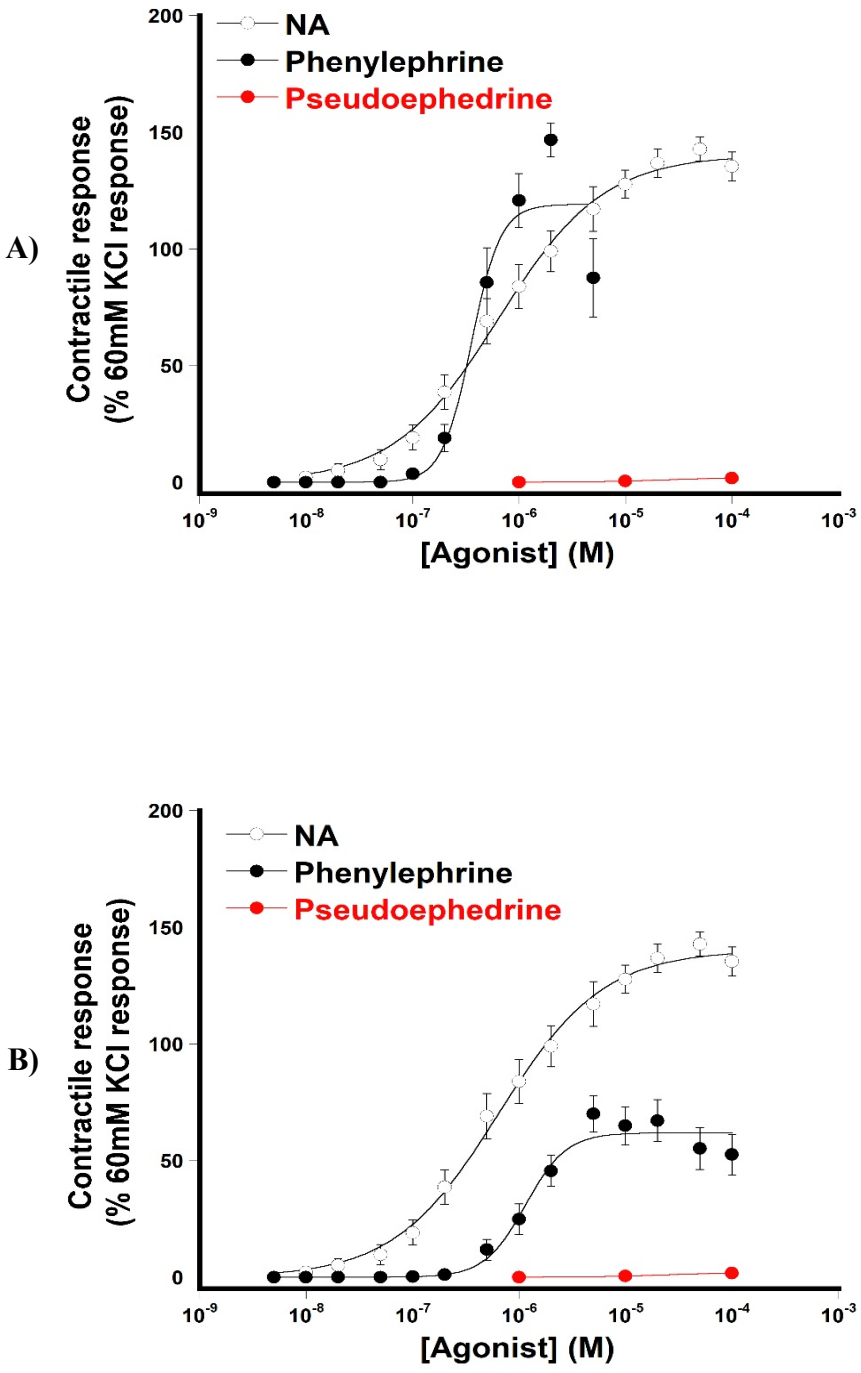


Figure 3.2 Comparison of the effect of various sympathomimetics on (A) the porcine isolated splenic artery and (B) the porcine isolated nasal artery. Effects of noradrenaline in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of 6-32 observations.

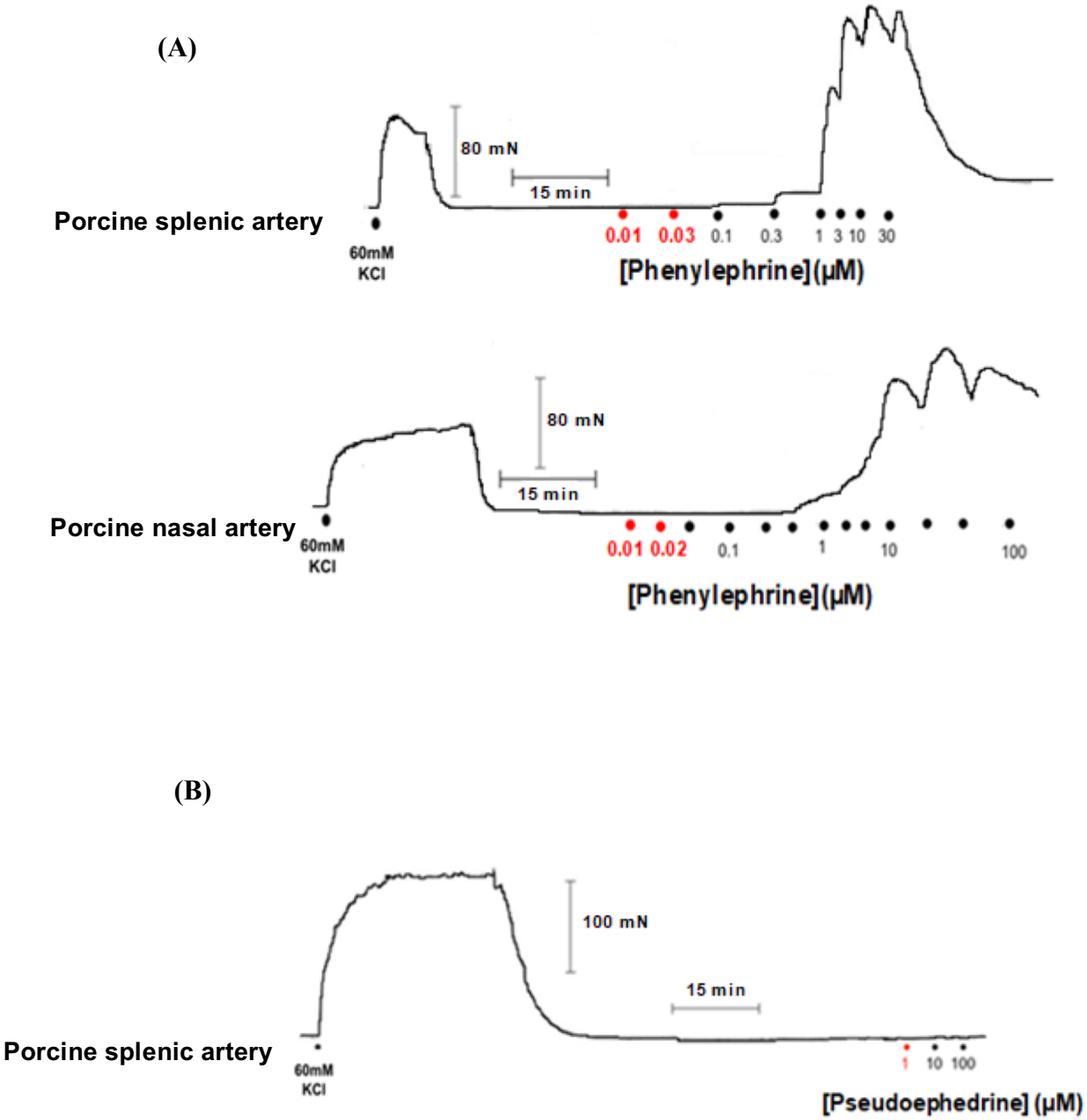


Figure 3.3 Representative trace recording of contractile responses of the porcine isolated splenic artery and nasal artery to 60mM KCl and the cumulative concentration of phenylephrine (UPPER, A) and representative trace recording of contractile responses of the porcine isolated splenic artery to 60mM KCl then to the cumulative concentration of pseudoephedrine (LOWER, B). Concentrations in RED are those known to occur *in vivo* following oral ingestion of the decongestants**.**

3.3.2 The electrical field stimulation of porcine blood vessels

Electrical field stimulation (EFS, 2-64 Hz) of the porcine splenic and the nasal artery at 10-minute intervals, for either 5s (Figure 3.4A) or 30s (Figure 3.4B) duration, caused frequency-dependent contractions. When stimulated at the highest frequency for 30s duration, (but not for 5s) the maximum response in the splenic artery was significantly greater than the corresponding response in the nasal artery, relative to the contraction to 60mM KCl (Figure 3.4).

However, at low frequencies of EFS neurogenic contractions in the nasal artery were larger than the corresponding response in the splenic artery, irrespective of the duration of stimulation. For example, 30s EFS at 4Hz in the nasal artery produced a contractile response equivalent to 39 ± 3% of the response to 60mM KCl (n=21), while for the splenic artery the response to 8Hz was only 24 ± 3% of the response to 60mM KCl (n=21) (see table 3.3).

These two stimulation parameters were used in further experiments. Figure 3.5 shows that for the nasal artery, the peak response to 4Hz, 30s EFS was attained before the cessation of stimulation, while for the splenic artery the response to 8Hz, 30s EFS developed much more slowly, with the peak response attained near the end of the period of stimulation. Furthermore, the time taken for responses to decline by 50% after cessation of stimulation (t_{50}) was 2-3 times longer in the splenic artery (28.3 ± 5.2s, n=21) compared to the nasal artery (9.3 ± 0.7s, n=21) (Figure 3.5 and see table 3.3).

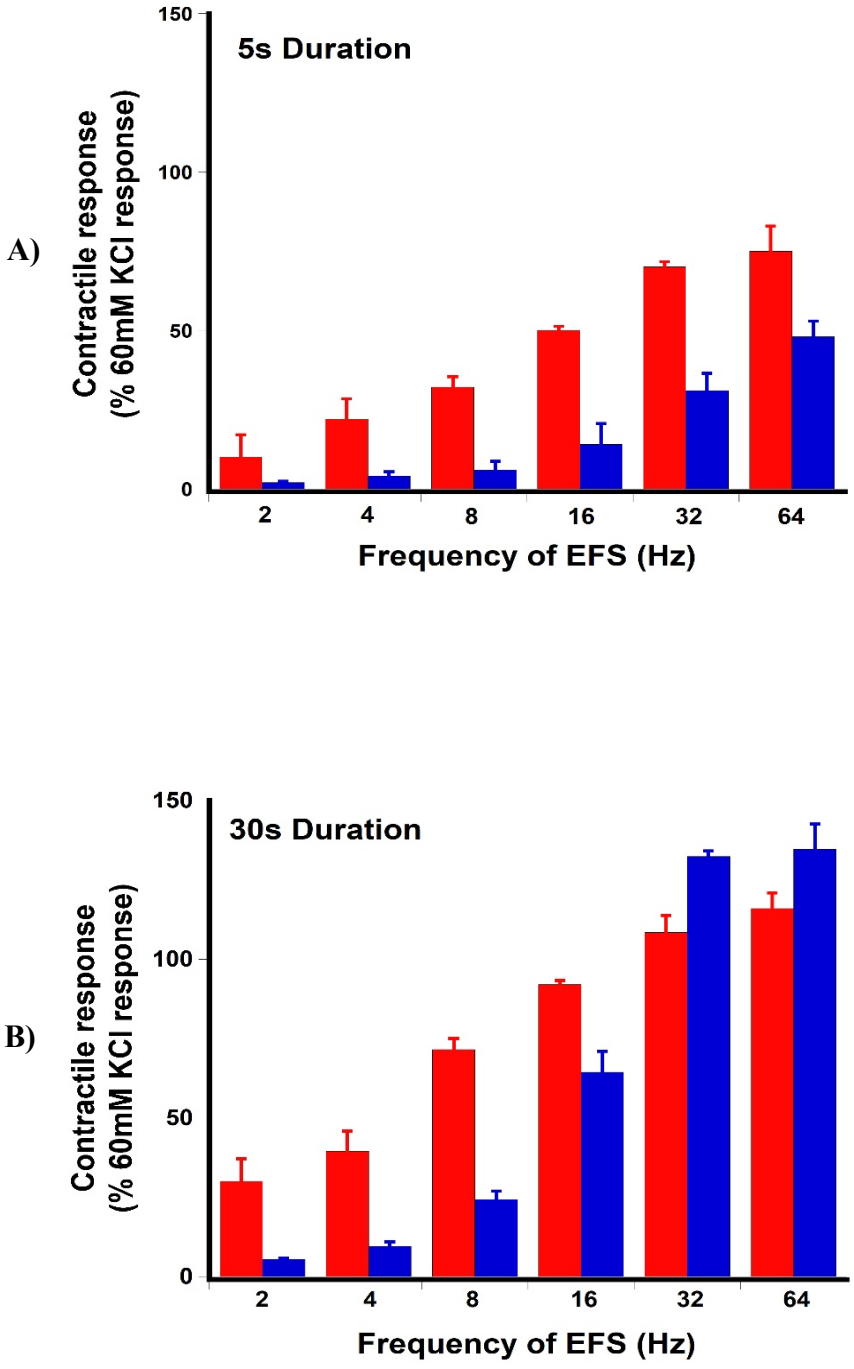


Figure 3.4 Comparison of the electrical field stimulation effects at different frequencies for (A) 5s duration and (B) 30s duration on the porcine isolated splenic artery (BLUE) and the porcine isolated nasal artery (RED). In both instances, the pulse width was 0.3ms and the current strength 200mA. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of twenty-one observations.

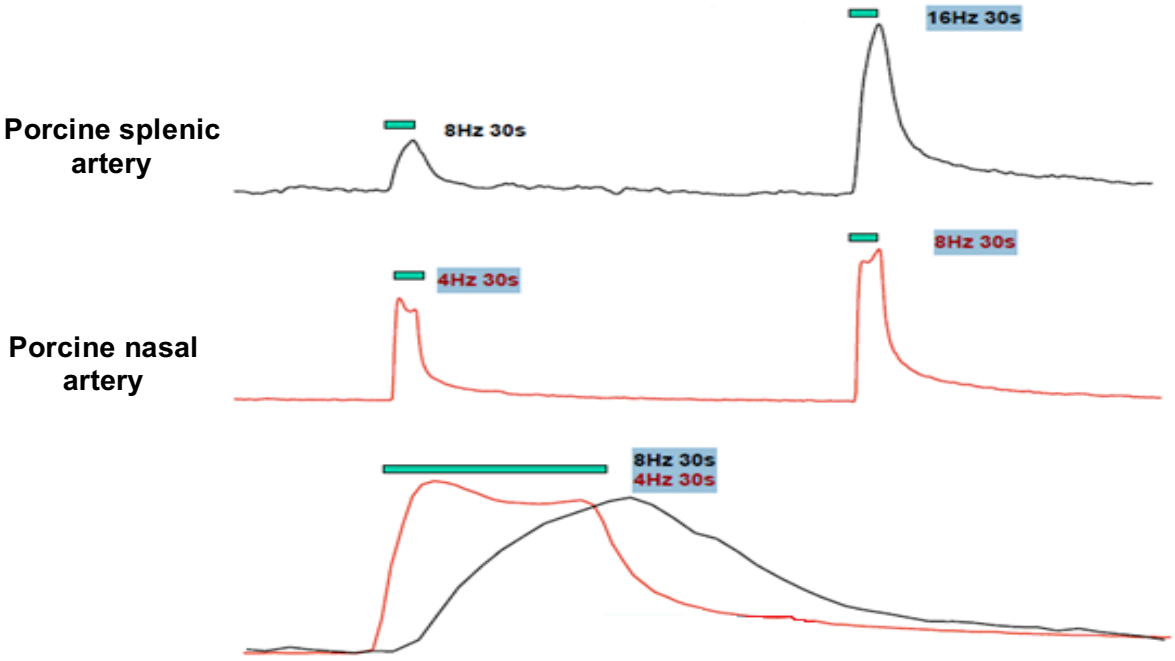


Figure 3.5 Representative trace recording of electrically evoked contractions of the porcine isolated splenic artery (8 and 16Hz, 200mA, 30s duration, BLACK, upper) and nasal artery (4 and 8Hz, 200mA, 30s duration, RED, middle).

The green bar denotes the period of electrical field stimulation (EFS). The lower trace shows expanded, normalised trace recordings of the response to the nasal artery (RED) and splenic artery (BLACK) following 30s EFS to 4 and 8Hz, respectively. Note that the response of the nasal artery reached a peak within the first 10s of EFS and declined rapidly after cessation of stimulation, while the response of splenic artery reached a peak at the end of a period of 30s EFS and declined more slowly.

3.3.3 The effect of cocaine on magnitude and duration of electrically evoked contraction in the porcine isolated nasal artery

This experiment performed to determine the effect of 10μM cocaine in mediating the magnitude and duration of electrically evoked responses of the nasal artery. Electrical field stimulation at 4Hz, 30s produced a contraction (33 ± 6% of the response to 60mM KCl) that was significantly enhanced to (48 ± 7% of the response to 60mM KCl), (P < 0.01, n=5) in the presence of 10μM cocaine. Therefore, the magnitude of responses (4Hz, 30s) of the nasal artery significantly increased by 67 ± 12% (P < 0.01, n=5) (Figure 3.6). Additionally, the presence of 10μM cocaine significantly increased the duration of the response: (t_{50}) of 9.9 ± 0.7s (control) to 18.8 ± 1.1s (10μM cocaine), (P < 0.01). In contrast, the duration of the electrically evoked contractions in the control segments did not change (t_{50}) of 13.9 ± 3.7s (the mean of the fisrt three consistent responses) to 14.3 ± 3.7s (the mean of the second three consistent responses). Figure 3.7 shows representative trace recordings that 10μM cocaine increased both the magnitude and duration of electrically evoked (4Hz, 30s) contraction of the nasal artery.

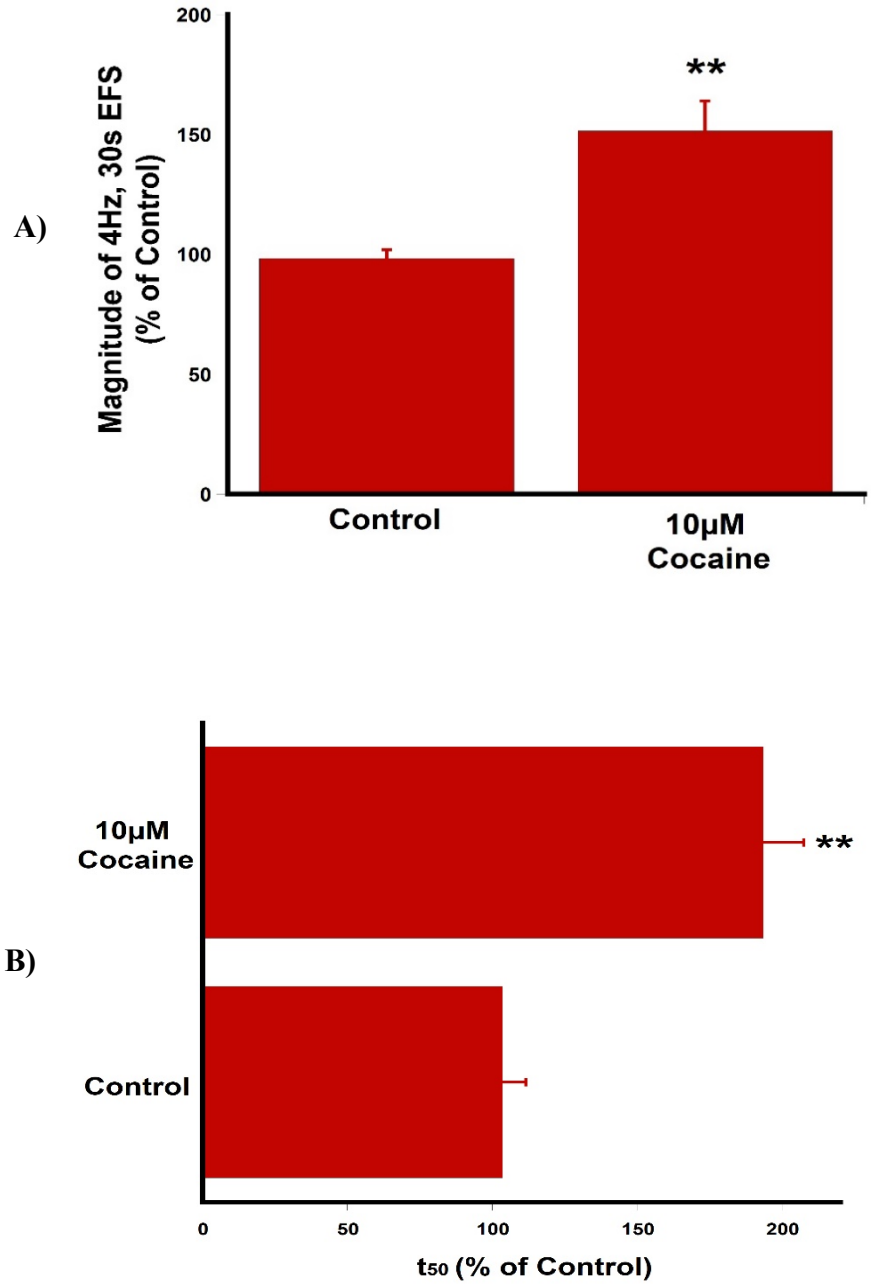


Figure 3.6 The effect of 10μM cocaine on (A) the magnitude and (B) the duration of the electrically evoked (EFS 4Hz, 0.3ms, 30s duration) contractions of the porcine isolated nasal artery. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of five observations.

** $P < 0.01$, Student's paired *t*-test.

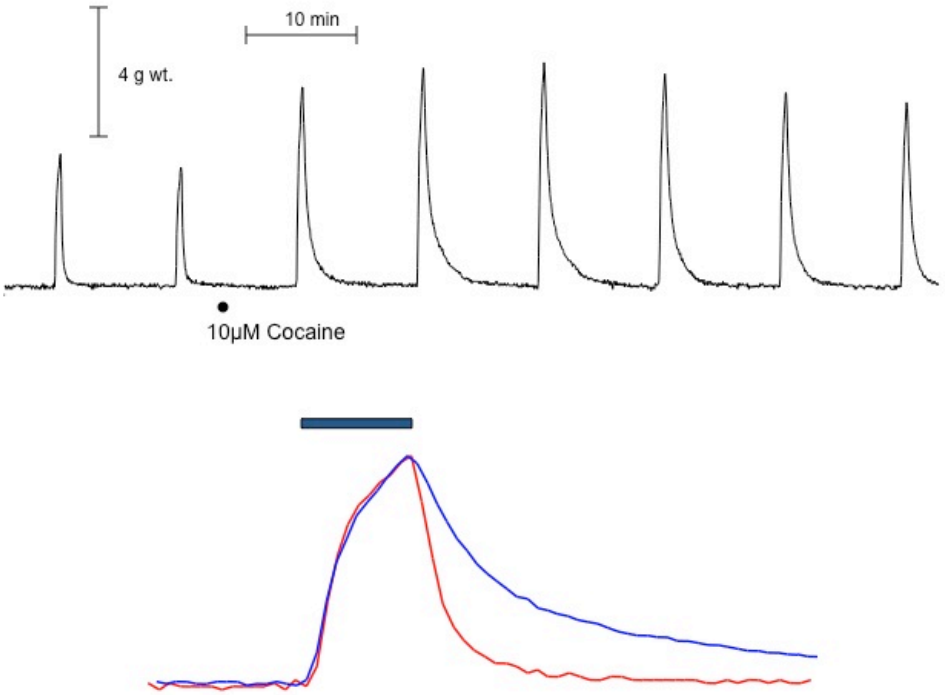


Figure 3.7 Representative trace recording of the effect of 10µM cocaine on the magnitude and time course of electrically evoked contractions of the porcine isolated nasal artery (4Hz, 200mA, 30s duration). The responses were shown before and after the addition of the drug.

The blue bar denotes the period of electrical field stimulation (EFS). The lower trace shows the pre (red) and post (blue) 10µM cocaine EFS responses normalised to highlight the effect of 10µM cocaine on the duration of electrically evoked (4Hz, 30s) contraction of the nasal artery.

3.3.4 The effect of tyramine on magnitude and duration of electrically evoked contraction in the porcine isolated nasal artery

The aim of this experiment was to examine the role of indirectly acting sympathomimetic amines tyramine used in three different concentrations (1, 10 and 100μM) on electrically stimulated segments of the porcine nasal artery.

Electrical field stimulation at 4Hz, 30s produced a contraction (39 ± 3% of the response to 60mM KCl) that was significantly enhanced to 43 ± 7% of the response to 60mM KCl ($P < 0.05$, n=6) and 44 ± 6% of the response to 60mM KCl ($P < 0.01$, n=6) in the presence of 10 and 100μM tyramine, respectively. However, no significant effect of 1μM tyramine has been noticed. Figure 3.8 showed that neither 1 nor 10μM tyramine affected the duration of electrically evoked contraction (4Hz, 0.3ms, 30s duration) of the porcine nasal artery (t_{50}), yet 100μM tyramine significantly increased the duration of electrically stimulated nasal artery contraction (Table 3.2).

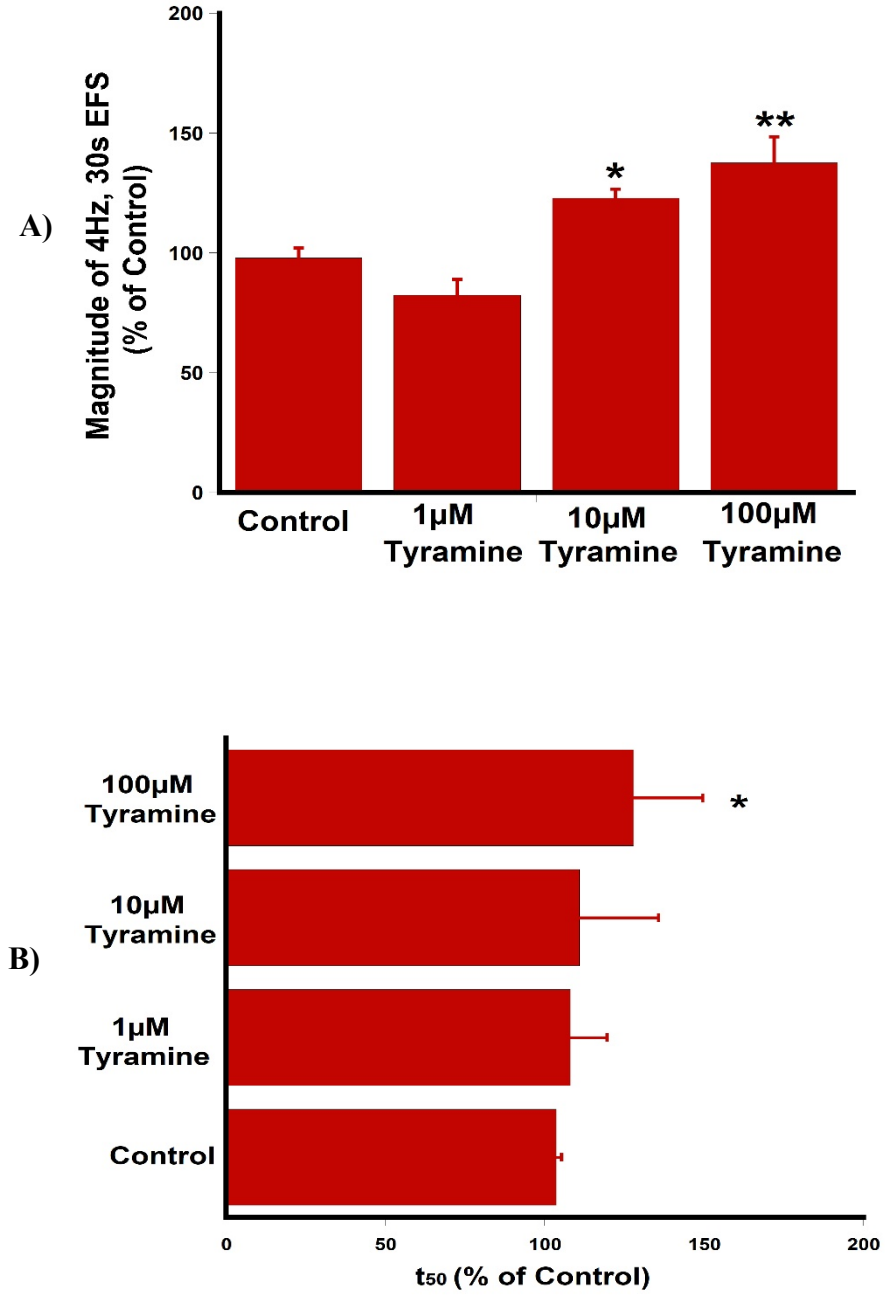


Figure 3.8 The effect of 1, 10 and 100μM of tyramine on (A) the magnitude and (B) duration of the electrically evoked (EFS 4Hz, 0.3ms, 30s duration) contractions of the porcine isolated nasal artery. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of six observations.

* $P < 0.05$, ** $P < 0.01$, one-way ANOVA followed by Dunnett's test.

Table 3.2 Comparison of the effect of tyramine (1, 10 and 100μM) on the post-stimulation decline of responses after electrical field stimulation (EFS) in the porcine isolated nasal artery. Results were shown as the mean ± S.E.M of six observations.
* ($P < 0.05$) - denotes a statistically significant difference from respective control segments, Student paired *t*-test.

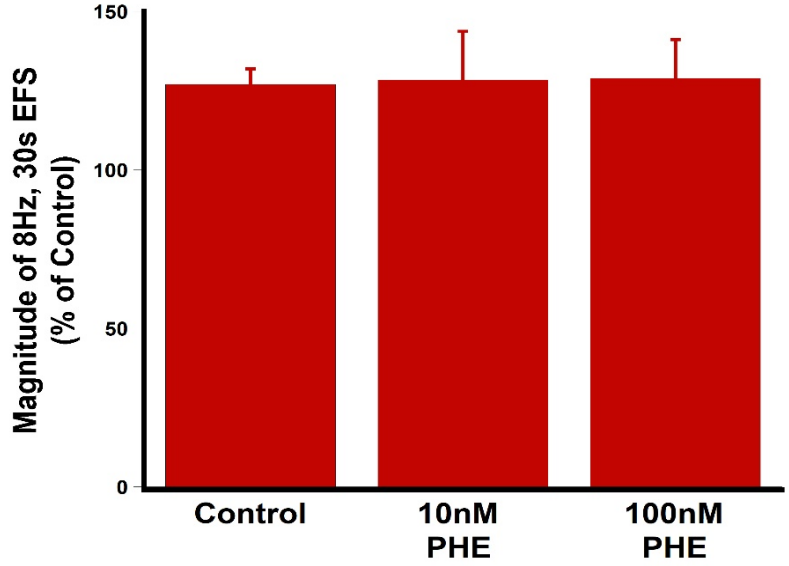
	First response	Second response
Control	14.1 ± 4.4	14.5 ± 4.4
Control **1μM Tyramine**	15.3 ± 6	16.2 ± 6
Control **10μM Tyramine**	15.1 ± 3.4	13.1 ± 2
Control **100μM Tyramine**	26.7 ± 1.8	33.3 ± 5*

3.3.5 The effect of phenylephrine on magnitude and duration of electrically evoked contraction in porcine blood vessels

As shown previously in figure 3.2, phenylephrine was not more potent in the nasal vasculature in the pig than the rest of the extra-nasal circulation. Therefore, a direct comparison of phenylephrine (10 & 100nM) as a constrictor agent in the porcine splenic artery and nasal artery against EFS responses has been evaluated. Porcine splenic artery results showed that phenylephrine 10nM and even increasing the concentration by 10-fold (100nM) failed to either contract the splenic artery (8Hz, 0.3ms, 30s duration) or enhance noradrenergic contractions. Similarly, neither 10 nor 100nM phenylephrine affected the magnitude or duration of electrically evoked contraction (4Hz, 0.3ms, 30s duration) of the porcine nasal artery (Figure 3.9). In conclusion, phenylephrine (10 or 100nM) did not affect electrically evoked contractions in either the nasal artery or the splenic artery (see table 3.3).

The significance of these observations is that 10nM phenylephrine (0.8-1.7ng/ml); (10-30nM) which is the peak plasma concentration achieved following oral ingestion of 10mg phenylephrine (Atkinson *et al*., 2014; Janin and Monnet, 2014; Geolette and Zimmerman, 2015) has no effect in the splenic and nasal arteries (Figure 3.10). Thus, our results showed that neurogenic activation of the nasal artery, which produces larger responses at lower frequencies, compared to the splenic artery, their contractile responses involve only noradrenaline release and the activation of α_1-adrenoceptors are not enhanced by the presence of phenylephrine.

A)



B)

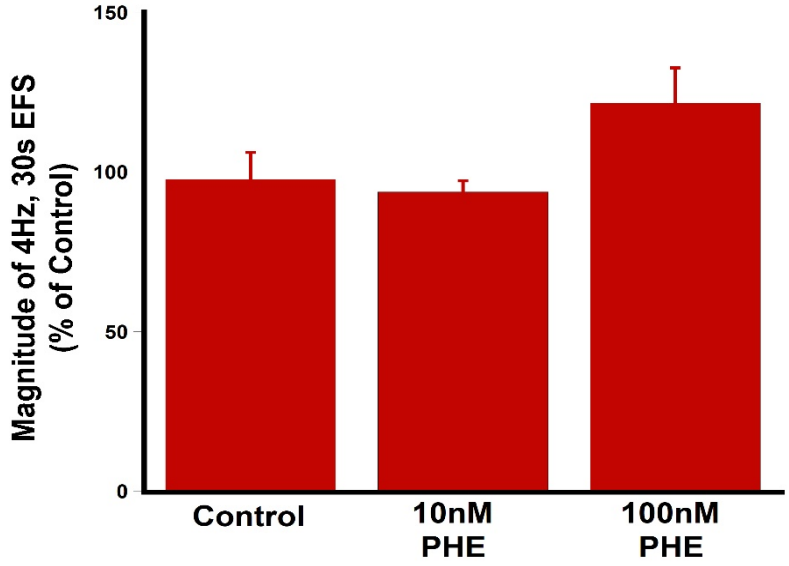
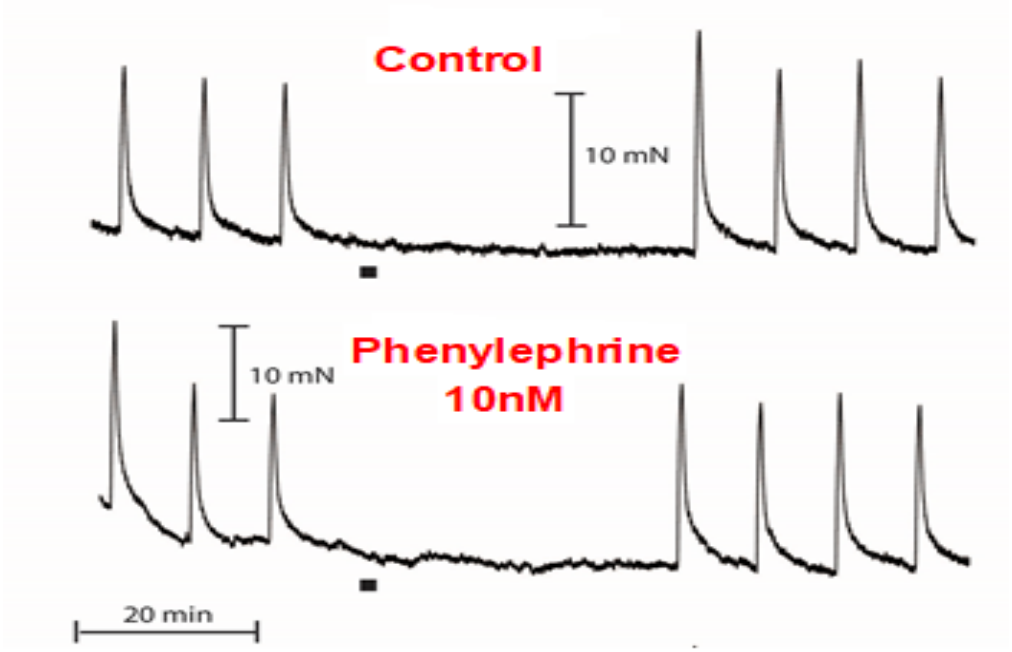


Figure 3.9 The effect of 10-100nM phenylephrine on the magnitude of electrically evoked contraction of (A) porcine splenic artery (8Hz, 200mA, 0.3ms, 30s duration) and (B) porcine nasal artery (4Hz, 200mA, 0.3ms, 30s duration). Responses were expressed as a percentage of the response before the addition of the drug and were shown as the mean ± S.E.M of 4-7 observations.

A)



B)

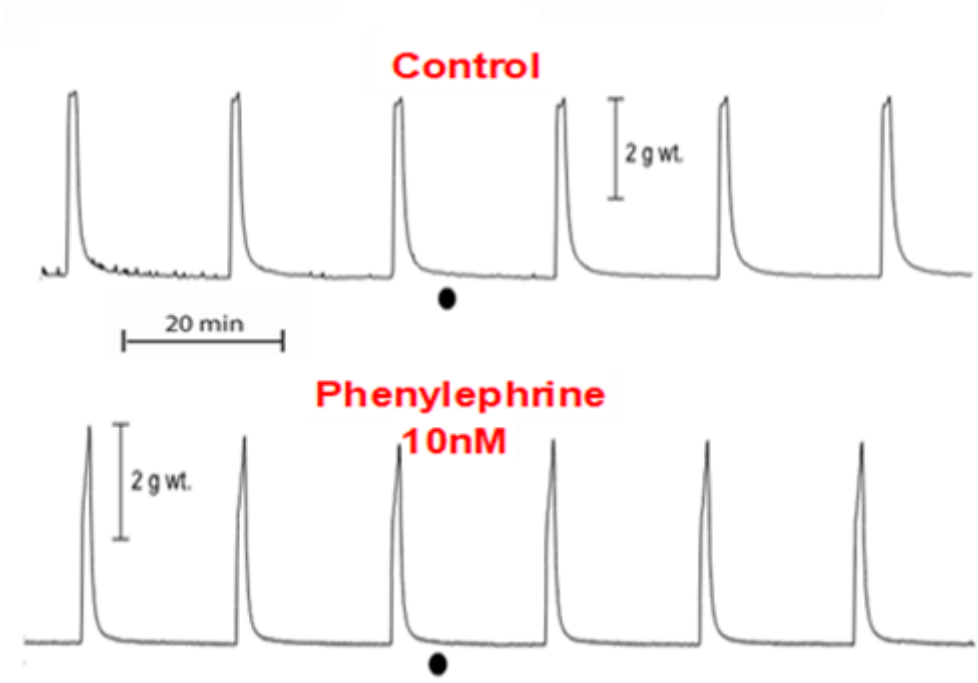


Figure 3.10 Representative trace recordings of the effect of 10nM phenylephrine on the magnitude of electrically evoked contraction of (A) porcine splenic artery (8Hz, 0.3ms, 30s duration at 10-minute intervals) and (B) porcine nasal artery (4Hz, 0.3ms, 30s duration at 10-minute intervals). Responses were shown before and after the addition of the drug.

3.3.6 The effect of pseudoephedrine on magnitude and duration of electrically evoked contraction in porcine blood vessels

In this experiment, we compared the indirect sympathomimetic action of pseudoephedrine, which is an essential orally-active nasal decongestant, on the porcine isolated splenic artery and nasal artery. In the first set of experiments, splenic artery preparations were electrically stimulated (8Hz, 30s, 300mA at 10-minute intervals) and exposed to pseudoephedrine (0.6 & 6μM) for 60 minutes. Pseudoephedrine (0.6 and 6μM) increased the magnitude and duration of responses to 8Hz, 30s EFS in the splenic artery (Figure 3.11A and table 3.3). Figure 3.12A showed representative traces recording of the response of porcine splenic artery to electrical field stimulation (8Hz, 0.3ms, 30s duration at 10-minute intervals), which has been enhanced by exposure to 0.6μM pseudoephedrine.

To confirm these results, we compared the action of pseudoephedrine (0.3 & 3μM) for 60 minutes in electrically stimulated nasal artery preparations (4Hz, 30s, 300mA at 10-minute intervals). Electrically stimulated nasal ring segments produced a contraction (39 ± 3% of the response to 60mM KCl) that was significantly enhanced to (51 ± 5% of the response to 60mM KCl), ($P < 0.01$, n=10) (Figure 3.11B and 3.12B) and increased in duration from 103 ± 0.6% to 203 ± 34% ($P < 0.01$, n=10) in the presence of 3μM pseudoephedrine only. Thus, 3μM pseudoephedrine significantly increased both the magnitude and the duration of responses to 4Hz, 30s EFS in the nasal artery (Table 3.3). The significant increase of contractions induced by 0.6-3μM pseudoephedrine in both porcine splenic and nasal arteries indicated that at concentrations known to occur *in vivo* (Kosoglou *et al*., 1997), pseudoephedrine enhanced the noradrenergic contractions but these pseudoephedrine concentrations failed to elicit a contraction of the porcine splenic and nasal blood vessels *per se*.

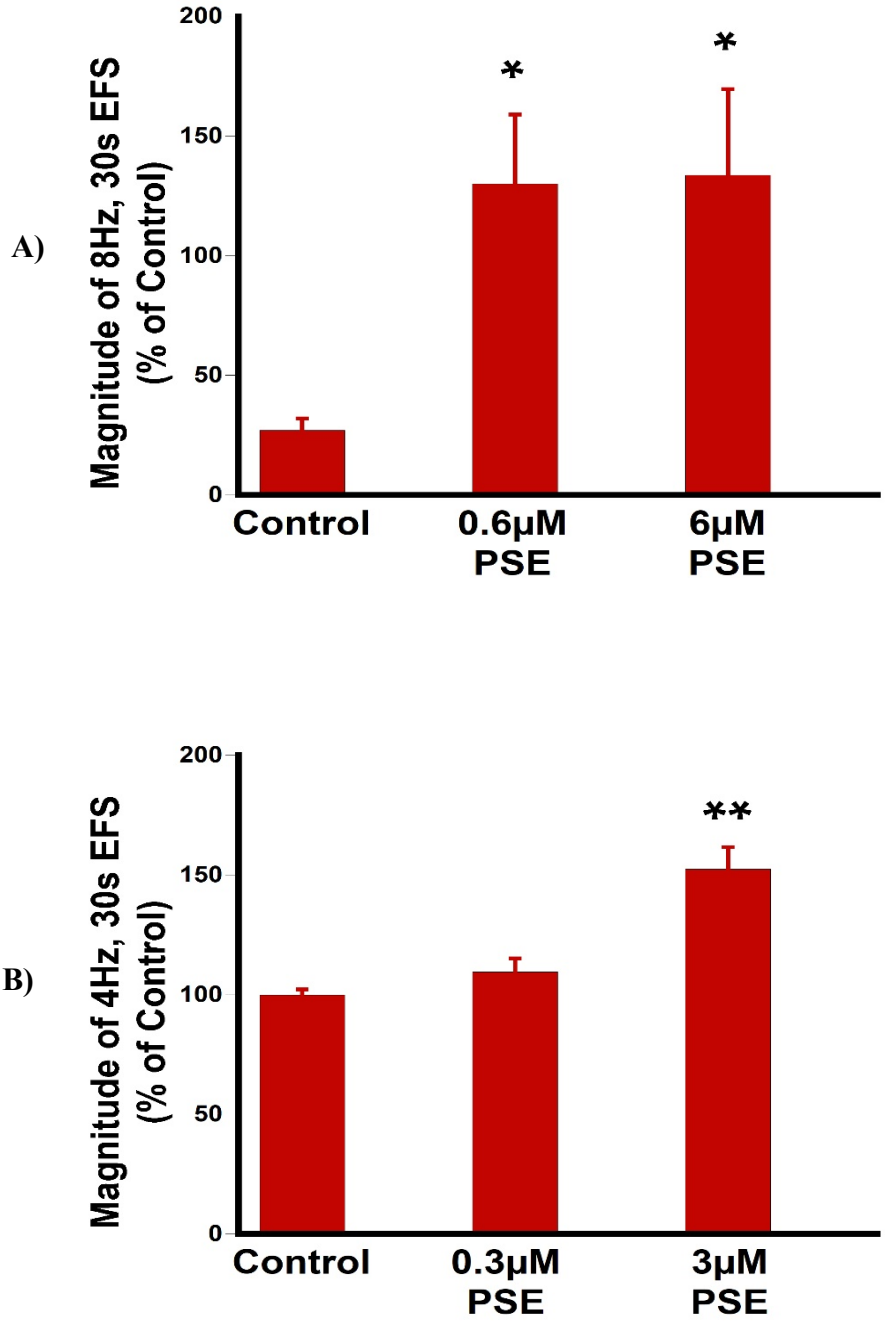


Figure 3.11 Comparison of the effect of pseudoephedrine on the magnitude of electrically evoked contraction of (A) porcine splenic artery (8Hz, 0.3ms, 30s duration) and (B) porcine nasal artery (4Hz, 0.3ms, 30s duration). Responses were expressed as a percentage of the response before the addition of the drug and were shown as the mean ± S.E.M of 6-12 observations.

* $P < 0.05$, ** $P < 0.01$, one-way ANOVA followed by Dunnett's test.

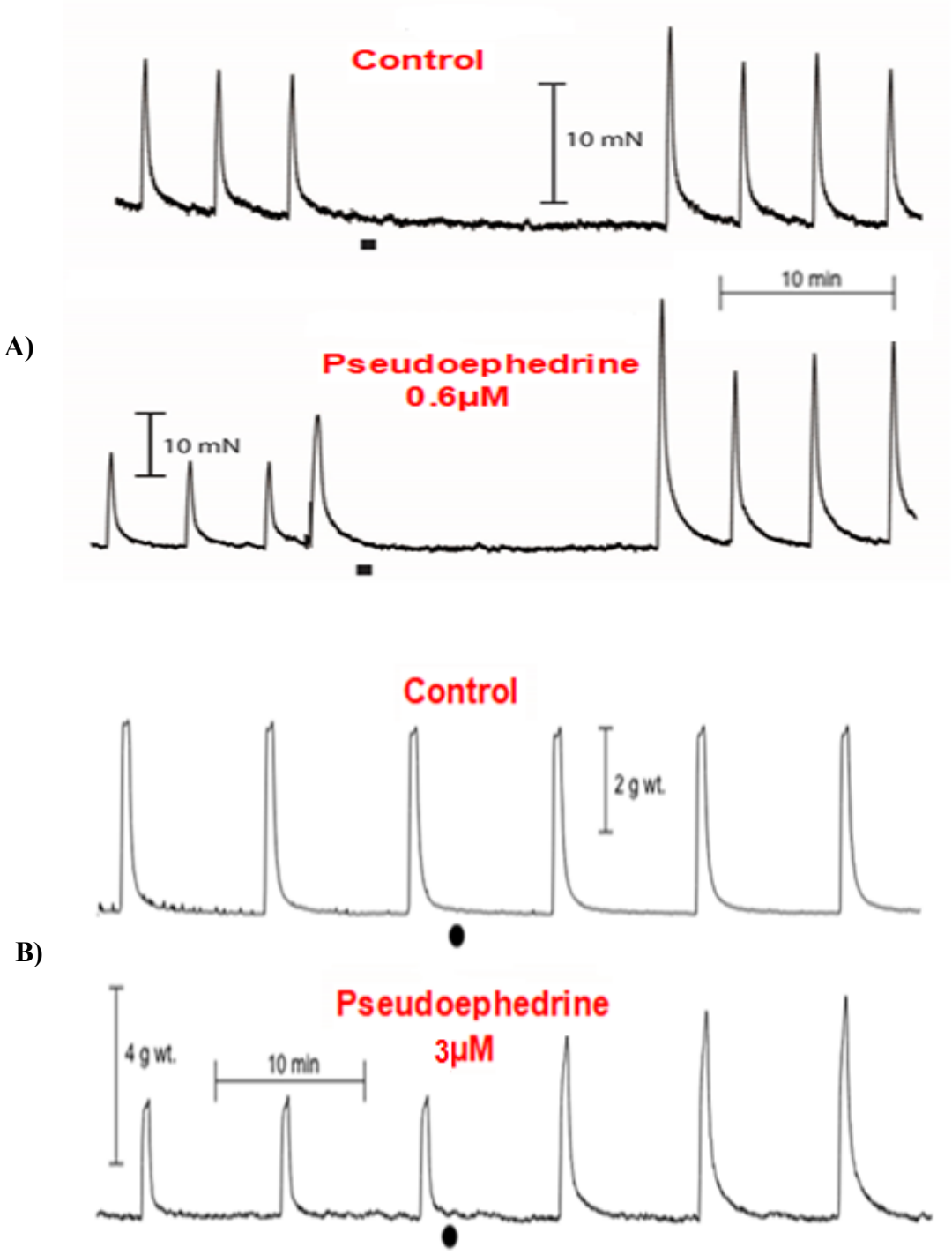


Figure 3.12 Representative trace recordings of the response of (A) porcine splenic artery to electrical field stimulation (8Hz, 0.3ms, 30s duration at 10-minute intervals) elicited after exposure to 0.6µM pseudoephedrine and (B) porcine nasal artery to electrical field stimulation (4Hz, 0.3ms, 30s duration at 10-minute intervals) elicited after exposure to 3µM pseudoephedrine. Responses were shown before and after the addition of the drug.

Table 3.3 Comparison of the effect of phenylephrine (10-100nM) and pseudoephedrine (0.3-6μM) on the magnitude and post-stimulation decline of responses after electrical field stimulation (EFS) in the porcine isolated splenic and nasal artery.

Results were shown as the mean ± S.E.M of 4-21 observations.

* ($P < 0.05$) and ** ($P < 0.01$) - denote a statistically significant difference from respective control segments, Student paired *t*-test.

Porcine Splenic Artery			**Porcine Nasal Artery**		
	EFS 8Hz 30s (g wt)	Time to 50% decline after EFS (s)		EFS 4Hz 30s (g wt)	Time to 50% decline after EFS (s)
Control (n=21)	2.71 ± 0.33 3.40 ± 0.41	28.3 ± 5.2 32.8 ± 6.7	Control (n=21)	4.85 ± 0.55 4.83 ± 0.57	9.3 ± 0.7 9.5 ± 0.5
Control (n=7) 10nM Phenylephrine	1.73 ± 0.36 2.18 ± 0.50	22.3 ± 1.8 28.3 ± 3.9	Control (n=4) 10nM Phenylephrine	7.08 ± 1.34 6.76 ± 1.45	8.7 ± 0.4 9.0 ± 0.6
Control (n=7) 100nM Phenylephrine	3.12 ± 0.47 3.75 ± 0.43	22.6 ± 1.4 40.3 ± 15	Control (n=4) 100nM Phenylephrine	5.02 ± 0.65 6.18 ± 1.08	8.1 ± 0.9 9.1 ± 0.9
Control (n=12) 0.6μM Pseudoephedrine	1.18 ± 0.14 2.55 ± 0.33*	26.4 ± 1.8 30.2 ± 2.1	Control (n=8) 0.3μM Pseudoephedrine	3.17 ± 0.65 3.34 ± 0.63	9.9 ± 1.4 12.0 ± 1.4
Control (n=6) 6μM Pseudoephedrine	2.43 ± 0.39 5.48 ± 1.29**	28.7 ± 3.6 54.6 ± 8.9**	Control (n=10) 3μM Pseudoephedrine	4.21 ± 0.78 6.07 ± 0.98**	9.1 ± 0.8 17.2 ± 1.5**

3.3.7 The effects of cocaine and pseudoephedrine on contraction to noradrenaline in the porcine isolated nasal artery

10μM cocaine blocks the neuronal uptake of sympathomimetic amines via targeting a specialised nerve membrane transport system, that block, in turn, potentiates the concentration of sympathomimetics at the appropriate site of receptors and finally leads to a reduction of the response (Chen *et al*., 1998). Since this study aimed to better understand the pharmacology of decongestants, 30μM pseudoephedrine effect on noradrenaline-induced contraction of the porcine isolated nasal artery was examined for a possible role like the effect of 10μM cocaine on contractions to the sympathomimetic agent (noradrenaline).

The present data showed that the influence of the uptake mechanism using 10μM cocaine was quite different from the effect of using 30μM pseudoephedrine. Responses to noradrenaline in the presence of 10μM cocaine (highest response was 10 ± 1% of the response to 60mM KCl with a pD_2 of 6.10 ± 0.03, n=8) were comparable to controls (highest response was 13 ± 3% of the response to 60mM KCl with a pD_2 of 5.50 ± 0.08, n=8) and 30μM pseudoephedrine (the highest response was 11 ± 1% of the response to 60mM KCl with a pD_2 of 5.60 ± 0.05, n=8). 10μM Cocaine produced a significant shift to the left in the concentration-response curve of noradrenaline and increased significantly the pD_2 value by 5-fold. However, 30μM pseudoephedrine did not cause any significant changes either in the concentration-response curve or in the potency value of noradrenaline (Figure 3.13). Comparing the results of maximum response, shifting in the curve and potencies of noradrenaline in the porcine nasal artery as a control and in the presence of 30μM pseudoephedrine showed that, they were quite similar but they were significantly different from 10μM cocaine either alone or in combination with 30μM pseudoephedrine.

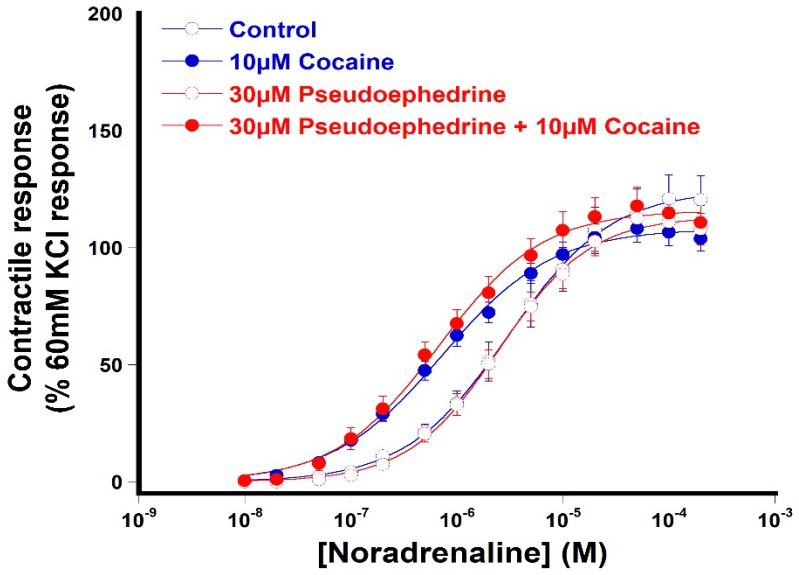


Figure 3.13 The effect of 10μM cocaine and 30μM pseudoephedrine on responses to the cumulative concentration of noradrenaline in the porcine isolated nasal artery. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of eight observations.

3.4 Discussion

The key findings from this study are that while the selective α_1-adrenoceptor agonist phenylephrine produced concentration-dependent contractions of both nasal and extra-nasal arteries from the pig, no evidence was found for preferential activation of the nasal vasculature; extra-nasal vessels were, in general, 4-fold more sensitive to this agonist. Also, noradrenaline was found to be 3-fold more potent in the splenic artery compared to the nasal artery.

Furthermore, at concentrations reported to be achieved *in vivo* (0.8-1.7ng/ml, 10-30nM) (Atkinson *et al*., 2014; Janin and Monnet, 2014; Geolette and Zimmerman, 2015) following oral ingestion of (10 or 12mg) phenylephrine failed to significantly affect *in vitro* direct vasoconstriction and neurogenic contractions in either the nasal vascular bed or extra-nasal artery from the pig. Taken together, these findings raise questions regarding the therapeutic rationale for the use of oral phenylephrine (10 or 12mg) as a decongestant. While pseudoephedrine was devoid of a direct vasoconstrictor action in both the nasal and splenic artery, we found evidence for enhancement of electrically evoked, noradrenergic contractions that was particularly evident in the nasal vessel at low frequencies of stimulation. Crucially, the latter effect was apparent at concentrations close to those known to occur *in vivo* following oral ingestion of decongestant dosage form of pseudoephedrine (Kosoglou *et al*., 1997).

It has been shown previously that the addition of cocaine can prevent monoamine transporters and block nerve endings from taking up neurotransmitters, thus releasing noradrenaline (Farmer *et al.*, 1963; Farrant, 1963). Later on, Hughes, 1972 found that combination of corticosterone (to block extra-neuronal uptake) and cocaine could

improve the release of noradrenaline after electrical field stimulation up to 3-fold in rabbit vas deferens and rabbit aorta and portal vein (Docherty *et al.*, 1982). These facts become consistent with the examination of the sympathetic supply of the nasal artery using electrical field stimulation and the effect of 10μM cocaine on responses of the nasal artery, which revealed that there is a good sympathetic supply in this blood vessel. Because of the blockade of noradrenaline uptake by neurons by the addition of 10μM cocaine, noradrenaline was more available at the receptors. This effect was visualised as a significant enhancement in the magnitude and duration of porcine nasal artery responses.

Tyramine acts indirectly by activation of α-adrenoceptors following selective uptake in adrenergic nerve endings and the release of noradrenaline (Patil *et al.*, 1967; Rothman *et al.*, 2003), leading to constriction of blood vessels and reduction in nasal mucosa volume (Jawad and Eccles, 1998; Eccles *et al*., 2005). Confirmation of the rich sympathetic supply to the nasal artery was indicated by responses to indirectly acting sympathomimetics (tyramine) (Patil *et al.*, 1967; Hayashi *et al.*, 1980) as the present study showed that tyramine at high concentration of 10 and 100μM significantly increased the magnitude, and 100μM increased the duration of porcine nasal artery responses. Taken together, these outcomes are consistent with the nasal artery being well sympathetically innervated.

Phenylephrine:

In relation to phenylephrine, the threshold concentration of phenylephrine to cause vasoconstriction in the nasal artery *in vitro* was 0.3μM, which was approximately 30-fold greater than the peak concentration of the drug reported after ingestion of 10mg as a decongestant.

For both nasal and splenic arteries, the action of phenylephrine appears to involve direct activation of α_1- adrenoceptors, since responses were sensitive to the selective α_1-adrenoceptor antagonist prazosin (Wright *et al*., 1995a).

To our knowledge, the nasal artery of the pig has not previously been studied, but others have investigated phenylephrine on the porcine mucosa to reveal comparable potency (pD_2 5.4; Corboz *et al*., 2003). To confirm these data and the outcome related to a direct comparison of the potency of phenylephrine on human nasal and extra-nasal blood vessels that I highlighted in the introduction section (Section 3.1, table 3.1 and figure 3.1) and because these studies were conducted by a variety of different investigators over a period of time. Therefore, similar observations were generated in our laboratory using porcine nasal and extra-nasal blood vessels and vasculature. The data performed by my colleague (Saif Yahya) on systemic extra-nasal vessels (mesenteric and renal arteries and splenic vein) from the pig and compared it with my results related to phenylephrine in the porcine nasal and splenic arteries and even with more recent observations on the nasal vascular bed from the pig performed by Denfria, (2016) and other investigators. The results show again systemic porcine extra-nasal blood vessels are generally more sensitive to phenylephrine than the nasal vascular bed (Table 3.4 and figure 3.14). Furthermore, the potency of phenylephrine in the porcine nasal artery (pD_2 5.30 ± 0.12, n=7) was similar to that reported by Johannssen and colleagues (1997) in human nasal resistance arteries. This vascular bed in man also appears to be less sensitive to phenylephrine than the mesenteric and skeletal vascular bed (Hui *et al*., 2015) or mammary artery (Rudner *et al*., 1999).

The results of this chapter obviously showed that noradrenaline was more potent than phenylephrine in the porcine splenic artery and even in the porcine nasal artery.

However, both agents share the same sensitivity for the porcine blood vessels in which they were more potent in the porcine (extra-nasal) splenic artery than in the porcine nasal artery. Denfria, (2016) previously performed the same findings on the porcine splenic artery and nasal artery, which also showed that noradrenaline and phenylephrine were more potent in the splenic artery than in nasal artery. Barbieri *et al*., (1998) also showed that noradrenaline was more potent than phenylephrine in the porcine splenic artery.

The lower sensitivity of the nasal vasculature to phenylephrine, compared to systemic vessels may account for the clinical observation in man that topical application of the drug to nasal mucosa, to aid nasotracheal intubation by reducing nasal mucosal volume, is associated with short-term elevation in blood pressure (Gross *et al*., 1984), presumably due to appreciable 'systemic overspill'. However, it is noteworthy that a steady-state concentration of 8ng/ml phenylephrine (approximately 40nM) can maintain blood pressure in women in spinal anaesthesia (Ngan Kee and Khaw, 2009a; Ngan Kee *et al.*, 2009b) and in patients undergoing haemorraghic shock (Hoen *et al*., 2005) or septic shock (Morelli *et al*., 2008) and thus raises the possibility that the conditions employed *in vitro* underestimates the true activity of phenylephrine at vascular α_1-adrenoceptors *in vivo.*

Nonetheless, these data collectively support the lack of selectivity of phenylephrine to the nasal vasculature and could question the known assumption that the nasal circulation is more sensitive to the nasal decongestant oral phenylephrine (Aviado *et al*., 1958; Aviado *et al*., 1959). Strong evidence and further analysis showed that oral phenylephrine cannot relieve symptoms of allergic rhinitis (Meltzer *et al.,* 2015) even when the dose was greater than before (30mg) (Meltzer *et al.,* 2016).

These findings further support the preceding notion over 50 years ago by McLaurin and assistants (1961) which also founded no improvement in symptoms of nasal congestion associated with phenylephrine. The present findings of no selective effect of phenylephrine on nasal blood vessels seem to be consistent with these researches. For drugs so widely used by the general public, this is an important and reassuring observation, but equally, there is a need for unambiguous evidence of therapeutic activity consistent with the medicinal claim.

Table 3.4 Published and unpublished data on the potency (pD_2) of phenylephrine in porcine isolated nasal and extra-nasal (systemic) blood vessels and vasculature.

Porcine Vessel	**Phenylephrine pD_2**
Extra-Nasal Vessels	
Aorta [a]	5.20
Splenic Artery Splenic Vein [b]	5.87 ± 0.07 5.67 ± 0.14
Renal Artery [b]	6.17 ± 0.06
Mesenteric Artery [b]	6.44 ± 0.07
Nasal Vessels and Vasculature	
Mucosal Strip [c]	5.38 ± 0.04
Nasal Artery Perfused Pig Snout [d]	5.33 ± 0.13 5.20 ± 0.19

a Wright *et al*., (1995b).
b Unpublished data (Saif Yahya).
c Corboz *et al*., (2003).
d Denfria *et al*., (2016).

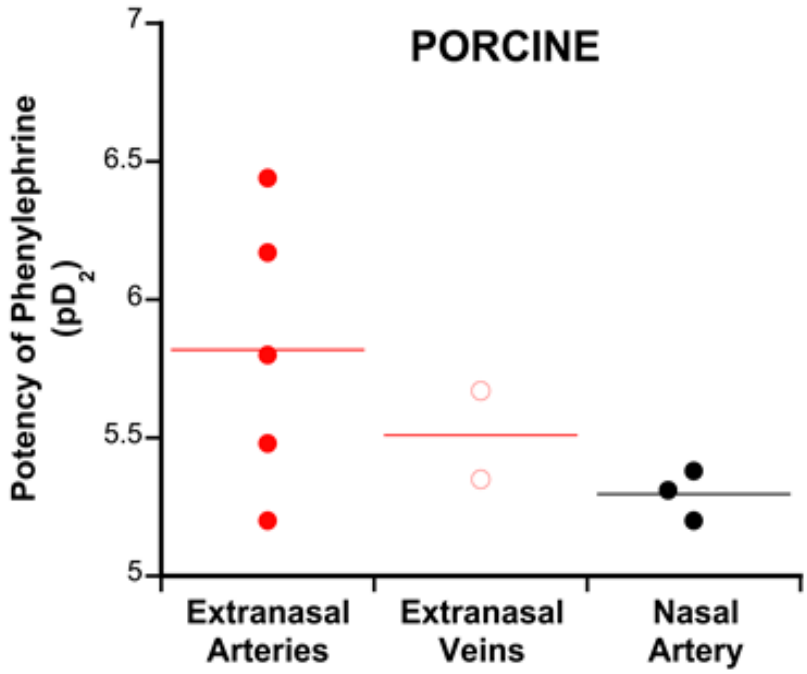


Figure 3.14 Comparison of the potency of phenylephrine (pD_2) in porcine isolated nasal and extra-nasal (systemic) blood vessels and vasculature.

Pseudoephedrine:

In relation to pseudoephedrine, our *in vitro* study also revealed surprising results. The decongestant activity of this drug is thought to involve an indirect activation of nasal α_1-adrenoceptors following selective uptake in adrenergic neurones and the release of noradrenaline (Patil *et al.,* 1967; Rothman *et al*., 2003), leading to constriction of blood vessels and a reduction of nasal mucosa volume and resistance (Jawad and Eccles, 1998; Eccles *et al*., 2005).

In our study, however, pseudoephedrine failed to elicit a contraction *per se* of either the splenic or the nasal artery, up to a concentration of 100μM. On the other hand, pseudoephedrine significantly enhanced electrically evoked contractions in both blood vessels and also selectively increased the duration of the porcine nasal artery responses at low frequency, comparable to that seen with either cocaine or tyramine. However, the two experiments with pseudoephedrine in the porcine splenic artery and nasal artery were performed in a slightly different way that is during 40 minutes before addition of pseudoephedrine, the tissue segments of the porcine splenic artery were not stimulated electrically opposed to the segments of the nasal artery, which were stimulated continuously. The effects of pseudoephedrine occurred at concentrations (0.6-3μM) close to those known to attain following oral ingestion of standard decongestant dose (Kosoglou *et al*., 1997).

Our results also show that both blood vessels were contracted following electrical field stimulation, but the nasal artery was clearly more responsive to low frequencies of stimulation than the splenic artery. Also, the nasal artery responded more quickly to electrical field stimulation and appeared to posses a more efficient uptake mechanism; neurogenic contractions waned more quickly after cessation of stimulation. It is

possible, therefore, that the combination of greater responsiveness to electrical field stimulation, together with a more avid uptake system, provides a functional basis for the decongestant activity of pseudoephedrine with minimal systemic side effects.

To understand the pharmacology of decongestants particularly pseudoephedrine, the effects on noradrenaline-induced contraction of the porcine isolated nasal artery were investigated for a possible role like the effect of 10μM cocaine. The results of this examination showed that there was a significant difference between 30μM pseudoephedrine and 10μM cocaine, which indicated that the mode of action of cocaine in causing attenuation of the vasoconstrictor tone was not similar to that of pseudoephedrine target.

In conclusion, pseudoephedrine appeared to enhance selectively both the magnitude and duration of low-frequency noradrenergic responses in the nasal vasculature and this may provide a functional basis for the recognised decongestant activity in man. For phenylephrine, however, experiments were unable to demonstrate any evidence of a selective action on the nasal vasculature, either direct or indirect, which contrasts with the long-held view about the therapeutic justification of giving a potent adrenoceptor agent to people by the oral route. This surprising observation is also response for the endogenous ligand noradrenaline, which showed greater potency in the porcine splenic artery (extra-nasal) compared with the porcine nasal artery, the results of the present study necessitate a further justification and investigation of the nasal and splenic arteries in-depth using the endogenous ligand noradrenaline.

Chapter Four

The effect of α-adrenoceptor antagonists against responses to α-adrenoceptor agonists in porcine blood vessels

4 The effect of α-adrenoceptor antagonists against responses to α-adrenoceptor agonists in porcine blood vessels

4.1 Introduction

The starting point for the work in this chapter was a desire to examine in more details the pharmacological characteristics of α_1-adrenoceptor-mediated contractions in the nasal and extra-nasal vasculature. Previous work (Chapter 3) has established that the nasal circulation is less sensitive to directly acting sympathomimetics (noradrenaline and phenylephrine) than the rest of the circulation, while the opposite is true for indirectly acting sympathomimetics (pseudoephedrine). One possible explanation for this striking difference is that the α_1-adrenoceptor subtype present in the two vascular beds are different, coupled to differential sympathetic innervation.

Over the past 20 years mounting evidence has consolidated the idea of pharmacological heterogeneity amongst α_1-adrenoceptors, in the cardiovascular system and other organs, leading to the view that there are at least three major subtypes, α_{1A}-, α_{1B}- and α_{1D}- (Alexander *et al*., 2017; Docherty 2019), each of which are similarly sensitive to the classic selective α_1-adrenoceptor antagonist prazosin. This concept of receptor heterogeneity has been largely driven by observations using genetic approaches, radioligand binding technique and functional studies with presumed selective agents, particularly antagonists, in a variety of cell-based systems, isolated tissue models and even whole animals. For example, radioligand binding studies in rat thoracic aorta and small mesenteric artery showed that there was a mixture of α_{1B}- and α_{1D}-adrenoceptors in rat thoracic aorta while α_{1A}- and α_{1B}-adrenoceptors predominate in mesenteric small arteries of the same species (Stassen *et al*., 1997).

Detailed pharmacological investigation has yielded potent antagonists selective for the α_{1A}-subtype, such as tamsulosin (Noble *et al*., 1997) and silodosin (Murata *et al*., 1999; Murata *et al*., 2000), the α_{1B}-subtype, (+)-cyclazosine (Stam *et al*., 1998) and shed light on previously unrecognized receptor selectivity for even long-established antagonists like WB-4101 and phentolamine (Hussain and Marshall, 1997; Bavadekar *et al*., 2008). It has also been argued that the clinical superiority of tamsulosin and silodosin for treating benign prostatic hypertrophy arises from their ability to exert tissue-selective effects (prostate versus cardiovascular) because of differential distribution of α_1-adrenoceptor subtypes in these organs. Thus, a commonly held view is that vascular α_1-adrenoceptors predominantly belong to the α_{1B}-subtype (Yang and Chiba, 2002; Errasti *et al*., 2003; Kaplan, 2005; Schilit and Benzeroual, 2009). This would account for the lower reported incidence of cardiovascular problems with tamsulosin and silodosin, compared to the older, non-selective antagonists related to prazosin, e.g. terazosin or alfuzosin (Wu *et al*., 2013; Roehrborn *et al*., 2017). However, it is noteworthy that is not a universal view of the characteristics of vascular adrenoceptor pharmacology, since several studies also suggest that the α_{1A}-adrenoceptor subtype can mediate contractions in specific blood vascular beds (Amadesi *et al*., 2001), including the porcine isolated splenic artery (Barbieri *et al*., 1998) and even guinea-pig nasal mucosa (Tanimitsu *et al*., 2000).

Another valuable approach for identifying receptor subtypes has been to examine agonist potency-ratio in the different systems. Indeed, this was the approach originally adopted by Alhquist (1948) in his original proposal of α- and β-adrenoceptors and later by Starke and colleagues (1975) in recognizing the subdivision of α-adrenoceptors. More recently, Smith *et al*., (1997) used this approach to shed light on the characteristics of α_1-adrenoceptor subtypes mediating contractions in rabbit

subcutaneous resistance arteries as the relative potency of A61603 to phenylephrine and to noradrenaline was 112 and 944, respectively, indicated the predominance of α_{1A}- and α_{1B}-adrenoceptors. This parameter has the distinct advantage of reducing the influence of experimental conditions, or tissue-specific factors, on the absolute potency of agonists, thereby making a comparison between different systems far more robust.

In the context of heterogeneity amongst α_1-adrenoceptors, it is noteworthy that Knepper and colleagues (1995) reported that the imidazoline derivatives *N*-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthlen-1-yl] methanesulfonamide hydrobromide (A61603) behaved as a full agonist at α_{1A}-adrenoceptors receptors expressed in LKY- cells and possessed a high degree of selectivity for this subtype relative to the α_{1B}- (30-fold) and α_{1D}-adrenoceptors (40-fold). Additionally, when the absolute potency of A61603 at each subtype was compared to that of noradrenaline and phenylephrine under the same conditions found that, the potency-ratio of A61603 (200-300-fold more potent than noradrenaline or phenylephrine, respectively) in the rat vas deferens and in the canine prostate (130-165-fold more potent than noradrenaline or phenylephrine, respectively) (Knepper *et al*., 1995). This outcome revealed that an agonist potency-ratio for these agents of 150 at α_{1A}-subtype compared to 40 at α_{1B}-subtype and, finally 0.03 for the α_{1D}-adrenoceptor subtype and provided distinguishing power between α_{1A}- and α_{1B}-adrenoceptor subtypes from α_{1D}-adrenoceptor subtypes (Knepper *et al*., 1995). Thus, agonist potency-ratio values offer greater discriminating power between receptor subtypes, particularly when combined with observations on selective antagonists.

In the present study, I have examined the pharmacological characteristics of α-adrenoceptor mediated contractions elicited by A61603 and noradrenaline in both the

splenic artery and the nasal artery from the pig. In light of the evidence of heterogeneity of α_1-adrenoceptors, even within individual vascular beds (Jaehnichen *et al*., 2004), several steps have been taken to improve the estimates of agonist potency and antagonists. First, in many experiments agonist potency has been determined from using a 1, 2, 5, 10 increment in serial dilutions to elicit responses, rather than the standard 1, 3, 10 schedules. Second, antagonist inhibition of vasoconstrictor responses to A61603 and noradrenaline has been based an examining the effect over a 1000-fold range using 3-fold increments in concentration. Third, I also undertook some experiments on the porcine isolated splenic vein. The changes to standard experimental protocol were prompted by recent contraction-based findings in this lab suggesting that neither phentolamine nor prazosin behaved in a manner consistent with competitive antagonism of noradrenaline at a single site in the splenic artery (Denfria, 2016).

4.1.1 Aims

The aims of this study were

1. Optimise the experimental conditions for examining the effect of the agonists in the porcine isolated splenic artery. Optimisation comprises three points:
   A) Examine the consecutive segments of the porcine isolated splenic artery behave in a similar manner.
   B) Examine the need to block $uptake_1$ for noradrenaline.
   C) Examine the time needed to incubate the porcine blood vessels with antagonists.
2. Compare the effects of different α-adrenoceptor agonists in the porcine isolated splenic artery and nasal artery.
3. Examine the effects of the non-selective α-adrenoceptor antagonist phentolamine against contractions elicited by noradrenaline and A61603 in the porcine isolated splenic artery and nasal artery.
4. Compare the effects of selective α_1-adrenoceptor antagonists against contractions elicited by noradrenaline and A61603 in the porcine isolated splenic artery.
5. Examine the effects of α-adrenoceptor antagonists against contractions elicited by noradrenaline in the porcine isolated splenic vein.

4.2 Materials and Methods

4.2.1 Materials

Materials used for the experiments of this chapter are listed in table 2.1.

4.2.2 Methods

Porcine segments from spleen and snout were collected from a local abattoir and transported to the laboratory. Dissection of the vessels was made and prepared for organ bath studies as mentioned in chapter 2 (section 2.1.1). Isometric tension measurement experiments were carried out in the same apparatus using the same buffer and general protocol mentioned in chapter 2 (section 2.1.2).

In some studies, segments of the porcine nasal and splenic artery were incubated with a single antagonist or a combination of antagonists according to the protocol developed for each experiment. All experiments with adrenaline and noradrenaline were carried out using cocaine (10μM) to inhibit uptake$_1$, corticosterone (30μM) to inhibit uptake$_2$ and propranolol (1μM) to inhibit β-adrenoceptors. However, only in optimisation of experiemtal condition experiments including the first three experiments (4.3.1, 4.3.2 and 4.3.3), the segments were exposed to adrenaline and noradrenaline without addition of cocaine, corticosterone and propranolol.

4.2.3 Data analysis

All values were expressed as the mean ± S.E.M in tissues from n different animals. The porcine blood vessels contraction to each agonist concentration was measured and stated as a percentage of the third response to 60mM KCl. The molar agonist concentration producing 50% of the maximum response (EC_{50}) was determined by use of the logistic equation (Kaleidagraph version 4.5.2 Synergy software, PA, USA) and expressed as the negative logarithm (pD_2) of the drug concentration that resulted in a 50% decrease or increase in vasoconstrictor tone. The agonist concentration ratio (CR) was determined from the concentration of the agonist causing 50% of the observed action in presence of the antagonist (EC_{50}'), compared to the agonist causing 50% of the observed action in absence of the antagonist ($CR=EC_{50}'/EC_{50}$).

In experiments with pharmacological inhibition produced by a series of antagonist concentrations, a Schild plot was produced by plotting log (CR-1) against the negative logarithm of antagonist concentration and the line of best fit for each tissue determined by regression analysis based on data obtained from tissues from each animal (Arunlakshana and Schild 1959). In some instances, a CR value for the highest concentrations of the antagonist could not be accurately estimated because the agonist concentration used did not produce a response greater than 75% of the estimated maximum. These values were not included in the Schild analysis. The intersection of the Schild plot with log (CR-1) = 0 with the log [Antagonist] x-axis is the pA_2 value. For the calculation of pK_B (the potency of a competitive antagonist) values, the calculation was done manually, by using the Gaddum equation as the formula given below where B is the concentration of antagonists used where $CR=EC_{50}'/EC_{50}$

$$Log\ K_B = Log\ [B] - Log\ (CR-1)$$

Statistically significant differences between the mean values were assessed using one-way ANOVA test for experiments on different antagonist incubation time and their controls. The results were considered statistically significant at a value of $P < 0.05$, where the null hypothesis was rejected. For RX-811059, the dissociation constant against noradrenaline contractions was estimated from the agonist-concentration ratio in the absence and presence of 1μM of the antagonist.

A Student's paired *t*-test was used to assess differences between mean values and deviation of the slope of Schild plots from unity. Differences between groups were considered significant if $P < 0.05$.

4.3 Results

4.3.1 The effects of contractile agents on contractions of the porcine isolated splenic artery

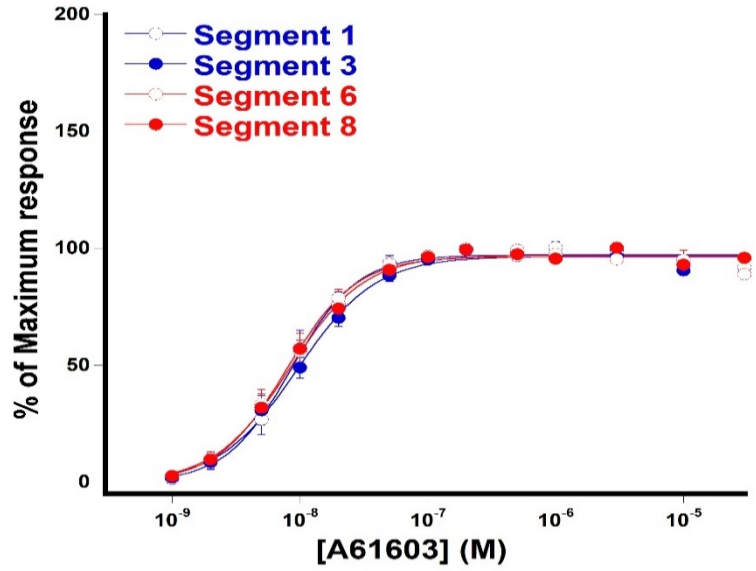
Two contractile agents with different mechanisms were used to cause contraction on the porcine isolated splenic artery. These agents were 30μM noradrenaline (a non-selective α-adrenoceptor), and vasoconstriction was additionally induced by depolarisation with 60mM KCl. The response to noradrenaline declined to less than 5% of its maximum response after 180 minutes, while KCl contraction was maintained between 80- 95% in responses to its peak response, after the same period.

4.3.2 The contraction of different segments of the porcine isolated splenic artery

In the initial preliminary experiments, 1-8 consecutive 5 mm segments were taken and numbered from the porcine splenic artery from proximal to distal and the effects of the selective α_{1A}-adrenoceptors (A61603) and noradrenaline were observed on it to determine its reproducibility across eight channels. In the first set of experiments, the segments were exposed to 60mM KCl and then to increasing cumulative concentrations of a selective α_1-adrenoceptor agonist A61603 and the responses to this agent were compared (one segment to another) (Figure 4.1A).

In the second set of experiments, the eight segments of the porcine splenic artery were exposed first to 60mM KCl and then to increasing cumulative concentrations of a non-selective α-adrenoceptor agonist noradrenaline and their responses to the endogenous agent were compared (one segment to another) (Figure 4.1B). The data from the above preliminary experiments demonstrated that in alternate 5 mm width segments of the porcine splenic artery, A61603 took almost 2-times longer to achieve contractions at steady-state compared to noradrenaline. The potency and the maximum contractions to noradrenaline and A61603 when expressed in % of the response to 60mM KCl, were similar in magnitudes and the mean concentrations which caused 50% of the maximum response across 4 cm lengths of the artery, did not vary by more than 0.1 of a log unit, therefore, segments were considered identical to each other and were used in our following experiments in a random manner (Table 4.1).

A)



B)

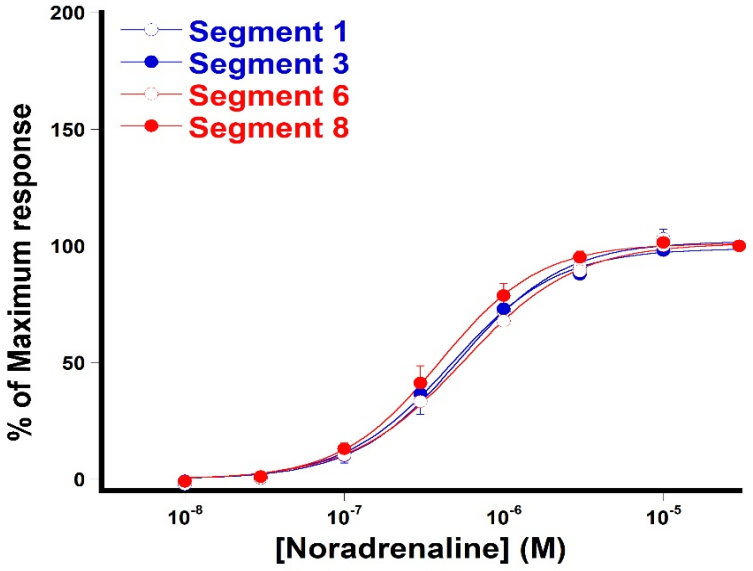


Figure 4.1 The response of different segments of the porcine splenic artery to increasing cumulative concentrations of (A) A61603 and (B) noradrenaline. Responses were expressed as a percentage of the response of each correspondent segment to a maximum response and were shown as mean ± S.E.M of 5-8 observations.

Table 4.1 Assessment of the effect of noradrenaline and A61603 on alternate segments of the porcine splenic artery from the same animal. Results were determined as a percentage of a former response to 60mM KCl and were expressed as the mean ± S.E.M of 5-8 observations.

Agonist	Segment Order	n	Maximum Response (% of 60mM KCl)	pD_2
Noradrenaline	1	5	153.2 ± 14.2	6.31 ± 0.04
	3	5	158.4 ± 7.1	6.37 ± 0.05
	6	5	167.9 ± 20.3	6.31 ± 0.04
	8	5	142.4 ± 11	6.39 ± 0.06
A61603	1	6	178.8 ± 14.2	8.08 ± 0.08
	3	8	178.9 ± 22.2	8.02 ± 0.06
	6	8	176.5 ± 4.1	8.10 ± 0.07
	8	8	173.1 ± 10.2	8.08 ± 0.08

4.3.3 The effect of 10μM cocaine on contractions to adrenaline and noradrenaline of the porcine isolated splenic artery

Cocaine inhibits the transport of sympathomimetics across the cell membrane, and thus, increases the interaction of sympathomimetics at the receptors (Chen *et al*., 1998). Therefore, this experiment was performed to determine the effects of cocaine on responses of the splenic artery to adrenaline and noradrenaline. A preparation of the porcine splenic artery was employed and arterial responses were generated and recorded in the presence and absence of a 10μM cocaine.

As shown in figure 4.2, cumulative additions of adrenaline and noradrenaline, produced concentration-dependent contractions in all preparations and the presence of 10μM cocaine shifted the concentration-response curve of catecholamines to the left without causing significant changes to their maximum response. The presence of 10μM cocaine caused a significant 5-fold increase in the sensitivity of the porcine splenic artery to noradrenaline, yet the potency for adrenaline did not change (Table 4.2). Therefore, in our experiments with noradrenaline and even adrenaline, 10μM cocaine was included in the bathing solution to inhibit neuronal uptake$_1$.

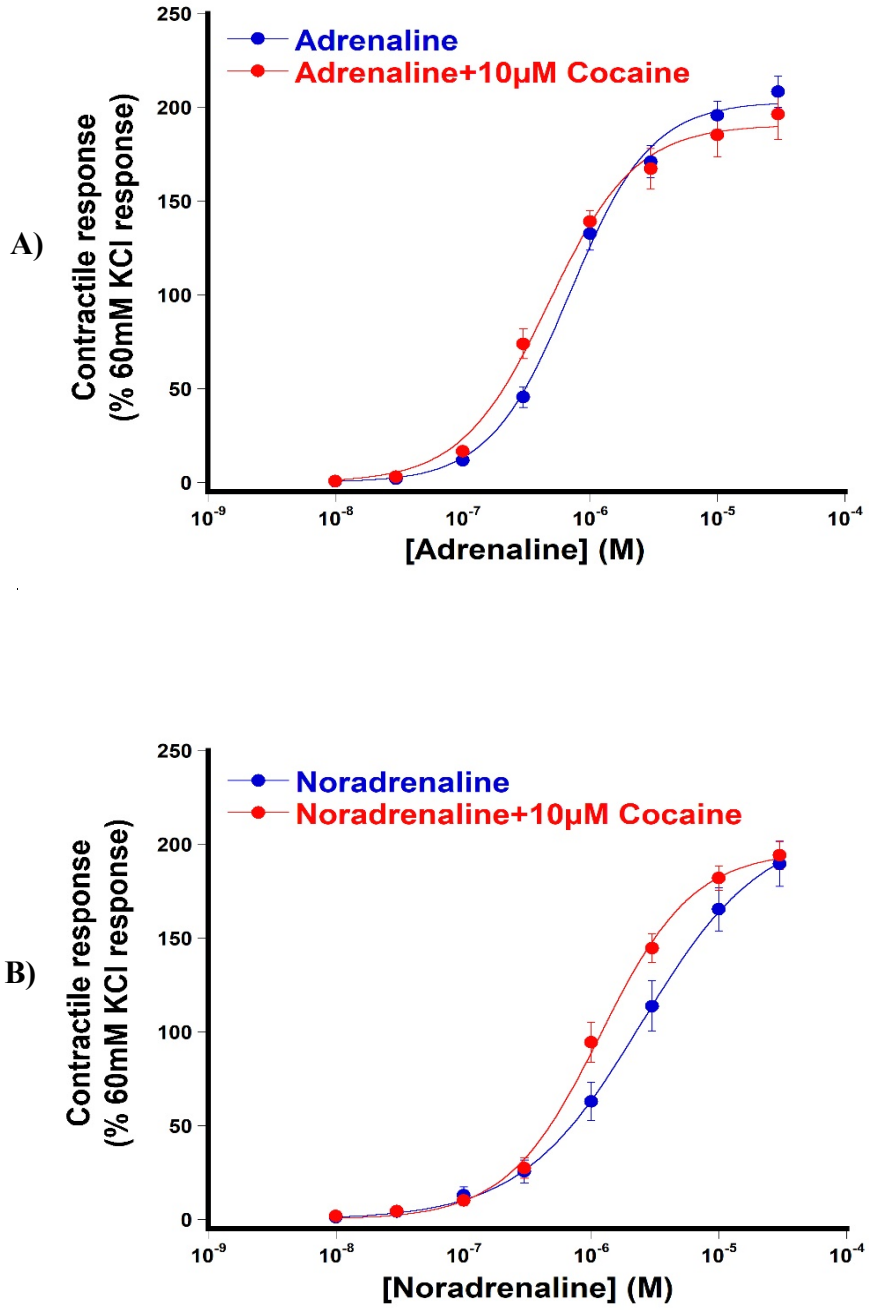


Figure 4.2 The effect of 10μM cocaine to increasing cumulative concentrations of (A) adrenaline and (B) noradrenaline in the induced contractions of the porcine isolated splenic artery. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of 6-13 observations.

Table 4.2 Comparison of the effect of 10μM cocaine to increasing cumulative concentrations of adrenaline and noradrenaline in the induced contractions of the porcine isolated splenic artery. Results were determined as a percentage of a former response to 60mM KCl and were expressed as mean ± S.E.M of 6-13 observations.

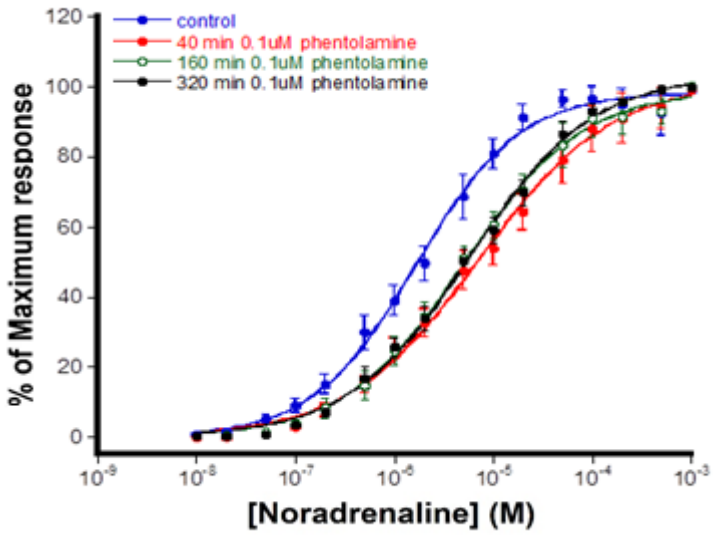
*($P < 0.05$) - denotes a statistically significant difference in potency in the presence and absence of 10μM cocaine, Student paired *t*-test.

	n	Maximum Response (% of 60mM KCl)	pD_2
Adrenaline	6	204 ± 9	6.20 ± 0.08
Adrenaline 10μM cocaine	6	190 ± 13	6.30 ± 0.05
Noradrenaline	13	234 ± 27	5.60 ± 0.09
Noradrenaline 10μM cocaine	13	191 ± 7	6.20 ± 0.1*

4.3.4 The effects of increasing time exposure to antagonists on the potency of noradrenaline-induced contractions of the porcine isolated splenic artery

This set of experiments represented a part of the optimisation of experimental conditions. Classically, I expect a slow upregulation over hours->days and the concentration-response curve for noradrenaline-induced contraction (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) is anticipated to shift towards left as the longer incubation time of antagonists will cause more receptors upregulation and assume to increase the noradrenaline sensitivity due to more available receptors. 0.03μM prazosin and 0.1μM phentolamine were incubated with the porcine splenic artery on increasing duration periods of 40, 160 and 320 minutes. The concentration of the antagonists was selected based on their ability to produce approximately 10-fold rightward shifts. The potency values of the control curve were consistent in both experiments (pD_2 = 5.77 ± 0.06). Figure 4.3A shows that, with increasing incubation times of 0.1μM phentolamine, the pD_2 values obtained were 5.08 ± 0.11, 5.23 ± 0.06 and 5.19 ± 0.12 (n = 5), respectively. The concentration-response curves for noradrenaline in the 3 different incubation time were very close. Figure 4.3B shows that, with increasing incubation times of 0.03μM prazosin, there were no significant differences among the pD_2 values obtained 4.80 ± 0.06, 4.73 ± 0.08 and 4.71 ± 0.22 (n = 6), respectively and no significant differences between the concentration-response curves. Table 4.3 shows the affinity of the prazosin and phentolamine antagonists to the receptors. Based on these results, statistically, there was no effect of the different increasing incubation time of antagonists on noradrenaline induced-contractions on the porcine isolated splenic artery. Furthermore, the increasing incubation time did not influence the apparent potency of noradrenaline and even the antagonist potencies were not variable. Therefore, the following experiments will be carried out using 40 minutes-incubation time for variable antagonists.

A)



B)

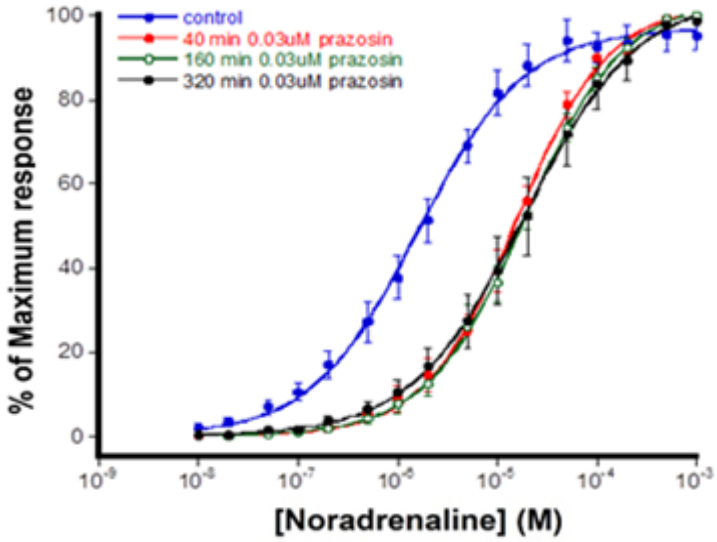


Figure 4.3 Responses of noradrenaline in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol on the porcine splenic artery incubated with (A) 0.01µM phentolamine and (B) 0.03µM prazosin at 40, 160 and 320 minutes. Responses were expressed as a percentage of the response of each correspondent segment to a maximum response and were shown as mean ± S.E.M of 5-6 observations.

Table 4.3 Quantitative parameters of the affinity of the antagonists to its receptors measured in pK_B values using the Gaddum equations. Results were expressed as the mean ± S.E.M of 5-6 observations.

Antagonist	n	pK_B		
		40 minutes	**160 minutes**	**320 minutes**
Phentolamine	6	7.5 ± 0.11	7.4 ± 0.05	7.4 ± 0.24
Prazosin	5	8.4 ± 0.11	8.5 ± 0.14	8.5 ± 0.26

4.3.5 The effects of various α-adrenoceptor agonists on contractions of porcine blood vessels

60mM KCl caused a sustained contraction of the porcine splenic artery and the porcine nasal artery equivalent to 15.2 ± 0.6 g wt. (n=45) and 12.3 ± 0.8 g wt. (n=40), respectively. Four different agonists were used to stimulate α-adrenoceptors in a porcine splenic artery and porcine nasal artery. These agonists included noradrenaline as a non-selective adrenoceptor agonist, phenylephrine and A61603 as selective α_1-adrenoceptor agonists and brimonidine as a selective α_2-adrenoceptor agonist.

Figure 4.4 and 4.5 showed that in both preparations, the imidazoline derivative A61603 produced slowly developing contractile responses at each concentration, often taking greater than 10 minutes to reach equilibrium and it was the most sustained contractions seen compared with other agonists. While, the maximum response to phenylephrine was approximately 80% of that to the other agonists, and at higher concentrations (3-10μM and above), the responses comprised a sharp increase in contraction over 3-4 minutes and then slowly declined at practically all concentrations of the agonist in the porcine splenic artery and porcine nasal artery. For noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol), equilibrium responses were observed at practically all concentrations, followed by a slow decline in contractions.

Table 4.4 showed and compared the effect of the agonists on both preparations, their magnitude of responses, the sensitivity of the splenic artery and the nasal artery to the different agonists and the relative potency-ratios. In this table, noradrenaline, phenylephrine and A61603 caused concentration-dependent contractions of the

isolated splenic artery and the nasal artery, with the latter agent at least (66 to 135)-fold more potent than the phenethylamine derivatives in the porcine isolated splenic artery and (389-933)-fold more potent in the porcine isolated nasal artery.

In contrast, the imidazoline derivative brimonidine produced contractions less than 10% of this elicited by noradrenaline in the porcine isolated splenic artery. However, noradrenaline was the most effective agonist in both preparations showing the highest maximum response. The most obvious finding from this table is that the porcine splenic artery was more sensitive to the actions of noradrenaline and phenylephrine, while the porcine nasal artery was more sensitive to the action of the most potent agonist (A61603). In the porcine isolated splenic artery, the magnitudes of contractions of noradrenaline, phenylephrine and A61603 were higher than that detected in the porcine isolated nasal artery.

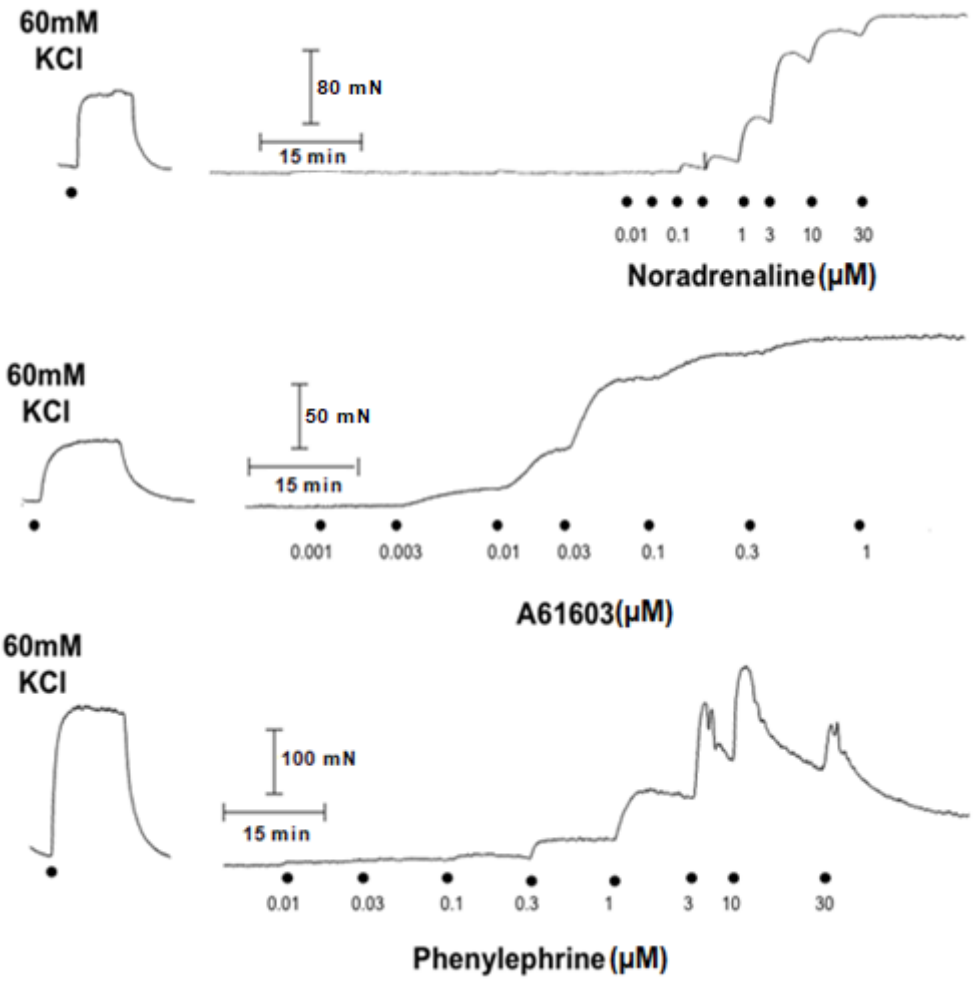


Figure 4.4 Representative trace recordings of contractile responses of the porcine isolated splenic artery to cumulatively increasing concentrations to noradrenaline, A61603 and phenylephrine.

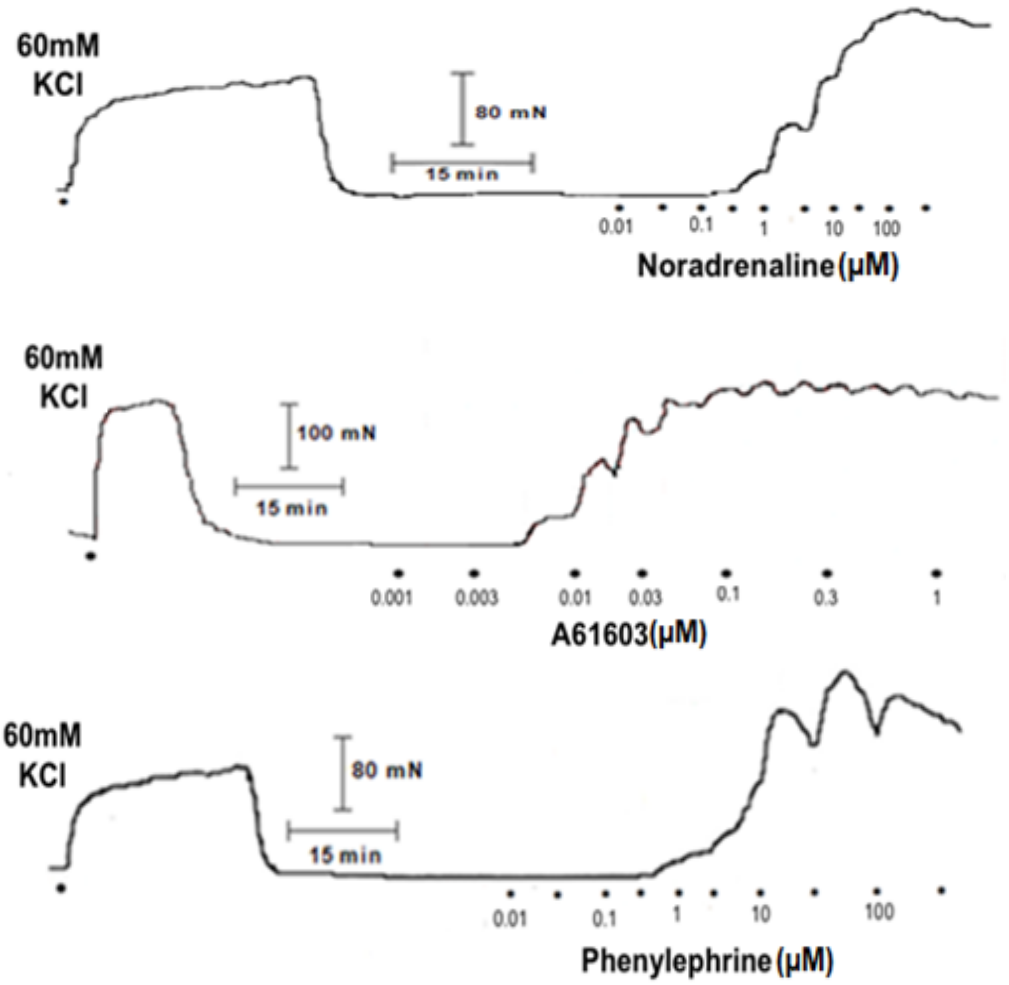


Figure 4.5 Representative trace recordings of contractile responses of the porcine isolated nasal artery to cumulatively increasing concentrations to noradrenaline, A61603 and phenylephrine.

Table 4.4 Comparison of the effects of various α-adrenoceptor agonists in the porcine isolated splenic artery and porcine isolated nasal artery. Effects of noradrenaline in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol. Results were determined as a percentage of a former response to 60mM KCl and were expressed as the mean ± S.E.M of 6-32 observations.

[a]- represents the potency-ratio of agonists relative to A61603 (1).

	Porcine splenic artery		Porcine nasal artery	
	Maximum Response (% of 60mM KCl)	**pD_2**	**Maximum Response (% of 60mM KCl)**	**pD_2**
Noradrenaline	234 ± 27 (n=32)	6.18 ± 0.09 (n=32) (66)[a]	126 ± 0.3 (n=6)	5.71 ± 0.15 (n=6) (389)[a]
Phenylephrine	141 ± 12 (n=20)	5.87 ± 0.07 (n=20) (135)[a]	60 ± 8 (n=7)	5.33 ± 0.13 (n=7) (933)[a]
A61603	191 ± 11 (n=13)	8.00 ± 0.04 (n=13) (1)	96 ± 7 (n=6)	8.30 ± 0.09 (n=6) (1)
Brimonidine	8 ± 3 (n=8)	6.30 ± 0.36 (n=8)	–	–

4.3.6 The effects of phentolamine against contractions to noradrenaline and A61603 of porcine blood vessels

Phentolamine (10nM-10μM) a non-selective α-adrenoceptor antagonist, caused concentration-dependent, parallel rightward shift of the responses to noradrenaline in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol, without changing the maximum response (Figure 4.6) of the porcine splenic artery and the same effect seen by the antagonist phentolamine to a selective α_1-adrenoceptor agonist A61603 (Figure 4.7).

For the endogenous ligand noradrenaline in the porcine splenic artery, Schild analysis of 10 separate observations based on the effect of seven different concentrations of phentolamine revealed that for 9 preparations the slope was less than 0.85 and in one preparation the slope was greater than 1. The mean slope of the Schild plot was significantly different ($P < 0.01$) from unity (Figure 4.6 and table 4.5). Moreover, in a separate experiment performed as the previous one but with the addition of a selective α_2-adrenoceptor antagonist (RX-811059 (0.1μM)) to phentolamine (10nM-10μM) and the present study found that RX-811059 did not affect the shifts of the response to noradrenaline and the slope remained as 0.67 ± 0.05 (n=6).

Schild analysis of six separate observations against A61603 in the porcine splenic artery revealed that for one preparation, the slope was less than 0.85 and two were greater than one. Against A61603 agonist, the pA_2 value for phentolamine was 8.51 and the slope of the Schild plot was not significantly different from unity (Figure 4.7 and table 4.5).

In order to examine wether the contractions for noradrenaline in the porcine nasal artery were affected by phentolamine in a similar manner to that previously observed in the splenic artery (slopes of the Schild plot less than unity) or not, a detailed re-examination of the effect of phentolamine (10nM-10μM) against noradrenaline-induced contractions in the porcine nasal artery was performed. As noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) caused concentration dependent contractions in the porcine nasal artery, the concentration-response curves to noradrenaline were displaced to the right with every increment in the concentration of phentolamine. The pA_2 value for phentolamine was less than that observed in the porcine splenic artery but the slope of the Schild plot was still significantly less than unity (Figure 4.8 and table 4.5). This observation showed that this unheralded phenomenon can be generalised to the rest of the circulation and it did not depend on the type of blood vessels being affected.

To determine the action of phentolamine (10nM-10μM) against A61603-induced contractions in a blood vessel of the nasal cavity, the porcine nasal artery segments were used. Like the isolated splenic artery, phentolamine (10nM-10μM) elicited a concentration-dependent rightward displacement of contractions to A61603 without changing the maximum response in the nasal artery segments. The slope of the Schild plot and the estimated pA_2 value were similar to that obtained with A61603 agonist against phentolamine in the porcine splenic artery (Figure 4.9 and table 4.5)

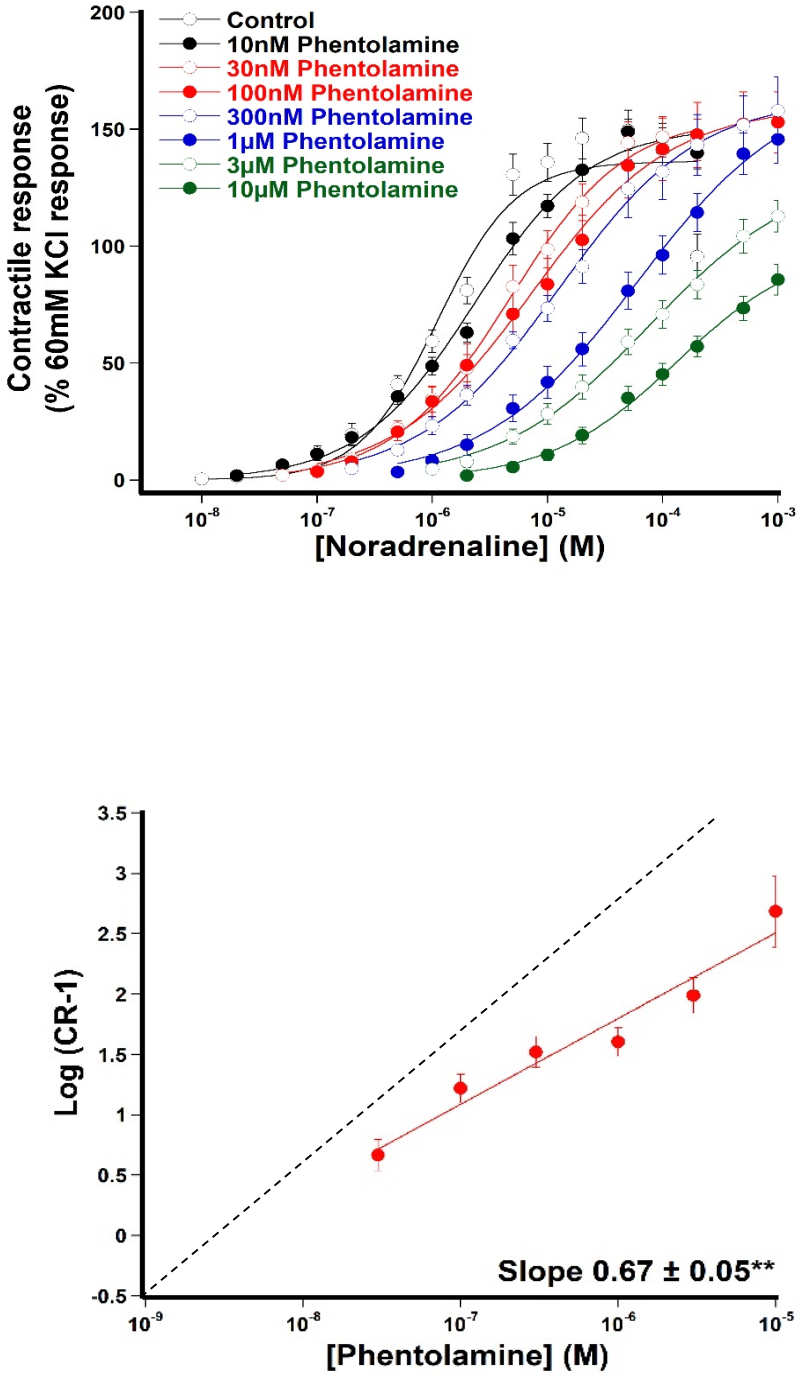


Figure 4.6 The effect of phentolamine against noradrenaline-induced contractions of the porcine isolated splenic artery in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol (n=10). Responses to noradrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

** ($P < 0.01$) - denotes that the slope of the Schild plot for each antagonist was significantly different from unity (the dashed line) based on single sample *t*-test.

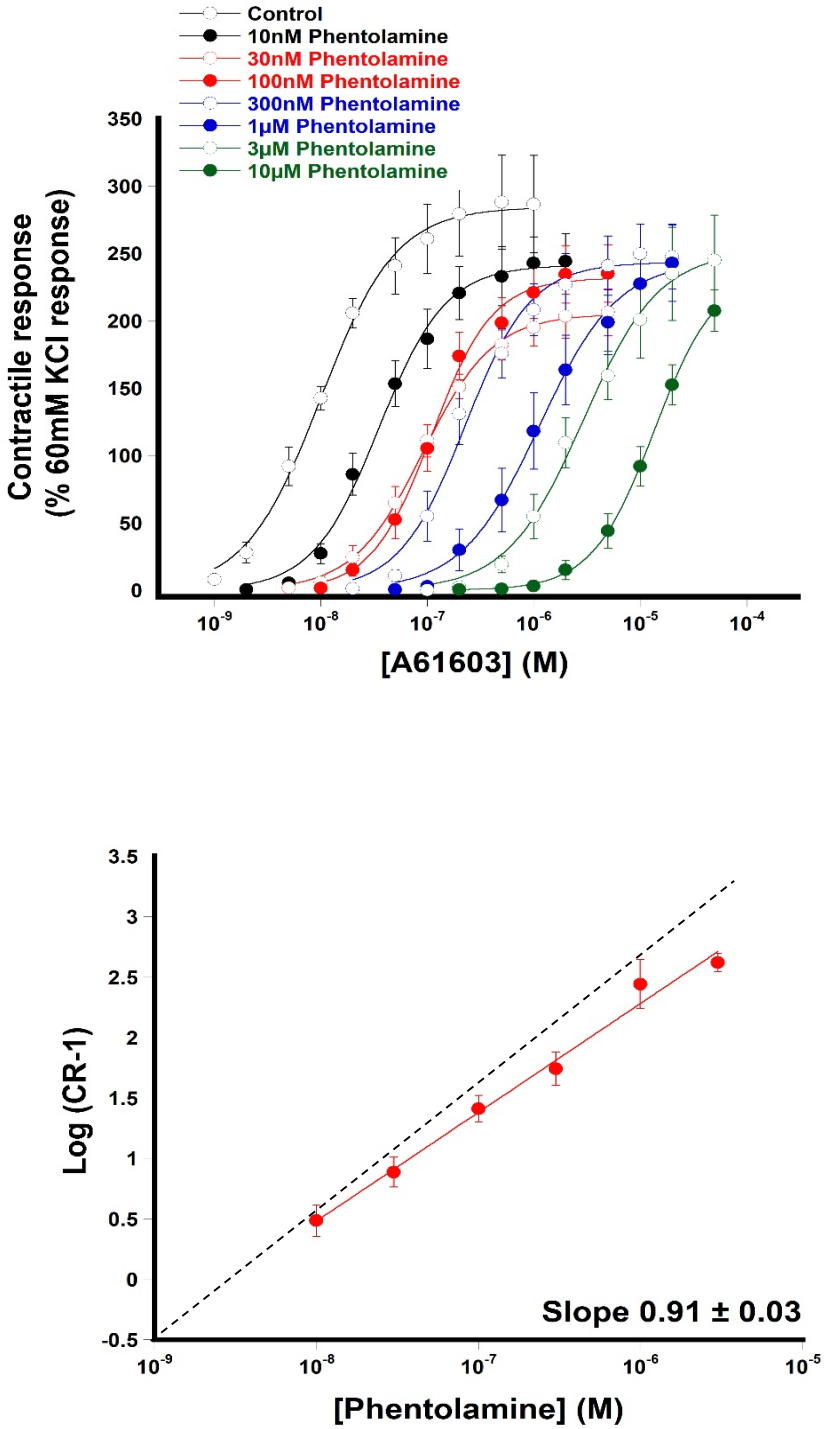


Figure 4.7 The effect of various concentrations of phentolamine against A61603-induced contractions of the porcine isolated splenic artery and the corresponding Schild plots related to it (n=6). Responses to the A61603 were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

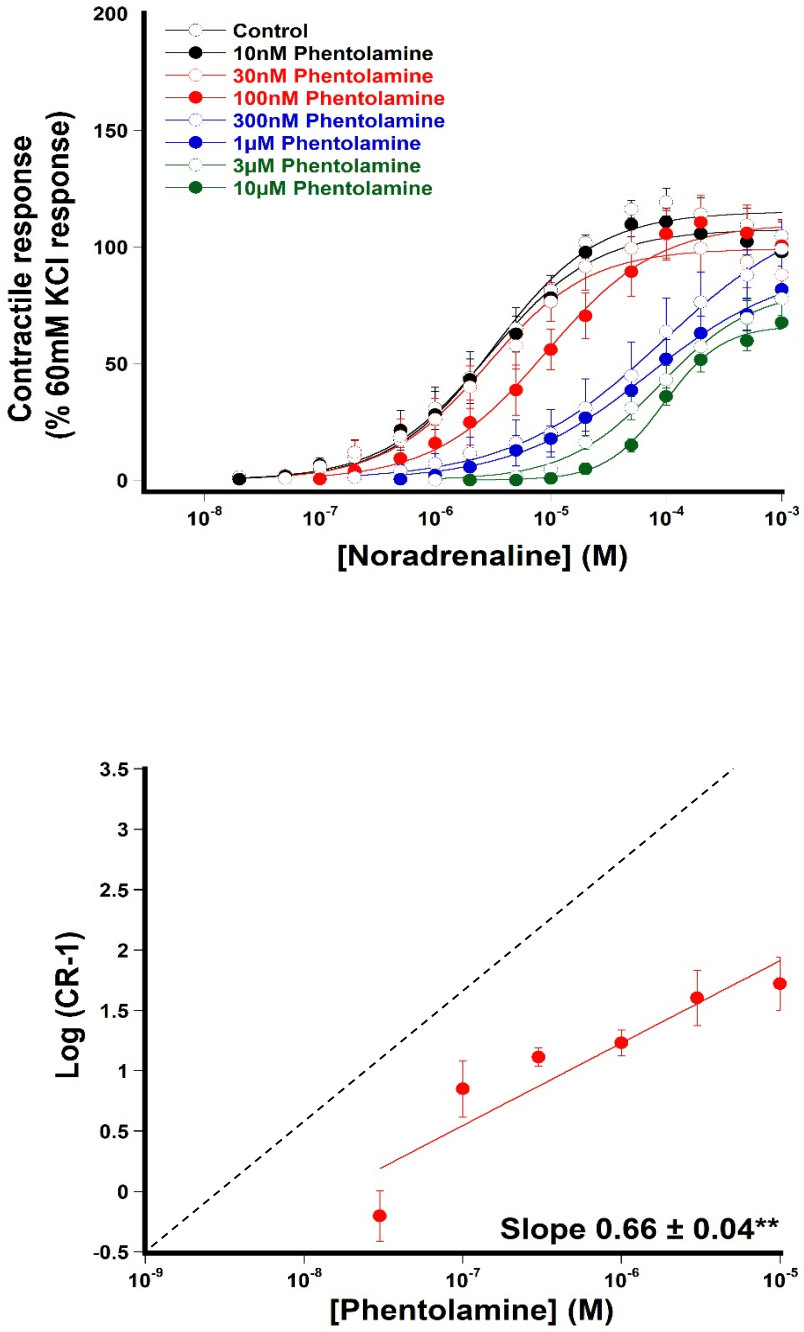


Figure 4.8 The effect of phentolamine against noradrenaline-induced contractions of the porcine isolated nasal artery in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol (n=6). Responses to noradrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.
** ($P < 0.01$) - denotes that the slope of the Schild plot for each antagonist was significantly different from unity (the dashed line) based on single sample *t*-test.

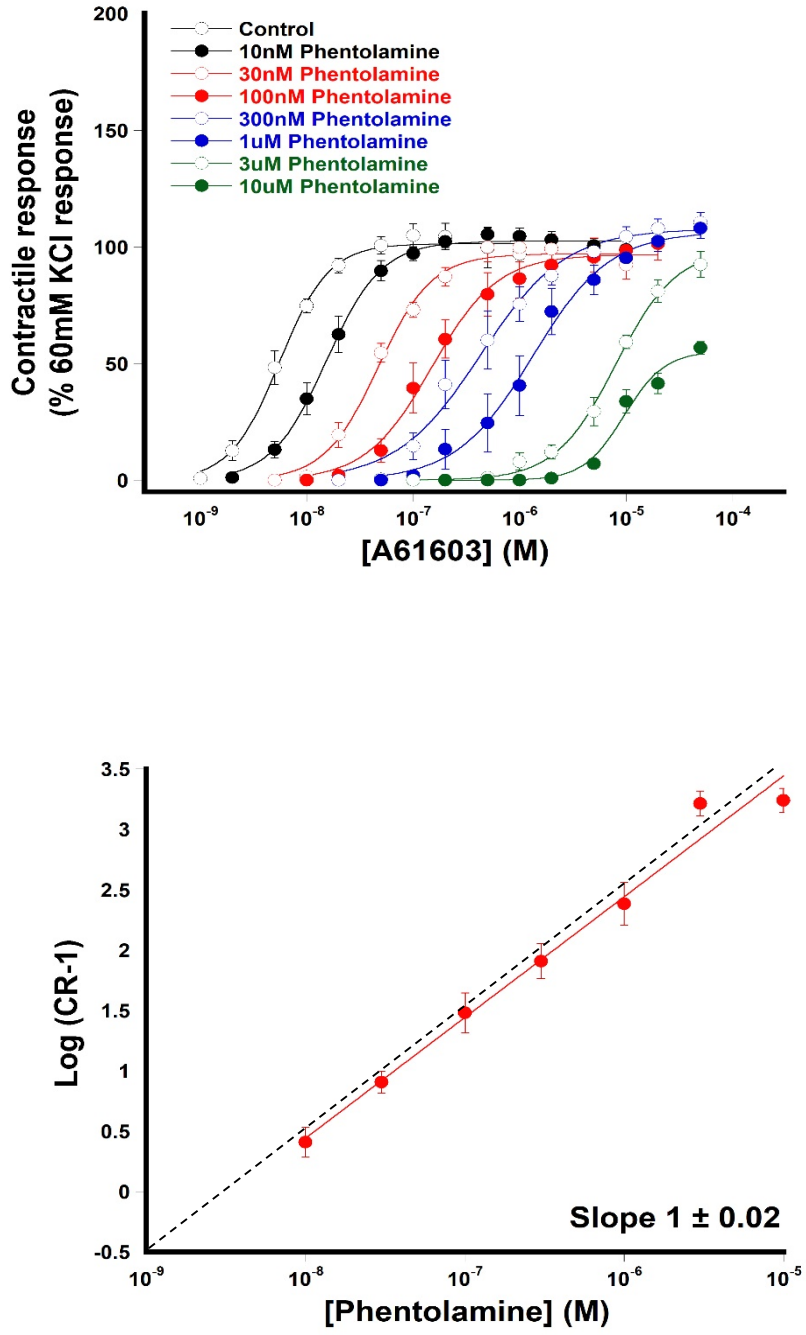


Figure 4.9 The effect of various concentrations of phentolamine against A61603-induced contractions of the porcine isolated nasal artery and the corresponding Schild plots related to it (n=6). Responses to the A61603 were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

Table 4.5 Schild analysis of the interaction between contractions to noradrenaline and A61603 and phentolamine antagonist in the porcine isolated splenic and nasal artery. Results were expressed as the mean ± S.E.M of 6-10 observations.

** ($P < 0.01$) - denotes a statistically significant difference from unity based on single sample *t*-test analysis.

	Noradrenaline		**A61603**	
	pA_2	Slope	pA_2	Slope
Splenic Artery	8.10 ± 0.13 (n=10)	0.67 ± 0.05** (n=10)	8.51 ± 0.10 (n=6)	0.91 ± 0.03 (n=6)
Nasal Artery	7.90 ± 0.18 (n=6)	0.66 ± 0.04** (n=6)	8.42 ± 0.13 (n=6)	1.00 ± 0.02 (n=6)

4.3.7 The effects of α_1-adrenoceptor antagonists against contractions to noradrenaline and A61603 of the porcine isolated splenic artery

In these experiments, different α_1-adrenoceptor antagonists were used against noradrenaline and A61603-induced contractions in the porcine isolated splenic artery. Prazosin (1nM-1μM) antagonist caused a concentration-dependent, parallel rightward shift of the responses to noradrenaline in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol, without changing the maximum response (Figure 4.10) and a similar behavior was observed with prazosin against contractions induced by A61603 (Figure 4.11). Analysis of the effect of prazosin, based on seven antagonist concentrations, showed that the apparent pA_2 value for prazosin against noradrenaline was 9.5 and the slope of the Schild plot was significantly different from unity (Figure 4.10 and table 4.6). However, the pA_2 value for prazosin against A61603 was 9.0 and the slope of the Schild plot was not significantly different from unity (Figure 4.11 and table 4.6).

Tamsulosin (0.1-100nM) produced non-parallel displacements of the concentration-response curves against noradrenaline-induced contractions (Figure 4.12). In contrast, tamsulosin (0.1-100nM) produced parallel concentration-dependent rightward shifts of the responses to A61603 (Figure 4.13). A marked decrease in the slope of the concentration-response curves with concentrations higher than 0.1nM tamsulosin against noradrenaline-induced contractions was observed, while the slope with A61603-induced responses fluctuated around one with increasing concentrations of tamsulosin. Schild regression analysis with tamsulosin against noradrenaline yielded a slope that differs significantly from unity and an apparent pA_2 value of 9.9, whereas Schild slope of tamsulosin with A61603 did not differ significantly from unity and yielded an apparent pA_2 value of 10.6 (Figure 4.12 and 4.13 and table 4.6).

Similar trends were observed with silodosin. The parallel shift of silodosin (1nM-1μM) was not observed with noradrenaline and the lower part (20%-30%) of the noradrenaline concentration-response curves appeared to exhibit resistance to the effects of silodosin (Figure 4.14) whereas silodosin (0.1-100nM) shifted the A61603 concentration-response curves in a parallel concentration-dependent dextral manner (Figure 4.15). As with tamsulosin, the noradrenaline concentration-response curves slope were reduced markedly in the presence of silodosin, in contrast to A61603 concentration-response curves slope were similar and varied slightly around one. Schild regression analysis of silodosin against A61603 yielded an apparent pA_2 value of 9.97 and a slope that did not differ significantly from unity. Parameters for 100nM silodosin against A61603 were excluded from the Schild regression analysis, as responses did not reach a true maximum. Since the Schild slope of silodosin against noradrenaline was substantially lower than one, a pK_B estimate of the lowest concentration of silodosin (1nM) was calculated to yield a value of 9.65 (Table 4.6).

Higher concentrations of tamsulosin and silodosin appeared to reduce the maximum contractile response to A61603 and noradrenaline. These observations were more apparent with responses to noradrenaline at concentrations higher than 10 and 30nM for tamsulosin (Figure 4.12) and silodosin (Figure 4.14), respectively.

Individual observation of the noradrenaline concentration-response curves in the presence of silodosin can be divided into two portions, where the lower half exhibited a less steep slope, especially seen at the higher concentrations of silodosin, while the upper portion displayed a steeper slope. Analysis of the time taken to reach 5-90% of maximum response showed that there were differences in the time taken to achieve steady-state between agonists with different selectivity's, in the presence of the lowest

concentration of silodosin (1nM). Noradrenaline displayed a slower, more sustained response in the presence of silodosin, hence increasing the time course by almost 2-fold, with respect to control. In contrast, there was no significant difference in time course for A61603-induced response in the presence and absence of silodosin.

In the same way, corynanthine (30nM-30μM), a selective α_1-adrenoceptor antagonist caused a concentration-dependent, parallel rightward shift of the responses to noradrenaline in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol, without changing the maximum response of the porcine splenic artery. Analysis of the effect of corynanthine, based on 7 antagonist concentrations, showed that the slope of the Schild plot for corynanthine was significantly different from unity (Table 4.6).

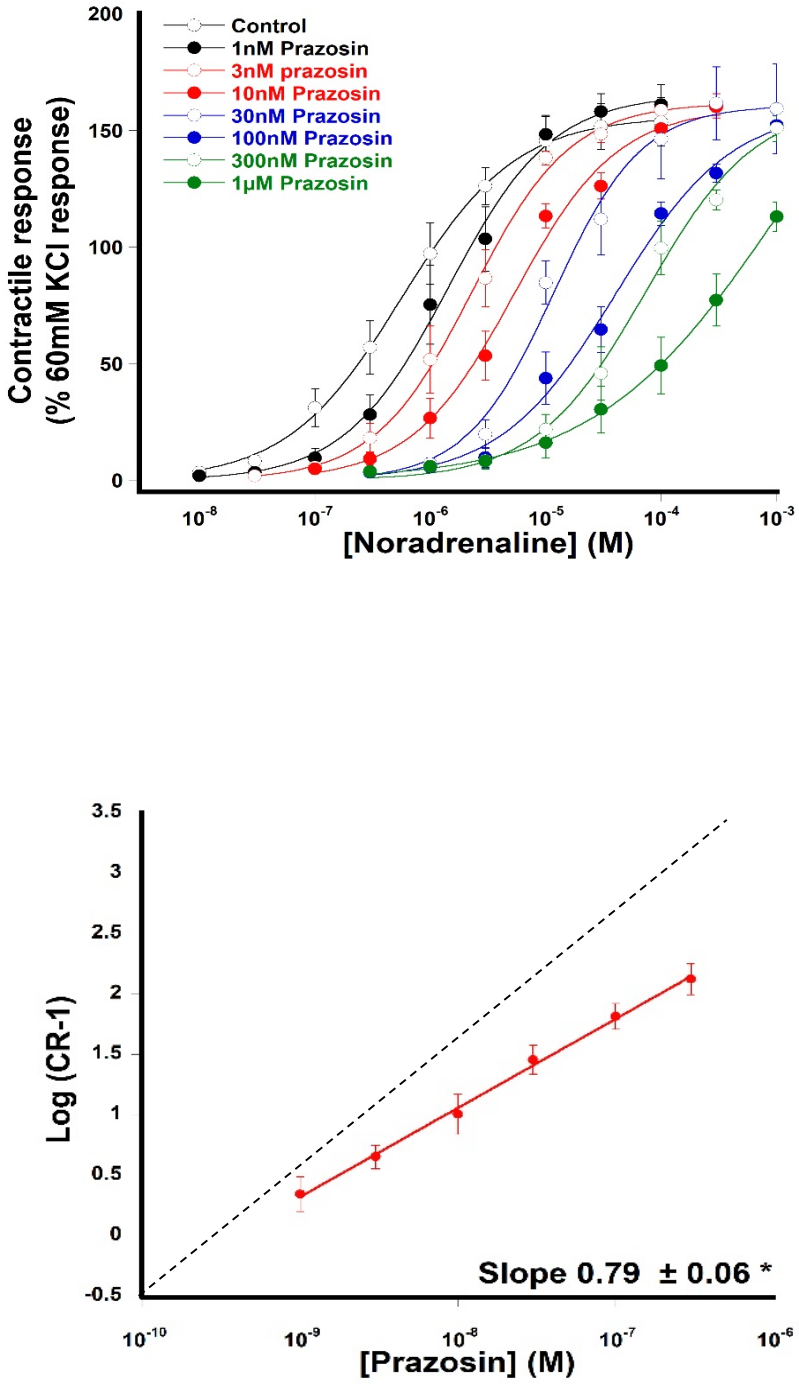


Figure 4.10 The effect of prazosin against noradrenaline-induced contractions of the porcine isolated splenic artery in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol (n=8). Responses to noradrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

* ($P < 0.05$) - denotes that the slope of the Schild plot for each antagonist was significantly different from unity (the dashed line) based on single sample *t*-test.

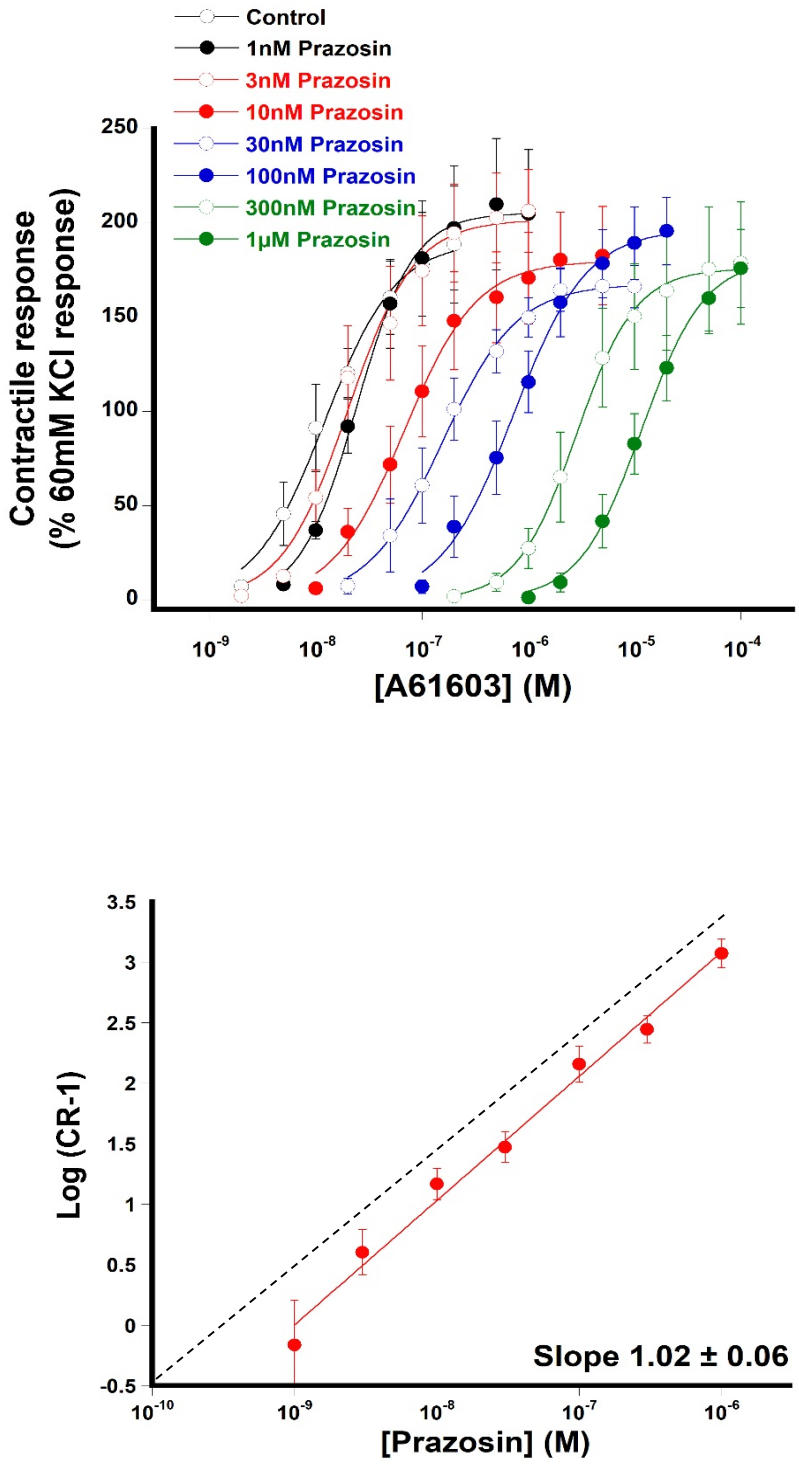


Figure 4.11 The effect of various concentrations of prazosin against A61603-induced contractions of the porcine isolated splenic artery and the corresponding Schild plots related to it (n=5). Responses to the A61603 were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

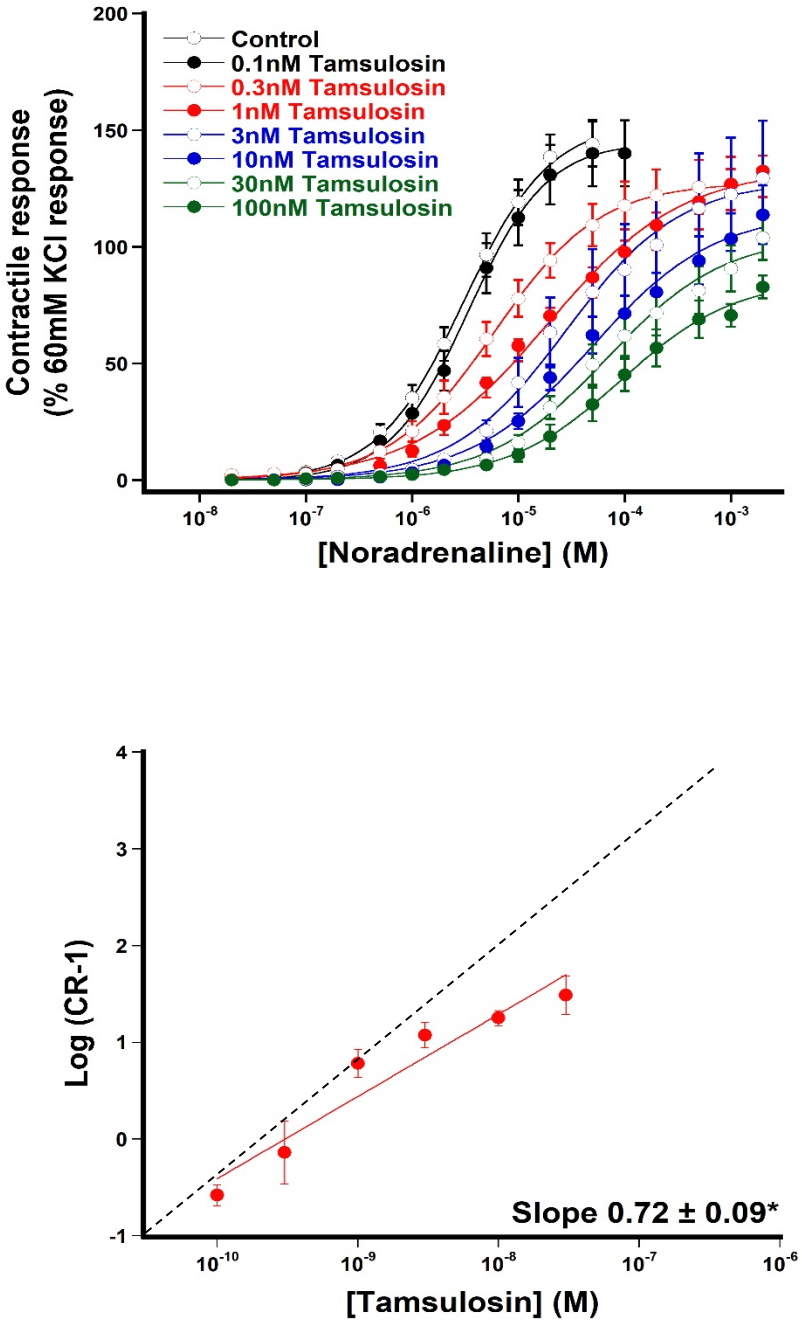


Figure 4.12 The effect of tamsulosin against noradrenaline-induced contractions of the porcine isolated splenic artery in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol (n=5). Responses to noradrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

* ($P < 0.05$) - denotes that the slope of the Schild plot for each antagonist was significantly different from unity (the dashed line) based on single sample *t*-test.

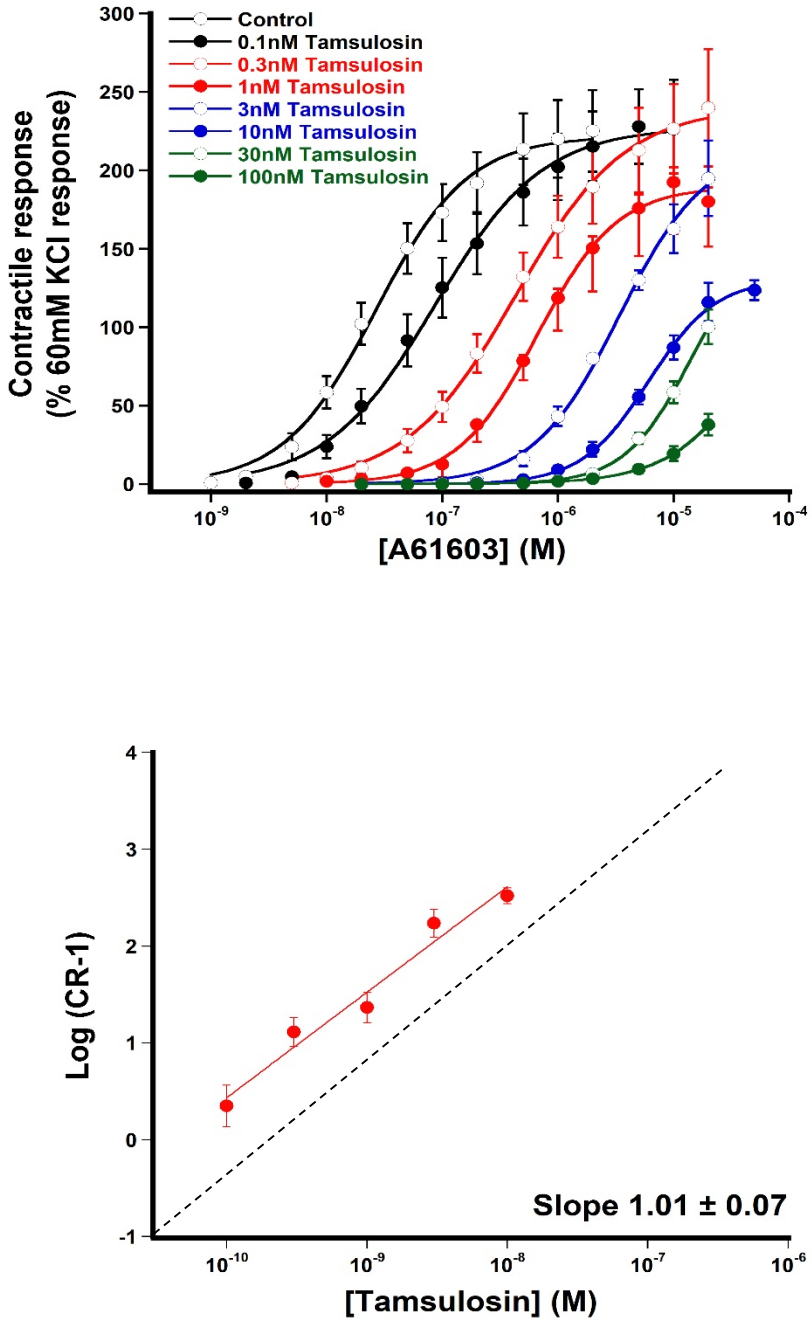


Figure 4.13 The effect of various concentrations of tamsulosin against A61603-induced contractions of the porcine isolated splenic artery and the corresponding Schild plots related to it (n=6). Responses to the A61603 were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

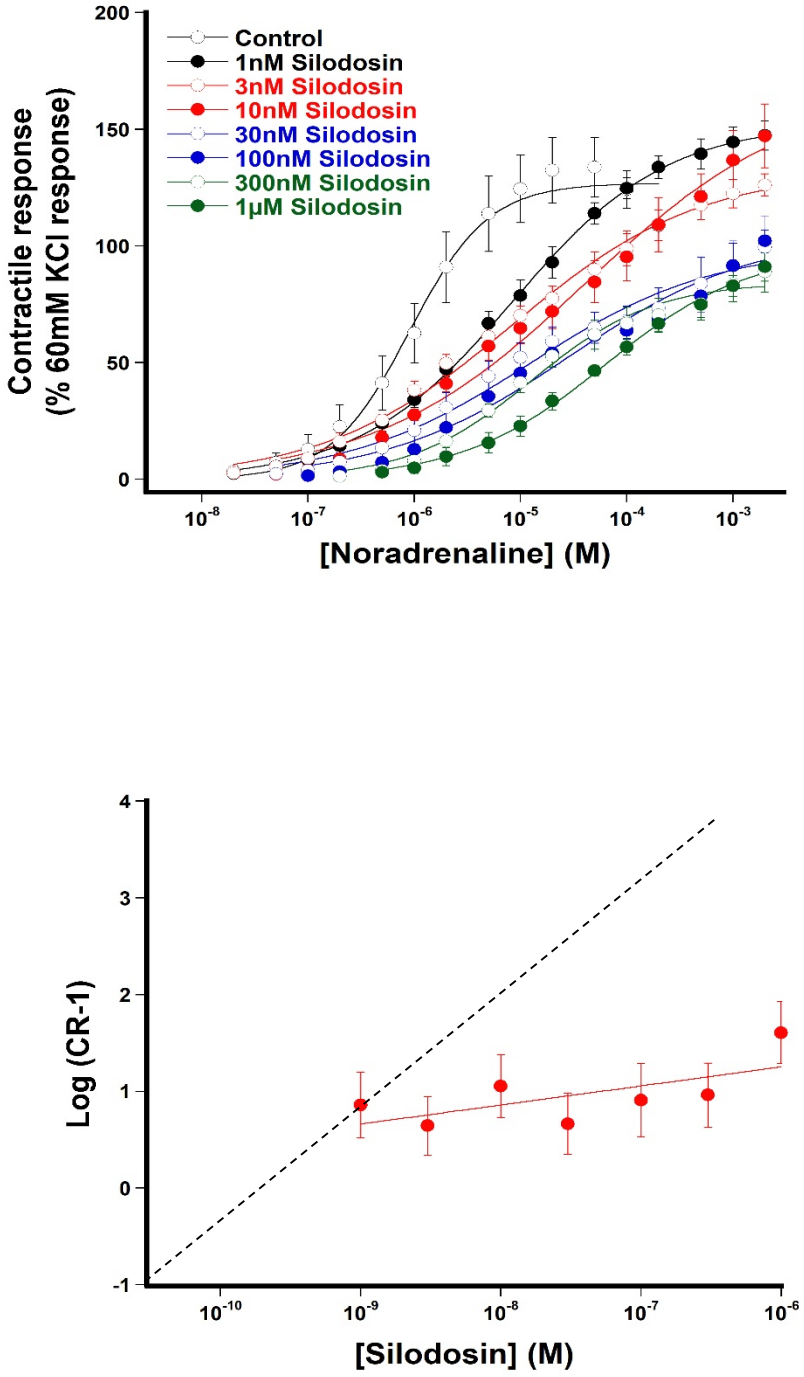


Figure 4.14 The effect of silodosin against noradrenaline-induced contractions of the porcine isolated splenic artery in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol (n=5). Responses to noradrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

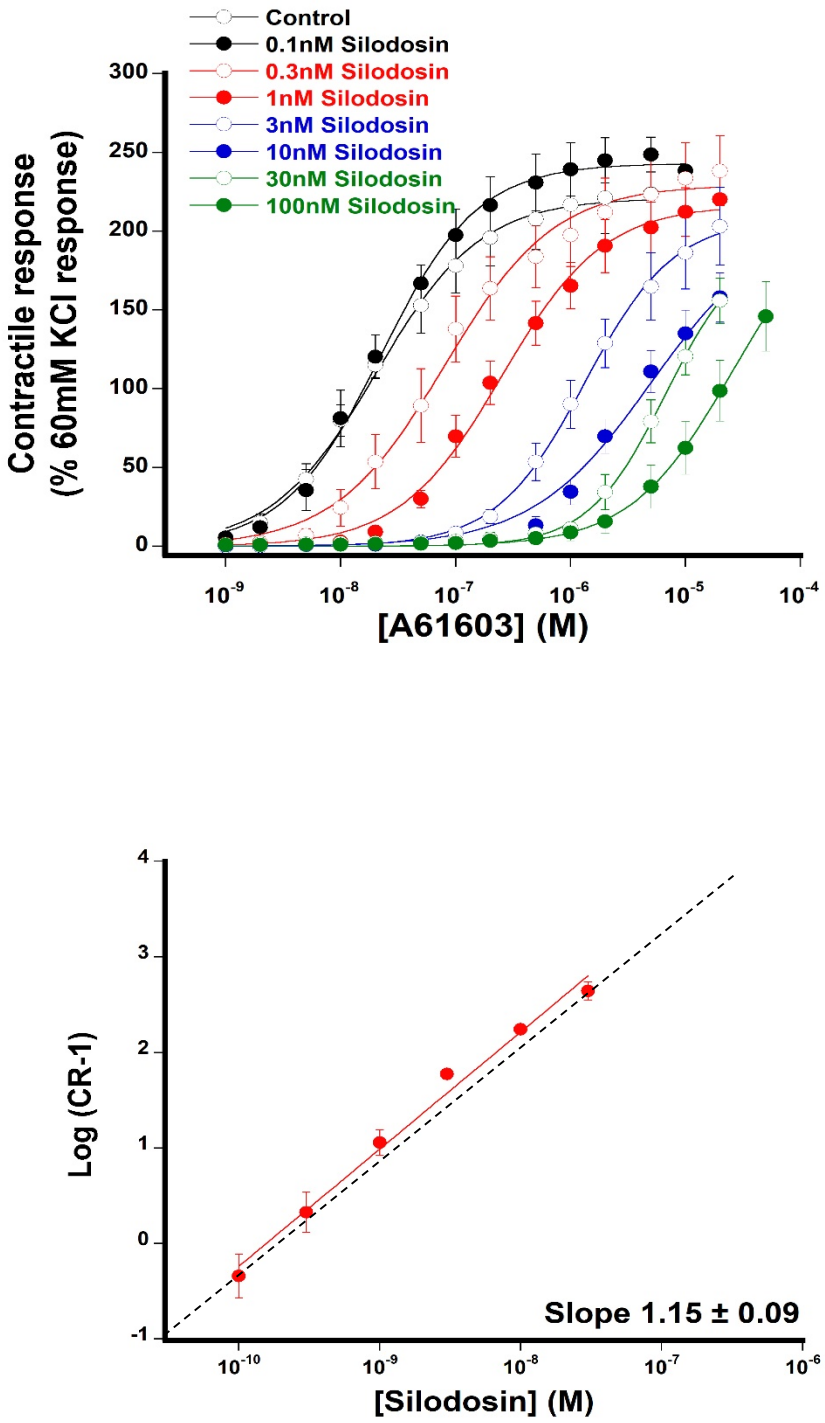


Figure 4.15 The effect of various concentrations of silodosin against A61603-induced contractions of the porcine isolated splenic artery and the corresponding Schild plots related to it (n=5). Responses to the A61603 were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

Table 4.6 Schild analysis of the interaction between contractions to various α-adrenoceptor agonists and different antagonists in the porcine isolated splenic artery. Results were expressed as the mean ± S.E.M of 5-8 observations.

* ($P < 0.05$) and ** ($P < 0.01$) - denote a statistically significant difference from unity based on single sample *t*-test analysis.

	Noradrenaline		**A61603**	
	pA_2/pK_B	Slope	pA_2	Slope
Prazosin	9.51 ± 0.25 (n=8) (1nM-1μM)	0.79 ± 0.06*	9.09 ± 0.2 (n=5) (1nM-1μM)	1.02 ± 0.06
Tamsulosin	9.97 ± 0.16 (n=5) (0.3-30nM)	0.72 ± 0.09*	10.62 ± 0.21(n=6) (0.1-10nM)	1.01 ± 0.07
Silodosin	9.65 ± 0.21 (n=5) (1nM)		9.97 ± 0.11 (n=5) (0.1-30nM)	1.15 ± 0.09
Corynanthine	8.04 ± 0.25 (n=6) (30nM-30μM)	0.63 ± 0.05**		

4.3.8 The effects of α-adrenoceptor antagonists against contractions to noradrenaline of the porcine isolated splenic vein

In a separate series of an experiment performed in another type of vascular bed (veins), noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) caused concentration-dependent contractions of the porcine isolated splenic vein (pD_2 6.07 ± 0.14, n=6). Phentolamine (10nM-10μM), a non-selective α-adrenoceptor antagonist, caused a concentration-dependent, parallel rightward shift of the responses to noradrenaline similar to that seen in the porcine isolated splenic artery and the porcine isolated nasal artery without changing the maximum response of the porcine splenic vein. The pA_2 value for phentolamine was similar to that observed in the porcine isolated splenic artery and the porcine isolated nasal artery (8.41 ± 0.32, n=6), furthermore, the slope of the Schild plot was significantly less than unity (0.77 ± 0.07, $P < 0.05$) (Figure 4.16).

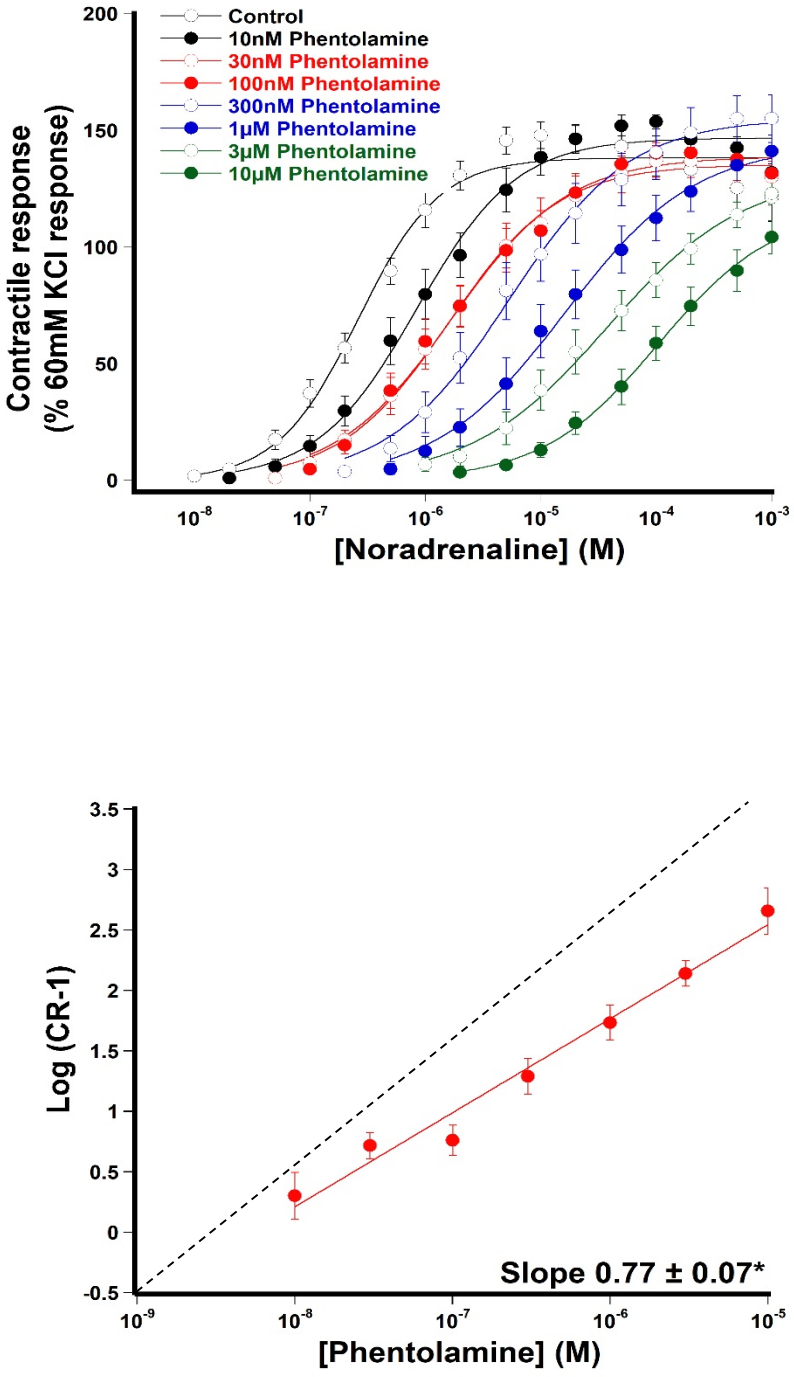


Figure 4.16 The effect of phentolamine against noradrenaline-induced contractions of the porcine isolated splenic vein in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol (n=6). Responses to noradrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist-concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

* ($P < 0.05$) - denotes that the slope of the Schild plot for each antagonist was significantly different from unity (the dashed line) based on single sample *t*-test.

The second experiment involved a comparison of the effect of a single concentration of 0.1μM prazosin, 30nM RX-811059 or a combination of 0.1μM prazosin and 30nM RX-811059 on the concentration-response curve of the porcine splenic vein to noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol). Antagonism with 0.1μM prazosin, an α_1-adrenoceptor antagonist, caused approximately 5-fold shifting of the concentration-response curve, reducing significantly the slope of the concentration-response curve compared to the control ($P < 0.05$, n=6) and decreased significantly the potency of the concentration-response curve to noradrenaline ($P < 0.01$), yet antagonist with prazosin did not affect the maximum response (Table 4.7). Conversely, blocking α_2-adrenoceptors with 30nM RX-811059 alone did not cause any alteration. Combination of 0.1μM prazosin and 30nM RX-811059 significantly shifted the concentration-response curve 27-fold to the right and reduced significantly the potency of the concentration-response curve to noradrenaline ($P < 0.001$, n=6). However, the maximum response and the slope of the concentration-response curve to noradrenaline did not show any variation (Figure 4.17).

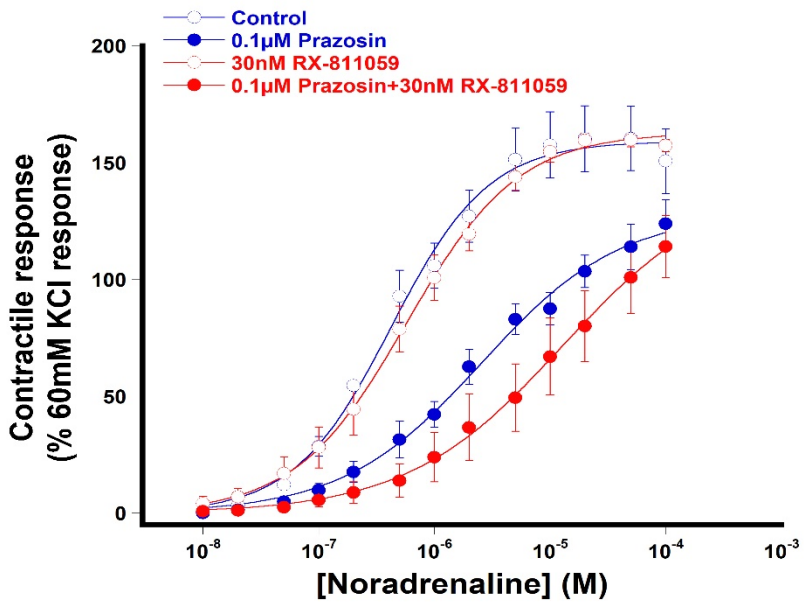


Figure 4.17 The effect of 0.1μM prazosin, 30nM RX-811059 and a combination of 0.1μM prazosin and 30nM RX-811059 antagonists against the noradrenaline-induced contraction of the porcine splenic vein in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of six observations**.**

Table 4.7 Quantitative parameters of the cumulative concentration-response of the porcine splenic vein to noradrenaline in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol in the absence and presence of 0.1μM prazosin, 30nM RX-811059 or a combination of 0.1μM prazosin and 30nM RX-811059. The table shows the maximum response, the negative logarithm of EC_{50} (pD_2) and the slope of the concentration-response curves. Results were determined as a percentage of a former response to 60mM KCl and were expressed as the mean ± S.E.M of 6 observations.
* ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) - denote a statistically significant difference from response control segments in the same vein, one-way ANOVA followed by Dunnett's test.

Porcine Splenic Vein				
	Maximum Response (% of 60mM KCl)	**pD_2**	**Slope**	**n**
Control	159 ± 9	6.35 ± 0.07	1.10 ± 0.06	6
0.1μM Prazosin	125 ± 7	5.68 ± 0.1**	0.78 ± 0.05*	6
30nM RX-811059	162 ± 3	6.21 ± 0.12	1.0 ± 0.06	6
0.1μM Prazosin + 30nM RX-811059	138 ± 9	4.92 ± 2.19***	0.96 ± 0.11	6

4.4 Discussion

Data of the definitive receptor properties and modulation of contraction of blood vessels on the nasal and extra-nasal circulation is critical for the understanding and development of decongestant medications for nasal congestion treatment. Therefore, a further study of the receptor properties was performed by histological examination of the porcine splenic artery and porcine nasal artery. This study examined the artery size, wall thickness, vascular smooth muscle and endothelium. The appendix 1 and 2 summarises the results of the histological studies of the porcine splenic artery and the porcine nasal artery. The figures show that there were no significant differences between the two arteries for example, both have a considerable and visible amount of smooth muscle cells in their tunica media and endothelial cells precipitate on the tunica intima and tunica adventia.

Because one of the starting points for this study was to provide a model to conduct research about the functional effects of α-adrenoceptor agonists and antagonists. Therefore, *in vitro*, comparative examinations of the effects of different antagonists have been assessed using blood vessels from the pig. A detailed pharmacological analysis of noradrenaline-induced contractions of the porcine isolated splenic artery established that the slope of the Schild plot for both selective and non-selective α-adrenoceptor antagonists was significantly less than unity, which was the same results obtained in the porcine isolated nasal artery with the non-selective α-adrenoceptor antagonist and absent with A61603-induced responses. Also, the presence of a component of the response resistant to the tamsulosin and silodosin antagonists and fluctuation in the concentration-response curves slope.

In addition to the fact of high pA_2 values for tamsulosin and silodosin antagonists as based on the estimated pA_2/pK_B values, which showed the rank order of potency for the antagonists was tamsulosin > silodosin > prazosin > corynanthine. The most intriguing findings were the higher sensitivity to A61603 in both preparations and differences in the relative potency-ratio of A61603 to noradrenaline and phenylephrine in the porcine splenic artery and nasal artery, which showed more evidence for a role of α_1-adrenoceptors in the porcine nasal artery compared to the splenic artery. All these facts provided strong evidence of the co-existence of α_1-adrenoceptor subtypes in both preparations and at least two receptors mediate contraction in response to exogenous noradrenaline in the porcine splenic artery and nasal artery, contrary to previous reports. The first type of receptor present was the main contractile mediating α_{1A}-adrenoceptor. The second minor population of receptors present which was resistant to the effects of tamsulosin and silodosin was likely to be the α_{1B}-subtype. One of the limitations of this explanation is that it does not explain why corynanthine yield a slope < 1 against noradrenaline-induced contractions in the porcine blood vessels. The existing account of no implication of α_2-adrenoceptors in *in vitro* contractions fails to resolve this contradiction.

Optimisation of experimental conditions:

In the beginning and in the first few experiments optimisation of the experimental approaches were carried out. First, vasoconstriction was induced by two pathways. It is obvious that vascular tone was modulated by a change in membrane potential. For example, changes in potassium channel properties (Hirst and Edwards, 1989, Nelson and Quayle, 1995, Nelson *et al.*, 1997), following nerve stimulation (Hirst and Edwards, 1989, Hill-Eubanks *et al.*, 2011) or alterations in chloride conductance (Nelson *et al.*, 1997; Jackson, 2000).

The membrane potential was regulated by ion channels in the plasma membrane of vascular smooth muscle causing subsequent opening or closing of L-type voltage-gated calcium channels to cause a contraction or relaxation, respectively (Yuan, 1995; Ghatta *et al*., 2006). In the present study, changes in membrane potential were induced by a depolarising agent, KCl, which produced sustained reproducible vasoconstriction without acting on the G protein-coupled receptor site (Ratz *et al.,* 2005). While noradrenaline contraction dropped gradually until reached the baseline after 180 minutes and this contraction was produced by directly activating a G protein-coupled receptor that causes stimulation of phospholipase C to yield IP_3 and DAG and finally this activation causes elevation of intracellular calcium and contraction (McKenzie *et al*., 2009). As the contraction to 60mM KCl was stable, therefore, it was used as a reference to standardise the contraction induced by all vasoconstrictors used in this thesis.

Mean responses induced by A61603 and noradrenaline in 1-8 various segments demonstrated that A61603 (pD_2 = 8.07 ± 0.07, mean ± S.E.M) was more potent than noradrenaline (pD_2 = 6.34 ± 0.04, mean ± S.E.M) in the porcine splenic artery by approximately 54-fold. Higher A61603 potency relative to a non-selective agonist was also observed in rabbit cutaneous arteries and human erectile tissue with predominant expression of α_{1A}-adrenoceptors (Knepper *et al*., 1995; Smith *et al*., 1997; Davis *et al.*, 2018). Hence, high potency values for A61603 indicated that contraction in the porcine splenic artery was mainly mediated by the α_{1A}-adrenoceptor. These observations were consistent with findings from Barbieri *et al*., (1998) who reported the presence of only the α_{1A}-subtype on this blood vessel, whereas α_{1B}-adrenoceptors were elusive, and no evidence for the α_{1D}-subtype.

At an early stage, it is essential to gain a thorough understanding of factors affecting the vasoconstriction produced by sympathomimetics and their binding with α-adrenoceptors. For example, the uptake of catecholamine at the nerve endings, as cocaine may inhibit the neuronal uptake of sympathomimetic amines and thus, it blocks a specialized nerve membrane transport system for sympathomimetics, that block, in turn, potentiates the concentration of sympathomimetics at the appropriate site of receptors and finally leads to attenuation of the response (Chen *et al*., 1998).

It has been found that using cocaine either in small concentration 3μM in rat isolated thoracic aorta (Brown *et al*., 1988) and in a 10μM concentration *in vitro* experiments with isolated blood vessels (Wright *et al*., 1995a) or in a high concentration of 5mg.kg^{-1} in the canine nasal mucosa (Berridge and Roach, 1986), can block the neuronal uptake of noradrenaline. In addition, 0.4mg/kg intravenous injection of cocaine can elevate plasma concentrations of adrenaline and noradrenaline in humans, which suggested that cocaine inhibits the reuptake of peripheral catecholamines and stimulates the central sympathetic system of humans (Sofuoglu *et al*., 2001).

The present data showed that 10μM cocaine shifted the concentration-response curves for noradrenaline and adrenaline to the left and increased the potency of noradrenaline in the splenic artery segments by 5-fold, due to blockade of neuronal uptake of noradrenaline at the α-adrenoceptor site. Yet, it had no effects on adrenaline responses. It is known that the neuronal reuptake mechanism shows saturatable kinetics and it is stereochemically selective, therefore it is in favour of L-isomer of noradrenaline and shows higher affinity to noradrenaline than to adrenaline and even to isoprenaline (Kalsner and Nickerson, 1969).

The present experiments of an increasing period of exposure to antagonists have been performed as part of the optimisation of experimental condition in which I examined the time required to incubate the tissue segments with the antagonist.

This examination follows on from McCune and co-workers study (2000) who observed that treatment with the phenylephrine-induced internalisation of the α_{1B}-adrenoceptor in transfected rat 1 fibroblast cells. However, after 24 hours treatment with prazosin and phentolamine antagonists, α_{1D}-adrenoceptors re-distributed from the perinuclear sites toward the cell membranes. Longer antagonist exposure to a receptor is believed to stabilise the receptors from being degraded and raised the possibility that the porcine isolated splenic artery is influenced by the increasing period of exposure to prazosin or phentolamine antagonists. The up-regulation of receptors is determined by the reduction in the pD_2 values of noradrenaline-induced contractions following the lowest time for the antagonist exposure. Therefore, these experiments examined isolated tissue segments, which are more complex than the cellular level and close reflecting the normal physiology using increasing duration periods of 40, 160 and 320 minutes of antagonist incubation time. The outcome confirmed that statistically there was no effect of the different increasing incubation time of (phentolamine and prazosin) antagonists on noradrenaline induced-contractions on the porcine isolated splenic artery.

The effect of different α-adrenoceptor agonists in the porcine isolated splenic artery and nasal artery:

By observing the concentration-response curves of different α-adrenoceptor agonists, it is clear that both the porcine splenic artery and the porcine nasal artery are highly sensitive to α_1-adrenoceptor agonists, which can be seen obviously with the most sensitive response to α_1-adrenoceptor A61603 (see table 4.4). This agent is a potent and

selective α_{1A}-adrenoceptor agonist and in the present study A61603 had a higher potency value than noradrenaline and phenylephrine in both preparations.

Examination of the agonist potency-ratio in two structures (nasal and splenic artery) under similar circumstances revealed that a massive difference between the values as the relative potency of A61603 to noradrenaline and phenylephrine (66-135) in the porcine splenic artery in compared to that of A61603 to noradrenaline and phenylephrine (389-933) in the porcine nasal artery. These ratios suggested that the role of α_1-adrenoceptor was more evident in the porcine nasal artery and the presence of a mixture of α_1-adrenoceptor subtype's involved in the contractile activity of both preparations.

These findings are entirely in keeping with the data showing that A61603 behaved as a full agonist approximately 100-fold more potent than noradrenaline in the porcine meningeal artery (Mehrotra *et al*., 2007) and in the rat caudal artery (Lachnit *et al*., 1997). Brimonidine, a selective α_2-adrenoceptor agonist, was largely devoid of contractile activity (Barbieri *et al*., 1998). Furthermore, the higher potency-ratio of A61603 to noradrenaline or phenylephrine in the rat vas deferens and in the canine prostate provided the distinguishing power between α_{1A}- and α_{1B}-adrenoceptor subtypes from α_{1D}-adrenoceptor subtypes (Knepper *et al*., 1995). There has been evidence of greater A61603 efficacy in comparing with phenylephrine in double knockout mice (Methven *et al*., 2009). A similar finding was also obtained from the potency value of A61603 relative to phenylephrine in the rabbit cutaneous resistance artery, which also revealed the presence of α_{1A}- or α_{1B}-adrenoceptor subtypes but not α_{1D}-adrenoceptor subtype (Smith *et al*., 1997). Higher efficacy with A61603 than noradrenaline in the porcine splenic artery was also observed in human skeletal muscle

resistance arteries, indicating the complex roles of functional α_1-adrenoceptor subtypes in regulating the human vasculature that has yet to be fully understood (McGrath *et al*., 2005).

The slope of Schild plot for phentolamine antagonist against noradrenaline and A61603:

Phentolamine is the antagonist of choice to help distinguish between α- and β-adrenoceptors for responses mediated by either noradrenaline or adrenaline. Radioligand binding and functional studies by Starke, 1981 and Bylund, 1992 confirmed and extended the finding that phentolamine does not discriminate between the two major subtypes of α-adrenoceptors; nevertheless, phentolamine was (10-30)-fold more potent at α_{1A}-adrenoceptor than α_{1B}-subtypes. From another aspect, based on the previous study by Barbieri and co-workers, (1998), who stated that the porcine splenic artery responded to noradrenaline and phenylephrine to reveal the presence of only one main subtype of α_1-adrenoceptor. The expectation was that this antagonist should cause a parallel rightward shift of the concentration-response curves and yield a pA_2 of approximately eight with a slope of the Schild plot not significantly different from unity (Apperley *et al*., 1976).

The choice of seven different concentrations over a 1000-fold range for all antagonists was based on the protocol adopted by De Mey and Vanhoutte (1981) in their seminal study of canine arteries and veins. In addition, these experiments included agonist concentrations at four data points per log cycle. It was anticipated that all these approaches should increase the accuracy of the data and allow for the detection of receptor heterogeneity contributing to constrictor responses to noradrenaline (Kenakin, 1984; Milnor, 1986; Kenakin, 1992).

However, the surprising result from this study that, the classic competitive, non-selective α-adrenoceptor antagonist phentolamine, a drug pivotal to the original identification of α- and β-adrenoceptors and subsequent subdivision of α_1 and α_2-adrenoceptors and even α_1-adrenoceptor subtypes (Furchgott, 1972; Starke, 1981; Bylund, 1992) showed specifically, the interaction with noradrenaline-induced contractions did not fulfill the accepted criteria for competitive antagonism in the porcine splenic artery (Arunlakshana and Schild, 1959; Kenakin, 1984).

In order to examine wether the contractions for noradrenaline in the porcine nasal artery were affected by phentolamine in a similar manner to that previously observed in the splenic artery (slopes of the Schild plot less than unity) or not, a detailed re-examination of the effect of phentolamine (10nM-10μM) against noradrenaline-induced contractions in the porcine nasal artery was performed. The pA_2 value for phentolamine was less than that observed in the porcine splenic artery but the slope of the Schild plot was still significantly less than unity. The same pattern was also detected with adrenaline against phentolamine, in which the slope of the Schild plot against adrenaline-induced contractions of the porcine splenic artery was also different from unity (0.81 ± 0.03**, $P < 0.01$, with a pA_2 value 8.48 ± 0.10, n=8) (see appendix 3). It seems unlikely that saturation of either $uptake_1$ or $uptake_2$ by noradrenaline can account for the low slope of the Schild plot (Kenakin, 1984), as all experiments were conducted in the presence of both 10μM cocaine and 30μM corticosterone to reduce the impact of these inactivation mechanisms. It should also be noted that the slope of Schild plots for the antagonists was uniform across the 1000-fold concentration range employed, with no evidence for two phases that could indicate sequestration and metabolism of the agonist (Langer and Trendelenburg, 1969).

The finding of phentolamine behaving as a non-competitive antagonist against noradrenaline in this blood vessel is not consistent with the heterogeneity idea related to the α-adrenoceptor subtypes as the antagonism with RX-811059 showed no differences in slope. Also based on the previous study by Denfria, (2016), the combined effect of a selective concentration of prazosin and RX-811059 under different conditions and pharmacological confirmation of the α_2-adrenoceptor only appear to contribute to noradrenaline-induced contractions in the porcine splenic artery in the presence of ancillary vasoactive agents (e.g. using both U46619 and forskolin). Thus, under standard *in vitro* conditions, co-stimulation of both α-adrenoceptor subtypes by noradrenaline in this non-peripheral artery does not afford any greater likelihood for detection of post-junctional α_2-adrenoceptors in contractile responses, compared to selective α_2-adrenoceptor agonists alone. This conclusion is consistent with the report that post-junctional α_1- and α_2-adrenoceptors in man operate in an independent manner (Muszkat *et al*., 2011).

The available data clearly indicate that this deviation from classical competitive inhibition by phentolamine is associated with responses elicited by the endogenous ligand(s). First, the contractile responses of the porcine isolated splenic artery and the porcine isolated nasal artery elicited by agonists selective for α_1-adrenoceptors, the imidazoline A61603 (Mehrotra *et al*., 2007) or even phenylephrine (Starke, 1981; Bylund, 1992) according to Denfria, (2016) were inhibited by phentolamine in a competitive manner; the slope of Schild plots were not significantly different from unity. Significantly, prazosin also behaved as a competitive antagonist against A61603 and phenylephrine (Denfria, 2016) in the porcine isolated splenic artery. Second, for phentolamine, the slope of the Schild plot against adrenaline-induced contraction of the splenic artery was also different from unity (appendix 3). Third,

these results were conducted in a repeat set of experiments against noradrenaline in the porcine nasal artery, in which also the agonist concentration curve involved 4 data points per log cycle to increase the accuracy of the estimate of pD_2, and the same non-competitive effect of phentolamine was observed.

Presently, the possible explanation for the lower than expected slope of the Schild plot for the classic α-adrenoceptor antagonist phentolamine against mainly noradrenaline and adrenaline in the porcine blood vessels is the possible involvement of more than one subtype of α_1-adrenoceptors, particularly α_{1A}-adrenoceptor as phentolamine interacts preferentially with α_{1A}-adrenoceptor (10-30)-fold more than α_{1B}-subtypes (Morrow and Creese, 1986; Bylund, 1992). Also, Hong and co-workers (2005), during their preliminary receptor binding study in human embryonic kidney and Chinese hamster ovary found that phentolamine had a higher binding affinity by 14-fold at α_{1A}-adrenoceptor than α_{2A}-adrenoceptors.

The slope of Schild plot for α_1-adrenoceptor antagonists against noradrenaline and A61603 and the heterogeneity of α_1-adrenoceptor in different blood vessels:

Examination of the Schild plot analysis was also performed with the selective α_1-adrenoceptor antagonists (prazosin, corynanthine, tamsulosin and silodosin) in the porcine splenic artery preparations against noradrenaline-induced contractions and again the slope of the Schild plot were less than unity, raising the possibility of receptor heterogeneity (Milnor, 1986). However, there was no evidence of any component of the response resistant to either prazosin or corynanthine.

Remarkably based on the Schild analysis alone, tamsulosin and silodosin did not

antagonise noradrenaline in a competitive manner, causing non-parallel dextral shifts in the concentration-response curves. At least 1nM of tamsulosin and silodosin was required to reduce the slope of the concentration-response curves for noradrenaline, which indicated that higher concentrations of the antagonist were required to produce small rightward displacements in the lower portion of responses to noradrenaline concentrations-response curves.

The magnitude of noradrenaline concentration-response curves decrease was more prominent with silodosin and may account for the substantial decrease in Schild slope. These results suggest the presence of a minor population of receptors that are resistant to the effects of the antagonists, in contrast to the observations that only α_{1A}-adrenoceptors were expressed on the porcine isolated splenic artery (Barbieri *et al*., 1998). It is possible that phenylephrine used by Barbieri and colleagues (1998) binds to the minor population of receptors with lower affinity than noradrenaline, therefore resistance to antagonists was not as pronounced.

There is evidence that noradrenaline is more potent than phenylephrine in tissues expressing α_1-adrenoceptors, but it cannot be ignored that phenylephrine can activate the α_{1B}-and α_{1D}-adrenoceptor subtype (Oriowo and Bevan, 1986; Knepper, *et al*., 1995). Reduction in concentration-response curves slope for noradrenaline by tamsulosin (at 10nM, but not 3nM) was reported in the human erectile tissue that exhibits α_{1A}-adrenoceptors and its functional phenotype α_{1L} (Davis *et al*., 2018). Davis and co-workers (2018) accounted these observations to the irreversible binding of tamsulosin to receptors due to its high affinity (>10) at α_{1A}-adrenoceptors, which subsequently leads to a reduction in maximum response, rather than the presence of another receptor. This phenomenon is not unique to high-affinity antagonists, silodosin

and tamsulosin (Yamagishi *et al*., 1996; Moriyama *et al*., 1997). High-affinity antagonists are thought to exhibit slow dissociation from the receptor, leading to an illusion of insurmountable antagonism likewise observed in this study at concentrations > 30nM and > 3nM for silodosin and tamsulosin, respectively.

However, due to the drastically prolonged time course of noradrenaline to silodosin, one might argue that responses have already achieved equilibrium, and even at the lowest concentration (1nM), high-affinity α_{1A}-antagonists are able to exhibit some sort of insurmountability. Individual analysis of silodosin experiments against noradrenaline strongly supports the expression of at least two receptors in the porcine splenic artery. It appears that concentration-response curve from individual experiments revealed that the silodosin produced a curve more similar to a two-site model, which separates the concentration-response curve into two distinct portions than a traditional one-site model observed with the noradrenaline concentration-response curves plots of mean data. The concentration-response curve for noradrenaline were resolved into a lower portion with a less steep slope, and a higher portion with a steeper slope similar to the control. The two-site model suggested that the effects of silodosin may be affected by receptor density or increased expression of the antagonist-resistant receptor. The varying potencies of silodosin may have masked the two-site model of the noradrenaline curve in the mean analysis of the concentration-response curves.

Functional experiments in animal models and human tissues with noradrenaline against α_{1A}-selective antagonists reported similar findings. Experiments in human skeletal muscle resistance arteries observed a diminished slope of the noradrenaline concentration-response curves to a higher concentration of α_{1A}-selective 5-methyl-urapidil (300nM) and reported that the concentration-response curves fit better

statistically and significantly with a two-site model. This observation caused the authors to conclude the presence of an unknown receptor other than the α_{1A}-adrenoceptor (McGrath *et al*., 2005). Similarly, the α_{1A}-selective antagonist RS-17053 also produced a non-parallel, biphasic, rightward shift of noradrenaline concentration-response curves in the rat caudal artery, but produced parallel dextral shifts with A61603 indicating the presence of α_{1A}-subtype (pK_B = 9.2) and at least one other receptor mediated contraction (Lachnit *et al*., 1997). The pharmacological profile of the subtype other than α_{1A}-adrenoceptor that mediates contraction was later characterised as α_{1B}-subtype in rat caudal arteries (Parés-Hipólito *et al*., 2006).

On this basis, silodosin has substantially greater selectivity for α_{1A}-adrenoceptors over α_{1B}-adrenoceptors than tamsulosin, it is plausible that the unknown receptor resistant to effects of silodosin, and less resistant to tamsulosin is the α_{1B}-adrenoceptor. Moreover, there has been evidence in the human prostate that silodosin was able to distinguish a small population of low-affinity sites that correlate with α_{1B}-adrenoceptors due to its high potency for α_{1A}-subtype over α_{1B}-subtype, whereas tamsulosin could not (Shibata *et al*., 1995). Nevertheless, with no involvement of α_2-adrenoceptors in contractions, still no reasonable explanation for the non-competitive inhibition and the lower than expected slope of the Schild plot by corynanthine. Binding studies showed that tamsulosin could discriminate between α_1-adrenoceptor subtypes, whereas prazosin and it's analogue could not (Bylund, 1992; Michel *et al*., 1996; Piascik *et al*., 1996). However, in functional studies, prazosin had different affinity for α_1-adrenoceptor subtypes and showed a predominant low potency for the α_{1A}-subtype and a high potency for the α_{1B}- and α_{1D}-adrenoceptors (Docherty, 2019). This observation clarified our *in vitro* functional observation of a non-competitive

antagonist action for prazosin against noradrenaline in the porcine splenic artery and improved the involvement of multiple α_1-adrenoceptors subtypes.

On the other hands, contractile responses of the porcine isolated splenic artery and the porcine isolated nasal artery was elicited by an agonist selective for α_1-adrenoceptors. The imidazoline A61603 (Mehrotra *et al*., 2007) was inhibited by phentolamine, prazosin, tamsulosin and silodosin in a competitive manner; the slopes of Schild plots were not significantly different from unity. In a similar manner phenylephrine, a selective α_1-adrenoceptor agonist (Starke, 1981; Bylund, 1992) was inhibited by phentolamine and prazosin in a competitive manner and the slopes were not significantly different from unity (Denfria, 2016).

Based on the estimated pA_2 values for antagonists against A61603, prazosin was (4-5)-fold more potent than phentolamine either in the porcine splenic artery or in the nasal artery, consistent with the presence of only α_1-adrenoceptors. The pA_2 values for prazosin against noradrenaline (9.5), A61603 (9.0) and according to Denfria, (2016) against phenylephrine (9.0), which are consistent with the known affinity for α_1-adrenoceptors (Starke, 1981), as it is for corynanthine (8.0). Moreover, the pA_2 value of tamsulosin (10.6) was higher than that of silodosin (9.9) against A61603. High pA_2 values of both antagonists against A61603 further support that α_{1A}-adrenoceptors are the major adrenoceptor subtype in the porcine splenic artery. Similarly, effects of these antagonists against phenylephrine-induced contractions demonstrated that tamsulosin was 3-fold more potent than silodosin in the rabbit thoracic aorta that predominantly expresses α_{1A}-adrenoceptors with a small population of α_{1B}-adrenoceptors (Yamagishi *et al*., 1996).

The effect of phentolamine and different α-adrenoceptor antagonists in the porcine splenic vein:

The present study showed the importance of interaction between phentolamine and noradrenaline, which was further underlined by observations in the porcine splenic vein, a preparation that represents the venous side of the circulation. Here again, in the porcine splenic vein, phentolamine caused a parallel rightward displacement of noradrenaline-induced contractions, but once again the slope of the Schild plot was less than unity. The present study also showed the significance of the interaction between the antagonists which is emphasised by the observations in the porcine splenic vein. Similarly, the marked 'prazosin- resistant' component of responses to noradrenaline was abolished by the presence of a low concentration of RX-811059.

In a number of other studies evaluating agonist/antagonist interactions, where surprising deviations from unity for the slope of the Schild plot have been reported for antagonists against either noradrenaline or phenylephrine contractions (Van der Graaf *et al*., 1996; Hussain and Marshall, 1997; Hussain and Marshall, 2000), it is noteworthy that this has been based on an analysis of at least five antagonist' concentrations over a 100-fold range. This analysis varies from the assumption of competitive inhibition in other vessels where fewer concentrations over a narrower range have been employed (Shoji *et al*., 1983; Koga *et al*., 1989; Wright *et al*., 1995b; Koga and Ikebe, 2008). Thus, a more stringent experimental design for pharmacological studies, particularly for vessels from large mammals that can allow up to seven antagonist concentrations over a 1000-fold range, appears to have greater potential to yield evidence for non-classical, agonist/antagonist interaction.

To my mind, these observations raise a paradoxical issue at the heart of Schild analysis - the likelihood of concluding an agent behaves as a competitive antagonist is actually

increased by limiting the number experiments conducted and/or limiting the range of antagonist concentrations employed. This would certainly explain why we previously described prazosin as a competitive inhibitor of noradrenaline-induced contractions of the porcine splenic artery (Wright *et al*., 1995a) and by Koga and colleagues (1989) in the canine femoral vein – despite the presence of both α_1 and α_2-adrenoceptors. In light of my experience, I would now recommend that when attempting to evaluate novel antagonists against the endogenous ligand in any type of isolated tissue, to predict likely activity *in vivo,* Schild analysis should be conducted with a minimum of 5 concentrations over a 100-fold range (DeMey and Vanhoutte, 1980; Van der Graff *et al*., 1996; Hussain and Marshall, 1997).

In summary, there is no straightforward explanation for the sensitivity of the splenic artery versus the nasal artery related to noradrenaline and phenylephrine, as there were no obvious differences detected in the receptor properties of both preparations. However, the most probable explanation for these observations can be related to the higher potency-ratio of A61603 to noradrenaline or phenylephrine in the porcine nasal artery in comparing with that in the splenic artery, which indicated that there was a more obvious role for α_1-adrenoceptors in the nasal artery. These findings also reflected differences either in the receptor subtype density or in the receptor coupling for the α_1-adrenoceptor in these blood vessels. Depending on this multiple information, the present study aim to use classical pharmacological approaches to establish whether post-junctional α_2-adrenoceptors in the porcine isolated splenic artery can be activated by the endogenous ligand, noradrenaline, and, therefore, contribute to contractile responses. These findings raise the possibility that standard *in vitro* experimental conditions may underestimate the role of post-junctional α_2-adrenoceptors in the vasculature *in vivo*.

Chapter Five

Do α_2-adrenoceptors play a role in vascular tone of the porcine isolated splenic artery either in normal or enhanced conditions?

5 Do α_2-adrenoceptors play a role in vascular tone of the porcine isolated splenic artery either in normal or enhanced conditions?

5.1 Introduction

It is becoming increasingly difficult to ignore the role of α_2-adrenoceptor in the action of nasal decongestants, especially in light of the large prevalence of α_2-adrenoceptors in the nasal vasculature (Stafford-Smith *et al.*, 2007). Also, recent evidence indicated that activation of α_2-adrenoceptors in the pig and human nasal mucosa, leading to a reduction in the nasal blood flow (Corboz *et al.*, 2007). For example, α_{2C}-adrenoceptors are located in nasal mucosal veins and the topical use of α_2-adrenoceptor agonist relieves congestion in cats (Corboz *et al*., 2013). Additionally, xylometazoline and oxymetazoline are the most widely used topical decongestants and proved to have an important agonist action at α_2-adrenoceptor (Haenisch *et al*., 2010). Traditionally, clonidine has been used in controlling blood pressure but historically, clonidine was originally developed as a peripheral vasoconstrictor agent for use as a nasal decongestant (Stahle, 2000) and, interestingly, a few case reports of overdose with this agent noted a major hypertensive crisis (Frye and Vance, 2000; Johnson *et al*., 2011; Perruchoud *et al*., 2012). The latter observation implies that the full vasoconstrictor action of clonidine has probably been underestimated because its principal cardiovascular effect is to reduce blood pressure.

α_2-Adrenoceptors have been identified in several blood vessels including arteries and veins. The high predominance of α_2-adrenoceptors have been observed in the venous side of the circulation, which they have also been engaged in their contraction

responses (Wang and Lung, 2003; Gyires *et al.*, 2009). For example, the dog saphenous vein, which has been considered as a classical model to study α_2-adrenoceptor mediated contractions (Flavahan *et al.*, 1984; MacLennan *et al.*, 1997; Kwan, 1999).

In the context of the arterial side of vasculature, α_{2A}-adrenoceptors are known to mediate contractions of the porcine ciliary arteries (Wikberg-Mattison and Simonsen, 2001) and endothelium-dependent relaxations in the coronary artery (Bockman *et al*., 1993), recent evidence suggested that the α_{2C}-adrenoceptor subtype is responsible for contractile responses in other vascular beds (Gavin *et al*., 1997; Rizzo *et al*., 2001). Additionally, porcine arteries (Jantschak and Pertz, 2012) and veins (Goernemann *et al*., 2009) were shown to have a sensitivity to α_{2C}-adrenoceptor agonists. it is noteworthy that the density of α_2-adrenoceptor binding sites are much lower than that reported in cell-based systems, but can vary from 500 fmol mg^{-1} protein in the porcine thoracic aorta (Wright *et al*., 1995a) to less than 20 fmol mg^{-1} protein in the porcine rectal artery (Blaylock *et al*., 2000).

The porcine isolated splenic artery is a non-peripheral vessel for which there is radioligand binding evidence of a sizeable population of post-junctional α_2-adrenoceptors (Wright *et al*., 1995a; Roberts *et al.*, 1999). Yet, the selective α_2-adrenoceptor agonist brimonidine was a poor vasoconstrictor agent in this vessel and only α_1-adrenoceptors appear to be involved in mediating contractions to noradrenaline: prazosin behaved as a competitive antagonist of responses (Wright *et al*., 1995a). Similarly, Barbieri and colleagues (1998) found no evidence for constrictor α_2-adrenoceptors in this blood vessel according to the low contraction percentage induced by the α_2-selective agonists, which were approximately reached to 31% and

13% of noradrenaline maximum effect. Additionally, authors of this study also found that blocking the α_2-adrenoceptors using the α_2-selective antagonist yohimbine against oxymetazoline- and phenylephrine-induced contractions yielded affinity (pA_2) values (6.80 and 6.74, respectively) consistent with α_1-adrenoceptor interaction only (Barbieri *et al*., 1998). A recent study by Denfria (2016) showed that α_2-adrenoceptors had a negligible unseen role in the porcine splenic artery. In a similar manner, the result of the previous chapter showed that there was no role of α_2-adrenoceptors when antagonising the effect of noradrenaline using RX-811059 in combination with phentolamine. All these negative observations raised the possibility that there is a hidden role in the participation of α_2-adrenoceptors in the contractile response in the porcine splenic artery.

Since it has been previously reported that α_2-adrenoceptor-mediated contractions in porcine blood vessels *in vitro* are enhanced in response to selective α_2-adrenoceptor agonists in veins, or uncovered in arteries, in the presence of a combination of a non-adrenoceptor vasoconstrictor (e.g. the thromboxane-mimetic U46619 to raise calcium level) and forskolin (to raise cellular cyclic AMP). This phenomenon has also been observed in the palmar lateral vein (Wright *et al*., 1995a), the ear artery (Roberts *et al*., 1999) and the rectal artery (Minyan *et al*., 2001), which implies that post-junctional α_2-adrenoceptors are more widely distributed than generally recognised from standard *in vitro* studies. However, in the presence of a combination of a thromboxane-mimetic (U46619) and forskolin, brimonidine produced a contraction equivalent to 25% of the maximum to noradrenaline (Roberts *et al*., 1999) that was insensitive to prazosin.

Thus, the most likely explanation for these seemingly contradictory observations is that standard *in vitro* conditions used for studying isolated blood vessels often

underestimates the role of α_2-adrenoceptors in the vasculature, even when activated by the endogenous ligand noradrenaline. From another aspect, the obligate role for ancillary vasoconstrictor agents in the porcine blood vessels, to reveal α_2-adrenoceptor-mediated contractions to noradrenaline, raises a question as to whether using of normal Krebs-Henseleit saline are optimal for revealing these responses. It is noteworthy that membrane potential is less negative in pressurised blood vessels *in vitro* (Murphy *et al*., 2007; Matthewson and Dunn, 2014) or vessels *in vivo* (Bartlett *et al*., 2000), and this may indicate that the routine inclusion *in vitro* of vasoactive ancillary agents could reveal functional responses to receptors that are not evident under standard experimental conditions. Earlier studies have shown that using L-NAME in the splenic artery was connected with the improvement of contractile tone (Dunn *et al.*, 1991; Lot *et al.*, 1993).

The action of α_2-adrenoceptor may be underestimated due to an absence of vasoconstrictor tone and/or the presence of endothelium-dependent inhibitory effects via NO. Hence, it could conceivably be hypothesised that α_2-adrenoceptors in the splenic blood vessels from the pig can also be influenced by other vasoconstrictors. However, it is unclear whether the use of other agents that either raise the intracellular calcium (KCl and S(-)-BayK8644) or remove the inhibitory influence of nitric oxide (L-NAME and ODQ) can uncover the α_2-adrenoceptor mediated contractions in the porcine splenic artery. Table 5.1 summaries the mechanism of action of the vasoconstrictor agents.

Table 5.1 The vasoconstrictor agents and their mechanism of action.

Vasoconstrictors	Mode of action	Reference
U46619 and forskolin	Direct activation of the Gprotein-coupled receptor specific for thromboxane A_2, while forskolin stimulates adenylyl cyclase (raise cellular cyclic AMP)	(Coleman *et al*., 1981)
(L-NAME) *N*ω-Nitro-L-arginine methyl ester hydrochloride	Remove basal release of nitric oxide by inhibition of nitric oxide synthase (endothelium)	(Schroeder and Kuo, 1995; Jiang *et al.*, 2000; Muzaffar *et al.*, 2003; Vitecek *et al.*, 2012)
ODQ (1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one)	Remove basal influence of nitric oxide by selective inhibition of soluble guanylyl cyclase activity	(Schrammel *et al.*, 1996; Hayabuchi *et al.*, 1998; Ellis *et al.*, 2003)
S(-)-BayK8644 (4S)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester.	Putative Ca^{2+} channel activator which enhances the influx of calcium through voltage-dependent and voltage-independent Ca^{2+} channels, (a dihydropyridine-based calcium agonist)	(Thomas *et al.*, 1985; Young *et al*., 1988; Szadujkis-Szadurska *et al*., 2013)
KCl	Direct depolarisation tone inducer	(Hirst and Edwards, 1989; Levitan *et al*., 1995; Nelson and Quayle, 1995; Nelson *et al.*, 1997; Macias *et al*., 2001)

Brimonidine was established as a full agonist at α_{2A}-adrenoceptors (Jansson *et al*., 1998; Alexander *et al*., 2017), and only elicits overt contractions via α_2-adrenoceptors in isolated vessels with a lower density of α_2-adrenoceptor binding sites when responses to it are 'uncovered' in vessels following pharmacological manipulation with a combination of a thromboxane-mimetic vasoconstrictor and forskolin (Roberts *et al*., 1999). Therefore, in the beginning, replication of what has been shown with brimonidine will be performed then the focus will be to examine what the endogenous ligand (noradrenaline) does at α_2-adrenoceptors. Also, the majority of α_2-adrenoceptor agonists possess an imidazoline structure, which is unrelated to the endogenous ligand (noradrenaline) for the receptor. Therefore, a comparative examination of the effect of selective α_2-adrenoceptor (brimonidine) and a non-selective α-adrenoceptors agonist, the endogenous ligand (noradrenaline) on non-peripheral blood vessels, is warranted.

The following chapter seeks to address the following question: can α_2-adrenoceptors interfere with the integrity and the relative vasoconstrictor activity, in the splenic artery from the pig either under standard *in vitro* conditions or the presence of other vasoactive agents (e.g. a combination of U46619 and forskolin). Moreover, from a therapeutic view, to understand and compare the role of the α_2-adrenoceptors agent is essential for enhancing the vascular tone of the blood vessels and improving approaches of direct precise therapies (more effective decongestants) using the α_2-adrenoceptors agent's incorporation with applicable substances and methods of pharmacological manipulation. For confirmation, this chapter will also examine the effects of the α_2-adrenoceptor antagonist RX-811059 (30nM), which maintains a high affinity and selectivity for α_2-adrenoceptors and has a 245-selectivity ratio of α_2/α_1 with a pA_2 value of (8.5) (Doxey *et al*., 1984; Mallard *et al*., 1992).

This value means that a concentration of 0.1μM RX-811059 may affect α_1-adrenoceptors, while a concentration of 30nM, which will be used in our experiments provides confidence in any inhibitory effect observed. Moreover, clarifying the effect of α_1-adrenoceptor antagonist either alone or in a combination with the α_2-adrenoceptor antagonist in the manipulated blood vessels will be carried out.

5.1.1 Aims

The aims of this study were

1. To examine the effect of various interventions on responses to brimonidine in the porcine isolated splenic artery and assess the role of post-junctional α_1-and α_2-adrenoceptors.
2. To examine the effect of various interventions on responses to noradrenaline in the porcine isolated splenic artery and assess the role of post-junctional α_1-and α_2-adrenoceptors.
3. To investigate the effects of α_2-adrenoceptor antagonist on the response of pharmacologically manipulated splenic artery of the pig to noradrenaline.
4. To investigate the effects of α_2-adrenoceptor antagonist if an α_1-adrenoceptor antagonist is incorporated into the response of pharmacologically manipulated splenic artery of the pig to noradrenaline.

5.2 Materials and Methods

5.2.1 Materials

Materials used for the experiments of this chapter are listed in table 2.1.

5.2.2 Methods

The collection of material and tissue preparation for contractility studies were described in chapter 2, section 2.1.1. Isometric tension measurement experiments were carried out in the same apparatus using the same buffer and the general protocol mentioned in chapter 2 (section 2.1.2). However, all experiments in this chapter related to the pharmacological manipulation are mentioned in detail in section 2.1.3. All experiments with noradrenaline are carried out using cocaine 10μM to inhibit $uptake_1$, corticosterone 30μM to inhibit $uptake_2$ and propranolol 1μM to inhibit β-adrenoceptors. In certain experiments, the role of raising vascular tone to approximately 40- 50% contraction of the third KCl response was examined by pre-contracting preparations with U46619. While using various vasoconstrictors including 10μM ODQ, 100μM L-NAME, 1μM S(-)-BayK8644 and 18mM KCl, which were added to the preparation at least 40-45 minutes before starting the agonist cumulative concentration-response curves caused an approximately elevation in the vascular tone (5-10%) of 60mM KCl.

Additionally, the effect of the antagonist(s) was incubated and left for at least 40 minutes before the segments of the porcine nasal and splenic artery were exposed to the agonist according to the protocol described for each experiment. In most of the experiments related to this chapter, the contraction of the porcine splenic artery, especially under control conditions was not sustained and showed evidence of rhythmic

activity after the addition of the increasing cumulative concentrations of agonist. Therefore, the measuring of the maximum responses depended on measuring the peak responses rather than the contraction prior to the next concentration. A number of experiments demonstrated non-saturation protocol in the concentration-response curves. This protocol showed that the responses failed to reach an asymptote within the range of concentrations employed. Therefore, agonist efficacy detected at the highest concentration of the agonist and potency value described a 20% contraction of 60mM KCl response.

5.2.3 Data analysis

All the results have been expressed as a mean ± standard error (SEM). The number of observations in different animals for each experiment was expressed as (n). In the majority of experiments, a Student paired *t*-test was used to compare differences between two groups and between every single point. In addition, ANOVA followed by *post hoc* Dunnett's test if more than two groups needed to be compared. All these analyses performed using Synergy KaleidaGraph software as already mentioned in chapter 2, section 2.4. A *P* value of less than 0.05% was considered statistically significant.

In many experiments described in this chapter, concentration-response curves failed to achieve an asymptote, meaning that pD_2 and R_{max} values could not be derived. In those instances, the contraction at the highest concentration of agonist was reported to reflect agonist efficacy, while a pD_{20} (the negative logarithm of agonist concentration that evokes a contraction, which was 20% of the 60mM KCl response) was used to reflect potency.

5.3 Results

5.3.1 Comparing the porcine isolated splenic artery responses to brimonidine either in normal or enhanced conditions

The present study sought to identify whether pharmacological manipulations have the ability to modify the vascular tone of the porcine splenic artery preparations and then to reveal the action of α_2-adrenoceptors using a selective α_2-adrenoceptor agonist. Therefore, the response of porcine splenic artery to brimonidine was examined by revealing the isolated ring segments to increasing cumulative concentrations (1nM-0.1μM) of it either in normal conditions or after pharmacological manipulations. The first one was the induction of a contraction by U46619 to approximately 40-50% of the response to 60mM KCl, which raised the basal tone up to 44 ± 5% (n=6) of the response to 60mM KCl above baseline. Then exposed to sufficient forskolin to lower tone back to 8.2 ± 0.5 (n=6), which was < 5-10% of the response to 60mM KCl.

The outcome of the combination of U46619 and forskolin indicates that these chemicals uncovered concentration-dependent contractions in the porcine arterial preparation as brimonidine at 10μM was significantly more efficient on pharmacological manipulation than on normal condition (54 ± 6% and 25 ± 5%, respectively, $P < 0.05$, n=6) (Figure 5.1). The use of a combination of U46619 and forskolin also significantly shifted the curve to the left and improved the sensitivity to brimonidine as they showed an effective contraction at 0.002μM, while preparations in basal condition showed a considerable contraction started with 0.02μM of brimonidine. Interestingly, porcine splenic segments in basal condition and with pharmacological manipulation showed a significant difference in their potency values (pD_{20} = 6.07 ± 0.07 and 6.45 ± 0.02, respectively $P < 0.01$, n=6) (Figure 5.2).

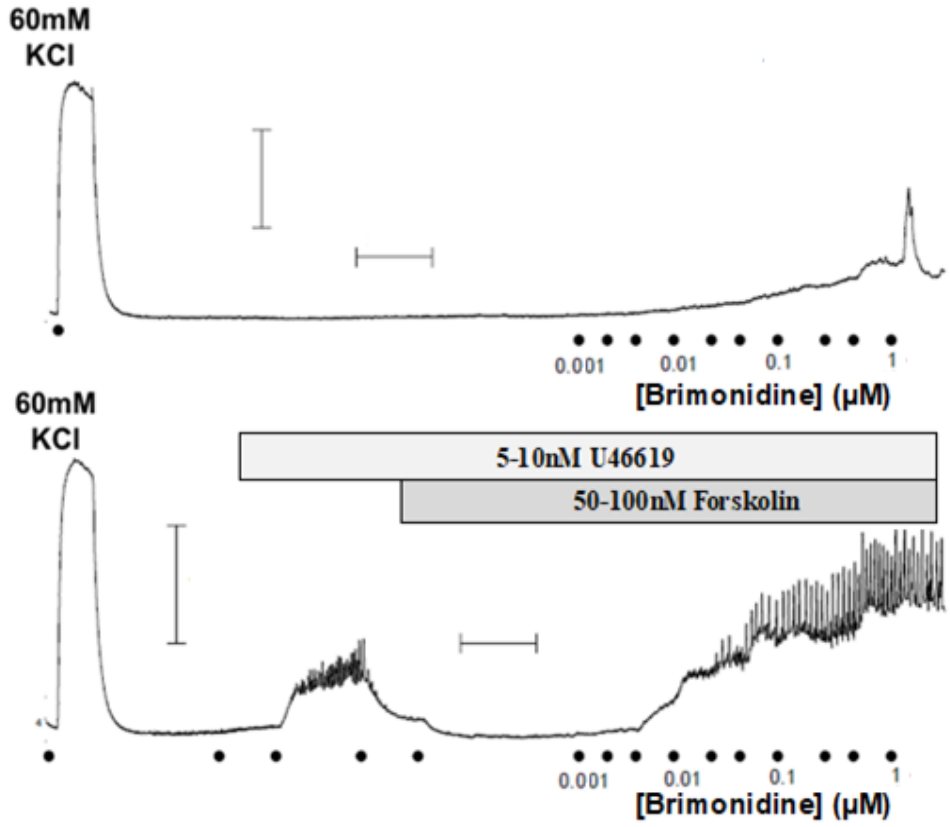


Figure 5.1 Isometric tension recording trace showing the response of porcine splenic artery to 60mM KCl followed by: (Upper) the response to a cumulative concentration of brimonidine added on the basal tone, (Lower) U46619-induced contraction followed by relaxation with forskolin and at the end of the experiment the preparations exposed to cumulative concentration of brimonidine. The vertical bar is equivalent to 10 g wt. and the horizontal bar denotes 30 minutes duration.

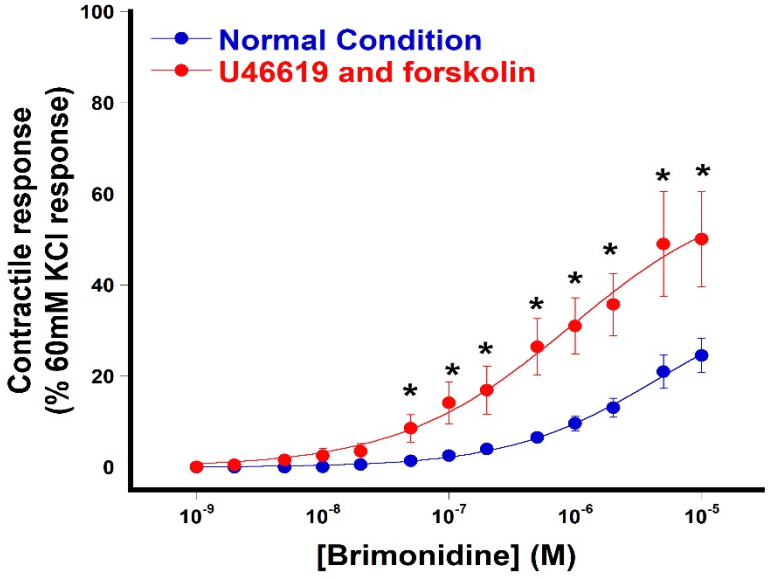


Figure 5.2 Response of porcine splenic artery to increasing cumulative concentrations of brimonidine in normal condition and after pharmacological manipulation. In the pharmacological manipulation group, the segments were contracted with U46619 then relaxed with forskolin before the exposure to brimonidine. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of six observations.

*$P < 0.05$, Student's paired *t*-test.

To examine the influence of alternative approaches of pharmacological manipulation on the response of porcine splenic artery using brimonidine. Manipulations were performed by raising the vascular tone using different vasoconstrictors including the following compounds (1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ). The tissue segments were tested by exposing two different groups to the cumulative concentration of brimonidine (1nM-0.1μM) in normal condition and after incubation with those contractile agents (Figure 5.3). Incubation and addition of the vasoconstrictors for 40-45 minutes caused an approximately 5-10% elevation in the vascular tone before being exposed to a cumulative concentration of brimonidine (1nM-0.1μM).

Table 5.2 summarised the quantitative parameters of the concentration-response curves of the porcine splenic artery to brimonidine. The table showed that incubation with 18mM KCl, 100μM L-NAME and 10μM ODQ elicited a significant increase in the vasoconstrictor response with brimonidine at 10μM incomparable to controls. Despite, a significant shifting of the concentration-response curves of the porcine splenic artery to brimonidine in the presence of all the vasoconstrictors, only 18mM KCl, 100μM L-NAME and 10μM ODQ caused a significant improvement in the potency values of the porcine splenic artery.

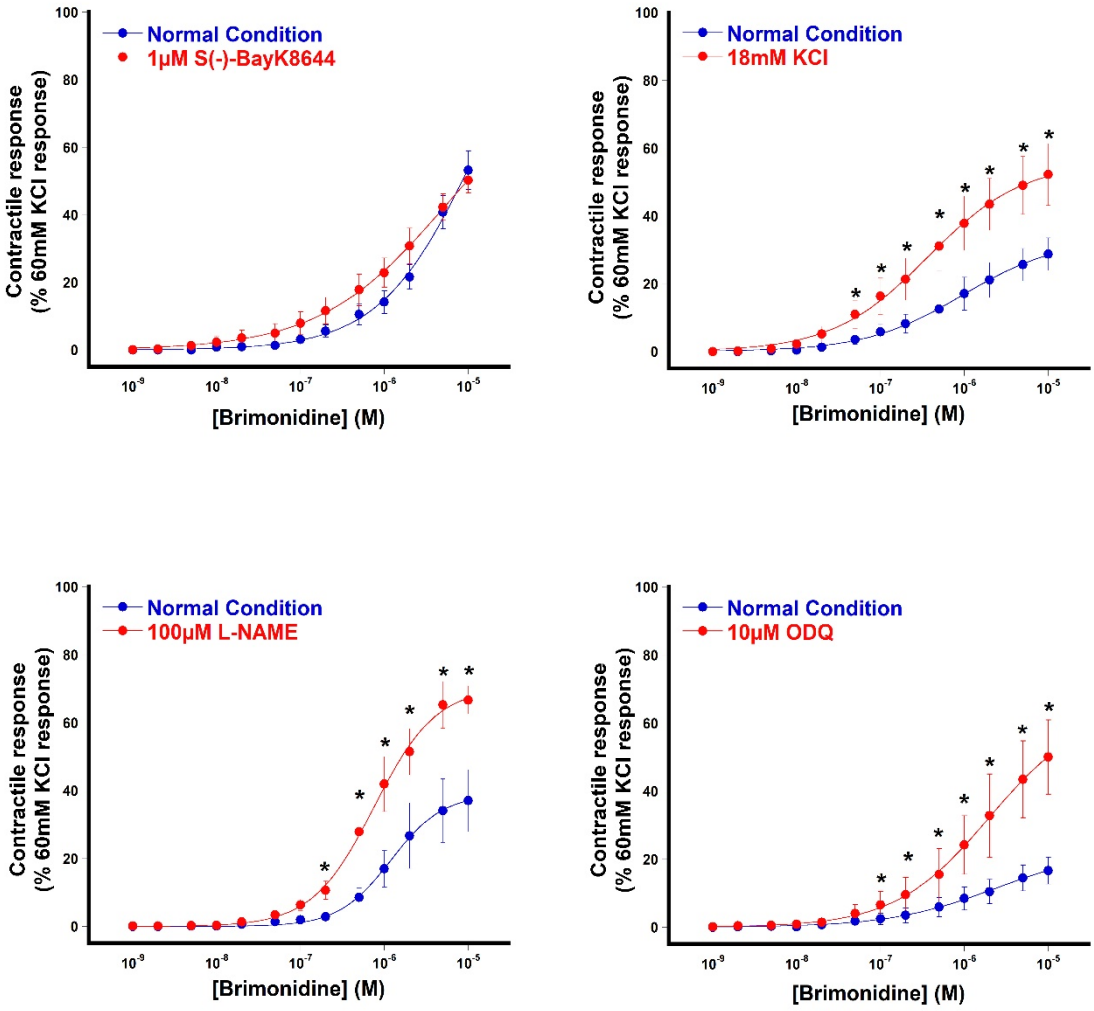


Figure 5.3 Responses of porcine splenic artery to increasing cumulative concentration of brimonidine in normal conditions and after pharmacological manipulations with various vasoconstrictors including 1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of 6-10 observations.

*$P < 0.05$, Student's paired *t*-test.

Table 5.2 Quantitative parameters of the concentration-response curves of the porcine splenic artery to brimonidine in normal condition and after addition of 1µM S(-)-BayK8644, 18mM KCl, 100µM L-NAME and 10µM ODQ. The table shows the maximum response, response at 10µM brimonidine, the negative logarithm of EC_{50} (pD_2) and the negative logarithm of EC_{20} (pD_{20}) as a percentage of a former response to 60mM KCl. Results were expressed as the mean ± S.E.M of 6-10 observations.

* ($P < 0.05$) and ** ($P < 0.01$) – denote a statistically significant difference from response control segments, Student's paired *t*-test.

[a]- Responses failed to reach an asymptote within the range of concentrations employed.

[b]- Responses reached an asymptote within the range of concentrations employed.

	n	Maximum Response (% of 60mM KCl)	Response at 10µM brimonidine (% of 60mM KCl)	pD_2	pD_{20}
Control[a]	10		56 ± 6		5.9 ± 0.1
1µM S(-)-BayK8644[a]	10		50 ± 4		6.1 ± 0.2
Control[b]	8	34.9 ± 4.5	29 ± 5	6.1 ± 0.1	6.2 ± 0.2
18mM KCl[b]	8	47.5 ± 4.6*	52 ± 9*	6.5 ± 0.1**	6.6 ± 0.2**
Control[b]	6	44.9 ± 7.2	39 ± 9	5.8 ± 0.07	5.8 ± 0.1
100µM L-NAME[b]	6	72.3 ± 4.5*	67 ± 4*	6.1 ± 0.1*	6.2 ± 0.1*
Control[a]	6		17 ± 4		5.6 ± 0.1
10µM ODQ[a]	6		50 ± 9*		6.1 ± 0.1**

5.3.2 Comparing the porcine isolated splenic artery responses to brimonidine in the presence of antagonists after pharmacological manipulation

By observing the results of the previous experiment and to further characterise the α_2-adrenoceptor on the porcine splenic artery, the response of these segments to brimonidine following pharmacological manipulation (using contraction with U46619 then relaxed with forskolin), was examined against the selective α_1-adrenoceptor antagonist (prazosin) in three different selected concentrations of 0.01, 0.1 and 1μM. Brimonidine in the presence of the combination of U46619 and forskolin caused an appreciable response in the splenic artery, which was inhibited by consequently increasing concentrations of prazosin.

The shifting of the concentration-response curves of brimonidine with 0.01 and 0.1μM prazosin was slight and similar, which revealed that a component of this contraction is resistant to prazosin. Yet, an obvious shifting was shown with 1μM prazosin, which caused rightward shifts of the concentration-response curve, reducing significantly the pD_{20} of the concentration-response curve to brimonidine by approximately 5-fold ($P < 0.05$) in comparison to the control. This shifting supports the idea of prazosin at only high concentration loses its selectivity and causes an interaction with the α_2-adrenoceptor binding site (Figure 5.4A). Additionally, the response at 10μM brimonidine when expressed as a % of the contraction to 60mM KCl showed an inhibition simultaneously with each addition of prazosin, significantly seen with (0.1 and 1μM) ($P < 0.01$, n=8) prazosin (Table 5.3).

The second set of experiments was performed to confirm and study the involvement of α_2-adrenoceptors in the contractile response of the porcine splenic artery to brimonidine after pharmacological manipulation (U46619 and forskolin) by antagonising the control group with 0.1μM prazosin and compared it by antagonism using 0.1μM RX-811059 in combination with 0.1μM prazosin. Figure 5.4B clearly showed that RX-811059 significantly shifted the response of porcine splenic artery to brimonidine and reduced the maximum response of brimonidine. The mean pK_B value for 0.1μM concentrations of RX-811059 against brimonidine in the porcine splenic artery was 9.02 ± 0.24 (n=7).

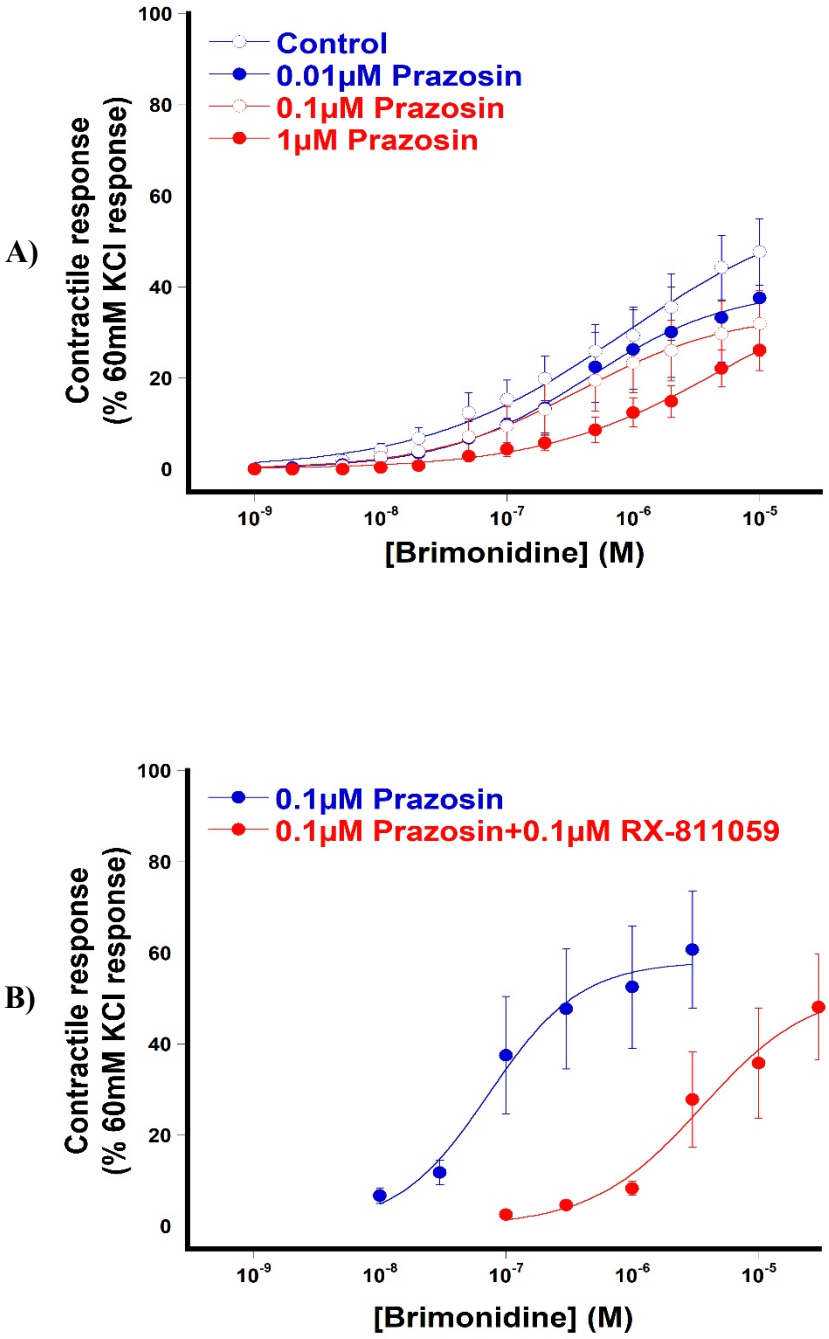


Figure 5.4 Antagonism of the response of the porcine splenic artery to increasing cumulative concentrations of brimonidine after pharmacological manipulation (contraction with U46619 followed by relaxation with forskolin) by (A) 0.01, 0.1 and 1µM prazosin and (B) 0.1µM prazosin alone and in a combination of 0.1µM prazosin and 0.1µM RX-811059. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of 7-8 observations.

Table 5.3 Quantitative parameters of the concentration-response curves of the porcine splenic artery to brimonidine in the presence of 0.01, 0.1 and 1μM of prazosin after pharmacological manipulation. The table shows the response at 10μM brimonidine and the negative logarithm of EC_{20} (pD_{20}) as a percentage of a former response to 60mM KCl. Results were expressed as the mean ± S.E.M of eight observations.

* ($P < 0.05$) and ** ($P < 0.01$) – denote a statistically significant difference from response control segments, one-way ANOVA followed by Dunnett's test.

	Response at 10μM brimonidine (% of 60mM KCl)	**pD_{20}**
Control	48 ± 8	6.21 ± 0.27
0.01μM Prazosin	39 ± 9	6.09 ± 0.3
0.1μM Prazosin	32 ± 7**	6.12 ± 1
1μM Prazosin	23 ± 4**	5.54 ± 0.15*

5.3.3 Comparing the porcine isolated splenic artery responses to noradrenaline in normal or enhanced conditions

The preceding experiment showed that responses to brimonidine were enhanced by 18mM KCl, U46619/forskolin, 100μM L-NAME and 10μM ODQ and only brimonidine responses in the presence of U46619/forskolin involved activation of α_2-adrenoceptors. However, in the following experiment and in order to examine the effect of pharmacological manipulation on the contractile response of noradrenaline, the preparation of isolated porcine splenic artery segments was used. Figure 5.5 showed that noradrenaline (10nM-100μM) elicited concentration-dependent contractions in the porcine splenic artery. This figure showed that, the enhancing of a contractions by 40-50% using U46619 to 40 ± 3% (n=9) above the basel tone according to the response to 60mM KCl, followed by relaxation and lowering the tone < 5-10% with forskolin to 5 ± 1% (n=9) compared to the response to 60mM KCl, enhanced the concentration-dependent contractions in the arterial preparations.

The addition of forskolin and sufficient U46619 to re-establish vasoconstrictor tone to 40-50% of the response to 60mM KCl, significantly shifted the concentration-response curve of the porcine splenic artery preparations and increased the potency to noradrenaline by 3-fold from 6.1 ± 0.14 to 6.57 ± 0.05 ($P < 0.001$, n=9). In relation to the maximum response, the response of the porcine splenic artery was significantly enhanced (control 145 ± 4%; pharmacological manipulation using U46619 and forskolin 159 ± 6% ($P < 0.05$, n=9).

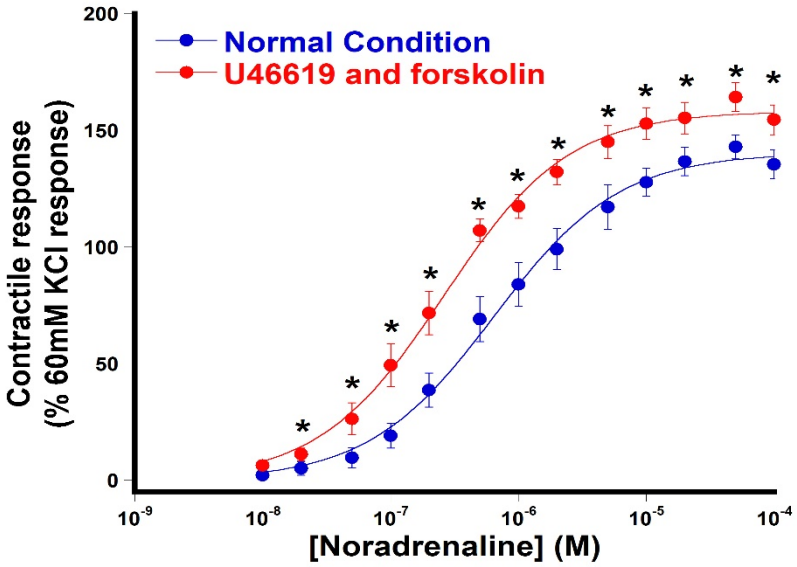


Figure 5.5 Response of porcine splenic artery to increasing cumulative concentrations of noradrenaline (in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol) in normal condition and after pharmacological manipulation. In the pharmacological manipulation group, the segments were contracted with U46619 then relaxed with forskolin before the exposure to noradrenaline. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of nine observations.

*$P < 0.05$, Student's paired *t*-test.

As described previously, the first method of pharmaceutical manipulation using U46619 and forskolin enhanced or uncovered the vasoconstrictor responses to noradrenaline in the splenic artery from the pig. Therefore, to try additional elucidate the possibility of relationships between α_2-adrenoceptors and the contractile response of the porcine splenic artery, another intervention of manipulation using the vasoconstrictor compounds (1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ), which modulate the vasoconstrictor activity by different mechanisms were explored using porcine splenic artery ring segments. Addition of the vasoconstrictors caused an approximately 5-10% elevation in the vascular tone and they were incubated 40-45 minutes before being exposed to a cumulative concentration of noradrenaline (10nM-100μM) (Figure 5.6). Exposure of the porcine splenic artery to noradrenaline (10nM-100μM) produced concentration-dependent contractions in response to 60mM KCl.

Figure 5.7 and table 5.4 showed that 1μM S(-)-BayK8644 caused a significant inhibition in the mean maximum response of noradrenaline contraction and even when the maximum response of every single point was compared ($P < 0.05$), which was particularly detected at over (5μM) concentration of noradrenaline. However, remarkably, the responses of the porcine splenic artery at (0.02-2μM) were significantly shifted to the left and increased by 100μM L-NAME and 10μM ODQ incomparable to controls ($P < 0.05$). From another perspective, 18mM KCl, 100μM L-NAME and 10μM ODQ caused non-significant changes in the mean largest contraction to noradrenaline in the porcine splenic artery with comparable to controls.

Comparing the pD_2 values of these compounds with a control found that 1μM S(-)-BayK8644, 100μM L-NAME and 10μM ODQ significantly increased the potency in

the porcine splenic artery. However, depolarisation with raised extracellular KCl caused a significant inhibition in the pD_2 value comparable to control in the porcine isolated splenic artery.

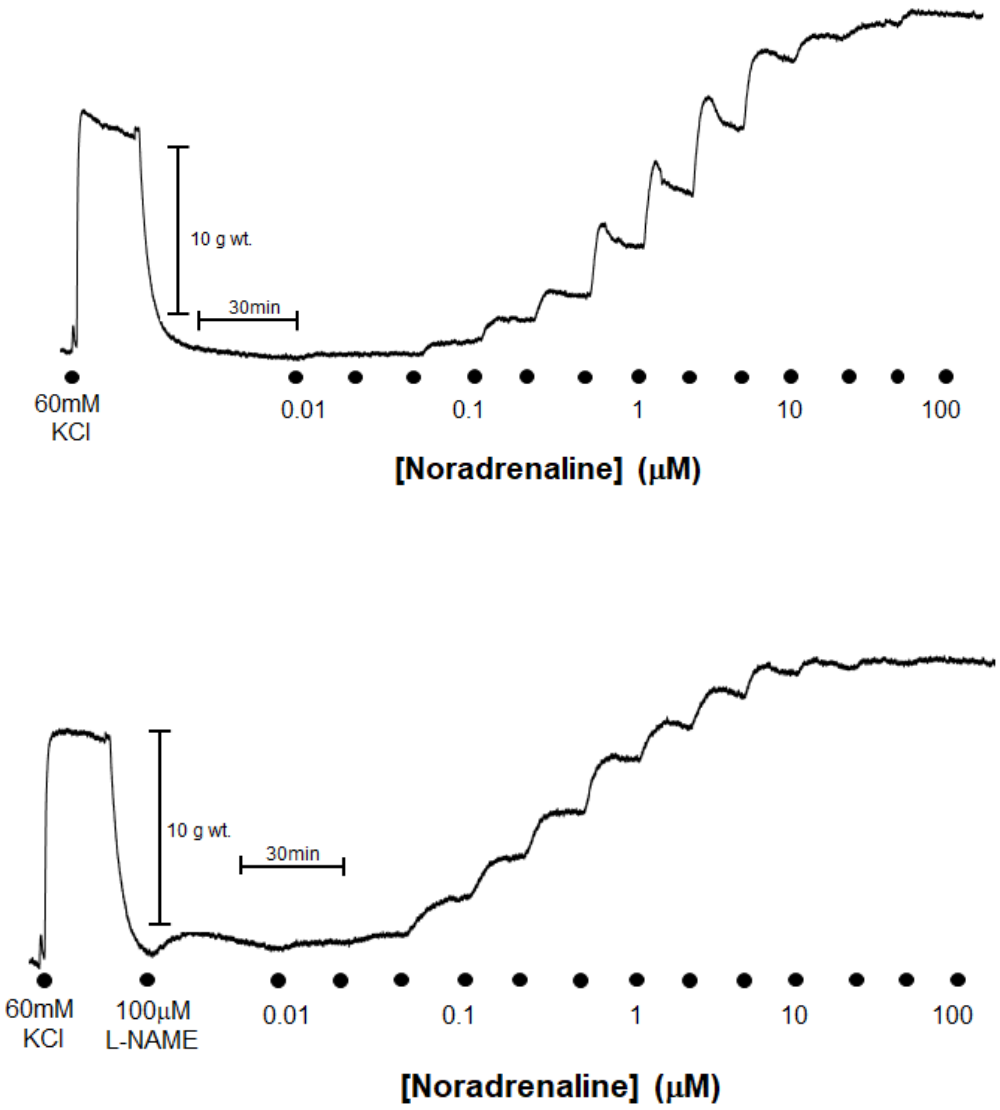


Figure 5.6 Isometric tension recording trace showing the response of the porcine splenic artery to 60mM KCl followed by: (Upper) the response to a cumulative concentration of noradrenaline added on the basal tone, (Lower) 100nM L-NAME then the preparations were exposed to increasing cumulative concentrations of noradrenaline. Effects of noradrenaline in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol. The vertical bar is equivalent to 10 g wt. and the horizontal bar denotes 30 minutes duration.

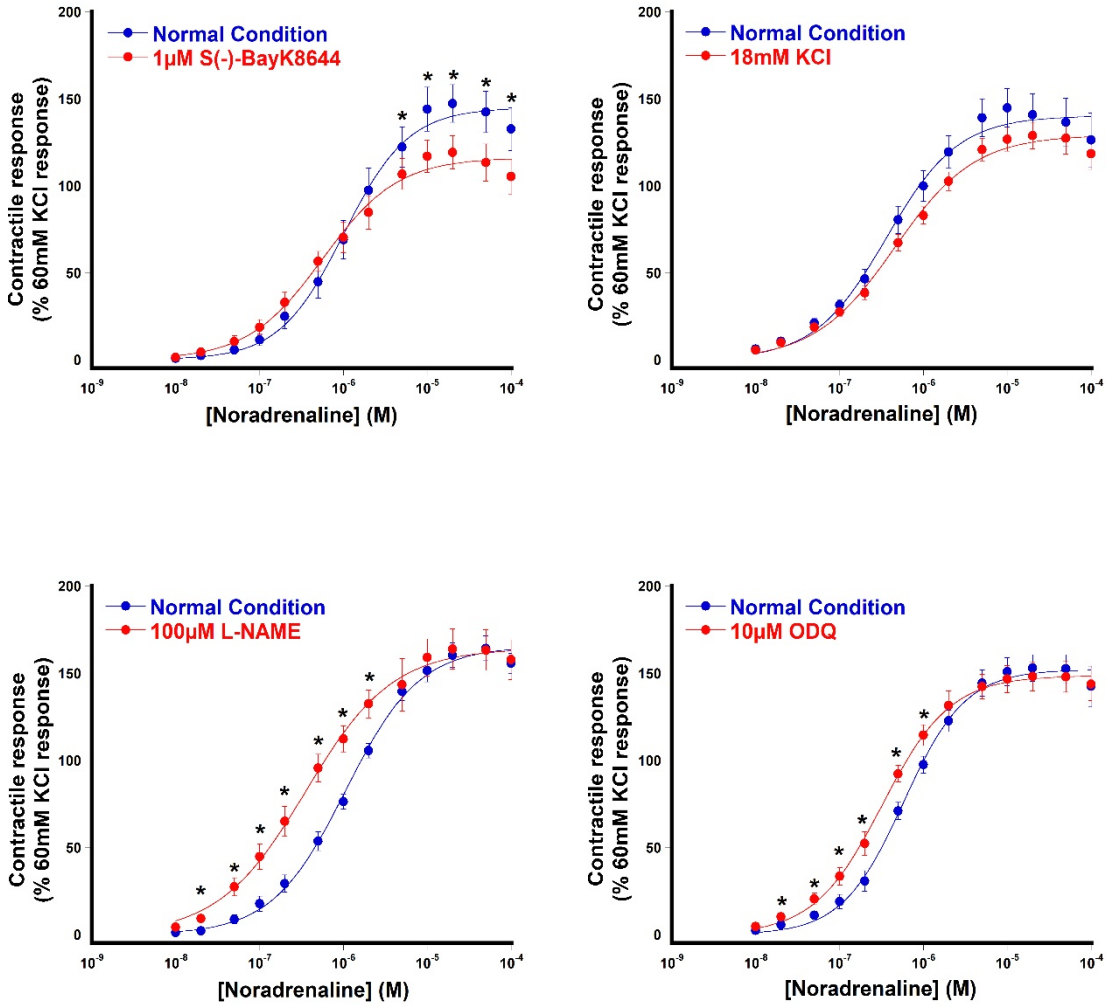


Figure 5.7 Response of porcine splenic artery to increasing cumulative concentrations of noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) in normal condition and after pharmacological manipulations with various vasoconstrictors including 1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of 6-10 observations.

*$P < 0.05$, Student's paired *t*-test.

Table 5.4 Quantitative parameters of the concentration-response curves of the porcine splenic artery to noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) in normal condition and after addition of 1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ. The table shows the maximum response and the negative logarithm of EC_{50} (pD_2) as a percentage of a former response to 60mM KCl. Results were expressed as the mean ± S.E.M of 6-10 observations.

* ($P < 0.05$) and ** ($P < 0.01$) – denote a statistically significant difference from response control segments, Student's paired *t*-test.

	n	Maximum Response (% of 60mM KCl)	pD_2
Control	10	156 ± 5	5.90 ± 0.1
1μM S(-)-BayK8644	10	127 ± 8**	6.11 ± 0.1*
Control	8	141 ± 3	6.40 ± 0.02
18mM KCl	8	130 ± 6	6.21 ± 0.05*
Control	6	165 ± 6	5.90 ± 0.06
100μM L-NAME	6	166 ± 2	6.41 ± 0.1**
Control	6	153 ± 8	6.20 ± 0.03
10μM ODQ	6	151 ± 9	6.50 ± 0.05**

5.3.4 Comparing the porcine isolated splenic artery responses to noradrenaline in the presence of an α_2-adrenoceptor antagonist after pharmacological manipulation

The present study set out to identify whether α_2-adrenoceptors were responsible for the contractile activity in the porcine splenic artery. Therefore, the following experiment manifests the specific role of α_2-adrenoceptor in the porcine splenic artery after pharmacological manipulation using various interventions, in which the action of non-selective adrenoceptor agonist (noradrenaline) was blocked in the second group using the highly selective α_2-adrenoceptor antagonist RX-811059 and compared with the control group. The first manipulation method was initiated with a U46619 contraction then sufficient relaxation with forskolin, porcine splenic artery showed concentration-dependent contractions to noradrenaline in both groups. However, the responses to noradrenaline still appeared to be resistant to blockade of α_2-adrenoceptors by RX-811059 in the porcine splenic artery (Figure 5.8).

Table 5.5 clarified that the highest responses and the negative logarithm of EC_{50} (pD_2) of the isolated porcine splenic artery were similar in both preparations which implied that RX-811059 had no significant effect on the response of the porcine splenic artery to noradrenaline in the presence of manipulation with U46619 and forskolin. In a similar manner, the classical noradrenaline-induced contraction with vasoconstrictors, in the presence of 30nM RX-811059, was assessed incomparable to the control. In general, 30nM RX-811059 had no significant impact following 40-45 minutes incubation on noradrenaline-induced contractions of the porcine splenic artery after addition of different kinds of tone inducers (Table 5.5 and figure 5.9).

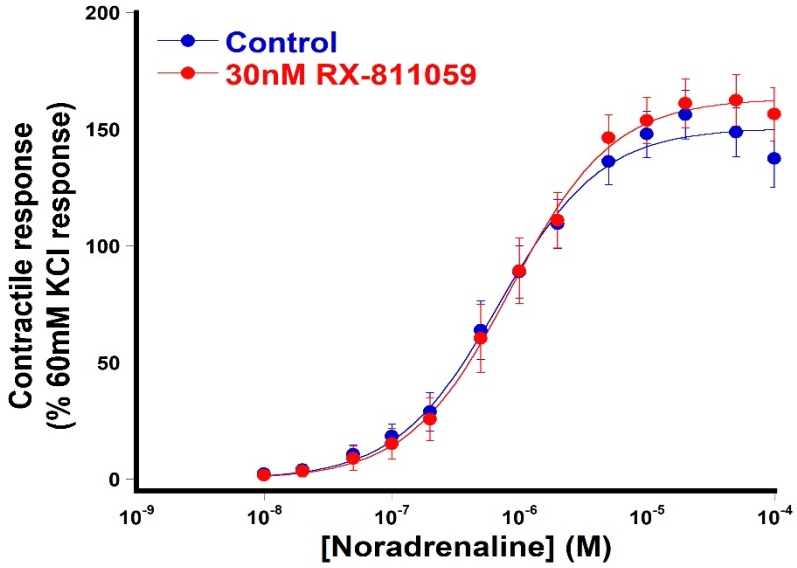


Figure 5.8 The effect of 30nM RX-811059 against a noradrenaline-induced contraction of the porcine isolated splenic artery (in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol) with the ancillary vasoactive agents U46619 and forskolin. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of eight observations.

Table 5.5 Quantitative parameters of the concentration-response curves of the porcine splenic artery to noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) after pharmacological manipulation using the ancillary vasoactive agents U46619 and forskolin, 1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ in absence and presence of 30nM RX-811059. The table shows the maximum response and the negative logarithm of EC_{50} (pD_2) as a percentage of a former response to 60mM KCl. Results were expressed as the mean ± S.E.M of 6-10 observations.

		n	Maximum Response (% of 60mM KCl)	pD_2
U46619 and forskolin	**Control**	**8**	**146 ± 8**	**6.16 ± 0.08**
	30nM RX-811059	**8**	**153 ± 7**	**6.07 ± 0.1**
1μM S(-)-BayK8644	Control	8	158 ± 2	5.90 ± 0.08
	30nM RX-811059	8	160 ± 2	5.90 ± 0.03
18mM KCl	**Control**	**10**	**139 ± 2**	**6.40 ± 0.04**
	30nM RX-811059	**10**	**140 ± 2**	**6.30 ± 0.06**
100μM L-NAME	Control	8	150 ± 1	6.60 ± 0.04
	30nM RX-811059	8	152 ± 1	6.40 ± 0.06
10μM ODQ	**Control**	**6**	**207 ± 1**	**6.50 ± 0.09**
	30nM RX-811059	**6**	**191 ± 2**	**6.50 ± 0.09**

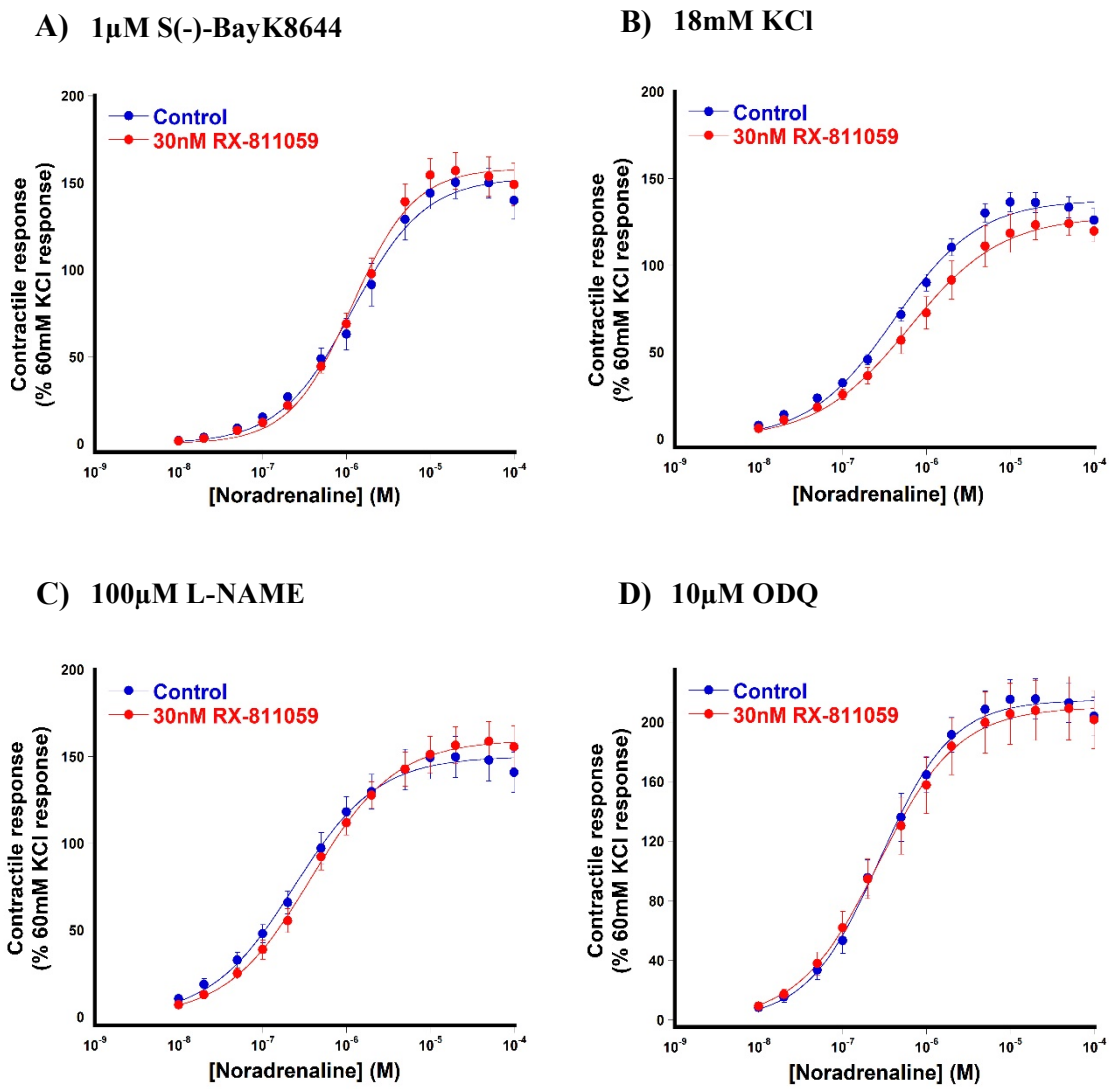


Figure 5.9 The effect of 30nM RX-811059 against a noradrenaline-induced contraction of the porcine isolated splenic artery (in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol) with various vasoconstrictors including (A) 1µM S(-)-BayK8644, (B) 18mM KCl, (C) 100µM L-NAME and (D) 10µM ODQ. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of 6-10 observations.

5.3.5 Comparing the porcine isolated splenic artery responses to noradrenaline in the presence of antagonists after pharmacological manipulation

This set of experiments performed using the same protocols of pharmacological manipulation using variable agents. However, in the first group after manipulation using the ancillary vasoactive agents U46619 and forskolin, the action of noradrenaline was antagonised with 30nM RX-811059 but in combination with 0.1μM prazosin (an α_1-adrenoceptor antagonist) and then compared to the response of the porcine splenic artery by blocking with 0.1μM prazosin alone.

The present results showed that the responses of the non-peripheral blood vessels were affected significantly by the antagonism using combined blockers incomparable to antagonism with 0.1μM prazosin only, which was seen obviously at 5-50μM concentration of noradrenaline based on comparing the maximum response of each single point ($P < 0.05$) (Figure 5.10). Yet, the contraction at 100μM noradrenaline of the response to 60mM KCl in both segments were similar (Table 5.6). The pD_{20} value was significantly inhibited by the combined form of the antagonists compared to 0.1μM prazosin. This remarkable result revealed the presence of α_2-adrenoceptors, which are involved in the contraction of the porcine splenic artery after pharmacological manipulation using U46619 and forskolin.

Concerning pharmacological manipulation using the vasoconstrictors, the present study showed that antagonism with 0.1μM prazosin in comparison to the combined forms of antagonists (0.1μM prazosin with 30nM RX-811059) caused a non-significant inhibition in the efficacy and pD_{20} values of noradrenaline concentration-response curves (Table 5.6 and figure 5.11).

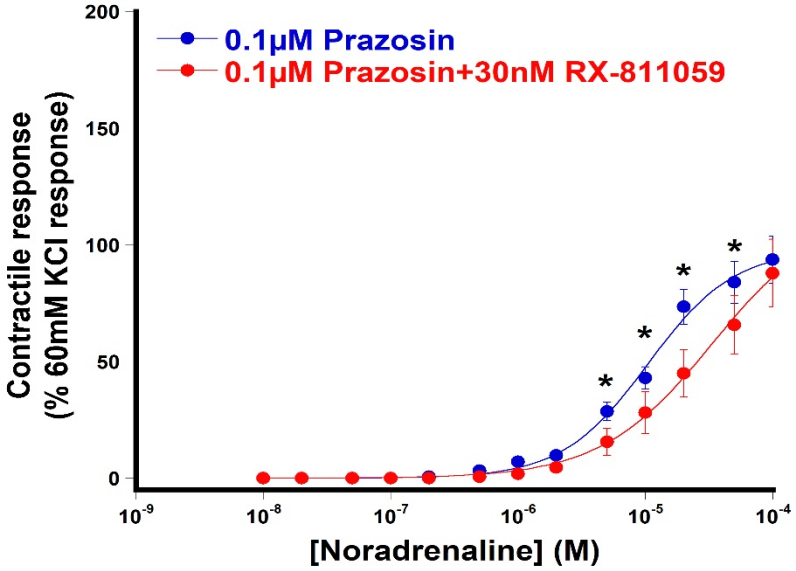


Figure 5.10 The effect of 0.1µM prazosin and a combination of the antagonists (0.1µM prazosin and 30nM RX-811059) against a noradrenaline-induced contraction of the porcine isolated splenic artery (in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol) with the ancillary vasoactive agents U46619 and forskolin. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of eight observations.

*$P < 0.05$, Student's paired *t*-test.

Table 5.6 Quantitative parameters of the concentration-response curves of the porcine splenic artery to noradrenaline (in the presence of 10μM cocaine, 30μM corticosterone and 1μM propranolol) after pharmacological manipulation using the ancillary vasoactive agents U46619 and forskolin, 1μM S(-)-BayK8644, 18mM KCl, 100μM L-NAME and 10μM ODQ in the presence of prazosin and a combination of 0.1μM prazosin and 30nM RX-811059. The table shows the response at 10μM brimonidine and the negative logarithm of EC_{20} (pD_{20}) as a percentage of a former response to 60mM KCl. Results were expressed as the mean ± S.E.M of 6-10 observations.

** ($P < 0.01$) – denotes a statistically significant difference, Student's paired *t*-test.

		n	Response at 100μM brimonidine (% of 60mM KCl)	pD_{20}
U46619 and forskolin	**0.1μM Prazosin**	**8**	**93 ± 8**	**5.44 ± 0.06**
	0.1μM Prazosin + 30nM RX-811059	**8**	**84 ± 9**	**5.05 ± 0.14****
1μM S(-)-BayK8644	0.1μM Prazosin	8	92 ± 5	5.30 ± 0.19
	0.1μM Prazosin + 30nM RX-811059	8	84 ± 4	5.20 ± 0.05
18mM KCl	**0.1μM Prazosin**	**10**	**111 ± 5**	**6.22 ± 0.14**
	0.1μM Prazosin+ 30nM RX-811059	**10**	**100 ± 2**	**6.15 ± 0.09**
100μM L-NAME	0.1μM Prazosin	8	129 ± 4	6.19 ± 0.19
	0.1μM Prazosin+ 30nM RX-811059	8	124 ± 3	6.18 ± 0.30
10μM ODQ	**0.1μM Prazosin**	**6**	**165 ± 4**	**6.06 ± 0.10**
	0.1μM Prazosin+ 30nM RX-811059	**6**	**164 ± 6**	**5.96 ± 0.14**

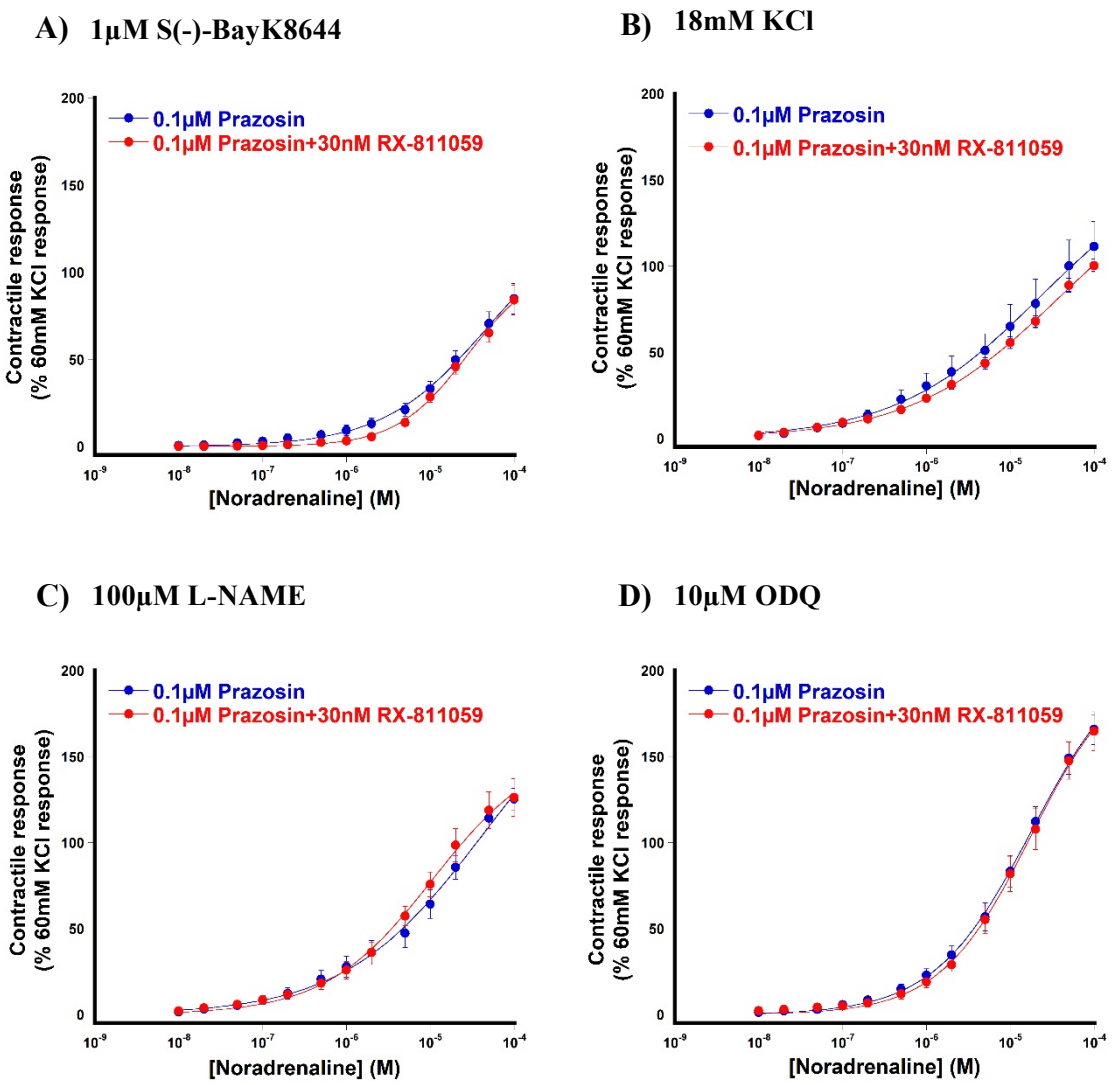


Figure 5.11 The effect of 0.1µM prazosin and a combination of the antagonists (0.1µM prazosin and 30nM RX-811059) against noradrenaline-induced contraction of the porcine isolated splenic artery (in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol) with various vasoconstrictors including (A) 1µM S(-)-BayK8644, (B) 18mM KCl, (C) 100µM L-NAME and (D) 10µM ODQ. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as mean ± S.E.M of 6-10 observations.

5.4 Discussion

The present chapter follows on from earlier work in our laboratory (Roberts *et al*., 1999; Denfria, 2016) in which post-junctional α_2-adrenoceptors mediating contractile responses were evident in the porcine splenic artery. Moreover, Wright *et al.*, (1995a) confirm that the porcine splenic artery contains α_2-adrenoceptors, which are contributing to their contractile responses. However, in all conditions α_2-adrenoceptors were demonstrated only if the former preparation was pharmacologically manipulated by an intervention requiring precontraction with U46619 and subsequent relaxation with forskolin. It is now possible that this study will provide further novel methods for uncovering the action of α_2-adrenoceptors mainly in the porcine splenic artery. Therefore, it has been recognised from the previous results (Wright *et al*., 1995a; Roberts *et al*., 1999) and even from the previous chapter that standard *in vitro* condition for isolated blood vessels often underestimates α_2-adrenoceptor-mediated constriction.

α_2-Adrenoceptors have dissimilar signal transduction pathway from α_1-adrenoceptors. α_1-Adrenoceptors bind and activate $G_{q/11}$-coupled receptors and thus initiate phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5, biphosphate to produce inositol trisphosphate that releases intracellular calcium, and diacylglycerol that acts with calcium to activate protein kinase C (Zhong *et al.*, 1999). While, α_2-adrenoceptors bind and activate $G_{i/o}$-coupled receptors (MacDonald *et al.*, 1997). Consequently, activation of α_2-adrenoceptors causes inhibition of both adenylate cyclase and cAMP levels. Yet, α_2-adrenoceptors activation did not affect the contractility of the blood vessels when the level of cAMP is at its basal level (Wright *et al.*, 1995b). Thus, elevating the level of cyclic AMP with forskolin could intensify the inhibition of cAMP level caused by activation of α_2-adrenoceptors and could

uncover the involvement of α_2-adrenoceptors in the contraction response when stimulated (Wright *et al.*, 1995b).

Based on these facts, a key part of this study has been to manipulate pharmacologically responses of the porcine splenic artery to allow better expression of responses. For example, a number of experiments with various methodological interventions using vasoconstrictor agents, which are assumed to increase the intracellular calcium ions and alter the cAMP level and furthermore to eliminate the inhibitory effect of nitric oxide, were conducted. The target for these experiments was to examine the vasoactive properties of the imidazoline-based α_2-adrenoceptor agonist (brimonidine) and the phenethylamine derivative (noradrenaline) as a non-selective α-adrenoceptor agonist in the porcine splenic artery and the impact of the density of vascular α_2-adrenoceptors in determining the biological activity of agonists. These examinations also have been based on an appreciation of the clinical use of α_2-adrenoceptor agents for a variety of clinical conditions like a new topical formulation of decongestant.

From a therapeutic point, if a combination of α_2-adrenoceptor agents with another vasoconstrictor agent causes a synergistic effect on topically applied decongestants by topical addition of those substances in a considerable clinical approved concentration that actually could enhance their effectiveness.

The effect of various interventions on responses to brimonidine and the effect of antagonists on those responses:

This study has shown as expected the presence of the combination of the thromboxane-mimetic U46619 and forskolin significantly enhanced contractions to brimonidine and noradrenaline in the porcine splenic artery preparations.

Based on these results and the fact that pharmacological manipulation will uncover the involvement of α_2-adrenoceptors to the contractile response of the porcine splenic artery (Roberts *et al.*, 1999). Confirmation and a strong indication of the involvement of α_2-adrenoceptors in the responses of the porcine splenic artery to brimonidine were provided by the routine inclusion of various concentrations of prazosin to prevent the activation of α_1-adrenoceptors. The finding that neither 0.01 nor 0.1μM prazosin caused a significant change in the sensitivity of the porcine splenic artery to brimonidine. Yet, 1μM prazosin was able to reduce the porcine splenic artery sensitivity by 5-fold suggests the involvement of α_2-adrenoceptors binding sites, which can be seen only at high concentration of α_1-adrenoceptor antagonism after pharmacological manipulation with U46619 and forskolin. Similarly, antagonism with 0.1μM RX-811059 in combination with 0.1μM prazosin allowed better expression of the α_2-adrenoceptors with pK_B=9.

A large and growing body of literature has found that α_2-adrenoceptor-mediated contractions could be aggravated by the presence of other methods of pharmacological intervention like vasoconstrictor agents (Dunn *et al.*, 1991; Roberts *et al.*, 1999). (see table 5.1). Moreover, these studies are highly dependent on the presence of extracellular calcium ions (Jim and Matthews, 1985; Daly *et al.*, 1990; Szadujkis-Szadurska *et al.*, 2013). Therefore, I used several substances that have been known to increase intracellular calcium ions. However, it is unlikely that S(-)-BayK8644 is implicated in the vasoconstriction responses of brimonidine-induced contractions in the porcine splenic artery as the efficacy and even the potency of the response to 60mM KCl were not significantly affected by the presence of this vasoconstrictor. The second vasoconstrictor (18mM KCl) demonstrated a significant enhancement of brimonidine concentration-responses curve and also in the potency value after inducing

depolarisation with raised extracellular KCl, this raises the possibility of a better expression of responses produced by 18mM KCl.

Nitric oxide (NO) is invented to raise cGMP level by the production of a soluble guanylate cyclase present in the smooth muscle cells. Thus, NO-stimulated activation of cGMP may reduce the vascular smooth muscle responses to exogenous catecholamines (Chies *et al*., 2003). The most commonly used nitric oxide synthase inhibitor is L-NAME (Schroeder and Kuo, 1995; Vitecek *et al.*, 2012). Several observations concluded a variation in the nasal congestion status happened simultaneously to changes in the nitric oxide, especially with one of its regulating molecules (ADMA) levels (Ohike *et al.*, 2005; Fidan *et al.*, 2012). However, this variation was thought to participate in the regulation of nasal blood flow.

Working on these bases, I examined and compared the influence of nitric oxide manipulation in the isolated porcine splenic artery using selective α_2-adrenoceptor (brimonidine). I anticipated that when basal blockage of the nitric oxide production in the ring preparations achieved by L-NAME (Al-Zobaidy *et al.*, 2011), then a spontaneous contraction of the porcine splenic artery (Lot *et al.*, 1993) or a higher sensitivity to directly or indirectly sympathomimetic drugs will detect (Dieguez *et al.*, 1998). The results of this study agree with the expectation that after incubation with 100μM L-NAME, the contraction of the porcine splenic artery to brimonidine clearly showed a significant enhancement in the efficacy and potency. An augmentation in the brimonidine-induced contractions of the porcine splenic artery can be seen with the presence of a specific inhibitor of soluble guanylyl cyclase, ODQ (Schrammel *et al.*, 1996), which improve the brimonidine efficacy and potency.

The effect of various interventions on responses to noradrenaline and the effect of antagonists on those responses:

Pre-treatment of the porcine splenic artery with S(-)-BayK8644 showed an alteration of noradrenaline-induced contractions and S(-)-BayK8644 did not facilitate the receptor-response coupling of non-selective α-adrenoceptor agonist via α_2-adrenoceptors, rather than via α_1-adrenoceptors. However, several available studies demonstrated that S(-)-BayK8644 had a superior influence on the potentiation of α_2-adrenoceptor responses (Cheung, 1985; Eskinder and Gross, 1987; Lippton *et al.*, 1987; Dunn *et al.*, 1991; Aleixandre *et al.*, 1992). Also, S(-)-BayK8644 enhanced vasoconstrictions in the feline mesenteric artery (Lippton *et al.*, 1987), the canine saphenous artery (Sulpizio and Hieble, 1987), and the rat, rabbit and canine saphenous veins (Cheung, 1985; Eskinder and Gross, 1987; Dunn *et al.*, 1991). S(-)-Bayk8644 similarly improved the potency and maximum response to α_1-adrenoceptor agonists in rat vessels (Wilffert *et al.*, 1984) and human hand vein (Arner *et al.*, 1988). This result is in agreement with our result, which showed a significant increase in the pD_2 value.

Yet, Dunn and co-workers (1991) showed that S(-)-BayK8644 did not affect noradrenaline-induced contractions in the rabbit ear vein, which contained a large population of α_2-adrenoceptors and the basis of the discrepancy between these studies is unclear.

There was a lack of effect by a depolarisation mechanism using 18mM KCl on noradrenaline-induced contractions of the porcine splenic artery. This finding does not support our current results with brimonidine using 18mM KCl. Similarly, previous studies showed a significant role of depolarisation by KCl in promoting the noradrenaline release in several blood vessels (Kirpekar and Wakade, 1968). It seems

possible that these studies used a higher concentration of KCl (Gibson and Pollock, 1973; Blaustein, 1975; Araujo and Bendhack, 2003). Therefore, it is possible that the concentration of KCl used in the present study is not quite enough to enhance contractions to endogenous non-selective α-adrenoceptor noradrenaline.

Neither L-NAME nor ODQ enhanced the mean maximum response to noradrenaline in the porcine splenic artery. This finding was consistent with several published studies, which showed that L-NAME inhibited noradrenaline-evoked contractions in the rat intact aortic rings (Stanke-Labesque *et al*., 2003). This also accords with the observations, which showed that neither L-NAME nor ODQ altered the vasoconstrictor response in the bovine supernumerary arteries (Bunton *et al*., 2000). However, the most interesting finding was a significant positive correlation between every single point of the maximum response and the modulation of the vascular tone using L-NAME and ODQ. This outcome improved that removal of the inhibitory influence by NO using either L-NAME or ODQ caused significant activation of the α_1-adrenoceptors role in the porcine splenic artery. Similarly, the pD_2 values were improved by L-NAME and ODQ in this non-peripheral blood vessel.

It was anticipated that pharmacological manipulation using a combination of U46619 and forskolin would make the response of the porcine splenic artery to noradrenaline more sensitive to the α_2-adrenoceptor antagonist RX-811059. However, surprisingly antagonism with RX-811059 has no significant inhibition to the noradrenaline response using this latter method of pharmacological manipulation. Similarly, the responses of noradrenaline-induced contractions with vasoconstrictors in the porcine splenic artery were not influenced by the presence of 30nM RX-811059. This result confirms that the role of α_2-adrenoceptors cannot reveal contractions to noradrenaline in the porcine

splenic artery using α_2-adrenoceptor antagonist alone (Berridge and Roach, 1986; Blaylock and Wilson, 1995) even after enhancing the vascular tone via various pharmacological interventions.

The most interesting finding was largely based on the ability of RX-811059 to inhibit significantly the pD_{20} value of the splenic artery to noradrenaline when combined with prazosin compared to antagonism with prazosin only, which provide strong evidence for the contribution of α_2-adrenoceptors in the responses of the porcine splenic artery after preconstruction with U46619 and subsequent relaxation with forskolin. Additionally, comparing the maximum response for every single point showed significant changes, especially seen at high concentration of noradrenaline (5-50μM). While antagonism of α_1-adrenoceptors with 0.1μM prazosin compared to using both α_1-adrenoceptor and α_2-adrenoceptor blockers resulted in substantial inhibition of noradrenaline response at 100μM of the response to 60mM KCl in the porcine splenic artery when manipulated with various vasoconstrictors. Additionally, no significant changes in the potency were observed with all vasoconstrictors after blocking with these antagonists.

As a summary, the results of the present chapter confirmed that the action of these vasoconstrictors are quite different with selective and non-selective α-adrenoceptors in the porcine splenic artery as their effects were more pronounced on the contraction at the highest concentration of brimonidine while with noradrenaline it was evident on the potency values. The key outcomes from this chapter found that using many interventions, which either raised calcium or removed the inhibitory influence of NO enhanced the α_1-adrenoceptor contractile responses in the porcine splenic artery for

both brimonidine and noradrenaline, but α_2-adrenoceptors responses are revealed via raising both calcium and cAMP levels for both brimonidine and noradrenaline agonists.

This chapter demonstrated that the α_2-adrenoceptors could be present and involved in the response of the porcine splenic artery to brimonidine and to the same extent that its involvement to noradrenaline-induced contraction responses in the same blood vessels. The pattern of finding that using different vasoconstrictors were less potent than using a combination of U46619 and forskolin in providing evidence of α_2-adrenoceptors in the splenic artery of the pig. Moreover, the vasoconstrictor properties of clinically used α_2-adrenoceptor agonist and the endogenous ligand noradrenaline are better manifests *in vitro* if cellular cyclic AMP is elevated, was perhaps surprising since all substances enhanced the vascular tone to the same extent.

Another important unpublished data from our laboratory (V.G. Wilson) showed that the sensitivity of superficial vessels (palmar lateral vein, tail artery and digital artery) to brimonidine in the presence of forskolin and U46619 was approximately 5-fold greater than central vessels (splenic and rectal artery), supporting the idea that selective α_2-adrenoceptors agonists have the potential to move blood from peripheral to central organs. Similar data confirmed that the potency value for brimonidine in the porcine nasal artery after manipulation with U46619 and forskolin 7.08 ± 0.32 (n=5) (Denfria, 2016) was higher than that detected in this chapter in the porcine splenic artery 6.4 ± 0.19 (n=6) by 5-fold. This profile of action, which is critically dependent on the distribution of α_2-adrenoceptors in the vasculature, could be of importance in countering the development of nasal decongestants. Examination of the effect of pharmacological manipulation using (U46619 and forskolin) in the porcine nasal artery with the endogenous ligand noradrenaline was also performed and the results showed

an enhancement of the concentration-dependent contractions in the nasal arterial preparations. The maximum response to noradrenaline

was increased significantly after this manipulation in the porcine nasal artery (control 101 ± 6%; U46619 and forskolin 127 ± 11%, $P < 0.05$, n=7). The same pattern of improvement in the potency value of the porcine splenic artery by 3-fold is detected in the porcine nasal artery after interventions with U46619 and forskolin, which also significantly increased the potency to noradrenaline from 6.07 ± 0.07 to 6.53 ± 0.04 ($P < 0.001$, n=7) (Appendix 4).

Appendix 5 showed the effect of antagonism with 30nM RX-811059 in the porcine nasal artery after manipulation the responses to noradrenaline with U46619 and forskolin still appeared to be resistant to the blockade of α_2-adrenoceptors by RX-811059. However, the same appendix showed that the mean maximum responses of the porcine nasal artery were affected significantly by the antagonism using combined blockers incomparable to antagonism with 0.1μM prazosin only. Yet, the pD_2 was similar in both groups. Considerably more work needs to be done to determine the current topics and future studies with more focus on the porcine nasal artery with the other methods of interventions using the vasoconstrictors are therefore suggested. Based on the present outcome, we could not easily reintroduce new decongestant medications by using the clinically accepted concentration of vasoconstrictor substances (e.g. S(-)-BayK8644, KCl, L-NAME and ODQ) along with α_2-adrenoceptor agents to improve approaches of activity or duration of the usual precise therapies. A reasonable approach to tackle more understanding of the works of nasal decongestant in the present study could be to use a different technique and intervention in the following chapter.

Chapter Six

The effects of lithium ions on porcine blood vessels and perfusate nasal mucosa

6 The effects of lithium ions on porcine blood vessels and perfusate nasal mucosa

6.1 Introduction

In the previous chapters, work concentrated on the pharmacological characteristics of the porcine blood vessels. The final part of this thesis gives an account to increase the understanding of the vital role of the nasal decongestant medications, which act as α-adrenoceptor agonists using the porcine blood vessels and the nasal mucosa. It has been reported previously, that the action of the decongestant particularly α-adrenoceptor agonist involves contraction of the vascular smooth muscle, attributable to a cascade of intracellular sequences following receptor activation. These events are assumed to contain activation of the phosphatidylinositol system, which causes a generation of inositol trisphosphate and the release of intracellular calcium from the sarcoplasmic reticulum then finally subsequent admittance of extracellular calcium to aid sustain the vascular contraction (Villalobos-Molina *et al.,* 1982; Hicks *et al.,* 1991).

These actions are in keeping with the use of phenylephrine as a contractile agent for the evaluation of endothelium-dependent and endothelium-independent vasodilator substances *in vitro* (McCulloch *et al*., 1997; Novakovic *et al*., 2017). This is not true for all smooth muscles. However, in the sheep isolated internal anal sphincter, a preparation that is endowed with α_1-adrenoceptors and developed myogenic tone (Rayment *et al*., 2010); phenylephrine caused a remarkable contraction of the smooth muscle that was not sustained. In the latter case, contractions to maximally effective concentrations of the agonist declined by nearly 80% over 60 minutes (Rayment *et al.,* 2010).

In a similar manner, submaximal contractions of the porcine renal interlobar artery to phenylephrine declined by as much as 60% over 20 minutes (Derkach *et al*., 2000; Ihara *et al*., 2001).

Working on the basis that lithium ions are known to inhibit inositol monophosphatase and elevate the second messenger (IP_3) in the smooth muscle (Fox *et al.,* 1985), together with a study by Dehpour and co-workers (1993) showing that lithium ions enhance methoxamine (α_1-adrenoceptor agonist)-induced contractions of the rabbit anococcygeus smooth muscle. The latter study has been suggested that methoxamine influenced either the accumulation of inositol triphosphate and/or the availability of other active metabolites in the phosphatidylinositol cycle.

A recent study by Rayment *et al.,* (2010) reported that 0.5-3mM lithium ions prevented the decline in contractions to phenylephrine and methoxamine without changing either the potency of the agonists or the magnitude of the maximum response. Significantly, the effect of lithium ions in the sheep isolated internal anal sphincter was not mimicked by L-690,488, a synthetic inhibitor of inositol monophosphatase (Atack *et al*., 1994). However, the study of Mantelli *et al*., (1988) which concluded that lithium ions had an antagonistic effect on the inotropic effect induced by α_1-adrenoceptors in the guinea-pig ventricular myocardium, do not support the previous research mentioned so far, leaving unclear both the mechanism underlying the decline in responses to the agonists and the nature of the interaction with the cation. Additionally, it has been found that the action of many receptors such as muscarinic, α-adrenoceptor, vasopressin (V_1 receptor) and histamine receptor can interfere with lithium ions as these receptors act by stimulating intracellular calcium and affecting the inositol second messenger pathway to yield their responses (Putney, 1981).

Isometric tension recording studies are considered as a routine conducted method for studying the pharmacological actions of nasal decongestants in the isolated nasal mucosa (Corboz *et al.*, 2003; Varty *et al.*, 2004; Chu *et al.*, 2006; Corboz *et al.*, 2013). However, an alternative technique, intended to evaluate the porcine nasal mucosa and assess the pharmacological contractions of the nasal decongestants, is the perfusion of the porcine nasal mucosa. This technique involves a perfusate injected into a sample of the pig snout through the vasculature of the nasal mucosa with good observation of the backpressure. Variations in the pressure inside the perfusion system will represent variations in the nasal mucosal blood vessels volume. In comparison with the isometric tension recording studies, this method was not familiar and even more complicated. By way of illustration, the dissection of the forehead to cannulate the sphenopalatine artery was essential to perform the perfusion of the nasal mucosa of hooded seals (Folkow, 1992).

Depending on this multiple information, the present study aimed to follow the pharmacological methodology developed in our research laboratory by Denfria, (2016) to examine the response of the perfused porcine snout to different contractile agents and compare the action of the commonly used decongestant medications with that in the porcine blood vessels. Moreover, evaluate in-depth the time course of contractions to maximum, and sub maximum concentrations of the classical oral decongestant (phenylephrine). Additionally, based on the use of lithium ions as an analysis tool in studies interfere with the phosphatidylinositol cycle, the work described in this chapter will attempt to understand the impact of lithium ions by examining its selective effect on the potency and magnitude of the contractile agents in the porcine blood vessels and the perfused snout. Furthermore, in this study, we will

evaluate the effect of lithium on the time course of contractions to maximum and sub maximum concentrations of agonists in the porcine vasculature and the perfused snout. This chapter was undertaken to design a new topical formulation of nasal decongestant using for example phenylephrine with the topical incorporation of the therapeutic concentration of lithium ions to increase the duration or to improve the effectiveness of the former and the classically used medications.

6.1.1 Aims

The aims of this study were

1. To examine the effect of lithium ions on the potency and magnitude of various constrictor agents in the porcine isolated splenic and nasal artery.
2. To examine the time course of contraction to some constrictor agents in the porcine isolated splenic and nasal artery in the presence and absence of lithium ions.
3. To examine the consistency of the porcine nasal artery as a developed technique to perfuse the porcine snout and maintain a stable pressure.
4. To examine the response of the perfused porcine snout to various constrictor agents.
5. To examine the effect of lithium ions on the potency and magnitude of some constrictor agents in the perfused porcine snout.
6. To examine the time course of contraction to phenylephrine in the perfused porcine snout in the presence and absence of lithium ions.

6.2 Materials and Methods

6.2.1 Materials

Materials used for the experiments of this chapter are listed in table 2.1.

6.2.2 Methods

The collection of material and tissue preparation for contractility studies were described in chapter 2, section 2.1.1. Isometric tension measurement experiments were carried out in the same apparatus using the same buffer and the general protocol mentioned in chapter 2 (section 2.1.2). While, experiments related to the perfusion of the porcine snout, the protocol was mentioned in details in chapter 2 (section 2.3 and 2.3.1). In some experiment related either to an isometric tension recording system or to the perfusion of the porcine snout, the time course of contractions to the addition of a single maximally and sub-maximally effective concentration of an agonist was monitored for up to 60 minutes. Additionally, 1mM lithium sulphate was added either to one paired segment in isometric tension recording system experiments or to one unpaired segment in perfusion experiments for at least 40 minutes before vasoconstrictor agent was added.

6.2.3 Data analysis

Contractions produced by the agonists were expressed as milliNewtons forces (mN). Responses elicited by either the concentration-response curve and the time course protocol were expressed as a percentage of the contraction to 60mM KCl. All data were analysed using Synergy KaleidaGraph software as already mentioned in chapter 2, section 2.4. The logistic equation according to (Kaleidagraph version 4.5.2 Synergy software) was used to draw the best-fit curve for the data from which the following parameters were obtained: R max (maximum response to cumulative addition of an agonist), EC_{50} (concentration causing 50% of the maximum responses). The latter was used to determine the negative logarithm of the concentration required to produce 50% of the maximum response (pD_2).

All the results have been expressed as a mean ± standard error (S.E.M). The number of observations in different animals for each experiment was expressed as (n). In the majority of experiments, either a Student paired *t*-test was used to compare differences between two groups related to the same animal or a Student unpaired *t*-test in case of perfusion experiments was used to compare differences between two unrelated animal groups. A *P* value of less than 0.05% was considered statistically significant.

6.3 Results

6.3.1 The effect of lithium ions on the cumulative concentration of various constrictor agents in porcine blood vessels

In the porcine isolated splenic artery and nasal artery, 60mM KCl produced a sustained contraction equivalent to 14.1 ± 0.8 g wt. (n=30) and 8.1 ± 1 g wt. (n=24), respectively. To illustrate the effect of lithium ions in the porcine isolated splenic artery and the porcine isolated nasal artery, a series of the experiment were carried out using a number of agonists. Cumulative addition of phenylephrine, metaraminol, guanfacine, and xylometazoline caused concentration-dependent contractions of the porcine isolated splenic artery and the porcine isolated nasal artery. For all agonists, responses to less than 1μM were generally slow in onset requiring at least 10 minutes to reach equilibrium, while for phenylephrine contractions at 3μM and above often comprised a rapid increase in tension over 3 minutes followed by a decline over the subsequent 10 minutes period of exposure. In particular, at the highest concentrations of phenylephrine (10-30μM), contractions declined towards baseline over a 30 minutes period of continued exposure (Figure 6.1). In contrast, contractions to all concentrations of guanfacine, xylometazoline, and metaraminol slowly developed over 10 minutes and often required up to 15-20 minutes to attain equilibrium.

In order to address the influence of lithium ions on the vascular activity, 1mM lithium sulphate was added which did not affect the basal tension of either the porcine isolated splenic artery or the porcine isolated nasal artery, but in the presence of this cation all agonists produced slow-developing, sustained contractions to cumulatively increasing concentrations (Figure 6.2 and 6.3). Table 6.1 and 6.2 clarified that the incubation of the porcine splenic artery and nasal artery preparations with 1mM lithium sulphate were

associated with a significant increase in the maximum response to phenylephrine, metaraminol and guanfacine in both preparations. While there was no significant difference in the maximum response to xylometazoline in the porcine splenic artery and nasal artery preparations.

What is interesting in this data is that the effect of lithium ions was more pronounced in the porcine splenic artery in compared with the nasal artery, especially for guanfacine agent. It is apparent from the tables that on average, lithium ions were shown to cause a reduction in the estimated potency of all agonists in both preparations. A significant decline in the pD_2 values was observed with phenylephrine and guanfacine in both preparations. Also, a decline was detected in the pD_2 values with xylometazoline in the splenic artery and metaraminol in the nasal artery. The most striking result to emerge from Table 6.1 and 6.2 is that metaraminol and xylometazoline were the most potent agonists in the porcine nasal artery and showed greater efficacy in comparison with other agonists, especially the classic widely used oral decongestant (phenylephrine) even without lithium ions.

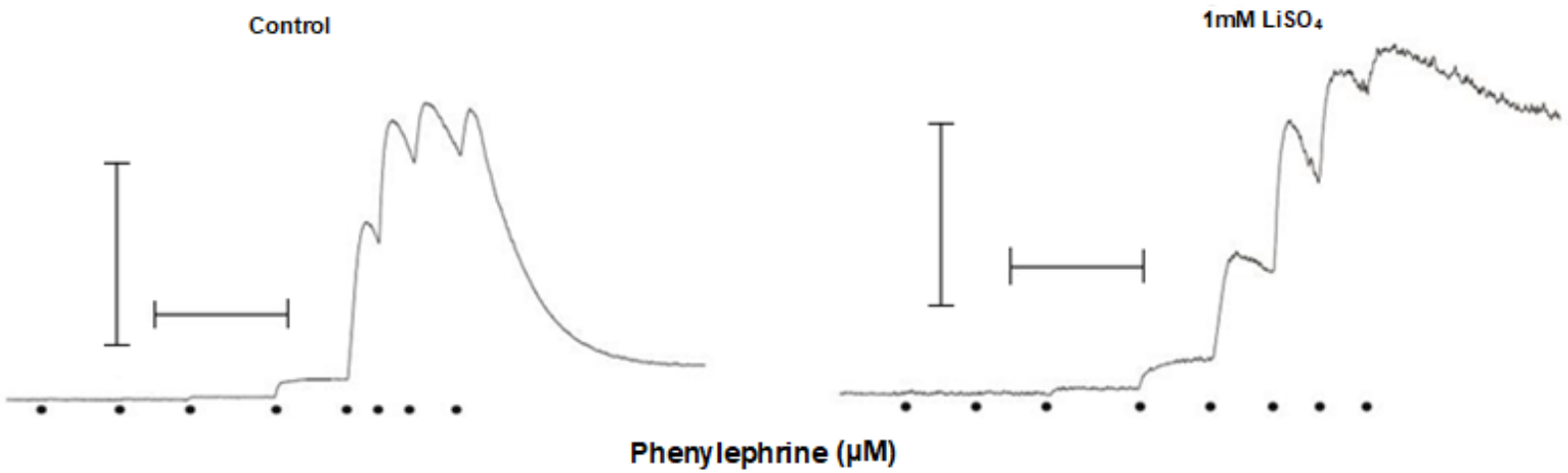


Figure 6.1 Isometric tension measurement trace shows the response of porcine isolated splenic artery to cumulative concentration of (0.01µM-30µM) of phenylephrine in the presence and absence of lithium ion. The vertical bar is equivalent to the magnitude of 60mM KCl, while the horizontal bar is the equivalent of 30 minutes.

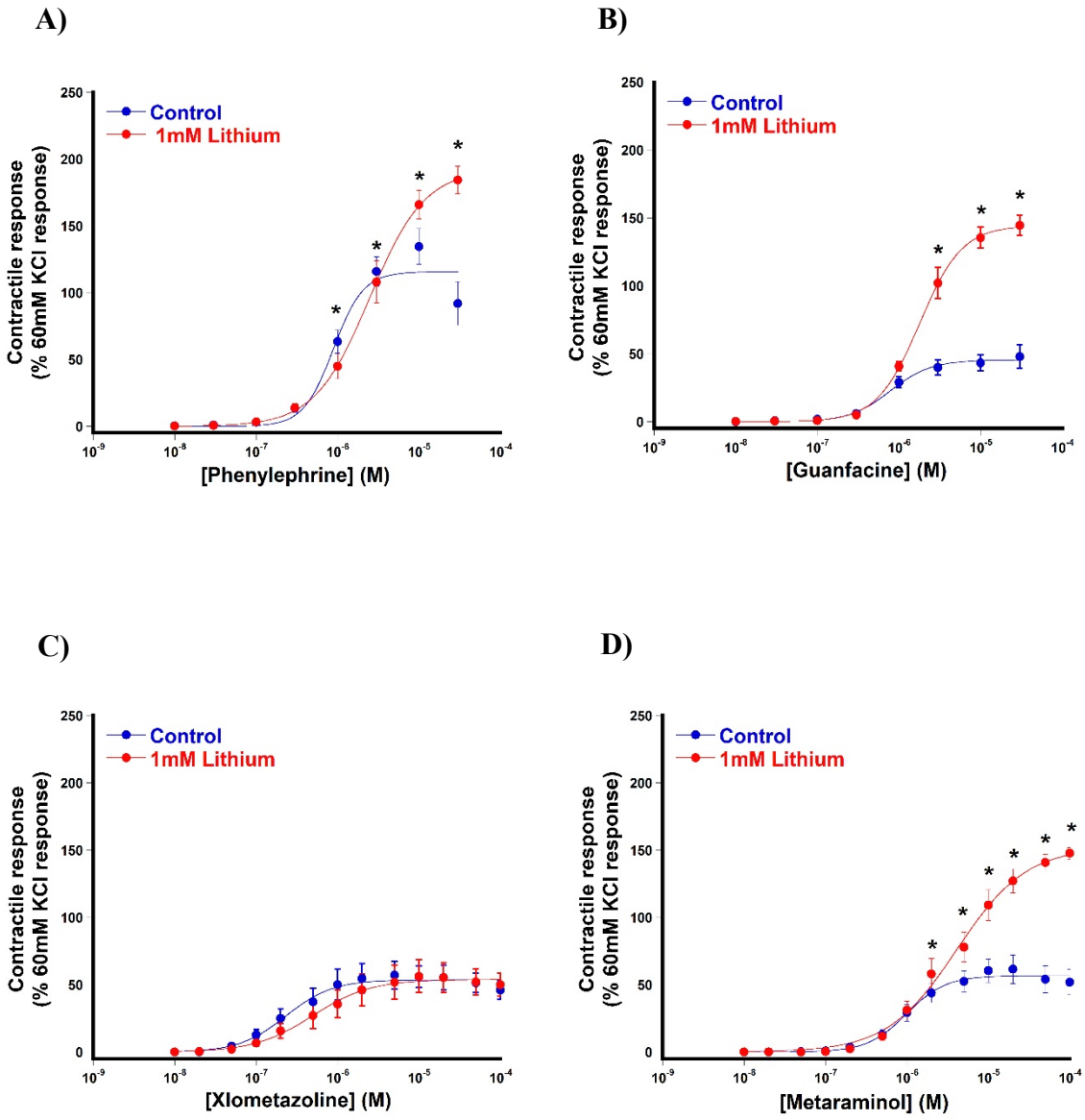


Figure 6.2 The effect of 1mM lithium sulphate on the cumulative concentration-response curves to (A) phenylephrine (B) guanfacine (C) xylometazoline and (D) metaraminol in the porcine isolated splenic artery in presence and absence of 1mM lithium sulphate. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of 6-8 observations.

*$P < 0.05$, Student's paired *t*-test.

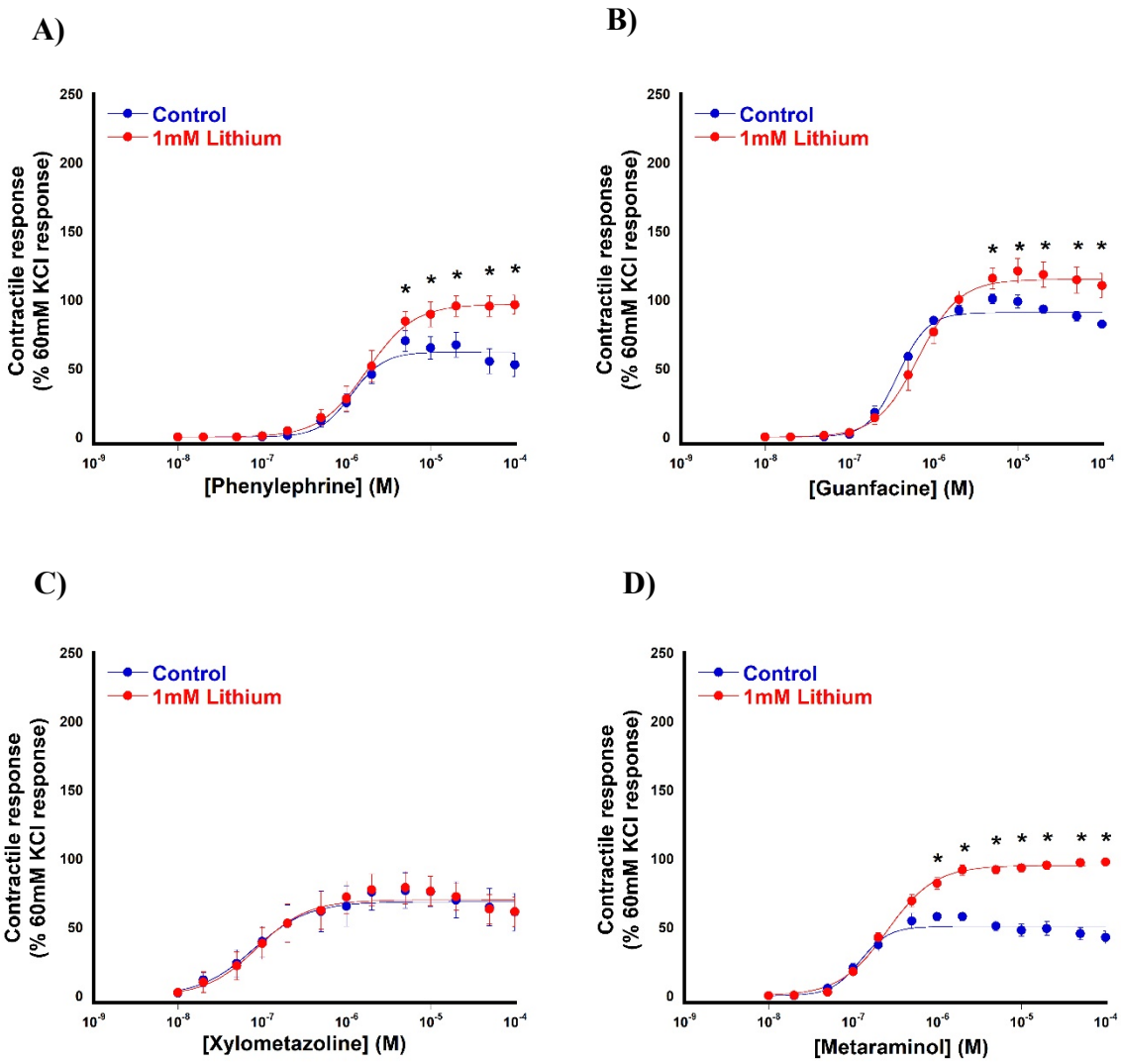


Figure 6.3 The effect of 1mM lithium sulphate on the cumulative concentration-response curves to (A) phenylephrine (B) guanfacine (C) xylometazoline and (D) metaraminol in the porcine isolated nasal artery in presence and absence of 1mM lithium sulphate. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of 5-7 observations.

*$P < 0.05$, Student's paired *t*-test.

Table 6.1 Comparison of the potency (pD_2) and maximum response to various α-adrenoceptor agonists in the porcine isolated splenic artery in the presence and absence of 1mM lithium sulphate. Results were determined as a percentage of a former response to 60mM KCl and were expressed as the mean ± S.E.M of 6-8 observations.

** ($P < 0.01$) and *** ($P < 0.001$) – denote a statistically significant difference in the presence and absence of 1mM Lithium Sulphate, Student paired *t*-test.

	n	Control		1mM Lithium Sulphate	
		Maximum Response (% of 60mM KCl)	pD_2	Maximum Response (% of 60mM KCl)	pD_2
Phenylephrine	6	117 ± 12	6.00 ± 0.03	193 ± 10**	5.63 ± 0.08**
Metaraminol	8	79 ± 9	5.50 ± 0.3	155 ± 7***	5.40 ± 0.1
Guanfacine	8	47 ± 8	6.10 ± 0.08	147 ± 7***	5.70 ± 0.05**
Xylometazoline	8	53 ± 8	6.50 ± 0.1	50 ± 9	6.10 ± 0.1**

Table 6.2 Comparison of the potency (pD_2) and maximum response to various α-adrenoceptor agonists in the porcine isolated nasal artery in the presence and absence of 1mM lithium sulphate. Results were determined as a percentage of a former response to 60mM KCl and were expressed as the mean ± S.E.M of 5-7 observations.

* ($P < 0.05$) and ** ($P < 0.01$) – denote a statistically significant difference in the presence and absence of 1mM Lithium Sulphate, Student paired *t*-test.

	n	Control		1mM Lithium Sulphate	
		Maximum Response (% of 60mM KCl)	pD_2	Maximum Response (% of 60mM KCl)	pD_2
Phenylephrine	7	60 ± 8	5.40 ± 0.05	98 ± 7**	5.11 ± 0.08**
Metaraminol	5	54 ± 4	6.90 ± 0.05	96 ± 3**	6.50 ± 0.05**
Guanfacine	6	91 ± 3	6.40 ± 0.02	115 ± 9**	6.07 ± 0.1*
Xylometazoline	6	78 ± 7	6.90 ± 0.1	86 ± 7	6.80 ± 0.2

6.3.2 The effect of lithium ions on the time course of various constrictor agents in porcine blood vessels

To determine whether the contractions caused by 3 and 30μM phenylephrine were affected by the addition of 1mM Li_2SO_4 or not in the porcine splenic artery and the porcine nasal artery, one paired segment were exposed to lithium ions for at least 40 minutes while the second one was not, before the addition of maximum and sub maximum concentration of the vasoconstrictors. 1mM Li_2SO_4 produced a significant change in the time course curve of 3 and 30μM phenylephrine in the porcine isolated splenic (Figure 6.4) and the nasal artery (Figure 6.5). Concerning peak response in the splenic and in the nasal artery preparations, 3μM phenylephrine did not show any remarkable difference in both the control and 1mM Li_2SO_4 groups. While 30μM phenylephrine showed a significant increase in relation to its maximum response in both preparations (Table 6.3). Lithium ions significantly enhanced the magnitude and maintained the response to 3 and 30μM phenylephrine in both isolated splenic and nasal preparations after 60 minutes.

The second set of experiments determined the possible effect of 1mM lithium ions on contributing to the maintenance of the magnitude and the response to 3 and 30μM metaraminol in the porcine splenic and the nasal artery segments. As shown in table 6.3 and figure 6.6 the highest response and response after 60 minutes to 3μM sub maximum and 30μM maximum concentrations of metaraminol in the porcine splenic artery were enhanced significantly. However, in the porcine isolated nasal artery, the peak response and response after 60 minutes were significantly improved with 30μM metaraminol only (Table 6.3 and figure 6.7).

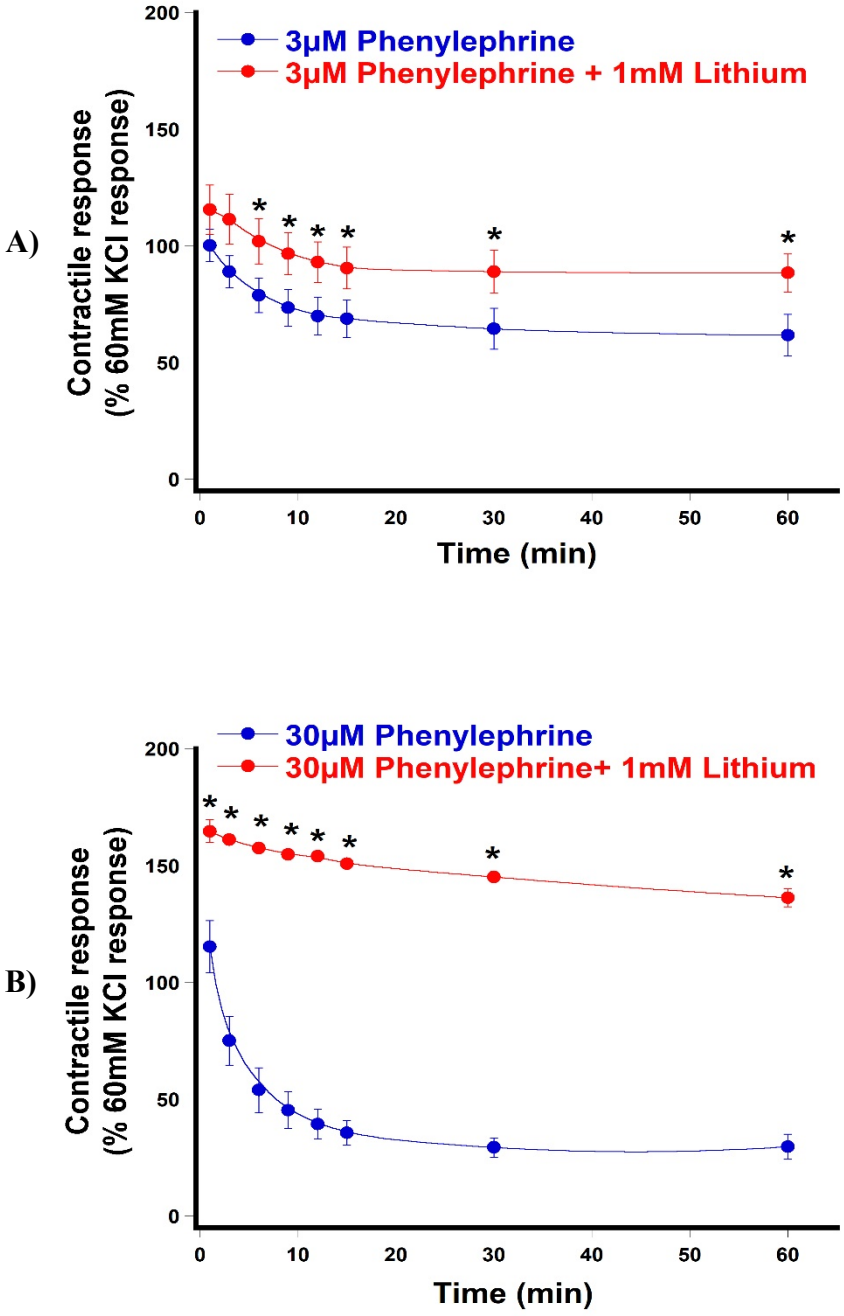


Figure 6.4 The time course of contractions of (A) 3μM and (B) 30μM phenylephrine in the porcine isolated splenic artery in the presence and absence of 1mM lithium sulphate. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of six observations.

*$P < 0.05$, Student's paired *t*-test.

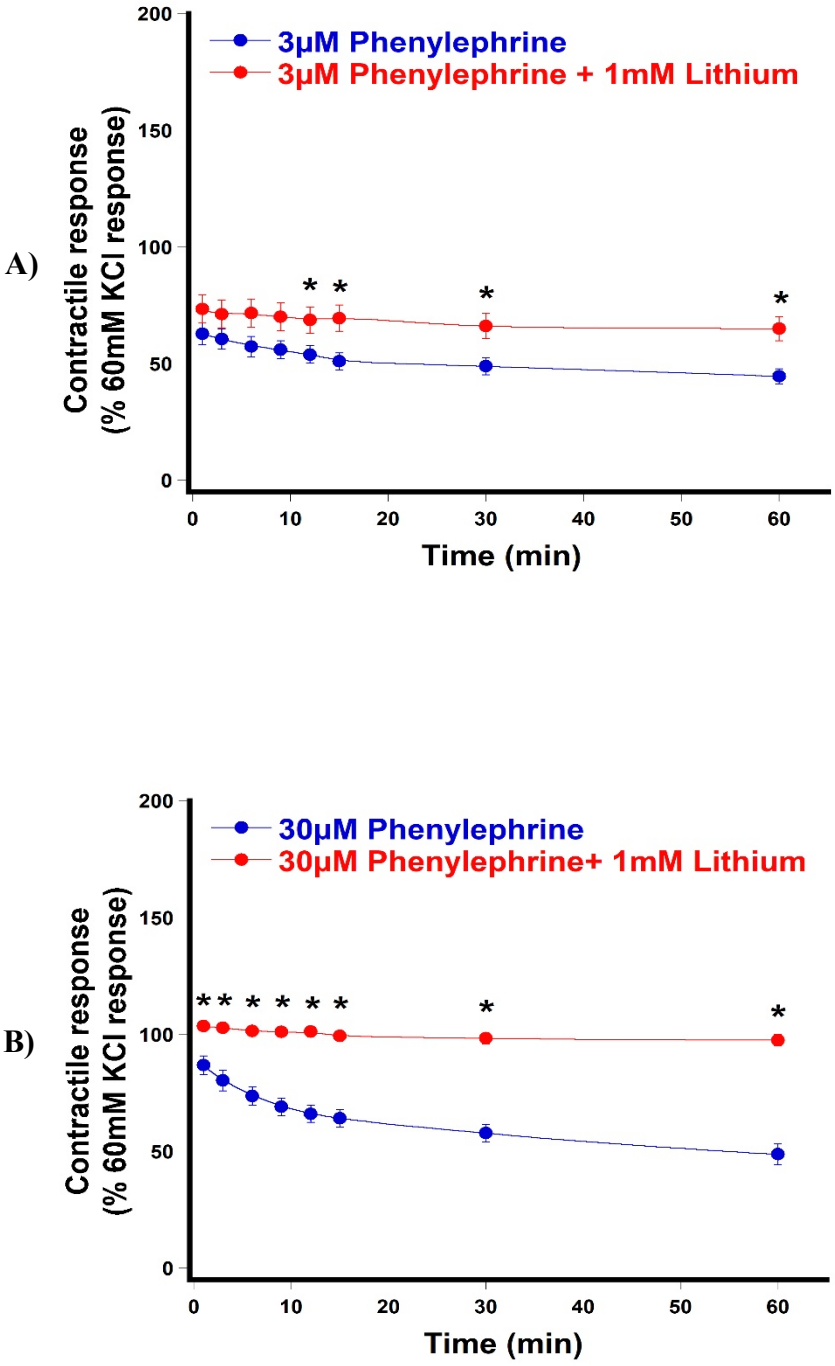


Figure 6.5 The time course of contractions of (A) 3μM and (B) 30μM phenylephrine in the isolated nasal artery in the presence and absence of 1mM lithium sulphate. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of eight observations.

*$P < 0.05$, Student's paired *t*-test.

Table 6.3 Comparison of the peak response and magnitude of contraction after 60 minutes of the porcine isolated splenic and nasal arteries to various vasoconstrictors in the presence and absence of 1mM lithium sulphate. Results were shown as the mean ± S.E.M of 6-11 observations.

* ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) – denote a statistically significant difference in the presence and absence of 1mM Lithium Sulphate, Student paired *t*-test.

		Peak Response (% of 60mM KCl)		Response after 60 minutes (% of 60mM KCl)	
		Control	1mM Lithium Sulphate	Control	1mM Lithium Sulphate
Phenylephrine	**Porcine**				
3µM	**splenic**	100 ± 7	116 ± 11	62 ± 9	88 ± 9*
30µM	**artery**	115 ± 11	165 ± 5**	30 ± 5	136 ± 4***
	(n=6)				
Phenylephrine	**Porcine**				
3µM	**nasal**	63 ± 5	73 ± 6	49 ± 4	98 ± 3***
30µM	**artery**	87 ± 4	104 ± 1**	45 ± 3	65± 5**
	(n=8)				
Metaraminol	**Porcine**				
3µM	**splenic**	76 ± 8	155 ± 5***	27 ± 2	139 ± 7***
30µM	**artery**	70 ± 9	172 ± 8***	22 ± 3.5	156 ± 6***
	(n=6)				
Metaraminol	**Porcine**				
3µM	**nasal**	33 ± 9	38 ± 10	21 ± 6	30 ± 10
30µM	**artery**	70 ± 8	106 ± 5***	41 ± 5	99 ± 6***
	(n=11)				

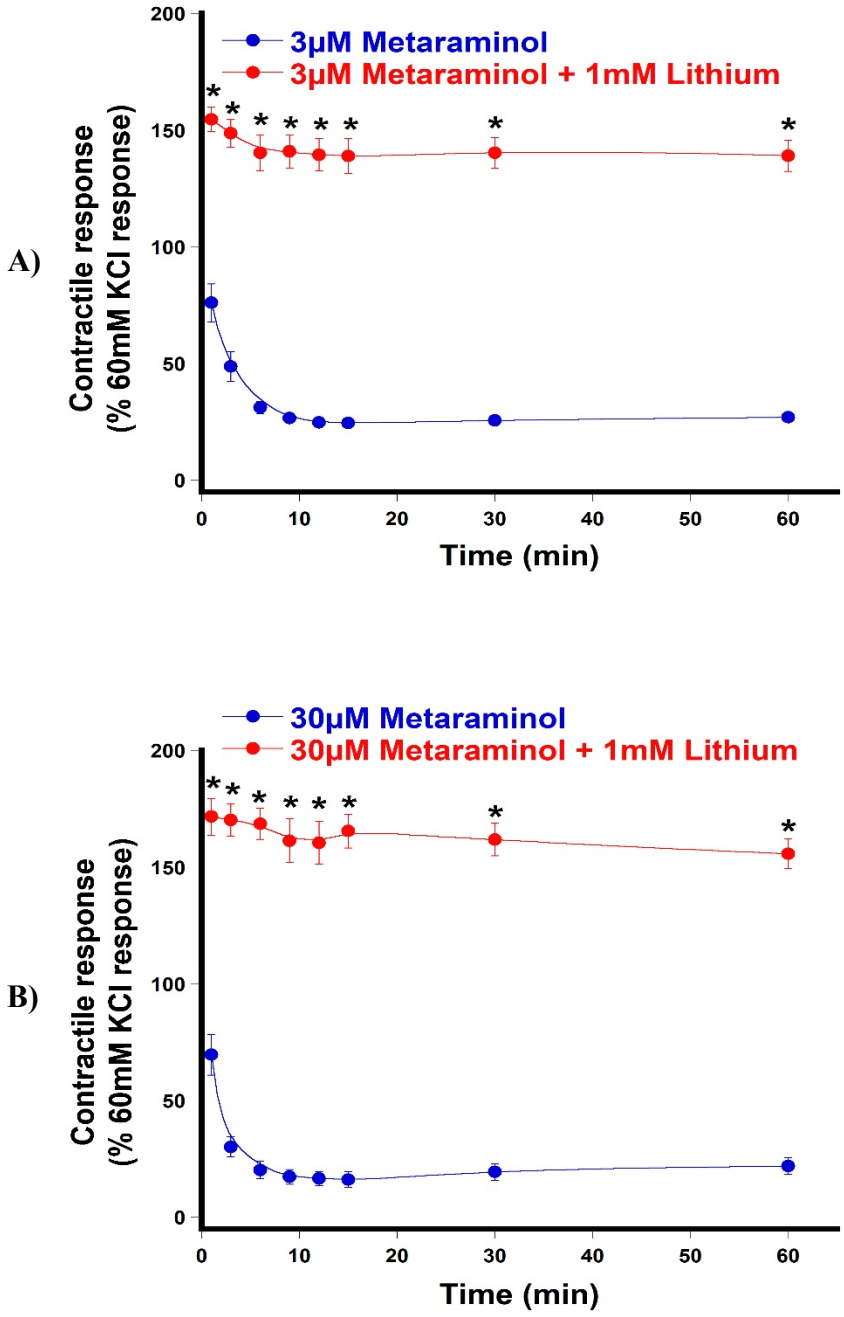


Figure 6.6 The time course of contractions of (A) 3μM and (B) 30μM metaraminol in the porcine isolated splenic artery in the presence and absence of 1mM lithium sulphate. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of six observations.

*$P < 0.05$, Student's paired *t*-test.

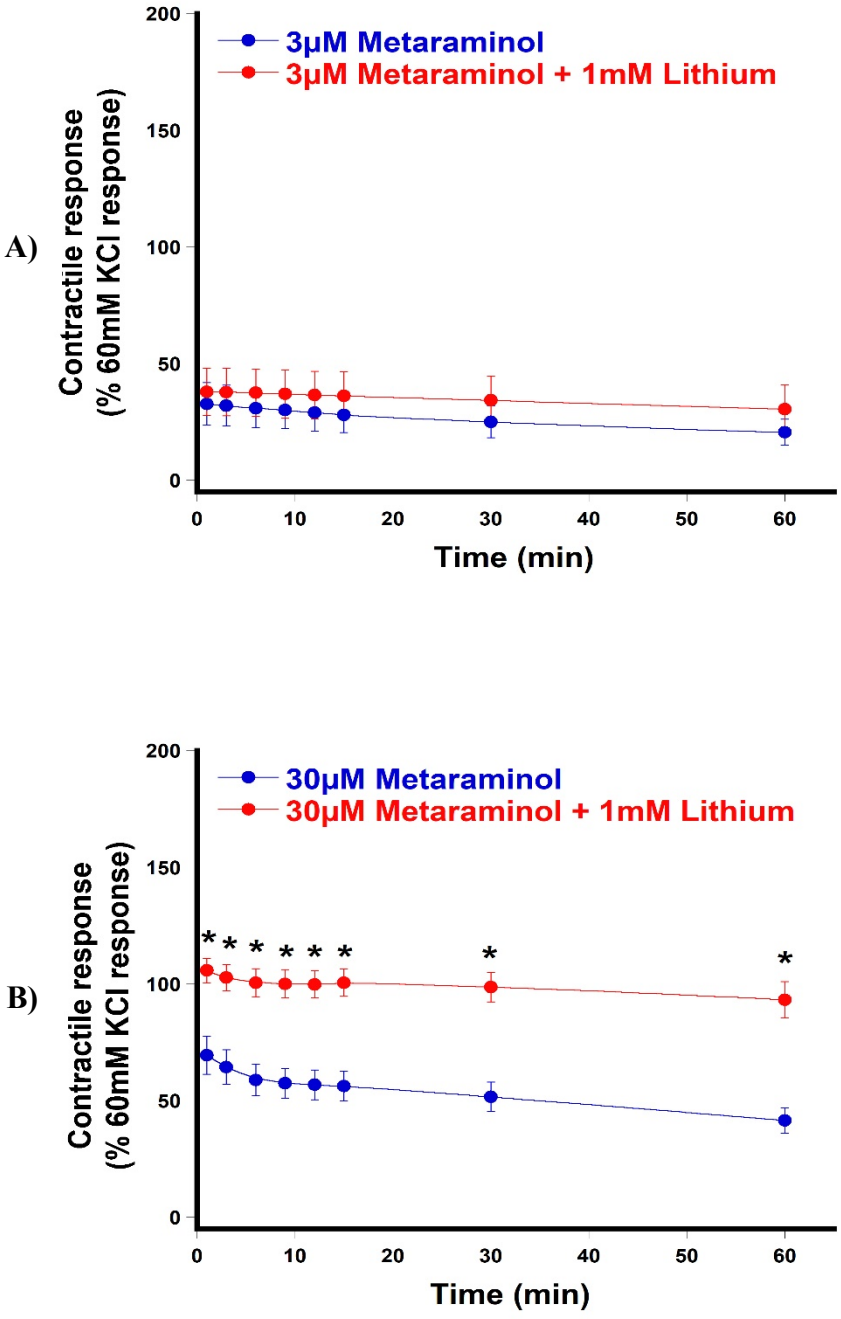


Figure 6.7 The time course of contractions of (A) 3μM and (B) 30μM Metaraminol in the porcine isolated nasal artery in the presence and absence of 1mM lithium sulphate. Responses were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of eleven observations.

*$P < 0.05$, Student's paired *t*-test.

6.3.3 Responses of the perfused porcine nasal mucosa to the cumulative concentration of various constrictor agents

Cannulation of the nasal artery in the intact porcine snout and perfusion with modified Krebs-Henseleit solution at 2.5ml/minute generated a resting perfusion pressure of 40.6 ± 7.8 mmHg (n=38). Exposure of the perfused porcine snouts to 60mM KCl produced pressor responses equivalent to 74 ± 3.9 mmHg (n=38). In each set of the experiment, the porcine nasal mucosa was perfused with cumulatively increasing concentrations of different vasoconstrictors including noradrenaline, adrenaline, phenylephrine, metaraminol, guanfacine, and xylometazoline. First, the pressure responses of the perfused porcine nasal mucosa to 10nM-100μM noradrenaline and adrenaline were detected and measured in four to six different samples. The mucosa of perfused snouts responded noticeably to minimum concentrations of both agents at 10nM and attained an equilibrium within 5-7 minutes of perfusion. Adrenaline showed the largest pressure response among all the agonists (see table 6.4). Figure 6.8 and table 6.4 showed the pressure response and the effective potency of noradrenaline and adrenaline in the perfusate porcine nasal mucosa.

In a separate series of experiments, the pressure responses were examined in several samples for phenylephrine and imidazolines (metaraminol, guanfacine, and xylometazoline). The agonists have added to the perfusion buffer fluid in half log unit cumulatively increasing concentrations started from 1nM-100μM for phenylephrine and guanfacine and from 10nM-100μM for metaraminol and xylometazoline. All the vasoconstrictors produced a concentration-dependent increase in the perfusion pressure of the porcine nasal mucosa that reached equilibrium within 5-10 minutes of perfusion.

Figure 6.9 showed the recording of the pressor response trace to phenylephrine and it was clear that the tissue similarly responded to 60mM KCl at the beginning and the end of the experiment. The threshold pressor concentration for phenylephrine was 0.1µM and the true maximum response (to 300 µM) was 142.5 mmHg with a potency of 5.40. While the pressure response to all imidazolines started earlier at 0.03µM. The maximum responses to metaraminol and xylometazoline were quite similar. In spite of the maximum response to α_2-adrenoceptor guanfacine was smaller than all other compounds and just reached 20.6 mmHg, but it showed the most effective potency among them (Figure 6.10 and table 6.4).

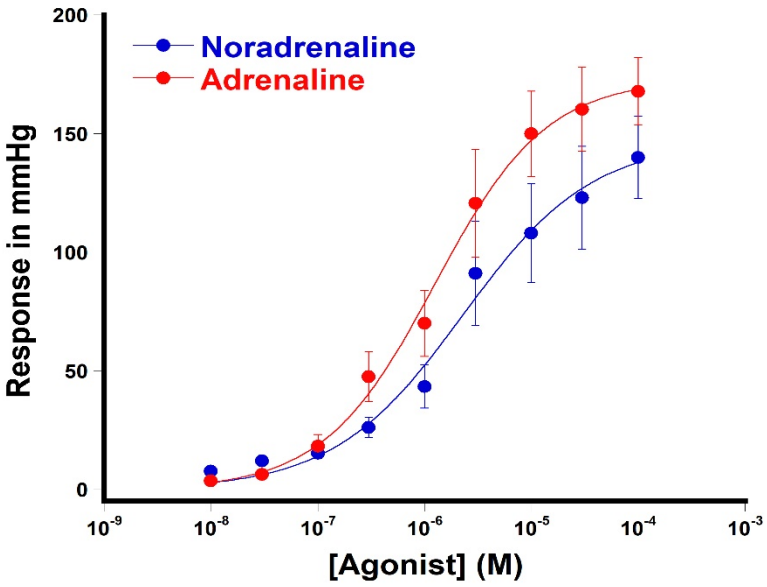


Figure 6.8 Comparison of the concentration perfusion pressure curve to noradrenaline and adrenaline of the perfused porcine snout. Results were expressed as raise in pressure inside the perfusion system measured in mmHg and were shown as the mean ± S.E.M of 4-6 observations.

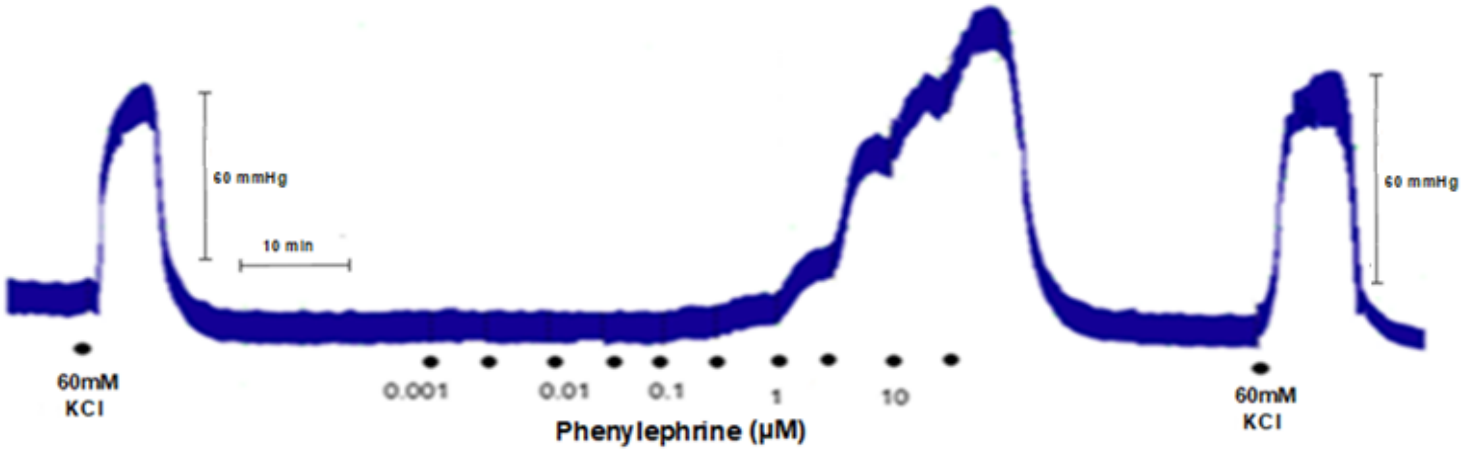


Figure 6.9 Representative trace recording of the perfusion pressure of the perfused porcine snout before and after increasing concentration of phenylephrine (1nM-300µM). It shows the response to 60mM KCl and return to modified Krebs-Henseleit buffer solution then to cumulative concentration of stimulant followed by a cessation of stimulant effect and return again to modified Krebs-Henseleit buffer solution and at the end of the experiment the preparation exposed to the second 60 mM KCl.

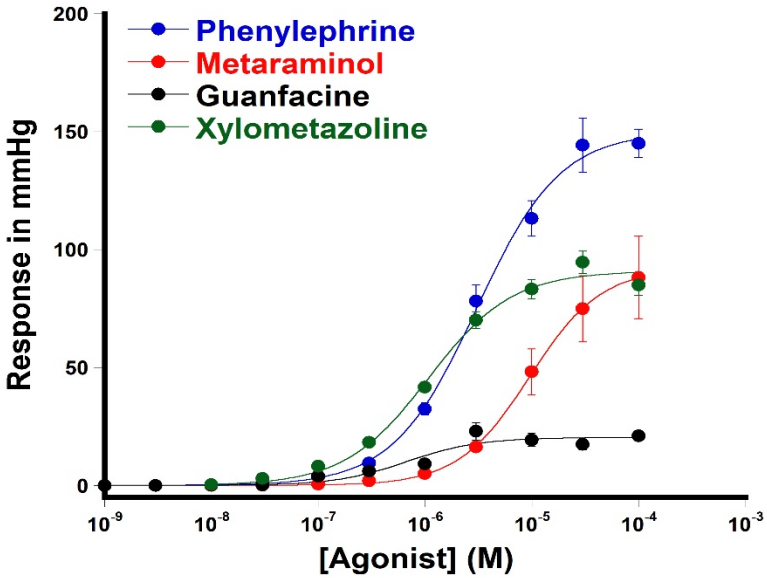


Figure 6.10 Comparison of the concentration-perfusion pressure curves to different agonists of the perfused porcine snout. Results were expressed as raise in pressure inside the perfusion system measured in mmHg and were shown as the mean ± S.E.M of 6-10 observations.

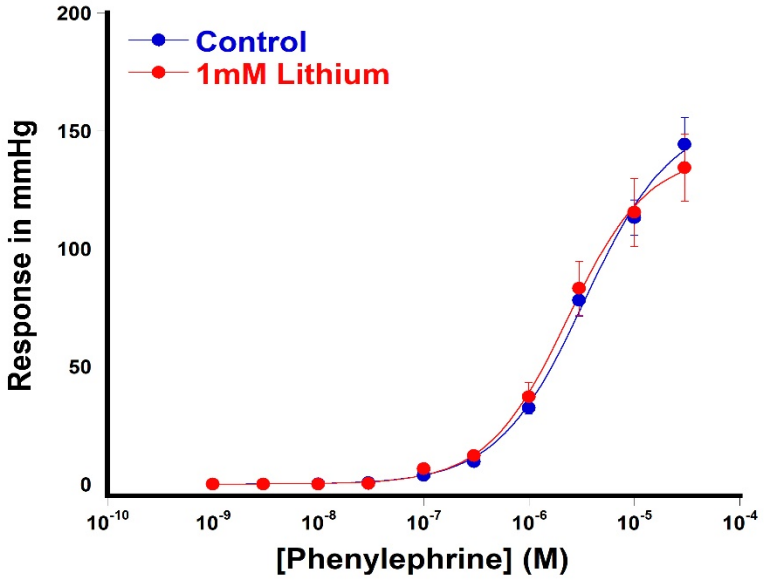
Table 6.4 Comparison of the potency (pD_2) and maximum pressure response of different agonists in the perfused porcine snout. The pressure responses were shown as the mean ± S.E.M of observations in unpaired 4-10 animals.

	Maximum Pressure Response	pD_2
Adrenaline	167.6 ± 17.7 mmHg (n=6)	5.80 ± 0.1 (n=6)
Noradrenaline	141.4 ± 9.6 mmHg (n=4)	5.50 ± 0.1 (n=4)
Phenylephrine	142.5 ± 10.3 mmHg (n=10)	5.40 ± 0.1 (n=10)
Metaraminol	94.1 ± 19.9 mmHg (n=6)	5.20 ± 0.09 (n=6)
Xylometazoline	82.9 ± 11 mmHg (n=6)	6.01 ± 0.1 (n=6)
Guanfacine	20.6 ± 2.3 mmHg (n=6)	6.20 ± 0.03 (n=6)

6.3.4 The effect of lithium ions on the cumulative concentration of various constrictor agents in the perfused porcine nasal mucosa

As described previously, a significant effect of lithium ions was detected on the response of the porcine blood vessels to a cumulative concentration of phenylephrine and guanfacine. Therefore, to clarify the effect of 1mM lithium sulphate on the cumulative addition of the most commonly used decongestants phenylephrine and guanfacine on the perfused porcine nasal mucosa, another set of experiments were conducted. After stabilisation of the pressure response, the first porcine snout was exposed to lithium ions for at least 40 minutes then to a cumulative concentration of agonist, while the other unpaired snout was exposed directly to a cumulative concentration of agonist. The addition of lithium ions did not affect the basal tone of the porcine nasal mucosa but surprisingly the addition of this cation to a group of perfused snout did not show any significant increase in the maximum response and estimated potency of both agonists in comparing with the unrelated control groups (Figure 6.11 and table 6.5).

A)



B)

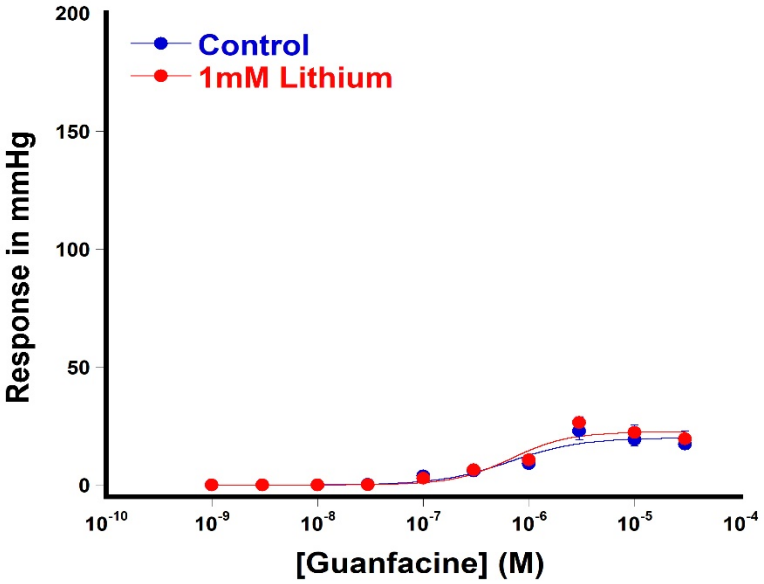


Figure 6.11 The effect of 1mM lithium sulphate on the cumulative concentration-response curves to (A) phenylephrine (B) guanfacine in the perfused porcine snout in the presence and absence of 1mM lithium sulphate. Results were expressed as raise in pressure inside the perfusion system measured in mmHg and were shown as the mean ± S.E.M of 4-10 observations.

Table 6.5 Comparison of the potency (pD_2) and maximum response to phenylephrine and guanfacine in the perfused porcine snout in the presence and absence of 1mM lithium sulphate. The pressure responses were shown as the mean ± S.E.M of observations in unpaired 4-10 animals.

	Control		1mM Lithium Sulphate	
	Maximum Pressure Response	**pD_2**	**Maximum Pressure Response**	**pD_2**
Phenylephrine	142.5 ± 10.3 mmHg (n=10)	5.40 ± 0.1 (n=10)	136 ± 15.7 mmHg (n=10)	5.60 ± 0.09 (n=10)
Guanfacine	20.6 ± 2.3 mmHg (n=6)	6.40 ± 0.03 (n=6)	22.9 ± 3.1 mmHg (n=4)	6.10 ± 0.05 (n=4)

6.3.5 The effect of lithium ions on time course of various concentration of phenylephrine in the perfused porcine nasal mucosa

To try and further elucidate the role of lithium ions on the magnitude and pressure response to sub-maximum and maximum concentrations of phenylephrine in the perfused porcine nasal mucosa, two unpaired groups of perfused porcine nasal mucosa were used. The first two groups were exposed to 3 and 30μM phenylephrine respectively, and the second group of the perfused porcine nasal mucosa was exposed to lithium ions for at least 40 minutes before being exposed to single either sub-maximum (3μM) or maximum (30μM) concentrations of phenylephrine.

The pressure response in two distinct groups produced rapid-developing, sustained contractions to 3μM and 30μM phenylephrine (Figure 6.12 and 6.13). The addition of lithium ions at the first 10 minutes significantly increased the pressure response of perfused nasal mucosa to 3 and 30μM phenylephrine from 98.6 ± 8.2 mmHg to 125.3 ± 7 mmHg ($P < 0.05$, n=9) and 103.9 ± 11.6 mmHg to 148.4 ± 9.1 mmHg, ($P < 0.05$, n=6), respectively (Figure 6.14). Furthermore, at the end of experiment (60 minutes) and in the presence of 1mM lithium sulphate, the pressure responses were significantly enhanced for 3μM and 30μM concentrations of phenylephrine from 44.9 ± 8.1 mmHg to 73.9 ± 7.9 mmHg ($P < 0.05$, n=9) and 53.5 ± 10.9 mmHg to 116.9 ± 9.7 mmHg, ($P < 0.01$, n=6).

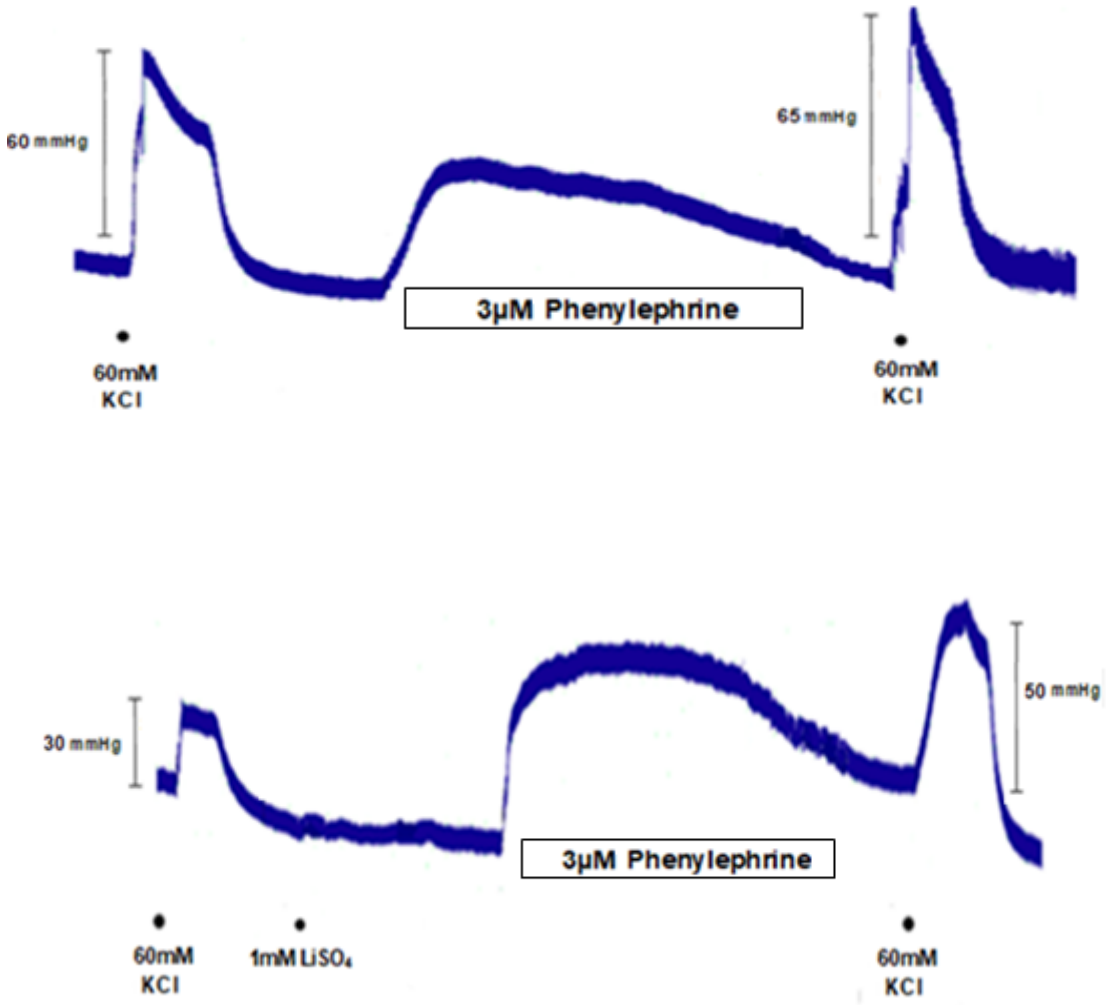


Figure 6.12 Representative trace recording of the perfusion pressure of the perfused porcine snout before and after infused with 3µM phenylephrine in the presence and absence of 1mM Lithium sulphate. It shows the response to 60mM KCl and return to modified Krebs-Henseleit buffer solution then to sub maximum concentration of phenylephrine for 60 minutes duration followed by a cessation of stimulant effect and return to modified Krebs-Henseleit buffer solution and at the end of the experiment, the preparation exposed to the second 60mM KCl.

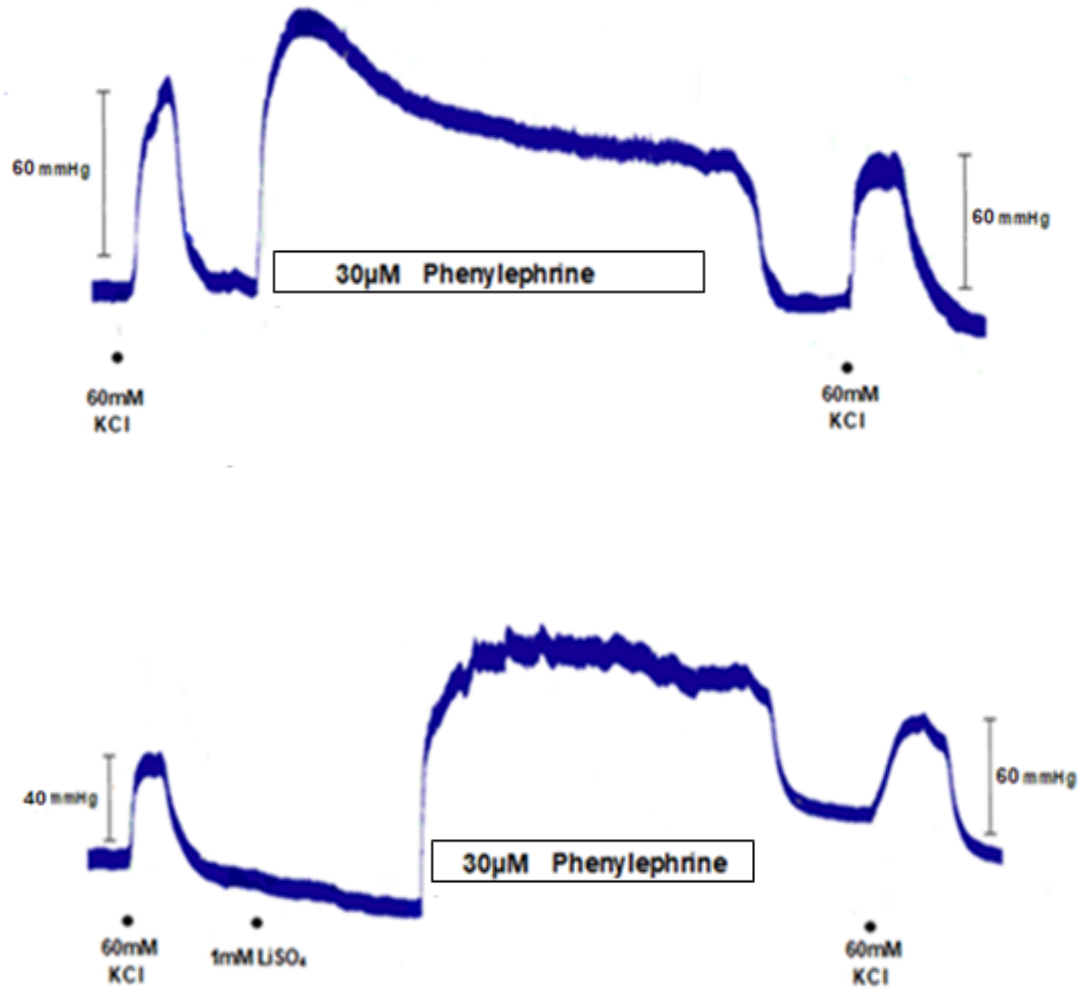
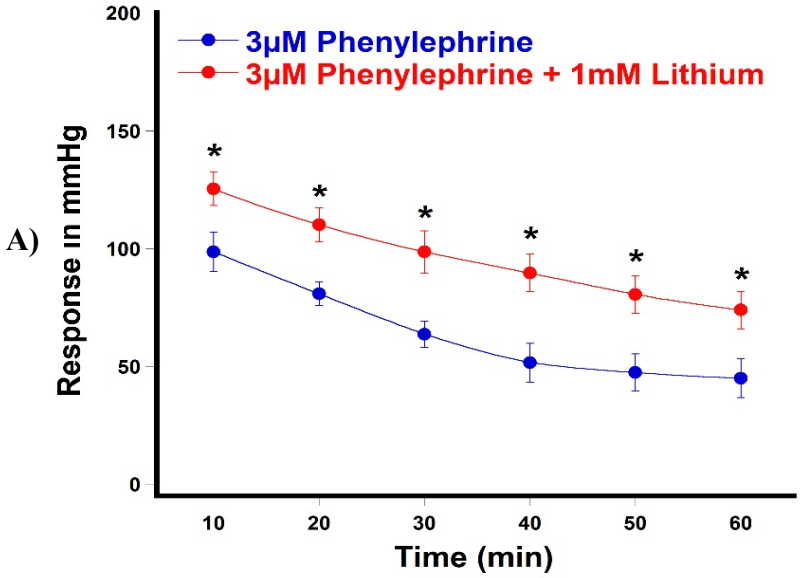


Figure 6.13 Representative trace recording of the perfusion pressure of the perfused porcine snout before and after infused with 30µM phenylephrine in the presence and absence of 1mM Lithium sulphate. It shows the response to 60mM KCl and return to modified Krebs-Henseleit buffer solution then to the maximum concentration of phenylephrine for 60 minutes duration followed by a cessation of stimulant effect and return to modified Krebs-Henseleit buffer solution and at the end of the experiment, the preparation exposed to the second 60 mM KCl.



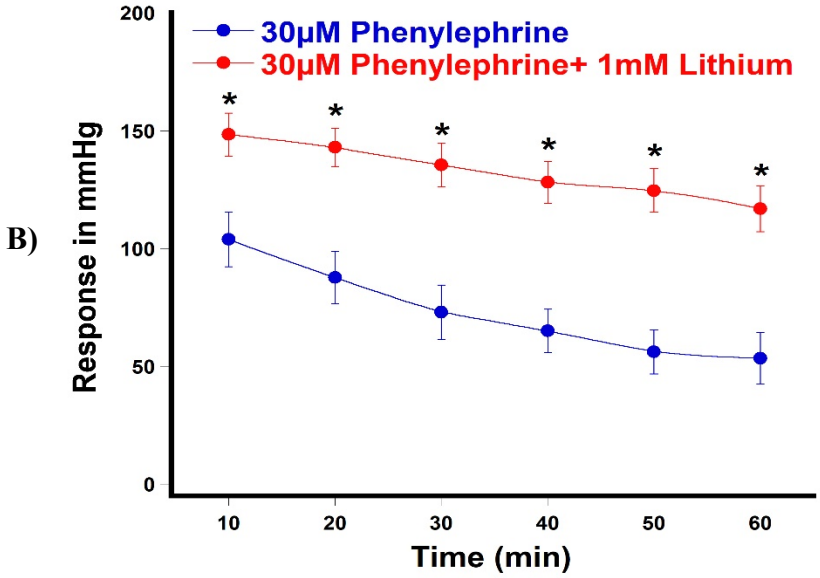


Figure 6.14 The time course of the perfusion pressure of (A) 3μM and (B) 30μM phenylephrine in the perfused porcine snout in the presence and absence of 1mM lithium sulphate. Results were expressed as raise in pressure inside the perfusion system measured in mmHg and were shown as the mean ± S.E.M of 6-9 observations.

*$P < 0.05$, Student's unpaired *t*-test.

6.4 Discussion

The main novel observation from this study is that lithium ions can enhance selectively the contractile responses of the porcine vascular smooth muscle to various α-adrenoceptor agonists, especially the phenethylamines. The effect has been established conventionally, by examining the interaction using cumulative increments in agonist concentration, to reveal enhanced maximal contractions. In addition, we have also employed single exposure to a high concentration of the agonists to demonstrate that lithium ions also increase the duration of responses.

These particular observations are qualitatively similar to that previously reported in the guinea-pig ileum, which observed that lithium ions at a concentration lower than 0.01M induced contraction on electrically stimulated longitudinal muscle preparation and then blocked contraction at a concentration more than 0.02M (Hirsch *et al*., 1978). These observations are supported by Menkes *et al*., (1986) who showed that lithium ions at therapeutic concentration (0.3-1mM) could prolong the physiological response to neurotransmitters that work on phosphatidylinositol system. Similarly, other studies showed that lithium inhibited the degradation of inositol trisphosphate leading to augmentation of the inotropic forces of α_1-adrenoceptors and this stimulatory effect of α_1-adrenoceptors was lithium dependent in guinea pig left atria (Molderings and Schumann, 1987) and in rat atria (Schmitz *et al*., 1987; Scholz *et al*., 1988). Additionally, Skomedal *et al*., (1991) reported that lithium increased in a dose-dependent manner the inotropic responses of α_1-adrenoceptors, but there was no significant effect on the potency of those agents, which is in line with our results.

This also accorded with observations in sheep isolated internal anal sphincter with α_1-adrenoceptor agonists and lithium (Rayment *et al*., 2010), which represent an

experimental paradigm that better fits with the clinical situation of looking the interaction between single doses of two different drugs. It is also noteworthy that our findings are qualitatively similar to those reported in the rabbit anococcygeus muscle by Dehpour and co-workers (1993). Crucially, this effect of lithium ions in the porcine vascular smooth muscle occurs at concentrations within the therapeutic plasma range (0.3-1mM) achieved when used for treating bipolar disorders (Severus *et al*., 2008; Can *et al*., 2014).

The effect of lithium ions on various constrictor agents in porcine blood vessels:

Lithium ions were found to increase the maximum response to phenethylamines including phenylephrine, metaraminol, and guanfacine by as much as 100-150%, without affecting response to the imidazoline agonist (xylometazoline) in the porcine splenic artery and nasal artery. The impact of lithium ions on the maximum response was observed more clearly in the porcine splenic artery than in the nasal artery, especially for guanfacine agonist. Associated with this change in the apparent 'efficacy' of different agents, is a reduction in the estimated potency (pD_2) of the four agonists in both porcine blood vessels. These observations suggest that the key parameters defining α-adrenoceptors agonism in the porcine vasculature may be altered by the presence of lithium ions. While the basis of this selective effect is still unclear, our observations warrant the assessment of other phenethylamine-based and imidazoline-based agonists at α-adrenoceptors in these preparations.

Furthermore, strong evidence of xylometazoline, guanfacine and metaraminol higher potencies mainly in the porcine nasal and to a lesser extent in the splenic artery was detected compared with phenylephrine without effect of lithium ions. Xylometazoline, which has been used as a topical nasal decongestant for more than five decades to act

directly and mainly on α_2-adrenoceptors and thus reduces the nasal airway resistance in dose-dependent manner (Johannssen *et al.*, 1997; Desjardins and Berlin, 2007; Haenisch *et al.*, 2010), was more potent (pD_2 = 6.90) than the standard decongestant phenylephrine (pD_2= 5.40) in the porcine nasal artery. A similar present data was found in the nasal mucosal vasculature (pD_2= 6.01 for xylometazoline versus 5.40 for phenylephrine).

Interestingly, the presence of lithium ions increased both the magnitude and duration of responses to phenylephrine and metaraminol in the porcine blood vessels. Phenylephrine, and to a lesser extent other agonists, have been used in vascular research for decades. One of the criticisms of much of the literature on phenylephrine is that why non-sustained contractions to high concentrations of the agonist in the porcine arteries and veins, has not been previously reported. Even with submaximal concentrations of phenylephrine, there is evidence of a decline of contractions over time (which is less apparent in the presence of lithium ions), but this phenomenon is most pronounced with the highest concentrations of the agonist. We believe that the routine use of cumulative concentration-response curves for studying α_1-adrenoceptors, with a focus on attaining an equilibrium response for each concentration (2-3 minutes) may mitigate against establishing whether each response is sustained for a significant period. Remarkably, Kanaide and colleagues demonstrated that submaximal contractions of the porcine renal interlobar artery to phenylephrine declined by as much as 60% over 20 minutes, without comparable changes in the cytoplasmic concentration of free intracellular calcium ions (Derkach *et al*., 2000; Ihara *et al*., 2001). Taken together, these findings are consistent with the notion of some form of desensitisation taking place over a much shorter period than that generally associated with changes in the receptor number in cells (Yang *et al*., 1999) and this process that lithium ions appear to affect selectively.

Perfusion of porcine nasal mucosa and the effect of lithium ions on various constrictor agents in perfused porcine nasal mucosa:

Various methods have been used in humans and animals to study the pharmacology of nasal decongestant such as, nasal geometry and nasal airway resistance or nasal blood flow monitoring (Lacroix and Lundberg, 1989a; Lacroix and Lundberg, 1989b; Cervin *et al*., 1999; Maeda *et al*., 2004; Zang *et al*., 2009; Vaidyanathan *et al*., 2012). In this study, a novel approach of porcine snout perfusion was conducted, to examine the vasoconstriction in nasal mucosal vasculature produced by phenylephrine and other agonists, which was reflected in the pressure changes of the perfusion system. This technique follows on from Denfria, (2016) who started this novel method of perfusion porcine nasal mucosa. In spite of similar perfusion rate (2.5ml/minute) used by Denfria, (2016) study, a higher baseline pressure with 40.6 ± 7.8 mmHg (n=38) was detected. To provide stability of the experiments, perfusions with cumulative increasing concentration of endogenous ligands (noradrenaline and adrenaline) were performed in the beginning to find the most efficacious and consistent approach to perfuse the porcine snouts.

The experiments in this chapter established that noradrenaline and adrenaline were the most effective agent in performing vasoconstriction in perfused pig snouts as its effect stared with the lowest concentration (10nM). The endogenous ligands were found to enhance the pressor tone almost eight times higher than guanfacine, which was one of the common used nasal decongestant. The rank order of maximum pressure responses of the agonists was adrenaline> noradrenaline > phenylephrine> metaraminol > xylometazoline> guanfacine. By contrast, the rank order of potencies was guanfacine> xylometazoline> adrenaline> noradrenaline > phenylephrine> metaraminol. This finding corroborates the results of Berridge and Roach, (1986) in anesthetised dog nasal mucosa, who found a similar rank order of potency for the endogenous ligands and phenylephrine.

Although, this study also demonstrated that adrenaline is more potent than xylometazoline, which could be accountable to variances in animal and technique used. There is considerable evidence demonstrating that, in anesthetised pig's nasal mucosa, phenylephrine and adrenaline has the same potency (Lacroix and Lundberg, 1989b), which qualitatively disagrees with our finding.

The interesting result of the perfusion snout study was obtained with phenylephrine, which revealed evidence for α_1-adrenoceptors at the level of the microcirculation, and this agent showed a similar sensitivity incomparable to the conduit artery used for isometric tension recordings (pD_2=5.40). This present outcome supports previous data with phenylephrine into the porcine mucosa, which also yield a potency of 5.4 (Corboz *et al*., 2003). However, contrary to the present study, Denfria, (2016) demonstrated that phenylephrine has lower potency (5.20 ± 0.19, n=6) on the perfused porcine nasal mucosa. The potency value of phenylephrine in the perfused snout, which is still lower than the potency values of other decongestants are consistent with the present results in the porcine blood vessels.

Among the α-adrenoceptor agonists used in the present study with perfusion experiments, guanfacine as a vasoconstrictor had the highest potency with a pD_2 value of 6.20, but with the lowest pressure response. There are similarities between the pD_2 value expressed by guanfacine in this study using porcine blood vessels and nasal snout. Interestingly, it is noticeable that guanfacine had a different contractile activity on different vessels, which could depend on the species and the vascular beds. Such as, *in vitro* experiments on rat isolated aortic strips, guanfacine behaved as a full agonist (with pD_2 of 6.1) but only as a partial agonist in the rat portal vein (with pD_2 of 4.4) (Digges and Summers, 1983).

Guanfacine was found to induce contractions of vascular rings of canine arteries and veins (Scholtysik *et al*., 1975), but did not affect rat isolated mesenteric arteries (Bognar and Enero, 1984).

Observations in the perfused porcine nasal mucosa with lithium ions, using cumulative increment concentrations afford no effect on phenylephrine and guanfacine suggesting that lithium ions effect on nasal mucosa is quite different from that on the nasal artery as it showed a considerable effect. While the presence of lithium ions in the perfused buffer solution before perfusion with maximum and sub maximum concentrations of phenylephrine showed that lithium ions increased both the magnitude and duration of responses to 3 and 30μM phenylephrine, which fits with the present results in the porcine splenic artery and nasal artery. In light of the present finding that a qualitatively similar interaction also occurs in the vasculature, there is a case to investigate whether phenylephrine, for example, may become a more effective decongestant (Johnson and Hrick, 1993), if combined with clinically acceptable levels of lithium ions. Evaluation of the effect of lithium ions on phenylephrine-induced contractions in human blood vessels is warranted.

In conclusion, we report that contractions of the porcine arteries and perfused nasal mucosa to high concentrations of phenylephrine are not sustained, unlike those to other α-adrenoceptor agonists, but declines over 30-60 minutes time frame. The presence of therapeutic levels of lithium ions not only prevents this effect but also selectively enhances responses to this agonist. Therefore, topical phenylephrine with lithium ions in therapeutic accepted concentration will provide better congestion relief.

Chapter Seven

General Discussion

7 General Discussion

Nasal congestion is a common medical problem causing discomfort and difficulty in breathing throughout the world. Nasal decongestants are one of the medications, usually given without seeking medical consultation, to relieve nasal congestion and to aid breathing. These medications lead to activation of α-adrenoceptors associated with the vasculature of the nasal mucosa and the systemic circulation mainly for the oral form, either directly or indirectly, causing vasoconstriction. This vasoconstriction action depends on the distribution of α-adrenoceptors in the nasal mucosa and subsequently to reduce nasal mucosal blood flow and swelling (Vaidyanathan *et al.*, 2012), thereby increasing the flow of air through the nasal cavity.

In general, both oral and topical decongestants act as vasoconstrictors. However, the topical route has shown significant advantages that include a non-invasive route of medication delivery, rapid onset of action, higher sensitivity, avoidance of the first-pass metabolism by the liver and targeting drugs directly to the nasal cavity without causing systemic side effects such as increased blood pressure (Touitou and Illum, 2013) and systemic vascular resistance (Morelli *et al*., 2008).

In my study, I utilized porcine blood vessels (splenic artery and vein) as an extra-nasal blood vessel because it has numerous benefits over many arteries for studying contractility, as it is long enough and uniforms in terms of internal diameter. This benefit provides the chance of understanding and examining the pharmacology of receptor features in-depth and intact ideal tissue with 3-fold increment in concentration.

Additionally, it is not possible to use the same tissue characteristics from small laboratory animals. As nasal mucosal vessels are more sensitive *in vivo* to the action of nasal decongestant than the rest of the circulation (Aviado *et al.*, 1958; Aviado *et al.*, 1959), the porcine nasal vasculature was used in my experiments.

Comparison of phenylephrine and pseudoephedrine on the nasal and extra-nasal blood vessels from the pig and their effects of noradrenergic-mediated contractions: Phenylephrine and pseudoephedrine are commonly used orally-active nasal decongestants, but the clinical efficacy and selectivity for the nasal vasculature of the former have been questioned (Eccles *et al*., 2005; Eccles *et al*., 2006; Horak *et al*., 2009). The work described in this thesis investigated the role and the activity of nasal decongestant compounds on nasal and extra-nasal blood vessels from the pig and their effects on noradrenergic-mediated contractions with particular emphasis on the characteristics of α_1-adrenoceptors in different vascular beds.

This thesis has clearly demonstrated that phenylephrine can produce direct vasoconstriction of porcine blood vessels. However, the nasal circulation (nasal artery and nasal mucosa) *in vitro* is less sensitive to directly acting sympathomimetics (noradrenaline and phenylephrine) than the rest of the circulation. The porcine splenic artery was (3-4)-fold more sensitive to noradrenaline and phenylephrine, respectively than the nasal artery. The concentration (0.8-1.7ng/ml, 10-30nM) (Atkinson *et al*., 2014; Janin and Monnet, 2014; Geolette and Zimmerman, 2015) described to be attained *in vivo* after ingestion of oral phenylephrine cannot affect significantly direct vasoconstriction and even neurogenic contractions in porcine nasal and extra-nasal blood vessels.

Despite the finding, that pseudoephedrine (indirectly acting sympathomimetics) did not produce direct vasoconstrictor action in either the nasal and splenic artery, it was able to enhance the electrically evoked noradrenergic contractions, which was chiefly apparent in the nasal artery and at concentrations similar to those achieved *in vivo* following oral ingestion of typical dose of pseudoephedrine (Kosoglou *et al*., 1997).

It has been shown that the porcine nasal artery was more sensitive to the electrical stimulation and revealed enhancement of electrically evoked noradrenergic contractions, which was chiefly obvious in the nasal vessel at low frequencies of stimulation. Furthermore, the porcine nasal artery has good sympathetic innervation and supply evoked by the significant intensification in the magnitude and duration of electrically evoked contractions in responses to 10μM cocaine and the indirectly acting sympathomimetic 100μM tyramine.

Interestingly, the unheralded change that has been promoted by the FDA in formulation over the last 15 years of previously topically administered agents as decongestants, phenylephrine and pseudoephedrine, to ones now included in numerous oral OTC formations at least in the UK and USA. As identified by Eccles (2006) and Hatton *et al.,* (2007), the re-introduction of oral phenylephrine as a decongestant by the FDA was based on: (i) the necessity to reduce the general availability of pseudoephedrine because of its potential to be used as a precursor for the production of methamphetamine and (ii) the finding of a meta-analysis of studies on oral phenylephrine generated more than twenty-five years earlier. It was not based on contemporary evidence of clinical efficacy using current research guidelines.

On examination of the literature after phenylephrine reintroduction, three researches have formally (re)-assessed the effectiveness of a standard oral dose of phenylephrine by evaluating either the nasal airway resistance or improvement in nasal congestion symptoms. Horak and colleagues (2009) showed that phenylephrine was no better than placebo, while as might be expected of a decongestant, a standard dose of pseudoephedrine reduced the nasal airways resistance. In the same way, recently two trials with oral phenylephrine failed to provide symptomatic relief of allergic rhinitis (Meltzer *et al.,* 2015) even when the dose was increased to 30mg (Meltzer *et al.,* 2016). All three studies are consistent with the conclusion of the study by McLaurin and colleagues (1961) over 50 years ago, based on an evaluation of symptoms associated with nasal congestion. Therefore, the main conclusion is no justification for the claim that this potentially hypertensive agent (phenylephrine) can act as a 'selective' nasal decongestant when given at (10-12mg) orally.

The absence of any major haemodynamic changes associated with the oral phenylephrine and also pseudoephedrine agrees with several other studies that attest to the general safety of these drugs in a young healthy population (Empey *et al*., 1980; Chua *et al*., 1989; Gelotte and Zimmerman 2015). Furthermore, the choice of the standard dose of phenylephrine in the OTC oral products is one that avoids obvious systemic side effects by simply being too low to do anything. By contrast, I think there is a rational basis for the oral use of pseudoephedrine as an orally-active nasal decongestant as it enhanced the magnitude and duration of effect of electrically evoked contraction in the vasculature and exerted a selective effect on nasal blood vessels. In a summary, this present study concurs with Thomas and colleagues (1991), who concluded that ‘in view of the small and brief effects of phenylephrine on peripheral resistance it is hard to believe that this dose (10mg) can have a therapeutic effect on the nasal vasculature ….”. The obvious potential for

phenylephrine to elevate blood pressure appears to have led to the choice of an oral dose, for widespread public consumption, devoid of cardiovascular side effect, but at the expense of any potential for decongestant activity.

Detailed examination of α-adrenoceptor mediated contraction of the porcine nasal and extra-nasal blood vessels:

The second major area of my work has been to investigate in detail the pharmacological characteristics of the α-adrenoceptors in the non-peripheral artery and nasal artery. Phentolamine is the classical non-selective antagonist, which does not discriminate between α_1 and α_2-adrenoceptors and has no effect on β-adrenoceptors (Furchgott, 1972; Starke 1981). Therefore, the expectation was that phentolamine should behave in a competitive manner resulting in a parallel rightward shift of the concentration-response curves against noradrenaline-induced contractions in the porcine blood vessels. This antagonist should also produce a pA_2 value of approximately 8 with a slope of the Schild plot not significantly different from unity (Applerley *et al*., 1976).

To improve both the accuracy of the slope of the Schild plot and the estimation of the antagonist pA_2 value. The detailed pharmacological examinations were conducted on porcine blood vessels with a large number of observations using different agonists and antagonists and based on using half log unit increments in antagonist concentrations and increments in agonist concentrations were increased to 4 concentrations (1, 2, 5, 10) per log cycle.

The empirical findings of a detailed examination of α-adrenoceptor mediated contraction of the porcine blood vessels have revealed unexpected observations that phentolamine and even the selective α_1-adrenoceptor antagonists did not behave as

classic competitive antagonists against contractions to noradrenaline. The slopes of the Schild plots (0.7-0.8) for the selective α_1-adrenoceptor antagonists (prazosin, corynanthine, tamsulosin and silodosin), also for phentolamine were significantly less than unity. This deviation from a straight line, which was not consistent with antagonism at a single site, suggested heterogeneity in the receptors contributing to responses in the porcine nasal and extra-nasal blood vessels.

Yet, phentolamine and the selective α_1-adrenoceptor antagonists inhibited contractions to A61603 a selective α_{1A}-adrenoceptor agonist (Knepper *et al*., 1995; Smith *et al*., 1997; Mehrotra *et al*., 2007; Davis *et al*., 2018) in a competitive manner – the slope of the Schild plot was not significantly different from unity in the porcine splenic and nasal artery. All these results are difficult to explain, but it strongly supports the expression of at least two α_1-adrenoceptors in the porcine blood vessels are present, which denotes the complicated role of functional α_1-adrenoceptor subtypes. One of this contractile receptor is α_{1A}-adrenoceptor. This outcome based on the higher sensitivity of the porcine nasal and extra-nasal blood vessels to A61603 agonist and differences in the relative potency-ratio of A61603 to noradrenaline and phenylephrine, respectively in both preparations. Furthermore, high pA_2 values for tamsulosin and silodosin antagonists as well as a component of the concentration-response curves to noradrenaline was resistant to the action of the latter antagonists. Interestingly, the other one seems to be the α_{1B}-subtype. However, the rather discrepancy results were corynanthine, which has approximately 10-fold selectivity for the α_1-adrenoceptors and inactivates it irrespective of its subtype (Doxey *et al*., 1994) yielded a slope < 1 against noradrenaline-induced contractions in the porcine blood vessels. These findings still cannot be explained by either α_2-adrenoceptors role or involvement of two subtypes of α_1-adrenoceptors.

Confirmation of the involvement of α_{1B}-subtype in the contractile activity of the porcine blood vessels requires to do further experiments using a selective α_{1B}-adrenoceptor antagonist together with α_{1A}-subtype. However, to date, there is no agreement about the availability of a selective antagonist for α_{1B}-adrenoceptor in functional studies.

As most of them including risperidone and cyclazosin showed significant different binding properties, which can be seen with ligand binding studies but not with functional studies (Yoshiki *et al*., 2014; Docherty, 2019).

Several studies have been used hydrophilic alkylating agent chlorethylclonidine (CEC), which shows preferential inactivation of the α_{lB}-adrenoceptor to identify this subtype (Muramatsu, 1991; Schwietert *et al*., 1991; Bultmann and Strake, 1993; Oshita *et al*., 1993; Low *et al*., 1994; Daniel *et al*., 1996). For example, in the rabbit thoracic aorta, prazosin inhibited the concentration-response curves for noradrenaline in the CEC-untreated in an uncompetitive manner and the Schild plots for prazosin were significantly less than unity showing two distinct affinity constants (9.71 and 8.74 for the rabbit thoracic aorta). However, after CEC pretreatment, contraction to noradrenaline was attenuated moderately, the high-affinity site for prazosin was abolished resulting in a competitive antagonism and Schild plots analysis were not significantly different from unity (Oshita *et al*., 1993). Yet, CEC pretreatment value has been questioned (Schwietert *et al*., 1991; Bultmann and Strake, 1993; Low *et al*., 1994; Daniel *et al*., 1996).

Barbieri *et al*., (1998) identified a slight inhibition in the maximum response and non-significant effect on the potency value of phenylephrine upon exposure of the porcine splenic artery to CEC, which revealed that the contraction response of phenylephrine was insensitive to CEC and there was a minor functional role for α_{1B}-adrenoceptors in this

blood vessel. Moreover, Barbieri and co-worker (1998) reported different affinity values with a varying subtype of selective α_1-adrenoceptor antagonists (WB-4101 (9.46), 5-methyl-urapidil (8.26), benoxathian (9.06) and BMY 7378 (6.91). Additionally, these selective α_1-adrenoceptor antagonists behaved competitively and yielded slopes of the Schild plot were not different from unity against phenylephrine-induced contractions in the porcine splenic artery. Based on these values, Barbieri *et al*., (1998) concluded the involvement of functional α_{1A}-subtype but not α_{1B}-adrenoceptors.

Examination of the conditions required to uncover post-junctional α_2-adrenoceptors in porcine blood vessels:

Currently, due to the importance of α_2-adrenoceptors, which was evident in many studies dealing with α_2-adrenoceptor function (MacDonald *et al.*, 1997; Corboz *et al.*, 2003) and availability (Stafford-Smith *et al.*, 2007) in the nasal mucosa. Moreover, many imidazoline-based decongestants activate α_2-adrenoceptors like oxymetazoline and xylometazoline (Haenisch *et al.*, 2010). Therefore, the present study tried to investigate the effect of various interventions on responses of porcine blood vessels to uncover the action of α_2-adrenoceptors.

In the porcine isolated splenic artery, it has been shown that augmentation of the calcium level by 1μM S(-)-BayK8644 and 18mM KCl or removing the inhibitory influence of NO via 100μM L-NAME and ODQ (10μM), improve the contraction response to α_1-adrenoceptors. However, the findings remarkably revealed that pharmacological confirmation of the involvement of post-junctional α_2-adrenoceptors was more pronounced by elevating calcium and cAMP levels as in the presence of ancillary vasoactive agents (a combination of U46619 and forskolin) in the porcine splenic artery rather than using other interventions. Therefore, we have established that therapeutically

active α_2-adrenoceptor agonist (brimonidine) can contract the porcine splenic artery, which may be undesirable for an orally-active agonist of high efficacy to be used as a nasal decongestant but not for the topical consumption.

Finally, our conclusions are largely based on the use of efficacy, maximum response and response at 20% of the former response to 60mM KCl, rather than other values. It would be interesting to determine the current topics of pharmacological manipulation with more focus on the porcine nasal artery, especially using other interventions are therefore suggested. For example, L-NAME, as based on information, gleaned from the understanding that the level of nitric oxide is affected and reduced by some nasal decongestants (Gusovsky *et al.*, 1986). Moreover, it may be worth investigating the potential for increasing the intracellular calcium by using either 1μM S(-)-BayK8644 or 18mM KCl and then increasing the cellular cyclic AMP level via forskolin on responses to brimonidine and noradrenaline on the porcine splenic and nasal artery. Future research on the topical application of α_2-adrenoceptors as a nasal decongestant in combination with the topical therapeutic accepted concentration of other methods of pharmacological manipulation to improve the quality and action of the common available nasal decongestants are essential.

Assessment of the vasoconstrictor properties of α-adrenoceptor agonists in the porcine perfused snout and their interaction with lithium ions:

Traditionally, lithium has been extensively used for decades to treat the affective disorder (Chiu and Chuang, 2010). Despite, its long clinical success, lithium has many adverse effects and a narrow therapeutic index (i.e. the difference between the therapeutic level (0.5-1mM) and the toxic level (2mM) is small), therefore, close monitoring of the plasma concentration of lithium is necessary (Schou, 1976). Although John Cade first recognised

lithium in 1949, and its mode of action interferes with various physiological functions such as ion channels, blood homeostasis, electrolytes levels and number of second messenger systems, still exact mechanism of action of lithium in the central nervous system is not obvious (Hirsch *et al*., 1978; Brown and Tracey, 2013).

Most of the studies about lithium adjusted upon its significant role in the pathophysiology of affective disorder and its actions as an antidepressant agent and this role includes its influence on biogenic amine neurotransmitters in which lithium uncompetitively inhibits the inositol monophosphatase enzyme and might slow down the phosphatidylinositol (PIs) cycle (Skomedal *et al*., 1991). The PIs cycle is considered as the main second messenger system responsible for mediating the contractile response to neurotransmitters in the vascular smooth muscles and by inhibition of inositol monophosphatase enzyme, lithium alters the vascular tone. However, this influence of lithium in relation to its therapeutic effect is still unclear, because this effect is shown only in higher concentration, which is more than the therapeutic concentration (Menkes *et al*., 1986).

To my knowledge, no one has ever examined the relevance of the interaction between lithium ions and α-adrenoceptor agonists in nasal and extra-nasal blood vessels, in addition to the perfused porcine snout. To address this, the effect of 1mM lithium ions on the cumulative increments in agonist concentrations and the potency values. Moreover, the magnitudes of a single exposure of the agonists were also detected using blood vessels and snouts from the pigs.

The novel observation in this study is that significantly lithium ions are capable of increasing the contractile response of the porcine splenic and nasal artery to various α-adrenoceptor agonists, especially the phenethylamines. Additionally, lithium ions can

increase and maintain the time course of contraction induced by phenylephrine and metaraminol in the porcine blood vessels particularly the porcine nasal artery. The most interesting finding was that lithium can enhance and maintain the time course of contraction caused by phenylephrine in the perfused pig snouts. This finding showed that a combination of phenylephrine and lithium ions in a therapeutic level could enhance the duration of action of phenylephrine. The outcomes of the current study are consistent with those of (Dehpour *et al*., 1993; Rayment *et al*., 2010). These studies confirm that lithium ions are associated with enhancement of the α_1-adrenoceptor agonists mediated contraction and sustainability of the time course. Considerably more work needs to be done to investigate the effect of lithium ions in human blood vessels. Additional concentration on the investigation of the definite mechanism of lithium action(s) on the porcine vascular smooth muscle with various α-adrenoceptor agonists should not remain obscure.

Our findings in the perfusion snout are in agreement with the results of the previous chapters that noradrenaline and phenylephrine have less sensitivity to the nasal vasculature and nasal mucosa and still, the extra-nasal vessels were, in general, (3-4)-fold more sensitive to these agonists than the nasal artery and nasal mucosa. Thus, no part of the nasal vasculature and mucosa are more sensitive to phenylephrine and noradrenaline than any of the extra-nasal vessels examined. Moreover, collectively the present study supported that phenylephrine was still the lowest effective agent in the porcine nasal artery and the perfused porcine nasal mucosa comparing with other common nasal decongestants even without lithium effects.

In this respect, the strong advocacy of phenylephrine as an orally-active decongestant (Desjardins and Berlin, 2007; Koller *et al*., 2007) from a pharmacological perspective, is

illogical. Additionally, there is no reasonable justification for the removal of the topical form of phenylephrine (Fenox)® from the market. The only possible clarification is that either the orally-active nasal decongestants are designed to have more affinity to the nasal vasculature than systemic circulation as postulated by (Aviado *et al.*, 1958; Aviado *et al.*, 1959) or presumably to avoid conflict about the presence of two different routes for the same drug and replaced by xylometazoline and oxymetazoline nasal spray. However, another important practical implication is that whether phenylephrine, if combined topically with clinically acceptable levels of lithium ions, may turn into a more effective topical decongestant medication. Hopefully, this combination will overcome the problems associated with phenylephrine and other decongestants, which provide effective topical nasal decongestant with long duration of action.

Finally, a number of important limitations must be considered. First, the inability to focus on the pharmacological manipulation using various interventions on the porcine nasal artery. Second, it was not possible to test some of the intriguing observations that were noted in the porcine blood vessels and vasculature using the human blood vessels. The synergistic interaction between α-adrenoceptor agonists and lithium could have a potential clinical application, especially the combined form of topically applied phenylephrine with a therapeutic level of lithium ions. Another limitation of this study was the inability to define the appropriate mechanism of action(s) of lithium with α-adrenoceptors.

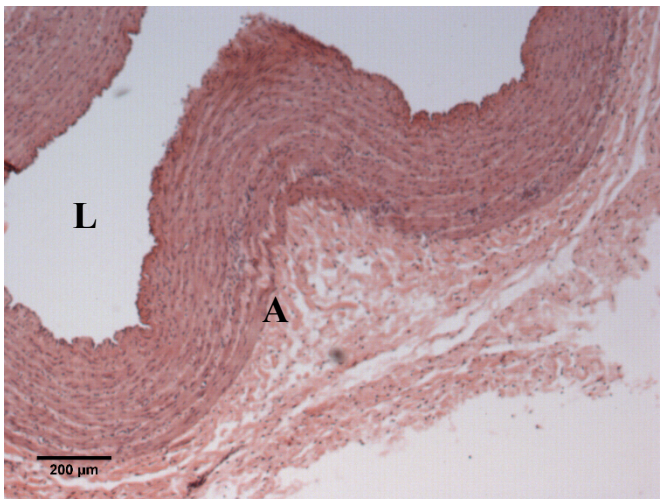
Conclusion

Our findings attest to the fact that phenylephrine can constrict nasal artery and nasal mucosa, which provides the basis for its topical use as a decongestant. This finding accords with its consumer to assist nasotracheal intubation (Christensen *et al*., 2017). However, oral phenylephrine (10mg) medication has no pharmacological basis for the claimed efficacy as a decongestant to alleviate symptoms of nasal congestion without attendant peripheral side effects. While the clinical efficacy of pseudoephedrine has been approved. The reason of higher sensitivity of the splenic artery versus the nasal artery and the nasal mucosa related to noradrenaline and phenylephrine is not clear but it may have something belonging to variation in either receptor density or receptor coupling for the α_1-adrenoceptor in porcine vasculature. The perfused porcine nasal snout possessed the same sensitivity to phenylephrine as the nasal artery.

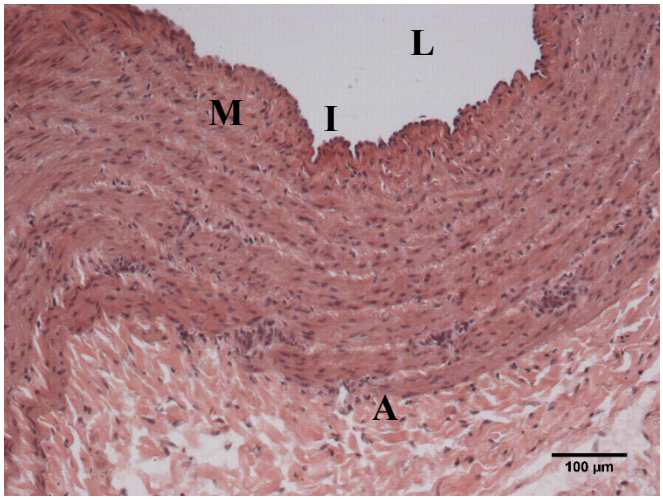
This thesis increases the understanding of the role of α_1-adrenoceptors in which under standard *in vitro* conditions, co-stimulation of both α-adrenoceptor subtypes by noradrenaline afford greater likelihood for the involvement of at least two subtypes of α_1-adrenoceptors and revealed no role for post-junctional α_2-adrenoceptors in contractile response *in vitro*. The latter effect and function can be seen only with pharmacological manipulation using raising calcium and cAMP levels in the porcine splenic artery and nasal artery. This conclusion is consistent with the report that post-junctional α_1- and α_2-adrenoceptors in man operate in an independent manner (Muszkat *et al*., 2011). Another important novel outcome of the present work is that the therapeutic levels of lithium ions can selectively enhance the contractile response of porcine vascular smooth muscle and the time course of porcine blood vessels and nasal mucosa to various α-adrenoceptor agonists, particularly the phenethylamines. Investigations are required to assess the impact of lithium ions on those agonists in the human blood vessel.

Appendix

A)



B)



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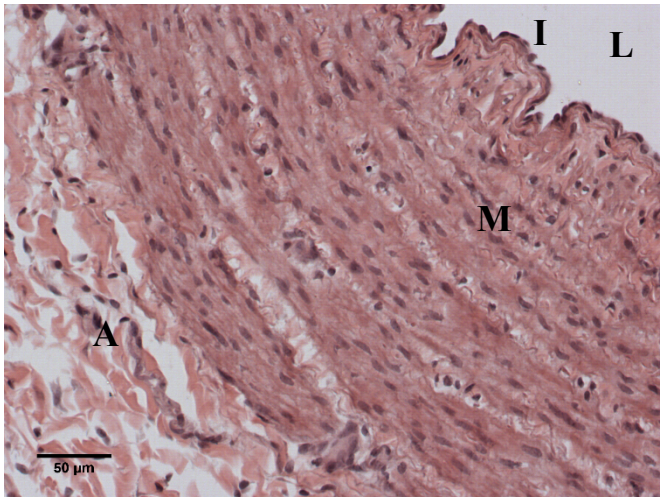
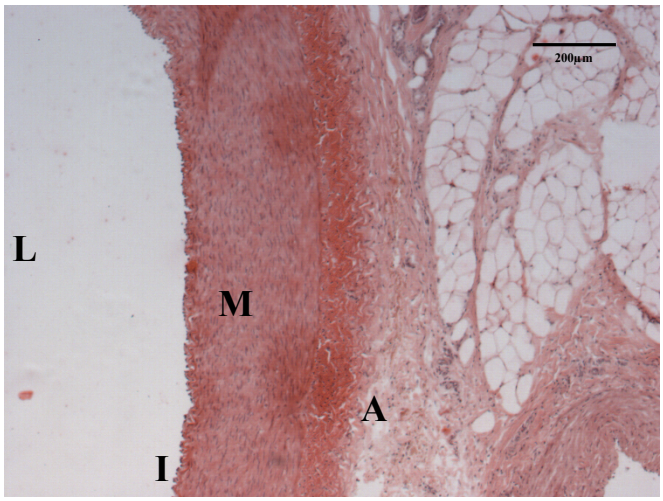


Appendix 1 Images from light microscope illustrated histology of the porcine nasal artery. Samples were cut into 8 microns thick slices and stained with H&E. Images were taken at three different powers of magnification (A) 5X, (B) 10X and (C) 20X. Letters were used to refer to different layers of the vein, L= lumen, I= tunica Intima, M= tunica Media, A= tunica Adventitia and the measurement scale units (um) refered to microns.

A)



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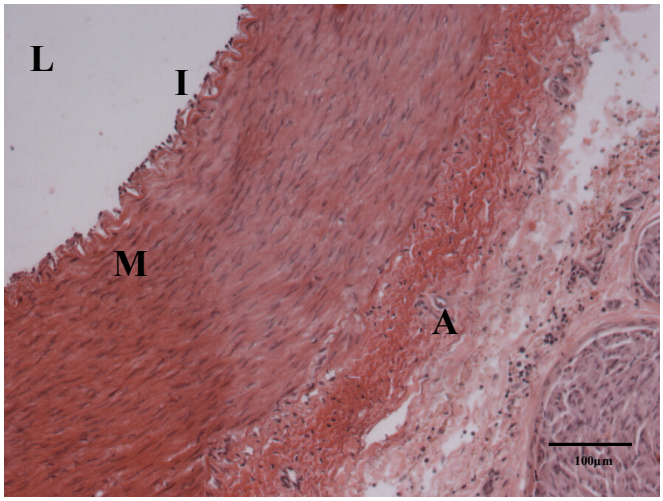
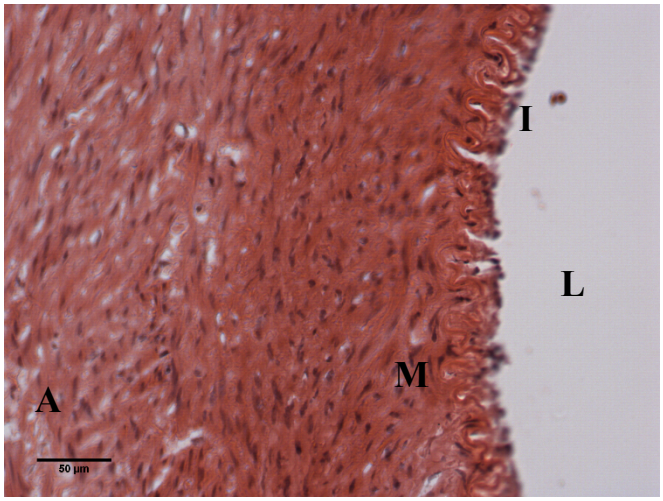
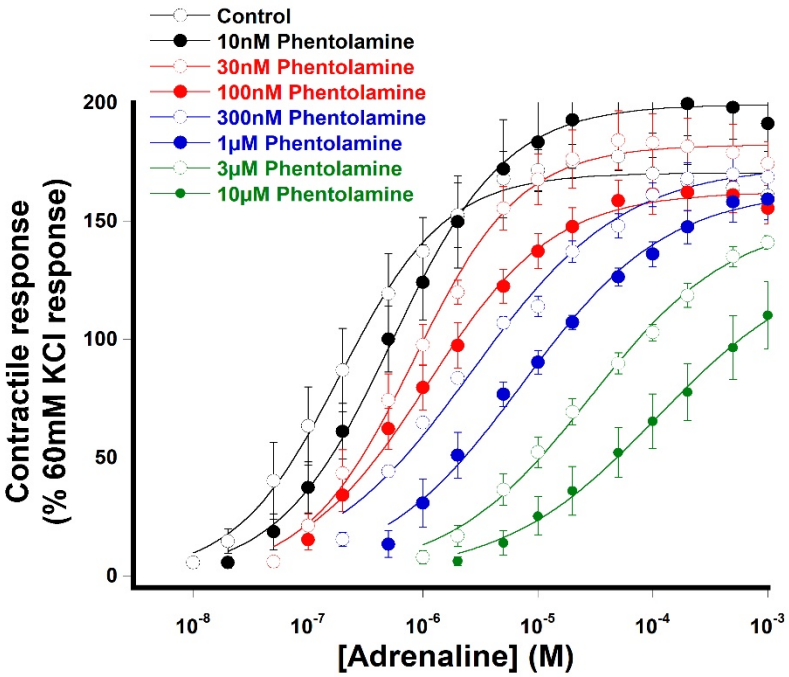
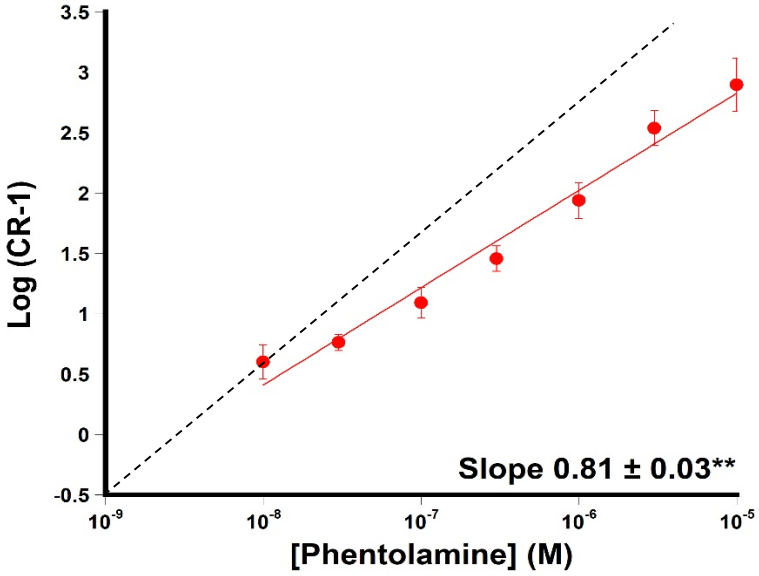


C)



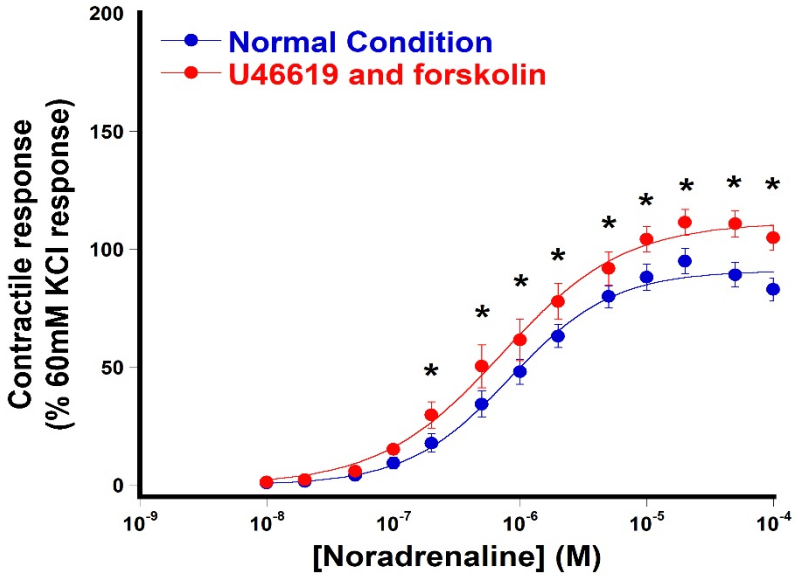
Appendix 2 Images from light microscope illustrated histology of the porcine splenic artery. Samples were cut into 8 microns thick slices and stained with H&E. Images were taken at three different powers of magnification (A) 5X, (B) 10X and (C) 20X. Letters were used to refer to different layers of the vein, L= lumen, I= tunica Intima, M= tunica Media, A= tunica Adventitia and the measurement scale units (um) refered to microns.





Appendix 3 The effect of phentolamine against adrenaline-induced contractions of the porcine isolated splenic artery in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol (n=8). Responses to adrenaline were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M, as were agonist- concentration ratios. The line of unity on the Schild plot was shown as a dashed line.

** ($P < 0.01$) - denotes that the slope of the Schild plot for each antagonist was significantly different from unity (the dashed line) based on single sample *t*-test.



Appendix 4 Response of the porcine nasal artery to to increasing cumulative concentrations of noradrenaline in normal condition and after pharmacological manipulation. In the pharmacological manipulation group, the segments were contracted with U46619 then relaxed with forskolin before the exposure to noradrenaline in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol. Results were expressed as a percentage of the contraction to 60mM KCl and were shown as the mean ± S.E.M of seven observations.

*$P < 0.05$, Student's paired *t*-test.

Appendix 5 Quantitative parameters of the concentration-response curves of the porcine nasal artery to noradrenaline (in the presence of 10µM cocaine, 30µM corticosterone and 1µM propranolol) after pharmacological manipulation using u46619 and forskolin firstly in the absence and presence of 30nM RX-811059. Secondly, in the presence of 0.1µM prazosin and a combination of 0.1µM prazosin and 30nM RX-811059. The table shows the maximum response and the negative logarithm of EC_{50} (pD_2) as a percentage of a former response to 60mM KCl. Results were expressed as the mean ± S.E.M of six observations.

*($P < 0.05$) – denotes a statistically significant difference, Student's paried *t*-test.

		Maximum Response (% of 60mM KCl)	**pD_2**
U46619 and forskolin	**Control**	107 ± 8	6.03 ± 0.12
	30nM RX-811059	109 ± 10	6.01 ± 0.03
	0.1µM Prazosin	76 ± 5	4.70 ± 0.01
	0.1µM Prazosin + 30nM RX-811059	70 ± 6*	4.60 ± 0.14

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