# The role of angiotensin II on human and partial bladder outlet obstructed rabbit corpus cavernosal contractility: modulation of nitric oxide-mediated relaxation and relevance to erectile dysfunction

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# ABSTRACT

The interaction between angiotensin II (Ang II), a smooth muscle constrictor peptide and nitric oxide (NO) a vasodilator, as well as the role of oxidative stress (OS), have been investigated in human and chronic partial bladder outlet obstructed (PBOO) rabbit corpus cavernosal tissue. The PBOO rabbit model is characterised by an increase in corpus cavernosal collagen deposition and a marked reduction and impaired relaxation of corpus cavernosal smooth muscle (CCSM) cells, making it a useful model for erectile dysfunction (ED).

Immunohistochemical analysis identified Ang II peptide distribution in human corpus cavernosal tissue, while organ bath studies determined the Ang II/NO interaction. OS was determined using apocynin and diphenylene iodonium chloride (DPI), inhibitors of NAD(P)H oxidase, which inhibit superoxide production and superoxide dismutase (SOD, the enzyme that accelerates the breakdown of superoxide).

Human penile Ang II was distributed in the arteriolar endothelium, the endothelium lining sinusoids and CCSM cells. The peptide caused a dose dependent contraction of CCSM strips that was inhibited by losartan (AT1 receptor antagonist) and apocynin. In contrast, CCSM relaxation induced by either sodium nitroprusside (SNP, an NO donor) or electrical field stimulation (EFS) was potentiated by losartan. The Ang II contractile response was enhanced in CCSM strips taken from PBOO rabbits and inhibited by losartan, DPI and SOD. CCSM relaxation induced by SNP/EFS was impaired in this model and improved by vardenafil (PDE5 inhibitor) and losartan.

Taken together, these findings suggest that Ang II and NO interact to modulate human and rabbit penile smooth muscle tone. Moreover, the Ang II response involves the production of superoxide and the development of OS. The increase in Ang II-mediated CCSM contraction following PBOO is likely to be a pathological consequence of the condition. Importantly, AT1 receptor inhibition may be a therapeutic target for the treatment of ED associated with PBOO.

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| | |
|---|---|
| α | Alpha |
| µ | Micro |
| β | Beta |
| 5-HT | 5-hydroxytryptamine |
| ACE | Angiotensin converting enzyme |
| ACh | Acetylcholine |
| AGEs | Advanced glycation end products |
| Ang I | Angiotensin I |
| Ang II | Angiotensin II |
| Ang III | Angiotensin III |
| ARB | Angiotensin II receptor blocker |
| BPH | Benign prostatic hyperplasia |
| CC | Corpus cavernosum/cavernosal |
| CAD | Coronary artery disease |
| CGMP | Cyclic guanosine monophosphate |

| | |
|---|---|
| CNS | Central nervous system |
| CCSM | Corpus cavernosal smooth muscle |
| CVD | Cardiovascular disease(s) |
| DPI | Diphenylene iodonium chloride |
| DM | Diabetes mellitus |
| ED | Erectile dysfunction |
| EFS | Electrical field stimulation |
| GC | Guanylyl cyclase |
| $H_2O_2$ | Hydrogen Peroxide |
| H & E | Hematoxylin and eosin |
| HCC | Human corpus cavernosal/cavernosum |
| HOCl | Hypochlorous |
| Hr(s) | Hour(s) |
| Hz | Hertz |
| ICP | Intracavernosal pressure. |
| IDDM | Insulin-dependent diabetes mellitus |
| IgG | immunoglobulin G |
| IHC | Immunohistochemistry |
| KCl | Potassium chloride |

| | |
|---|---|
| L-NAME | L-NG-nitroarginine methyl ester |
| LUTS | Lower urinary tract symptoms |
| M | Molar |
| MMAS | Massachusetts Male Aging Study |
| MRNA | Messenger RNA |
| Mg | Milligram |
| Min | Minute(s) |
| ml | Millilitre |
| Mm | Millimolar |
| Ms | Millisecond |
| NA | Noradrenaline |
| NaCl | Sodium chloride |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate |
| $NaHCO_3$ | Sodium bicarbonate |
| $NaH_2PO_4$ | Monosodium phosphate |
| NANC | Non-adrenergic non-cholinergic |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |

| | |
|---|---|
| NPY | Neuropeptide Y |
| NS | Non-significant (statistical analysis) |
| $^{\bullet}O_2^-$ | Superoxide |
| $OH^{\bullet}$ | Hydroxyl radicals |
| $ONOO^-$ | Peroxynitrite |
| OS | Oxidative stress |
| PBOO | Partial bladder outlet obstruction |
| PDE-5 | Phosphodiesterase type-5 |
| PE | Phenylephrine |
| PG(s) | Prostaglandin(s) |
| PI3K | Phosphatidyl-inositol kinase |
| PKG | Protein Kinase G |
| RAS | Renin-Angiotensin system |
| ROS | Reactive oxygen species |
| S | Second(s) |
| SHR | Spontanously hypertensive rat |
| SNP | Sodium nitroprusside |
| SOD | Superoxide dismutase |
| V | Volt |

| | |
|---|---|
| VED | Vasculogenic ED |
| VIP | Vasoactive intestinal peptide |
| VSMC | Vascular Smooth Muscle Cells |

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Modulation of nitric oxide-mediated rabbit corpus cavernosal smooth muscle relaxation with angiotensin II:relevance to penile erection. Moderate poster presentation. AUA Annual congress USA, Orlando 2008.

# STATMENT OF ORIGINALITY

I, Hani Sedigh Ertemi, confirm that the work presented in this Thesis is original and my own, based on the analysis of results from my laboratory experiments, which I have performed with guidance from my supervisor, colleagues and senior scientists.

# Chapter one

# General Introduction

## 1.1 Anatomy of the penis

The human penis is a pendulous organ suspended from the anterior and lateral aspect of the pubic arch containing the distal part of the urethra. It is composed of three erectile bodies running in parallel; the corpus spongiosum, encompassing the urethra and terminating in the glans penis; and the two corpus cavernosa (CC) which function as blood filled capacitors providing the structure to the erect organ (Andersson and Wagner, 1995). The penile CC is a highly specialized vascular structure that is morphologically adapted to their function of becoming engorged during sexual arousal. The trabecular smooth muscle constitutes approximately 40-50% of tissue cross sectional area, as assessed by histomorphometreic analysis (Nehra *et al*, 1998). The cavernosal tissue is composed of a complex meshwork of interconnected cavernosal spaces, or sinusoids, lined by vascular endothelium. Each sinusoid is separated by trabeculae consisting of fibrous tissue, elastic fibres and plain muscular fibre this fibro-elastic network, which fills with blood during erection.

The relationship/architecture of these structures can be seen in the scanning electron micrographs of a normal rabbit corpus cavernosum shown in Fig 1.

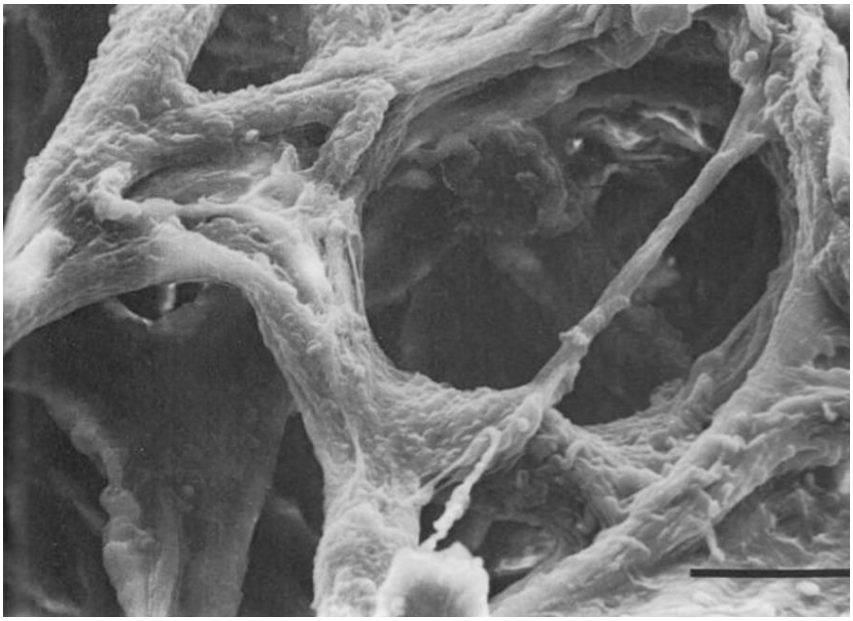

**Figure 1.** Scanning electron micrograph from a representative cavernosa of a 6 month control rabbit (scale bar 50µm). Adapted with permission from Dr C S Thompson.

### 1.1.1 Blood supply of the penis

There are three main arteries of the penis; cavernosal, dorsal and bulbourethral. All three arise from a shared branch of the internal pudendal artery and provide an extensive vascular network (Yiee, 2010).

Nowadays, there is a tendency to perform *in vitro* experiments using the pudendal artery to investigate pathophysiological aspects of penile function, since the artery is the major resistance to penile engorgement during sexual stimulation.

The CC arterial blood supply is mainly fed from the deep penile cavernosal artery (Andersson and Wagner, 1995), which cause corporal enlargement during erection, whereas the deep dorsal artery is more likely to cause glans enlargement.

The architecture of venous drainage is completely different to that of the arterial supply; there exists only one deep dorsal vein that runs alongside the dorsal arteries

(Fig 2) and nerves in Buck's fascia above the tunica albuginea, which is a multilayered structure where emissary veins pass (Moscovici *et al*, 1999).

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### 1.1.2 Nervous supply of the Penis

The innervation of the penis is both autonomic (sympathetic and parasympathetic) and somatic (sensory and motor). From the neurons in the spinal cord and peripheral ganglia, the sympathetic and parasympathetic nerves merge to form the cavernous nerves, which enter in the CC and corpus spongiosum to affect neurovascular events during the tumescence and detumescence (Dean and Lue, 2005).

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**Figure 2.** Penile anatomy. Adapted from Fazio and Brock (Erectile dysfunction:management update) , 2004

## 1.2 Physiology of penile erection

Penile erection is elicited by a physiologically unique sequence of events (Goldstein & Udelson, 1998; Andersson, 2001; Carson & Lue, 2005; Giulano & Rampin, 2004; Argiolas & Melis, 2005). On male sexual arousal higher cerebral centres in the paraventricular nucleus of the hypothalamus and limbic system are activated (Anderson & Wagner, 1995). Central neurotransmitters implicated in the control of erection include dopamine, serotonin, noradrenalin, opiates, oxytocin, prolactin and gamma-aminobutyric acid. The precise role of these central pathways in erectile physiology has been difficult to verify as the central nervous system (CNS) also controls other components of sexual activity, including arousal and copulation. However, evidence from animal experiments and studies in patients with spinal cord injuries has established, at least in part, that sacral spinal reflux pathways control erection (Giulano & Rampin, 2004; Argiolas & Melis, 2005). This spinal mechanism involves afferent pudendal nerve pathways communicating via interneurons to pre-ganglionic parasympathetic neurons in the dorsal horn of the sacral spinal cord (S2-S4) (Giulano &Rampin, 2004; Argiolas & Melis, 2005). These afferent parasympathetic (cholinergic) fibres reach the penis via the pelvic plexus and are implicated in both detumescence and ejaculation (Giulano & Rampin, 2004; Argiolas & Melis, 2005).

The erectile process also involves non-adrenergic non-cholinergic (NANC) neurotransmission, which causes smooth muscle relaxation of the penile arteries and corpus cavernosum. The principal neurotransmitter during this event is nitric oxide (NO). This has lead to the now widely held belief that this molecule is a highly important component of penile erection (Trigo-Rocha *et al*, 1993; Burnett 1997).

The unique haemodynamics of erection has previously been investigated (Shirai & Ishii, 1981; Fournier *et al*, 1987, Aboseif & Lue, 1988; Goldstien & Udelson, 1998). These studies demonstrated that during this process the compliance of the corpora increases and arterial resistance decreases, exposing sinusoids to systemic blood pressure leading to engorgement of blood in the CC. As the flow increase and sinusoidal smooth muscle relax, subtunical venules draining the corpus cavernosal tissue become compressed against the inelastic tunica albuginae, resulting in veno-occlusion and a rise in the intracavernosal pressure (ICP). This veno-occlusive mechanism is absent in the corpus spongiosum, which maintains pressure mainly through flow state in the glans penis. Relaxation of corpus cavernosal smooth muscle allows expansion of the corpora body with blood under pressure, allowing the penis to act as a capacitor. In the presence of adequate inflow and high outflow resistance, the capacitor function is limited solely by the stiffness of fibro-elastic elements of the penis. Detumescence occurs as a consequence of increased cavernosal smooth muscle tone, contraction of the sinusoids and a reduction in arterial inflow and venous resistance.

### 1.2.1 The central role of nitric oxide (NO)

Penile erection is a haemodynamic process involving increased arterial inflow and restricted venous outflow, co-ordinated with corpus cavernosum smooth muscle (CCSM) relaxation. Although this process is generally accepted to be under neuroregulatory control, biochemical mediators released locally from the cavernosal endothelium and/or smooth muscle also participates in initiating and maintaining an erection (Sullivan *et al,* 1999). Although several vasodilators have been implicated in erectile physiology, it is now well established that NO is the main vasodilator

involved (Leite *et al*, 2007; Toda *et al*, 2005). It is released by the endothelium of the arteries that supply the penis and the corpus cavernosum, as well as during NANC neurotransmission. In this capacity NO is derived from nitregic nerves (Ignarro *et al*, 1990; Rajfer *et al* , 1992; Toda *et al*, 2005). NO mediates CCSM relaxation following diffusion in to vascular smooth muscle cells (VSMCs), where it initiates a series of reactions (Fig 3). It activates soluble guanylyl cyclase (GC) to generate cyclic guanosine monophosphate (cGMP) (Kim *et al*, 1991) which activates protein kinase G (PKG) isoforms (Hofmann, 2005). PKG acts by phosphorylating proteins that modulate cellular function. Principally, the inhibition of calcium mobilization from intracellular stores (the sarcoplasmic reticulum), elicits CCSM and vascular smooth muscle relaxation, resulting in penile erection (Kim *et al* 1991; De Tejada 1992; Trigo-Rocha *et al* 1993; Hofmann, 2005).

Apart from smooth muscle relaxation the NO-cGMP-PKG system also controls other physiological functions that protect against vascular diseases. These include inhibition of adhesions molecule expression, lipid oxidation, matrix protein synthesis and deposition, VSMC proliferation and inhibition of oxidative stress (OS) ( Muzaffar *et al*, 2005).

The importance of the NO-cGMP-PKG axis in mediating erection is illustrated by the therapeutic action of phosphodiesterase type 5 (PDE5) inhibitors (sildenafil, vardenafil, tadalafil), which are commonly used to treat patients with erectile dysfunction (ED). These drugs act by inhibiting PDE5, the enzyme that breaks down cyclic GMP to inactive GMP. Importantly, this process augments cGMP levels and leads to CCSM relaxation (Sullivan *et al*, 1999).

NO is formed from the precursor amino acid L-arginine by enzymatic action of NO synthase (NOS), which exists in three main isoforms: neuronal NOS (nNOS),

inducible (iNOS) and endothelial NOS (eNOS). All three isoforms have been detected in penile tissue, although nNOS and eNOS are the main constitutively active NOS enzymes expressed (Burnett *et al*, 1993).

Blood flow is another variable that should be considered in CCSM relaxation, since it induces fluid shear stress in the penile vasculature stimulating phosphatidyl-inositol 3- kinase (PI3K), which phosphorylates protein kinase B (PKB), which in turn phosphorylates eNOS to also generate NO (Burnett, 2004). Given the dramatic alteration in penile flow during erection, this may represent a key mechanism that links nNOS to eNOS. It maybe that nNOS provides the initial dilatory trigger and NO derived from eNOS maintains the erection during sexual intercourse (Burnett, 2004; Hurt *et al*, 2006; Musicki &Burnett, 2006).

This may not be the full story pertaining to NOS activity; other contributing factors probably play a role adding to the complexity of the process. This is illustrated by the fact that nNOS and eNOS deficient mice are still able to maintain erectile function and reproductive capacity, (Burnett, 2004; Musicki & Burnett 2006). In addition, sildenafil failed to augment erections in nNOS deficient mice but was effective in eNOS deficient mice (Cashen *et al*, 2002).

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**Figure 3.** Physiology of erection, adapted from Mechanisms in Erectile Function and Dysfunction: An Overview, K P Nunes *et al,2012.*

### 1.3 Corpus cavernosal constrictor mediators

The erectile response is terminated when cGMP-specific phosphodiesterase catalyses the hydrolysis of cGMP to 5'- GMP, thus halting the cascade of reactions and leading to smooth muscle contraction and concomitant detumescence (Firoozi *et al*, 2005). There is also the concomitant activation of pro-contractile mechanisms/mediators, which keep the smooth muscle of the penile arteries and trabeculae contracted (Holmquist, *et al* 1992). Several constrictor mediators have been identified and are thought to play a role in terminating penile erection.

#### 1.3.1 Noradrenaline (NA)

Sympathetic noradrenergic fibres and parasympathetic cholinergic terminals innervate cavernosal tissue (Giuliano and Rampin, 2004). The sympathetic pathway causes detumescence via NA neurotransmission, which causes corpus cavernosal contraction.

#### 1.3.2 Serotonin (5-hydroxytryptamine, 5-HT)

5-HT, a monoamine transmitter has been found in the central and peripheral nervous system, as well as in a number of non-neuronal cells in the gut, cardiovascular system and blood. 5-HT is one of the oldest neurotransmitters in evolution and has been implicated in the aetiology of numerous disease states, including depression, anxiety, hypertension and irritable bowel syndrome.

It is well established that neuronally-released 5-HT participates in the control of sexual behaviour, both in humans and animals, whereby it acts as a major modulator of the central neuroregulatory control of erection (Carson III, 2007). Its actions on erectile function are via supraspinal and spinal neurotransmission and affects both sympathetic, parasympathetic, and somatic outflow mechanisms (Carson, III, 2007).

Generally, 5-HT exerts an inhibitory effect on male sexual behaviour (Bitran and Hull, 1987). However, it also has a facilitatory role depending upon its action on other 5-HT receptor subtypes located in the CNS (de Groat, 1993).

Neurogenic derived 5-HT has been reported to cause human CCSM contraction via 5-HT(1A), (1B), (2A) & (4) receptor subtypes (Lau *et al*, 2006). It also has a direct action on CCSM tissue, since it causes a profound contraction of human and rabbit CCSM tissue via 5-HT 2A receptor activation (Lau *et al*, 2006, 2007)

It is likely that the physiological role for 5-HT is to modulate penile flaccidity, especially as its levels are significant elevated in cavernous serum from normal men during the flaccidity/detumescence phase (Uckert *et al*, 2003).

### 1.3.3 Neuropeptide Y (NPY)

NPY has been identified in the human penis (Hauser-Kronberger *et al*, 1994), where it is co-localised with NA in sympathetic perivascular nerves and contribute to the vasoconstriction elicited by activation of sympathetic nerves (Ekblad *et al,* 1984).

Immunohistochemical studies have identified numerous NPY-immunoreactive nerves in erectile tissues, with a high density around helicine arteries (Wespes *et al*, 1988; Schmalbruch and Wagner, 1989;Kirkeby *et al*, 1991). Initially, NPY was suggested to

play a role in detumescence (Kirkeby *et al*, 1991). However, functional studies using human corpus cavernosal strips did not support this, as NPY was ineffective (Hedlund and Andersson, 1985). Interestingly, intracavernous injection of NPY increased ICP and led to penile tumescence in rabbits (Kirkeby *et al*, 1992).

### 1.3.4 Angiotensin II (Ang II)

The renin–angiotensin system (RAS) is a complicated hormonal system. Angiotensin II (Ang II) is the main effector of RAS and a regulator of important physiological functions. Angiotensinogen is the precursor of all angiotensin peptides, consisting of 453 amino acids. It is synthesized and released from the liver into the circulation. Renin, an acid protease produced by the kidney juxtaglomerular cells, cleaves amino acids from the N-terminus of angiotensinogen to generate angiotensin I (Ang I). Subsequently, Ang I is converted to the octapeptide Ang II by ACE, which occurs primarily in the lung. In addition, the formation of Ang II from Ang I is catalyzed by a chymotrypsin-like serine protease, chymase, which is released from mast cells. Ang II is metabolized by aminopepsidase A to angiotensin III (Ang III).

The traditional view of RAS as a systemic endocrine system has been extended in the past decade. Many organs and tissues, such as heart, brain, kidney, and blood vessels, contain components of RAS, functioning in a paracrine manner, that is mediators released/synthesised by the organ and acting locally (Tipnis *et al*, 2000). Tissue RAS synthesizes Ang II locally, which is modulated independently of systemic RAS. It plays an important role in the regulation of local tissue functions in addition to systemic RAS. The accumulated evidence suggests the existence of RAS in the penis.

### 1.3.4.1 Angiotensin II receptors

Ang II exerts its biological actions mainly through two subtypes of angiotensin receptors: type 1 (AT1) and type 2 (AT2). These receptors are seven transmembrane-spanning receptors that belong to the G-protein-coupled receptor superfamily but only have 34% sequence identity (Mukoyama *et al*, 1993). AT1 has two isoforms: AT1a and AT1b. AT1a is dominantly expressed in the smooth muscle and endothelial cells of the blood vessel wall, brain, and many other organs. The AT1b receptors are found mainly in the anterior pituitary and adrenal cortex (Gasc *et al*, 1994). Ang II is a potent ligand for AT1, whereas Ang I and Ang III have much lower affinity for AT1. The most commonly described physiological actions of Ang II are mediated by AT1, including vasoconstriction, cell growth and proliferation, salt and water retention, activation of the sympathetic nervous system and stimulation of vasopressin. Unlike AT1, which has been extensively studied, the physiological role of AT2 is much lesser known. AT2 is dominantly expressed in fetal tissues, which dramatically decreased after birth (Shanmugam *et al*, 1995; Mialn *et al* 1991). Consequently, it is difficult to study the function of AT2 in adult tissues, as expression levels are very low. Frequently in experimental settings, blocking AT1 is required to reveal the functions of AT2. Studies have shown that the actions of Ang II mediated by AT2 are mostly opposite to those of AT1. These physiological responses include vasodilatation, antiproliferation, and apoptosis (Zimpelmann *et al*, 2001).

### 1.3.4.2 Ang II signalling

Stimulation of AT1 triggers multiple signal transduction processes, leading to a variety of (patho) physiological actions. AT1-mediated vasoconstriction occurs through both calcium-dependent and independent mechanisms. The classic pathway is the G-protein-mediated activation of phospholipase, releasing inositol trisphosphate and diacylglycerol, thereby increasing intracellular calcium. Studies have also shown that Ang II activates the RhoA/Rho-kinase-mediated calcium independent pathway via AT1, leading to the inhibition of myosin light chain phosphatase ( Ryan *et al*, 2004; Ying *et al*, 2006).

Another important function of AT1 is to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, increasing reactive oxygen species (ROS) production. ROS not only quickly react with NO, to reduce NO bioavailability, but it also stimulates RhoA/Rho-kinase activity (Jin *et al*, 2006; Burnett *et al*, 2006). Moreover, AT1 initiates intracellular signalling cascades that are important in the regulation of cell growth and proliferation through receptor tyrosine kinases (e.g., epidermal growth factor receptors, platelet-derived growth factor receptors, and insulin receptors), non-receptor tyrosine kinases (e.g., Src and Janus kinase), and mitogen activated protein kinases (Nakashima *et al*, 2006; Touyz *et al*, 2002).

AT2-mediated vasodilatation is observed in both large conduct vessels, such as the aorta, and small resistance arteries including mesenteric, coronary, and uterine arteries. The activation of AT2 enhances NO production through modulation of endothelial eNOS protein expression and/or phosphorylation at positive regulatory sites on eNOS (Yayama *et al*, 2006).

#### 1.3.4.3 The physiological role of Ang II in the penis

Ang II exerts its physiological function/action in the corpus cavernosum in a paracrine manner, since the octapeptide is produced by the human corpus

cavernosum and acts locally (Kifor *et al*, 1997). Angiotensin peptides, including Ang I and Ang II, has been detected in human CCSM and endothelial cells (Kifor *et al*, 1997). Becker *et al*. (2001) reported that Ang II levels were 30% higher in the cavernous blood than in systemic blood. A comparison of the different penile phases, i.e. flaccidity, tumescence, rigidity, and detumescence, revealed that Ang II levels were significantly higher during detumescence (Becker *et al*, 2001). This finding highlights the importance of Ang II when the penis is not erect. The distribution pattern of Ang II containing cells has been identified in the endothelial and CCSM cells of the rat penis ((Kifor *et al*, 1997). Ang II binding sites have also been determined in rabbit corpus cavernosum by autoradiography. Support for the location of Ang II in the corpus cavernosum has been provided by immunostaining for ACE using dog corpus cavernosa, which found the enzyme in the endothelial cells (Iwamoto *et al*, 2001).

The functional response of Ang II has been reported; it was found to cause a profound contraction of isolated rabbit cavernosal muscle strips that was much stronger than Ang I (Park *et al*, 1997). In addition, they found ACE inhibition markedly reduced the contractile response elicited by Ang I, indicating that Ang I is converted to Ang II by ACE located in the cavernosal tissue (Park *et al*, 1997).

They also determined that Ang II-induced CCSM contraction was via AT1 receptor activation and not AT2 (Park *et al*, 1997). These observations have been confirmed using canine CCSM tissue, in addition, the effect of Ang II was augmented by NOS inhibition (Comiter *et al*,1997). Blockade of AT1 receptors also significantly reduced adrenergic agonist- and electrical field stimulation induced contractions, suggesting a possible role of Ang II in regulation of a-adrenergic activity in the penis. These data

are in line with previous findings that suggest RAS has a stimulatory influence on the sympathetic nervous system (Brasch *et al*,1993).

It would seem, therefore, that Ang II is a potent constrictor of CCSM and thus an essential modulator of erectile function. This role is clear to see following in vivo injections of Ang II into the cavernosal body of anesthetized dogs, which terminated spontaneous erections (Kifor *et al*, 1997). Interestingly, intracavernosal injection of losartan, the AT1 antagonist, increased the ICP in a dose dependent manner, resulting in penile erection (Kifor *et al*, 1997). These data reinforce the notion that the physiological role of Ang II is to increase CCSM tone to ensure the penis is kept in a flaccid state.

### 1.3.5 Reactive oxygen species, Ang II and nitric oxide

Molecular oxygen (dioxygen) is utilized by all mammalian cells, there being more than 200 enzymes that have oxygen as one of its substrate (Malmstrom, 1982). The metabolism of oxygen inevitably generates ROS. Amongst the ROS family are superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), hypochlorous (HOCl), hydroxyl radicals ($OH^{\bullet}$), reactive aldehydes and lipid radicals (Hensley *et al* 2000; Shah & Channon, 2004). There are also products from reactions between ROS and NO, for example, peroxynitrite ($ONOO^-$) (Jeremy *et al*, 2000; Patel *et al*, 2000). Sources of ROS include NADPH oxidase, the mitochondrial electron transport chain, xanthine oxidase, NOS, cyclooxygenase, lipoxygenase, haemoxygenases and haemoprotien, as well as by redox cycling of small molecules, hypoxia-re-oxygenation, auto-oxidation, hypoxia and oxygenation of haemoglobin. Oxidative injury, i.e. OS, occurs when the oxidative burden of the body exceeds its antioxidant capacity. Lipid peroxidation, protein oxidation, DNA oxidation, decreased synthesis and

bioavailability of eNOS and nNOS and the up-regulation of pro-inflammatory cytokines, growth factors and tissue-specific receptors have all been implicated in this form of injury (Slater, 1984). Ang II is a potent stimulator of the smooth muscle enzyme NAD(P)H oxidase, which stimulates the production of ROS such as $^{\bullet}O_2^-$ (Touyz*, et al* 2001). In fact, in human blood vessels the membrane associated NAD(P)H oxidase is thought to be the principle source of basal and Ang II-induced $^{\bullet}O_2^-$, and is implicated in virtually every cardiovascular disease (CVD) (Cai *et al*, 2003; Jeremy *et al*, 2004; Puntmann, *et al*, 2005). The expression of NADPH oxidase is upregulated by a disparate number of factors implicated in vascular and erectile pathology. These include; endotoxins, peptide growth factors, endothelin-1, Ang II, thromboxane A2, hypoxia and shear stress (Muzaffar et al, 2005).

$^{\bullet}O_2^-$ exerts a number of pathological actions that range from increased cell proliferation, apoptosis of endothelial cells, signal transduction activation, calcium mobilization and vasoconstriction (Young & Woodside, 2001; Jeremy *et al*, 2004). The up regulation of $^{\bullet}O_2^-$, production by Ang II in endothelial and vascular smooth muscle is thought to directly contributes to Ang II-induced smooth muscle contraction (Kawazoe *et al*, 2000). $^{\bullet}O_2^-$ also reacts readily with NO to produce $ONOO^-$ and other ROS (Patel *et al*, 2000). These reactions have two main consequences: 1) a reduction of NO bioavailability and 2) the generation of other toxic radicals (Folkerts *et al*, 2001; Dweik, 2005). An increase in the vascular formation of $^{\bullet}O_2^-$ and $H_2O_2$ has been reported in atherosclerosis, diabetes mellitus (DM), dyslipidemia, smoking, and hypertension (Jeremy *et al*, 1999 & 2004; Li *et al*, 2004).

## 1.4 Erectile dysfunction (ED)

ED is defined as the persistent inability to maintain or achieve a penile erection sufficient for satisfactory sexual performance. It is a widespread problem affecting many across all age groups. The anxiety and psychological burden can affect the well being of sexually active men and seriously impinge on their quality of life. Over 30 million men suffer from ED in the USA (Lue *et al*, 2000) and the prevalence is predicted to rise considerably over the next 25 years, impacting on 300 million men worldwide by 2025 ( Ayta *et al, 1999).*

ED is often associated with CVD (Hafez, 2005), including DM (McCulloch *et al*, 1980), hypercholesterolemia (HcH) (Martin-Morales *et al*, 2001; Wei *et al*, 1994), hypertension (Martin-Morales *et al*, 2001), and smoking (Jeremy and Mikhailidis, 1998), all of which are increasing globally. Although, the aetiology of ED is not fully understood, in the last 4 decades the elucidation of many aspects of the erectile mechanism has ushered in a new era of therapeutic options for erectile disorders (Mersdorf, 1991). New insight into erectile neurotransmission (Andersson *et al*, 1994) and the discovery of the importance of the NO pathway (Ignarro, 1990), has resulted in a rational alternative for treating ED (Andersson, 2011).

As discussed, Ang II is the primary effector of RAS, it is a multifunctional hormone that plays an important role in vascular function and the beneficial effect of Ang II receptor blockers in CVD (hypertension, heart failure and stroke) is well known (Schiffrin *et al*, 2000).

Since Ang II and NO signalling pathways mutually regulate each other by multiple mechanisms ( Yan *et al*, 2003) and the corpus cavernosum is essentially a modified blood vessel, it is reasonable to assume that Ang II has a role to play in ED.

### 1.4.1 Aetiology of erectile dysfunction

ED can be classified as organic or psychogenic (Melman & Gingell, 1999). The former constitutes around 80 % of causes, which include vasculogenic and neurogenic subgroups. However, it is becoming increasingly apparent that the aetiology of a significant proportion of these patients is of mixed origin. (1$^{st}$ Latin American Erectile Dysfunction Consensus Meeting, 2003).

#### 1.4.1.1 Psychogenic ED

Previously, psychogenic impotence was believed to be the most common type, with 90% of impotent men in this group. This was exemplified in the 1960's and 1970's when ED was generally managed by psychiatrists, and thought to be a psychological disorder (Morgantaler, 1999). However, since the introduction and improvement of diagnostic aids, and the use of intracorporal vasoactive drugs as therapeutic agents, this view is no longer held.

Sexual behaviour and penile erection are controlled by the hypothalamus, the limbic system, and the cerebral cortex (Dean *et al*, 2005).Therefore, stimulatory or inhibitory messages can be relayed to the "spinal erection centres" to facilitate or inhibit erection. Two possible mechanisms have been proposed to explain the inhibition of erection in psychogenic ED: direct inhibition of the spinal erection centre by the brain as an exaggeration of the normal suprasacral inhibition and excessive sympathetic outflow, which increases CCSM contraction and prevent its relaxation, a

process required for penile erection (Steers, 1990). Psychogenic ED is more prevalent in younger men, accounting for up to 50% of cases in under 40 years old (Caskurlu *et al*, 2004), compared to around 10% in those over 50 years old (Slag *et al*, 1983). It can be further classified into generalized and situational (Rosen, 2001), or primary and secondary (lifelong or acquired) (Anonymous, 1st Latin American Erectile Dysfunction Consensus Meeting, 2003). The common disorders associated with this type of ED include anxiety, psychotic disorders, and depression (Farre *et al*, 2004). Antidepressant can also further exacerbate ED in these patients (Nurnberg *et al*, 2002).

#### 1.4.1.2.Vasculogenic ED

Vasculogenic ED (VED), involving arteriogenic or veno-occlusive impairment, is the commonest underlying pathogenesis in ED. This is exemplified by the strong correlation between VED and CVD and its common risk factors. VED is more prevalent in those with coronary artery disease (CAD) (Kloner *et al*, 2004). Moreover, the severity seems to correlate with the number of coronary arteries affected (Greenstein *et al*, 1997). The Massachusetts Male Aging Study (MMAS) revealed that cigarette smoking doubled the risk of significant VED after 8-10 years, with slightly lower risks associated with hypertension, and dietary intake of cholesterol and unsaturated fat (Feldman *et al*, 2000). Atherosclerotic lesions were demonstrated in the iliac arteries of cholesterol-fed rabbits that developed subsequent impairment of erectile function (Azadzoi & Goldstein, 1992). Diabetic men are similarly at risk of developing VED (Feldman *et al*, 1994; Romeo *et al*, 2000), which manifests in over 50% of men 60 years old (Romeo *et al*,2000). In one

report, diabetic and older men were found to have a high incidence of fibrotic lesions in the cavernous artery, with intimal proliferation, calcification and luminal stenosis (Michal *et al*, 1982).

VED is now a recognized marker for vascular disease, which may not have manifested clinically yet (Sullivan *et al*, 1999; Montorsi *et al*, 2003; Kaiser *et al*, 2004;). Bocchio *et al* (2005) demonstrated that the carotid intima-media thickness, a marker of atherosclerosis, correlated significantly with the severity of VED in 270 men who had vascular risk factors but no clinical evidence of atherosclerotic disease. Equally, impaired endothelium-dependent, and independent, vasodilatation of the peripheral vasculature in men with VED without clinical CVD has also been demonstrated (Kaiser *et al*, 2004). Montorsi *et al*, (2003) also recognised VED as a surrogate marker for CAD. They reported that of the 300 patients presenting with acute chest pain associated with angiographically proven CAD, 67% had developed VED prior to cardiac symptoms, at a mean interval of 38.8 months. It is now clear that the common denominator that links VED with CAD is endothelial dysfunction (Sullivan *et al*, 1999).

#### 1.4.1.3 Neurogenic ED

Approximately, 10-19% of ED is of neurogenic origin (de Tejada Saenz *et al*, 2005). In fact, if one was to include, iatrogenic causes and mixed ED, the prevalence of neurogenic ED is probably much higher. While, the presence of a neurogenic disorder or neuropathy does not exclude other causes, confirming ED is indeed of neurogenic origin can be challenging. Common causes include Parkinson disease, Alzheimer disease, epilepsy and cerbrovascular events (Lue, 2001). ED in these

conditions tend to be manifested at the initiation of the erectile response. The effect of Parkinsonism is thought to be due to an imbalance in the dopaminergic pathway (Lue *et al*, 1992).

In men with spinal cord injuries, their erectile function depends largely on the nature, location and extent of the spinal lesion. In addition to ED they may also have impaired ejaculation and orgasm. Reflexogenic erection is preserved in 95% of patients with complete upper cord lesions, whereas only about 25% of those with complete lower cord lesion can achieve an erection (Eardley *et al*, 1991).The close association/location between cavernous nerves and pelvic organs, can mean that surgery on these organs is a frequent cause of ED, which is very common after radical prostatectomy (27-75%) (EAU guideline, 2010). However, nerve sparing surgical techniques/procedures can reduce the risk of ED significantly (Hatzichristou *et al*, 2004). Pelvic fracture can result in ED due to cavernous nerve injury or vascular insufficiency or both. Diabetic peripheral and autonomic neuropathy is now a well recognized disease process that also can lead to ED (Colakoglu *et al*, 1999; Brown *et al*, 2005).

#### 1.4.1.4 Endocrinological ED

Between 2% and 23% of men with ED have an underlying endocrine disorder (Morales & Heaton, 2003), although it is commonly unclear whether it is the primary aetiology (Salonia *et al*, 2003).

Hypogonadism is not an infrequent finding in ED. Androgens influence the growth and development of the male reproductive tract and secondary sex characteristics; their effects on libido and sexual behaviour are well established. In a review of

published articles from 1975 to 1992, Muligan and Schmitt (1993) concluded that testosterone enhances sexual interest, increase the frequency of sexual acts and increase the frequency of nocturnal erection. The suggested underlying pathogenesis includes increased smooth muscle apoptosis, increased build up of penile connective tissue, and reduced eNOS expression (Salonia *et al*, 2003).

Hyperprolactinemia whether from a pituitary adenoma or drug treatment, resulted in both reproductive and sexual dysfunction. Symptoms included loss of libido, ED, gynecomstia and infertility. It is also associated with low testosterone levels, which appears to be secondary to the inhibition of gonadotropin-releasing hormone secretion due to the elevated prolactin level (Leonard, 1989).

#### 1.4.1.5 Drug-induced ED

The incidence of drug related ED has been reported to be as high as 25% (Slag *et al*, 1983). However, this is complicated by the fact that most patients have an ongoing disease process, warranting the medication. Drugs that affect the neural, vascular or endocrine system, may lead to ED (Thomas, 2002). The commonest recognized drug-related ED association is caused by antihypertensives, including thiazide and β-blockers (Neaton *et al*, 1993). It is presumed that α and β blockers cause ED by disturbing the autonomic pathways involved in the erectile response (Thomas, 2002). Thiazides probably exacerbate ED by reducing blood flow to the corpora as a result of reducing systolic blood pressure (Gingell, 2004).

### 1.4.2 Oxidative stress (OS) in ED

The role of OS in ED has been the subject of several studies (Beckman & Koppenol, 1996; Agarwal *et al*, 2006). It is known that oxidative products stimulate the production of transforming growth factor-b1 (TGF-b1) and up-regulate fibronectin gene expression, which have a detrimental effect on the erectile response. Thus, treatment with antioxidant agents are beneficial in reducing ED. Barassi *et al* (2009) noted that plasma reactive oxygen metabolite concentrations were higher and plasma total antioxidant status was lower in patients with arteriogenic ED compared to those with non-arteriogenic ED. In addition, antioxidant treatment was found to correct OS in the former group and improve their condition. Another feature of these drugs is that they reduce erectile tissue fibrosis, often associated with ED (Agarwal *et al*, 2006).

OS also plays a role in NO metabolism/function, since it counteracts NO-mediated relaxation of intracavernous arteries and CCSM. This is supported by the finding that $^{\bullet}O_2^-$ destroys NO faster than its natural degradation rate, limiting its bioavailability (Kinlay et al, 2004). Additionally, NO has been shown to decrease the adhesion of platelets and leucocytes to the vascular endothelial cells (Radomski *et al,* 1987). A reduction in NO concentration was suggested to aggravate the adhesion of these cells to the endothelium and release substances such as thromboxane $A_2$, 5-HT and leukotrienes leading to vasoconstriction, exacerbating ED (Jeremy *et al*, 2000).

### 1.4.3 Partial bladder out flow obstruction and ED

It is well recognized that the incidence of ED in men increases with age (Panser *et al*, 1995) as does the incidence of benign prostatic hypertrophy (BPH). It is, therefore, likely that some men presenting with symptomatic BPH will have varying degrees of ED, purely on the basis of their age. However, it is also possible that

severe urinary symptoms secondary to BPH might be associated with ED as a result of sleep disturbance, psychological anxiety and perhaps even a physiological effect from the development of BPH. Although the prevalence of ED and ejaculatory dysfunction are similar (~ 50 % of men aged ≥ 50 years) and increase with age, these conditions are not inevitable consequences of aging (Rosen *et al*, 2003).

In addition to age and Lower Urinary Tract Symptoms (LUTS) other co morbidities described previously, (e.g. DM, hypertension, heart disease) are also risk factors for ED (Thompson *et al*, 2005).

Several clinical studies have demonstrated a relation between LUTS and ED. Namasivayam *et al* (1998) evaluated 168 men attending the prostate clinic for both LUTS and ED, they found that men with LUTS and BPH were more likely to suffer with symptoms of ED than those without prostatic symptoms. Data from the USA BPH Registry provided additional evidence of the significant association between LUTS and ED. As the severity of LUTS increased, so did the severity of ED (Rosen *et al,* 2009). Not surprisingly, these results support previous studies highlighting LUTS as an independent risk factor for ED (Rosen *et al*, 2003; Balnker *et al*, 2001).

### 1.4.4 The role of Ang II in ED

There is evidence that Ang II levels in the cavernous blood of men with organogenic ED is higher than in men with psychogenic ED or healthy subjects (Becker *et al* , 2001). This observation is supported by another study, which showed that angiotensin converting enzyme (ACE) activity in cavernous blood from diabetic patients with organogenic ED was higher than patients with psychogenic ED.

Interestingly; there was no difference in ACE activity in the systemic blood from both patient groups (Hamed *et al*, 2003).

In animals, ACE mRNA expression was up-regulated in a rat model of arteriogenic ED, whilst, expressed at very low levels in control rat penis (Lin *et al*, 2001). Other studies have shown that inhibition of ACE and AT1 improves erectile function, demonstrating the pathological effects of Ang II. Treatment with the ACE inhibitor captopril partially reversed ED and decreased blood pressure in stroke-prone spontaneously hypertensive rats (SHR) (Dorrance *et al*, 2002). One proposed mechanism for hypertension-associated ED in SHR is that Ang II causes vascular remodeling in the penis. Enalapril, another ACE inhibitor, significantly reduced vascular resistance in isolated penile vascular beds (Hale *et al*, 2001).

Treatment with the AT1 blockers candesartan and losartan not only lowered the blood pressure but also prevented the adverse morphology changes in penile tissue of SHR. Moreover, combination therapy of losartan and sildenafil, further improved penile structure when compared with monotherapy with sildenafil or losartan alone, this response was thought to be due to an increase in endothelium-dependent relaxation (Toblli *et al*, 2007).

It is plausible that the prevention of ED by blood pressure lowering drugs may be secondary to the correction of hypertension. However, in their study Jin et al (2008) found that apocynin treatment only slightly reduced blood pressure, whereas it restored most of the erectile function in an Ang II-infused hypertensive rat model. This suggests that blood pressure control may not be the only target to improve erectile function in these rats. This notion is supported by studies that compared the effects of AT1 blockers on erection with other blood pressure-reducing agents. Neither amlodipine, (a calcium antagonist), nor hydralazine, (a vasodilator), showed

any improvement on penile structure in SHR, despite similar blood pressure lowering action to that of AT1 blockers (Tobil *et al*, 2004; Mazza *et al*, 2006). Similarly, clinical data suggest that antihypertensive drugs such as diuretics, β-adrenergic antagonists, and calcium blockers either have no effect or worsen erectile function, while AT1 blockers may have beneficial effects (Fogari *et al*,2002; Manolis *et al*, 2008).

The signalling pathway activated by Ang II in the penis and the role of Ang II in the development of ED has been investigated *in vivo* by Jin *et al* (2008). They found that, chronic infusion of exogenous Ang II for 4 weeks induced ED in Sprague–Dawley rats. It was suggested that the excessive Ang II significantly increased NADPH oxidase protein expression as well as NADPH oxidase-dependent ROS/$^{\bullet}O_2^-$ generation in penile tissue isolated from Ang II-infused rats, which would undoubtedly lead to CCSM constriction and ultimately ED.

DM is a major risk factor for ED (Ahn *et al*, 2007; El-Sakka *et al*, 2007). Recent evidence suggests that inhibition of RAS improves cardiovascular function in DM. The effect of AT1 blockers on ED was investigated in streptozotocin-induced diabetic rats (Chen *et al*, 2007). Similar to the human studies, Ang II levels were elevated in the penile tissue of these animals. Erectile frequency induced by apomophine and erectile responses to the stimulation of major pelvic ganglion were significantly increased by the AT1 blocker valsartan, whereas another antihypertensive drug, spironolactone (an aldosterone receptor antagonist), did not improve erectile function in diabetic rats. These observations confirm that an increase in Ang II is a major contributor for DM associated ED.

#### 1.4.4.1 Clinical evidence of Ang II blockade in ED

Whilst some antihypertensive drugs may worsen erectile function, many clinical studies suggest those that target RAS can be beneficial or have no significant negative impact on erectile function and sexual activity (Doumas *et al*, 2006).

Initial studies of RAS intervention were focused on its effects on sexual activity. In a study of 164 hypertensive men with ED, 12-week therapy with losartan significantly improved sexual satisfaction and sexual activity; with no effect on those without a previous history of ED (Llisterri *et al*,2001). In a randomized, double-blind, crossover study, the effects of the ACE inhibitor lisinopril and the β-blocker atenolol were compared in non-treated hypertensive men. During the initial 4 weeks of treatment both lisinopril and atenolol reduced sexual function. However, those on prolonged lisinopril treatment (16 weeks) recovered sexual activity, which was not the case for those on prolonged atenolol treatment (Fogari *et al*, 1998).

Several clinical studies have shown that inhibition of AT1 and ACE improves erectile function. In a recent study, more than 1,000 hypertensive patients with hypertension and metabolic syndrome were enrolled for treatment with the AT1 blocker irbesartan alone or irbesartan with hydrochlorothiazide (a diuretic). At the end of 6 months those treated with irbesartan or irbesartan/hydrochlorothiazide, had a significant improvement in erectile function along with increased sexual desire and sexual contact (Baumhakel, 2008). Interestingly, those who received prior antihypertensive treatment with calcium channel blockers, diuretics or antidiabetic drugs, and statins had a negative influence on erectile function, whereas α-blockers, β-blockers, and ACE inhibitors did not affect erectile function. Although the mechanisms underlying

the positive effects of ACE and AT1 inhibitors on erectile function in humans are not clear, it seems that blood pressure reduction in itself is not enough to improve erectile function. This is supported by many studies, which have shown that other classes of antihypertensive drugs are not as beneficial (Manolis *et al*, 2008). Instead, it is more likely that the improvement of endothelial function by ACE and AT1 inhibitors contribute significantly to the recovery of erectile function. This is consistent with the finding that ACE and AT1 inhibitors have been shown to increase eNOS mRNA and protein expression and reduce ROS generation in patients with CVD, leading to an increase in NO bioavailability and thus smooth muscle relaxation (Billups *et al*, 2005).

## 1.5 Hypotheses: Angiotensin II, oxidative stress and NO/cGMP interact and play a pivotal role in the normal and pathological erectile process.

Ang II has been implicated in the termination of penile erection. This study investigates whether this process involves Ang II-mediated CCSM contraction and the development of OS interacting with NO/cGMP-mediated relaxation, the salient feature of penile erection. Moreover, whether the relationship between these mediators are disturbed in PBOO, a known model of ED, where NO bioavailability is reduced. I will evaluate further and test the hypothesis that inhibition of Ang II and OS and the preservation of the NO/cGMP pathway will have a beneficial effect on CCSM function using tissue from human and experimental animal models.

### 1.5.1 Aim

1) To characterise the effect of Ang II on CCSM function using exogenous Ang II and its antagonists and inhibitors of OS in human, as well as control, and PBOO rabbit models using organ bath methodology. In addition, the presence and distribution of Ang II within corpus cavernosal tissue, will be determine using immunohistochemistry.

2) To determine whether the beneficial actions of losartan (AT1 receptor antagonist) on CCSM function, is due to a pro-tumescence effect by inhibiting Ang II-mediated CCSM contraction.

3) To investigate whether the NO-mediated CCSM relaxation associated with PBOO is impaired and the effect of *in vitro* losartan treatment.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Purchase and maintenance of rabbits

Adult male New Zealand White rabbits weighing between 2.5 - 3.0 kg were purchased from Harlan UK (Bicester, Oxford), a UK Home Office accredited source. These normal healthy rabbits were kept in the animal house for at least a week before the start of the study to acclimatise them to the local surroundings. The rabbits were exposed to a 12-hr on and 12-hr off light/ dark cycle during that week and to the end of the study, prior to their sacrifice. All animal were fed ad libitum on rabbit maintenance, standard plain diet (Scientific Diet Supplies, Whitham, UK) and allowed free access to water.

## 2.2 Induction of partial bladder outlet obstruction (PBOO) in rabbits

Animal models of PBOO are well established (Khan *et al*, 1999; Calvert *et al*, 2001a; Lin *et al*, 2008; Beamon *et al*, 2009) and can mimic BPH and reproduces the urodynamics and structural changes in bladder pathophysiology (Calvert et al, 2001a ; Beamon et al, 2009).

To create PBOO, each rabbit received a general anaesthetic (1-2% halothane in $O_2$) and was then placed on a heating pad regulated at $37^0$C.

The abdominal fur was shaved and a urinary balloon catheter (Foley, C.R. Bard international Ltd, Crawley, UK) size 8 Fr gauge was inserted uretherally via the penis into the bladder and the balloon inflated. A lower midline laparotomy was then performed to expose the bladder, unlike man the whole of the rabbit bladder lays

superficially within the peritoneum. The bladder neck was identified and a 2-0 silk ligature was then placed around the urethra as distal as possible below the bladder neck but above the entrance of the prostatic ducts into the urethra. The silk ligature was placed snugly, without any tension, around the urethra so that the catheter can be easily removed without any resistance. This procedure was found to be reproducible and required approximately 20 min to complete.

At the end of the operation the peritoneum was irrigated liberally with about 50 ml of sterile normal saline, to prevent small bowel obstruction secondary to adhesion formation. The urinary balloon catheter was then removed and the laparotomy incision closed in layers. The peritoneum was closed with continuous 2-0 vicryl, rectus muscles were approximated using interrupted 2-0 vicryl sutures, rectus sheath closed using continuous 2-0 vicryl. The skin was closed with continous subcuticular 2-0 prolene sutures. Local anaesthetic (lignocaine 1%) was then applied to the wound site and the animal allowed to recover (Khan et al, 1999).

Pain medication (buprenorphine, 0.1 mg/kg im, twice daily for 2 days) and antibiotics (enrofloxacin, 10 mg/kg im, twice daily for 5 days pre- and post-operatively) was administered to each rabbit. Sham-operated rabbits underwent the same surgical procedure without tying the ligature around the urethra.

All procedures were conducted under an approved Home Office Project Licence.

## 2.3 Acquisition of control, sham-operated and PBOO rabbit penile tissues

After 8 weeks the sham-operated and PBOO rabbits were killed by cervical dislocation (using a method permitted by the Home Office). The penis was rapidly excised from each rabbit and placed in cold oxygenated Krebs solution at $4^0$C and transported to the laboratory for organ bath experiments (Thompson *et al,* 2001). The Krebs solution was made up of sodium chloride (NaCl) 133 mM, sodium bicarbonate ($NaHCO_3$) 16.4 mM, magnesium sulphate ($MgSO_4$) 0.6 mM, potassium chloride (KCl) 4.7 mM, calcium chloride ($CaCl_2$) 2.5 mM, monosodium phosphate ($NaH_2PO_4$) 1.4 mM and glucose 7.7 mM with a pH of 7.4.

Tissue preparations were investigated on the same day within 1h of acquisition.

Twenty animals were used for the control rabbit study (see Chapter 3). In other experiments 20 rabbits underwent PBOO with 12 age-matched, sham-operated rabbits (see Chapter 5).

## 2.4 Acquisition of human penile tissues for experiments

Human penile organs were obtained from 35 patients undergoing gender reassignment surgery at Charing Cross Hospital, London UK. Ethics approval was obtained (Riverside Ethics Committee Chelsea & Westminster Hospital, London) and all patients gave their informed consent prior to surgery. The patients undergoing gender reassignment surgery were on oestrogen treatment for approximately two years. However, this was discontinued for at least 2 months prior to surgery and experiments were conducted within 24 h of tissue acquisition (see Chapter 4).

Mirone *et al*, (2000) has reported that corpus cavernosal tissue obtained from gender reassignment surgery constitute a reliable/viable source of human tissue with functional integrity. This tissue can be used for up to four days to evaluate drug activity or to study corpus cavernosal pathophysiological mechanisms; a conclusion based on unchanged responses during this time, to chemical stimuli such as phenylephrine (PE), Ang II and potassium chloride (KCl), as well as acetylcholine (ACh; endothelium-dependent) and sodium nitroprusside (SNP; endothelium-independent), mediators of smooth muscle relaxation.

## 2.5 Organ bath experiments

### 2.5.1 Materials

The following chemicals were purchased from Sigma Chemical Co. (Poole, UK): KCl, atropine hydrochloride, guanethidine, indomethacin, phenylephrine (PE), diphenylene-iodoniuym chloride (DPI), superoxide dismutase (SOD), 4-hydroxy-3-methoxyacetophenone (apocynin), human angiotensin II (Ang II), PD123,319, L-$N^G$-nitroarginine (L-NAME). Angiotensin II antiserum (T-5001), host guinea pig for immunohistochemistry was purchased from Bachem (Bubendorf, Switzerland). Rabbit anti-guinea pig peroxidase conjugate antibody, mouse monoclonal anti CD34 and normal goat IgG was purchased from Dako UK, Ltd (Cambridgeshire, UK). SNP was purchased from BDH Chemical Ltd (Poole, UK. Losartan was a free gift from Merck Research Laboratories (New Jersey, USA).

### 2.5.2 Organ bath technique

Before the start of the experiments the buffer reservoir, thermoregulated circuit and 10 ml organ baths were washed twice with distilled water and Krebs. The buffer reservoir was then filled with the Krebs solution. Artery forceps were used to clamp the tubes that released Krebs into the organ bath from the reservoir, as well as the tubes that drained the buffer way. The apparatus was calibrated using the chart 4 software the system with a 4g weight.

The corpus spongiosum and urethra were excised and the corpus cavernosal muscle isolated from the surrounding tunica albuginea by careful dissection of rabbit and human penis and cut into strips (4-8 pieces) of approximately 1x3x1 mm and 5x5x6 mm, respectively. The size and weight of normal, sham-operated and PBOO rabbit cavernosal strips from each experimental group were similar.

Each tissue strip was attached at one end to the horizontal section of an L-shaped stainless steel rod with a piece of string. This anchorage was regarded as the tissue "fixed point" during the experiments. A piece of string was also tied to the other end of the tissue, which was long enough to be rapped around a hook attached to a transducer connected to a Grass Polygraph (model 7D; Astro-med Grass, Slough UK). This was the "dynamic point" of the set up, as either relaxation or contraction of the strip will cause a change in the tension, which was recorded by the polygraph. The tissue strips were mounted vertically into the organ baths and immersed into Krebs solution at pH 7.4, maintained at $37^0$C by the thermoregulated circuit and bubbled with a mixture of 95% $O_2$ - 5% $CO_2$.

A 2g tension was applied to the suspended tissue strips, which were washed twice and left for 1h to equilibrate and the tension recorded on the Grass Polygraph. It was noticed on some occasions that the tissue tension in the organ bath drop below 2g, however no attempt to re-equilibrate the tension was taken, as this might affect tissue contractility. The lay out of the apparatus can be seen in Fig 4.

In some experiments the L-shaped stainless steel rod was also equipped with two parallel platinum electrodes. The tissue was strung up the same as before, but mounted between these electrodes to allow electrical field stimulation (EFS). A stimulation frequency of 8Hz was used in all experiments, since it has been reported that this frequency produced the maximum relaxation (32.8%) of human cavernosal strips (Lau *et al*, 2009).

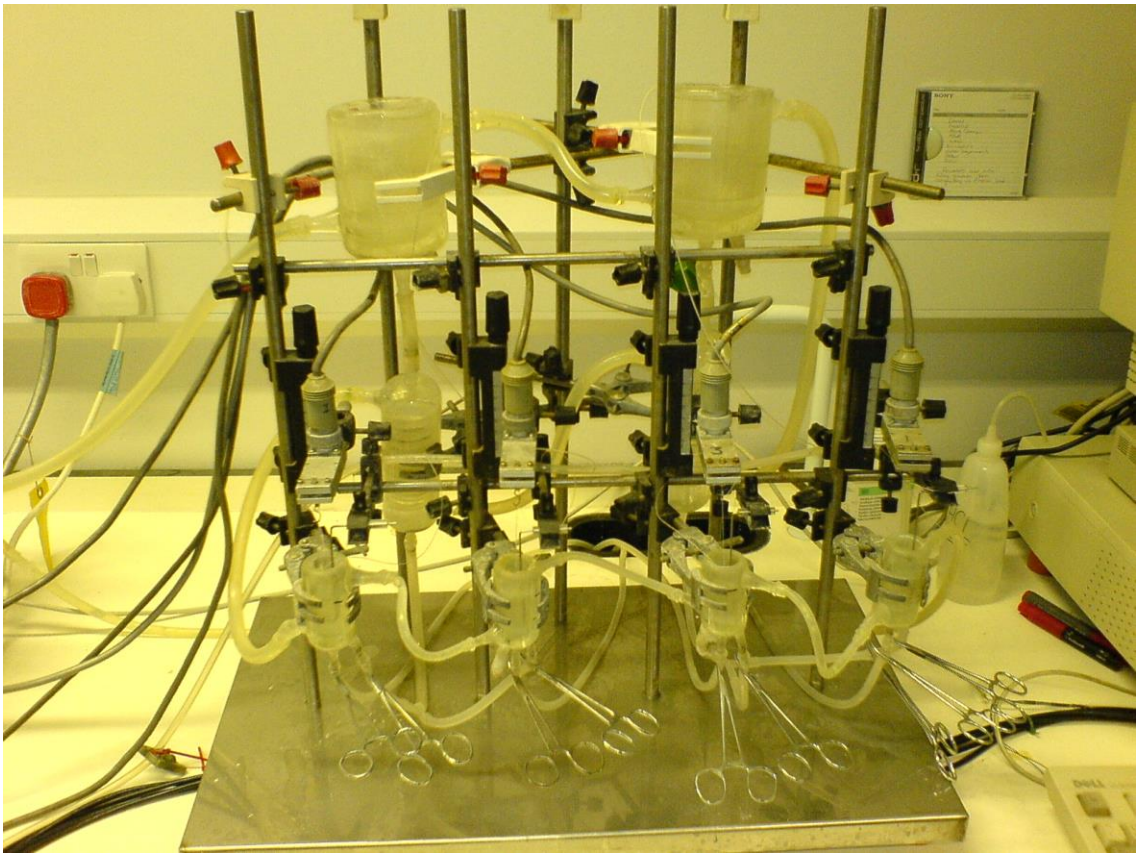

**Figure 4: Photograph showing 4 organ baths used to determine corpus cavernosal tissue function.**

## 2.6 Immunohistochemistry

Human CC tissue was fixed in formalin and embedded in paraffin wax. Serial 3μm tissue sections were cut to allow immunostaining of adjacent sections for Ang II, smooth muscle actin and an endothelial marker, CD34. Sections were rehydrated and endogenous peroxidase was blocked by 0.5% hydrogen peroxide in methanol. Antigen retrieval for Ang II and smooth muscle actin immunostaining was by microwaving at 900W for 20 min in Tris-EDTA buffer pH 9. Antigen retrieval for endothelial immunostaining was by microwaving at 600W for 10 min in citrate buffer pH 6. For smooth muscle actin immunostaining, sections were covered with mouse monoclonal anti smooth muscle actin (Dako) at 1:500, followed by anti mouse IgG conjugated to horseradish peroxidase using the NovoLink polymer system (Leica). For endothelial immunostaining, sections were covered with mouse monoclonal anti CD34 at 1:50, followed by anti mouse IgG conjugated to horseradish peroxidase as for smooth muscle actin immunostaining. For Ang II immunostaining, sections were covered with guinea pig anti Ang II at 1:100 in Tris buffer pH 7.6 for 30 min. After washing, sections were covered with rabbit anti guinea pig IgG conjugated to horseradish peroxidase at 1:500 for 30 min. After further washing, diaminobenzidine and hydrogen peroxide were added as substrates for peroxidase. The sections were counterstained with Mayer's haematoxylin. Control sections had omission of the first stage antibody.

# Chapter 3

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# The interaction between angiotensin II and nitric oxide on rabbit corpus cavernosal function.

## 3.1 Introduction:

The regulation of corpus cavernosal smooth muscle tone has been a subject of interest due to its importance in the process of penile erection. Most studies have focussed on mediators of CCSM relaxation in an attempt to develop new treatment strategies for ED and it is from this work the PDE 5 inhibitors have emerged as the front line therapeutic option. However, unlike most smooth muscle cells those of the corpus cavernosum spend the majority of the time contracted (Chang *et al*, 2002), which keeps the penis in a flaccid state. This is achieved by a variety of vasoconstrictor agents that maintain the balance between CCSM contraction and relaxation, as discussed in Chapter 1. Thus, the control of penile tone determines the functional status of the penis at any one time, which will range from; detumescence and flaccidity to tumescence and erection.

The vasoconstrictors agents that keep the penis in the flaccid/detumescence state are not very well understood. Angiotensin II (Ang II), a known modulator of regional blood flow in the vascular bed, is thought to be one such mediator of human CCSM contractility and tone (Becker *et al*, 2001).

A previous rabbit study demonstrated that Ang II causes a dose dependent contraction of CCSM tissue (Park *et al*, 1997). These authors carried out functional characterisation of the Ang II receptors on rabbit CCSM and found that the Ang II response was blocked by AT1 but not AT2 receptor antagonist. Although this was a very important finding, they did not explore the possible interaction of Ang II with NO an important mediator of CCSM relaxation. This was partially addressed in another study that examined the effect of the NOS inhibitor, nitro-$_L$-arginine methyl ester (L-NAME) on the Ang II response using canine CCSM (Comiter *et al*, 1997). In that

study Ang II again caused a dose-dependent contraction, which was augmented by L-NAME. The authors also noted that adding SNP at the peak of the Ang II contraction caused CCSM relaxation. However, this may be a direct effect of SNP-induced relaxation and not an interaction between Ang II and NO. This study, therefore, lacks a true demonstration of an interaction at the receptor level.

Thus, in this Chapter the focus will be on the physiological interaction of Ang II/NO, together with their relationship with ROS and OS on rabbit CCSM tone. This is particularly important, since along with its potent and well defined vasoconstrictive action, Ang II has been demonstrated to increase $^{\bullet}O_2^-$ production in endothelial cells, vascular smooth muscles, adventitial fibroblasts and mesangial cells through activation of NADH/NADPH oxidases (Touyz *et al* ,2002; Pagano *et al* ,1998; Jaimes *et al* ,1998; Lassègue *et al* ,2003). In addition, chemical antagonism between $^{\bullet}O_2^-$ and NO is recognized as a potentially important modulator of vascular reactivity as well as a source of $ONOO^-$ production, a potent oxidant (Beckman *et al*, 1990). These experiments have the potential to define the physiological role of Ang II in the erectile process.

## 3.2 Material and Method:

### 3.2.1 Tissue acquisition

See Chapter 2 for details of tissue acquisition and experimental procedure.

### 3.2.2 Ang II response

In preliminary experiments, multiple application of different concentration of Ang II were applied to each rabbit CCSM strip to create a cumulative dose response curve, an approach described by Park et al (1997). Unfortunately, this resulted in an undulating curve which was difficult to interpret. It was also noted that repeated applications of Ang II caused a marked desensitisation of the functional response (i.e. tachyphylaxis), which made the data even more unreliable. This phenomenon has been reported by others using isolated human arteries (Hidaka T *et al* 2005). Therefore, to avoid erroneous interpretation of the data, a single dose of Ang II / individual tissue, in a dose range of $10^{-8}$M – $10^{-5}$M was used to minimise the development of tachyphylaxis and create each dose response curve. This meant each Ang II concentration had to be repeated several times, which was time consuming and required the use of many tissue strips.

### 3.2.3 Effect of Ang II receptor antagonists and L-NAME

The effect of Ang II on tissue strips was determined; the tissue was then washed over a 30 min period and exposed to either losartan ($10^{-5}$ M; AT1 antagonist) or PD123,319 ($10^{-5}$ M; AT2 antagonist) for 20 min before repeating the Ang II response.

The effect of the vehicle (distilled water for 20 min) on the Ang II response was also determined. In other experiments, nitro-$_L$-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor, $10^{-4}$ M) was added to the bathing solution for 20 min and the effect on the Ang II ($10^{-6}$ M) response determined.

### 3.2.4 Effect of SNP

Tissue strips were pre-contracted with phenylephrine PE and cumulative response curves were constructed for SNP ($3x10^{-7}$ - $10^{-6}$ M), with each dose only being applied when the response had reached a plateau. The tissues were then washed several times followed by the addition of losartan to the organ bath for 20 min. The tissues were re-contracted with PE and cumulative response curves were again constructed for SNP.

### 3. 2.5 Electrical field stimulation (NANC neurotransmission)

In a series of experiments CCSM tissue strips from control rabbits were exposed to a mixture of guanethidine ($5 \times 10^{-6}$ M), atropine ($10^{-5}$ M) and indomethacin ($10^{-6}$ M), added to the bathing solution and left for 20 min. The purpose of this treatment was to inhibit the adrenergic, cholinergic and cyclo-oxygenase pathways, respectively, leaving the NANC pathway intact. Tissues were then pre-contracted with PE ($10^{-4}$ M) followed by EFS of penile nerves with a Grass S88 (Astro-med Grass, Slough UK) stimulator. The stimulator delivered single square waves (duration 0.4 ms; 100V) at a frequency of 8.0 Hz in 5 s trains.

### 3.2.6 Oxidative stress

**3.2.6.1.1 DPI :** The effect diphenylene iodonium chloride (DPI, $10^{-4}$M, NAD(P)H oxidase inhibitor, which inhibits $^{\bullet}O_2^-$ production) has on the Ang II-induced contraction ($10^{-6}$M) was determined.

**3.2.6.2 SOD:** The effect superoxide dismutase (SOD, 200 IU/ml; the enzyme that accelerates the breakdown of $^{\bullet}O_2^-$) has on the Ang II ($10^{-6}$M) response was also determined.

**3.2.6.3 DPI and SNP**

To demonstrate the effect of inhibiting OS on SNP-induced relaxation, DPI and losartan were used. SNP cumulative response curves were constructed using rabbit CCSM tissue. The tissue was washed several times before the addition of DPI and losartan to the organ bath for 20 min and re-contracted with PE and the SNP cumulative response curve repeated.

### 3.2.7.Electrical Field Stimulation (NANC neurotransmission).

In another series of experiments, the effect OS and AT1 receptor inhibition have on NANC neurotransmission was determined.

The triple blockers were added to the organ bath; tissues were then pre-contracted with PE ($10^{-4}$ M) followed by EFS of penile nerves, which resulted in CCSM relaxation. This was followed by the addition of losartan and DPI, which blocked AT1 receptors and OS, respectively. The tissues were re-contracted with PE and the EFS repeated.

### 3.2.8 Statistical Method

The raw data from Ang II pre- and post-losartan, PD123,319, L-NAME, DPI and SOD were expressed as % contraction of the PE contractile response. EFS and SNP tissue responses determined pre- and post- losartan, DPI were expressed as % relaxation of PE-induced tone. These were analysed and expressed as mean ± SEM using Graph Pad Prism 4.0 software (see examples, Tables 1-4). Comparisons of the resultant Ang II dose response curves and SNP cumulative dose response curves were made using analysis of variance (2 way ANOVA, $p < 0.05$). Student's unpaired and paired t-test statistical analysis was also determined on the raw data by the software package with statistical significance accepted at $p < 0.05$. The individual number of tissue strips used is included in the figure Legends. Strips from at least 5 animals were used in each experiment

## 3.3 Results:

### 3.3.1 Ang II and its receptors antagonists on corpus cavernosal smooth muscle contraction.

Ang II caused a dose dependent contraction ($10^{-8}$M – $10^{-5}$M) of CCSM strips. This effect was significantly (ANOVA; $P < 0.0001$) reduced by losartan (Table 1, Figs 5 & 5a) but not by PD123,319 (ANOVA ; $P= 0.79$, Table 2, Figs 6 & 6a) , confirming that the contractile effect of Ang II on rabbit CCSM is mediated mainly by AT1 receptor. The addition of water (vehicle) had no effect on Ang II-induce contraction.

**Table 1:** Ang II–induced contraction of corpus cavernosal strips pre- and post- losartan

| Strip No. | Ang II $10^{-8}$M % PE contraction | Ang II $10^{-8}$M post-los % PE contraction | Ang II $10^{-7}$M % PE contraction | Ang II $10^{-7}$M post-los % PE contraction | Ang II $10^{-6}$M % PE contraction | Ang II $10^{-6}$M post-los % PE contraction | Ang II $10^{-5}$M % PE contraction | Ang II $10^{-5}$M post-los % PE contraction |
|---|---|---|---|---|---|---|---|---|
| 1 | 30.0 | 20.0 | 17.0 | 5.0 | 39.0 | 17.0 | 44.0 | 22.0 |
| 2 | 24.0 | 13.0 | 11.0 | 3.0 | 42.0 | 10.0 | 68.0 | 30.0 |
| 3 | 12.0 | 10.0 | 24.0 | 12.0 | 42.0 | 13.0 | 61.0 | 9.0 |
| 4 | 5.0 | 4.0 | 33.0 | 18.0 | 42.0 | 18.0 | 90.0 | 18.0 |
| 5 | 40.0 | 24.0 | 50.0 | 30.0 | | | 48.0 | 30.0 |
| Mean | 22.20 | 14.20 | 27.00 | 13.60 | 41.2 | 14.5 | 62.2 | 21.8 |
| Std. Deviation | 13.9 | 7.9 | 15.3 | 10.9 | 1.5 | 3.7 | 18.3 | 8.8 |
| Std. Error | 6.2 | 3.6 | 6.8 | 4.9 | 0.75 | 1.8 | 8.2 | 3.9 |

**Figure 5**

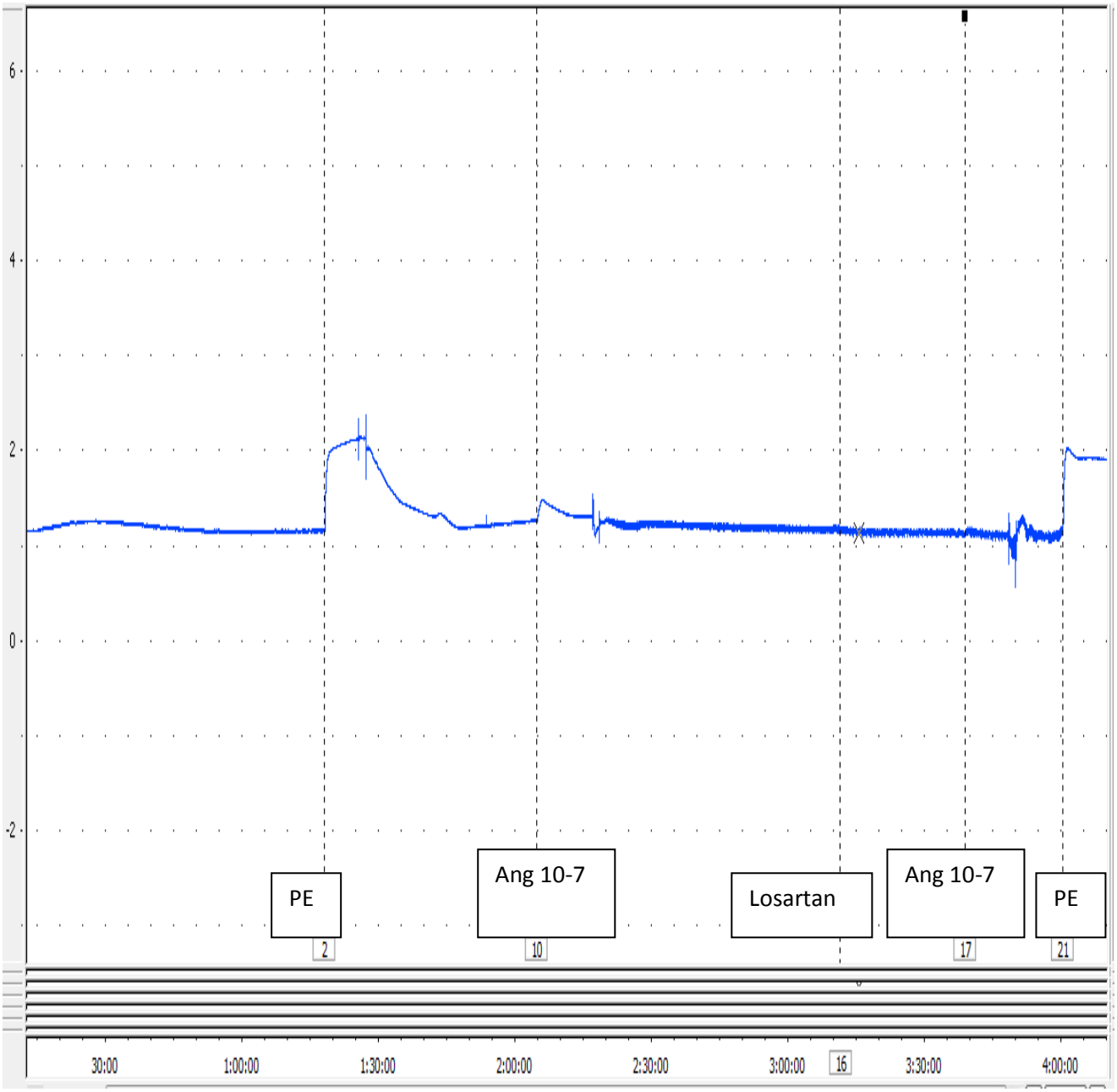


**Figure 5:** Representative tracing of Ang II ($10^{-7}$ M)–induced contraction of a corpus cavernosal strip pre- and post- losartan.

**Figure 5a**

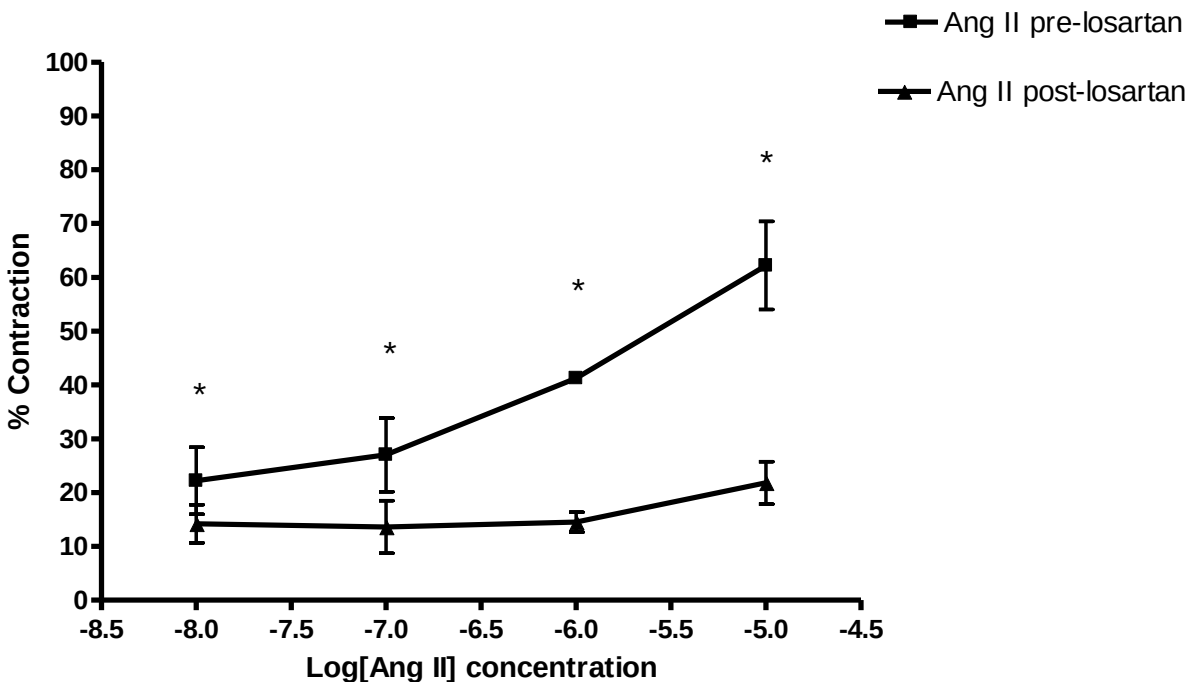


**Figure 5a.** Ang II-induced contraction of corpus cavernosal strips pre- (■) and post- (▲) losartan. *Denotes the data points where there was a significant difference in Ang II-induced contraction pre- and post- losartan (Ang II (M): $10^{-8}$ $p<0.05$, $10^{-7}$ $P<0.0026$, $10^{-6}$ $p<0.0014$, $10^{-5}$ $p<0.016$, paired Student's t-test, n =4).

**Table 2:** Ang II–induced contraction of corpus cavernosal strips pre- and post- PD123,319

| Strip No. | Ang II $10^{-8}$M % PE contraction | Ang II $10^{-8}$M post-PD123,319 % PE contraction | Ang II $10^{-7}$M % PE contraction | Ang II $10^{-7}$M post-PD123,319 % PE contraction | Ang II $10^{-6}$M % PE contraction | Ang II $10^{-6}$M post-PD123,319 % PE contraction | Ang II $10^{-5}$M % PE contraction | Ang II $10^{-5}$M post-123,319 % PE contraction |
|---|---|---|---|---|---|---|---|---|
| 1 | 23.0 | 21.0 | 41.0 | 40.0 | 74.0 | 65.0 | 70.0 | 72.0 |
| 2 | 20.0 | 14.0 | 64.0 | 60.0 | 53.0 | 50.0 | 82.0 | 77.0 |
| 3 | 38.0 | 44.0 | 26.0 | 20.0 | 42.0 | 40.0 | 70.0 | 72.0 |
| 4 | 26.0 | 16.0 | 28.0 | 35.0 | 54.0 | 58.0 | 38.0 | 39.0 |
| 5 | 14.0 | 20.0 | 34.0 | 27.0 | 35.0 | 60.0 | 48.0 | 58.0 |
| 6 | 12.0 | 24.0 | 42.0 | 70.0 | 15.0 | 10.0 | 32.0 | 21.0 |
| Mean | 22.1 | 23.2 | 39.2 | 42.0 | 45.5 | 47.2 | 56.7 | 56.5 |
| Std. Deviation | 9.4 | 10.8 | 13.8 | 19.3 | 19.9 | 20.2 | 20.6 | 22.2 |
| Std. Error | 3.8 | 4.4 | 5.6 | 7.9 | 8.1 | 8.2 | 8.2 | 9.0 |

**Figure 6**

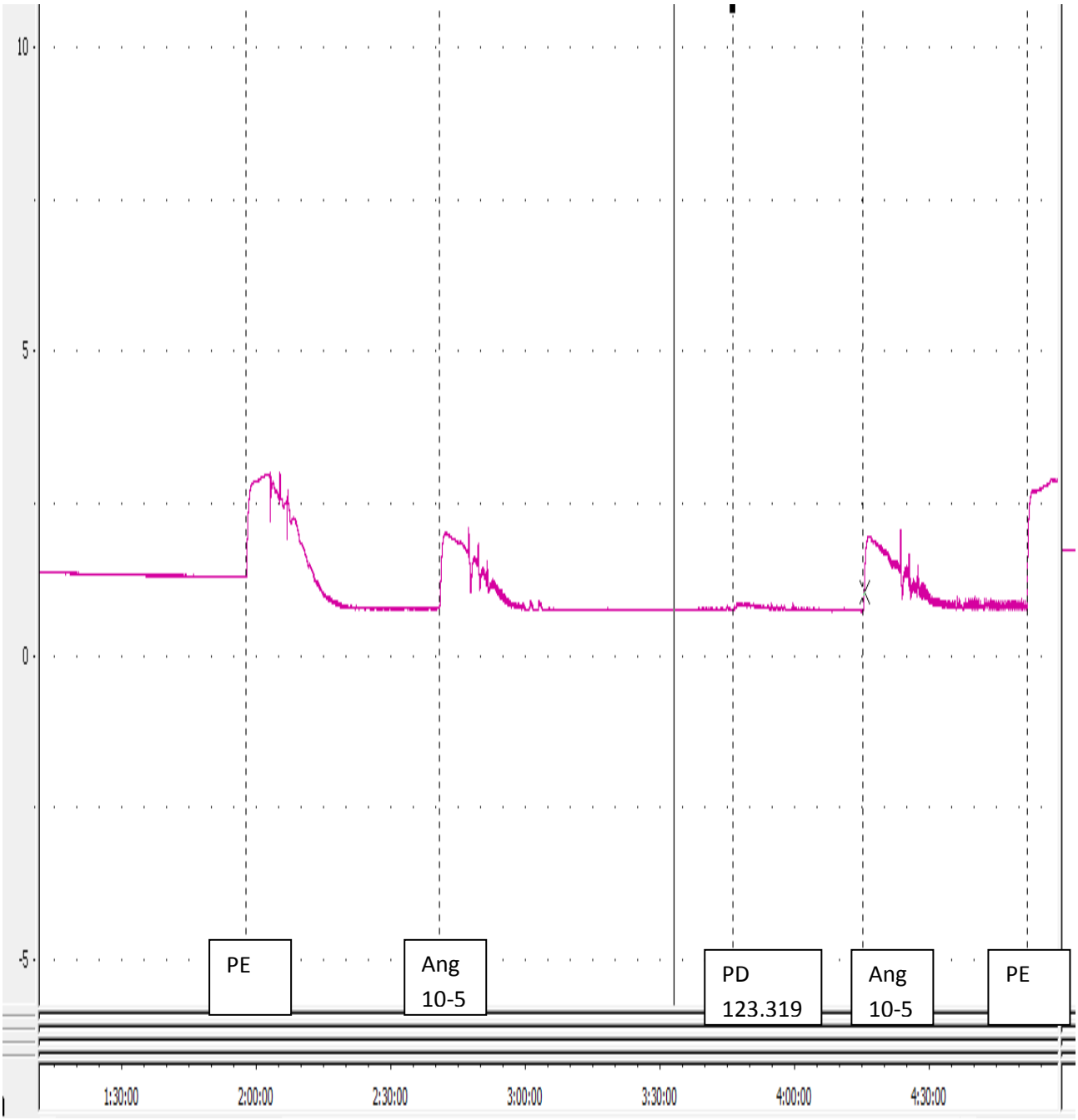


**Figure 6:** Representative tracing of Ang II ($10^{-5}$ M)–induced contraction of a corpus cavernosal strip pre- and post- PD123,319.

**Figure 6a**

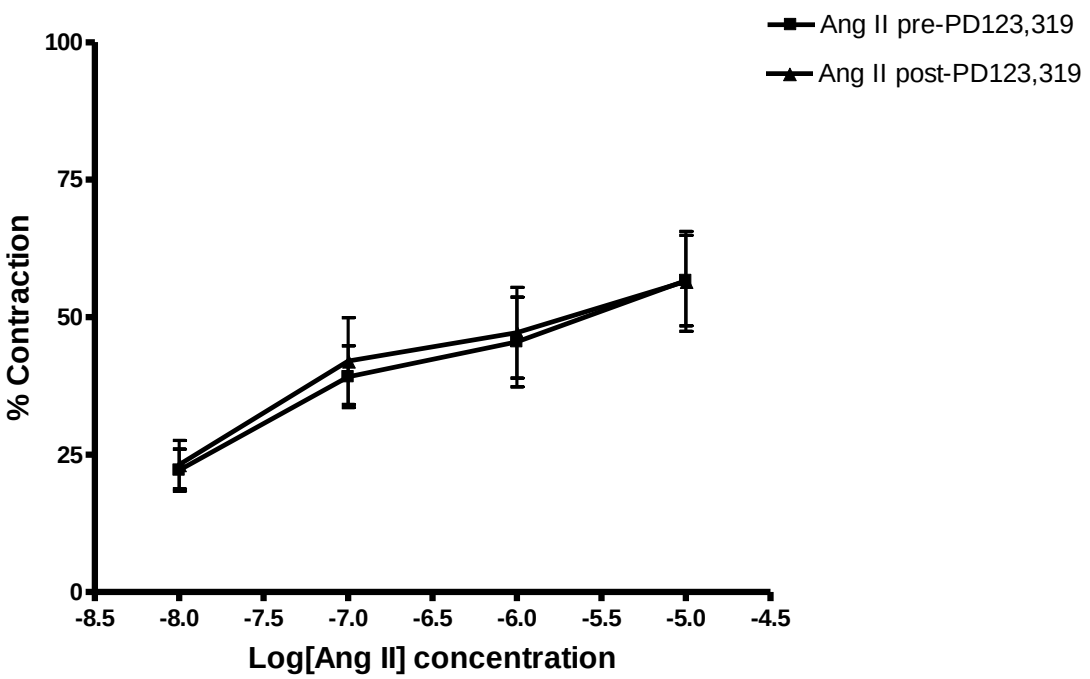


**Figure 6a.** Ang II-induced contraction of corpus cavernosal strips pre- (■) and post- (▲) PD123,319 (n = 6).

### 3.3.2 Effect of L-NAME

The contractile effect of Ang II was significantly (ANOVA; $P < 0.01$) enhanced by adding L-NAME to the bathing solution (Table 3, Figs 7 & 7a ).

**Table 3:** Ang II–induced contraction of corpus cavernosal strips pre- and post- L-NAME.

| Strip No. | Ang II $10^{-8}$M % PE contraction | Ang II $10^{-8}$M post-L-NAME % PE contraction | Ang II $10^{-7}$M % PE contraction | Ang II $10^{-7}$M post-L-NAME % PE contraction | Ang II $10^{-6}$M % PE contraction | Ang II $10^{-6}$M post-L-NAME % PE contraction | Ang II $10^{-5}$M % PE contraction | Ang II $10^{-5}$M post-L-NAME % PE contraction |
|---|---|---|---|---|---|---|---|---|
| 1 | 14.0 | 33.0 | 37.0 | 47.0 | 40.0 | 42.0 | 64.0 | 66.0 |
| 2 | 23.0 | 64.0 | 27.0 | 38.0 | 48.0 | 55.0 | 50.0 | 58.0 |
| 3 | 24.0 | 35.0 | 29.0 | 36.0 | 25.0 | 35.0 | 32.0 | 42.0 |
| 4 | 10.0 | 13.0 | 36.0 | 43.0 | 47.0 | 62.0 | 35.0 | 39.0 |
| 5 | 28.0 | 38.0 | 27.0 | 38.0 | 39.0 | 58.0 | 46.0 | 58.0 |
| 6 | 27.0 | 33.0 | 53.0 | 59.0 | 54.0 | 59.0 | 80.0 | 95.0 |
| 7 | | | | | 31.0 | 47.0 | 70.0 | 89.0 |
| 8 | | | | | 54.0 | 74.0 | 72.0 | 76.0 |
| 9 | | | | | 68.0 | 83.0 | | |
| Mean | 21.0 | 36.0 | 34.8 | 41.8 | 45.1 | 57.2 | 56.1 | 65.4 |
| Std. Deviation | 7.3 | 16.4 | 9.9 | 10.6 | 13.0 | 15.0 | 17.9 | 20.4 |
| Std. Error | 2.9 | 6.7 | 4.0 | 4.3 | 4.3 | 5.0 | 6.3 | 7.2 |

**Figure 7**

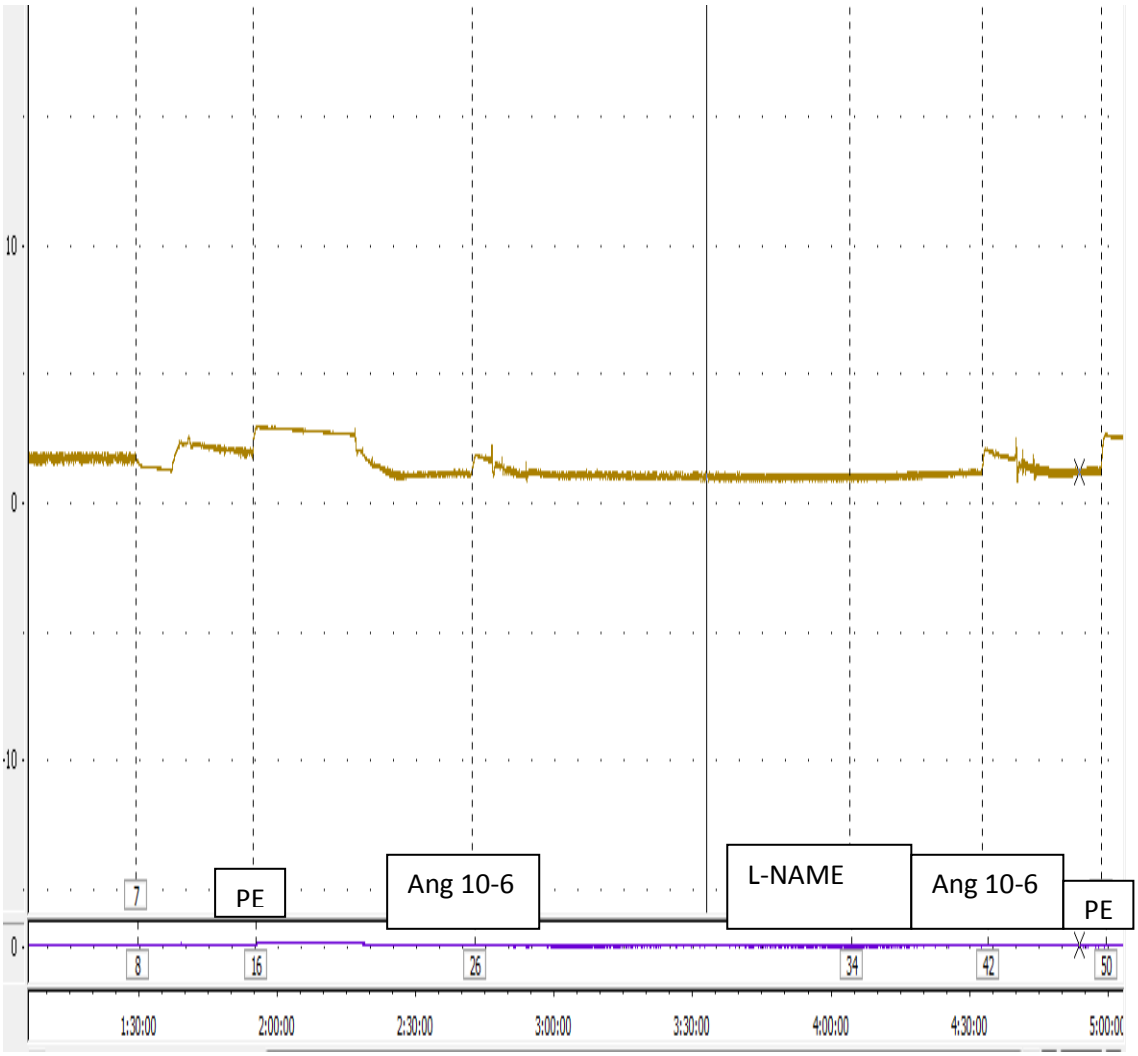


**Figure 7:** Representative tracing of Ang II ($10^{-6}$ M)–induced contraction of a corpus cavernosal strip pre- and post- L-NAME.

**Figure 7a**

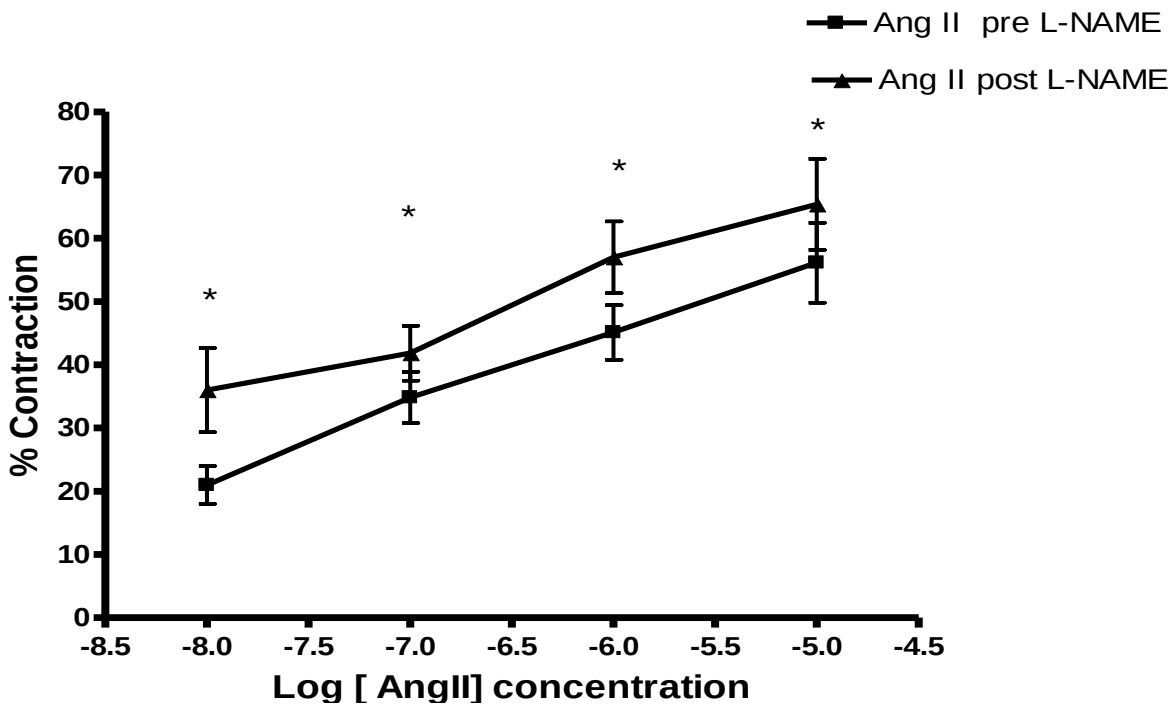


**Figure 7a.** Ang II-induced contraction of corpus cavernosal strips pre- (■) and post- (▲) L-NAME. *Denotes the data points where there was a significant difference in Ang II-induced contraction pre- and post- L-NAME (Ang II(M): $10^{-8}$ p<0.047, $10^{-7}$ P<0.0047, $10^{-6}$ p<0.0006, $10^{-5}$ p<0.0032 , paired Student's t-test, n =6).

### 3.3.4 SNP and EFS-induced corpus cavernosal smooth muscle relaxation

The cumulative dose response curve constructed for SNP from rabbit CCSM strips was significantly (ANOVA; P< 0.008) enhanced following the addition of losartan (Table 4, Figs 8 & 8a). Similarly, EFS-induced relaxation of CCSM strips was significantly improved following the addition of losartan (73.9+/-6.1 vs 82.5+/-5.9; P< 0.033, Fig 9).

**Table 4:** SNP-induced relaxation of corpus cavernosal strips pre- and post- losartan.

| Strip No. | SNP $10^{-7}$ M<br>% PE relaxation | SNP $10^{-7}$ M post –los<br>% PE relaxation | SNP $3x10^{-}$ M<br>% PE relaxation | SNP $3x10^{-7}$ M post-los<br>% PE relaxation | SNP $10^{-6}$M<br>% PE relaxation | SNP $10^{-6}$M post -los<br>% PE relaxation | SNP $3X10^{-6}$ M<br>% PE relaxation | SNP $3X10^{-6}$ M post-los<br>% PE relaxation |
|---|---|---|---|---|---|---|---|---|
| 1 | 11.0 | 13.0 | 23.0 | 25.0 | 46.0 | 53.0 | 52.0 | 76.0 |
| 2 | 23.0 | 25.0 | 41.0 | 50.0 | 61.0 | 62.0 | 69.0 | 73.0 |
| 3 | 15.0 | 15.0 | 26.0 | 26.0 | 48.0 | 51.0 | 49.0 | 76.0 |
| 4 | 12.0 | 15.0 | 25.0 | 26.0 | 47.0 | 51.0 | 50.0 | 75.0 |
| 5 | 12.0 | 31.0 | 26.0 | 38.0 | 72.0 | 80.0 | 72.0 | 82.0 |
| Mean | 14.6 | 19.8 | 28.2 | 33.0 | 54.8 | 59.4 | 58.4 | 76.4 |
| Std. Deviation | 4.9 | 7.82 | 7.3 | 10.9 | 11.4 | 12.4 | 11.1 | 3.36 |
| Std. Error | 2.2 | 3.5 | 3.0 | 4.9 | 5.0 | 5.5 | 5.0 | 1.5 |

**Figure 8**

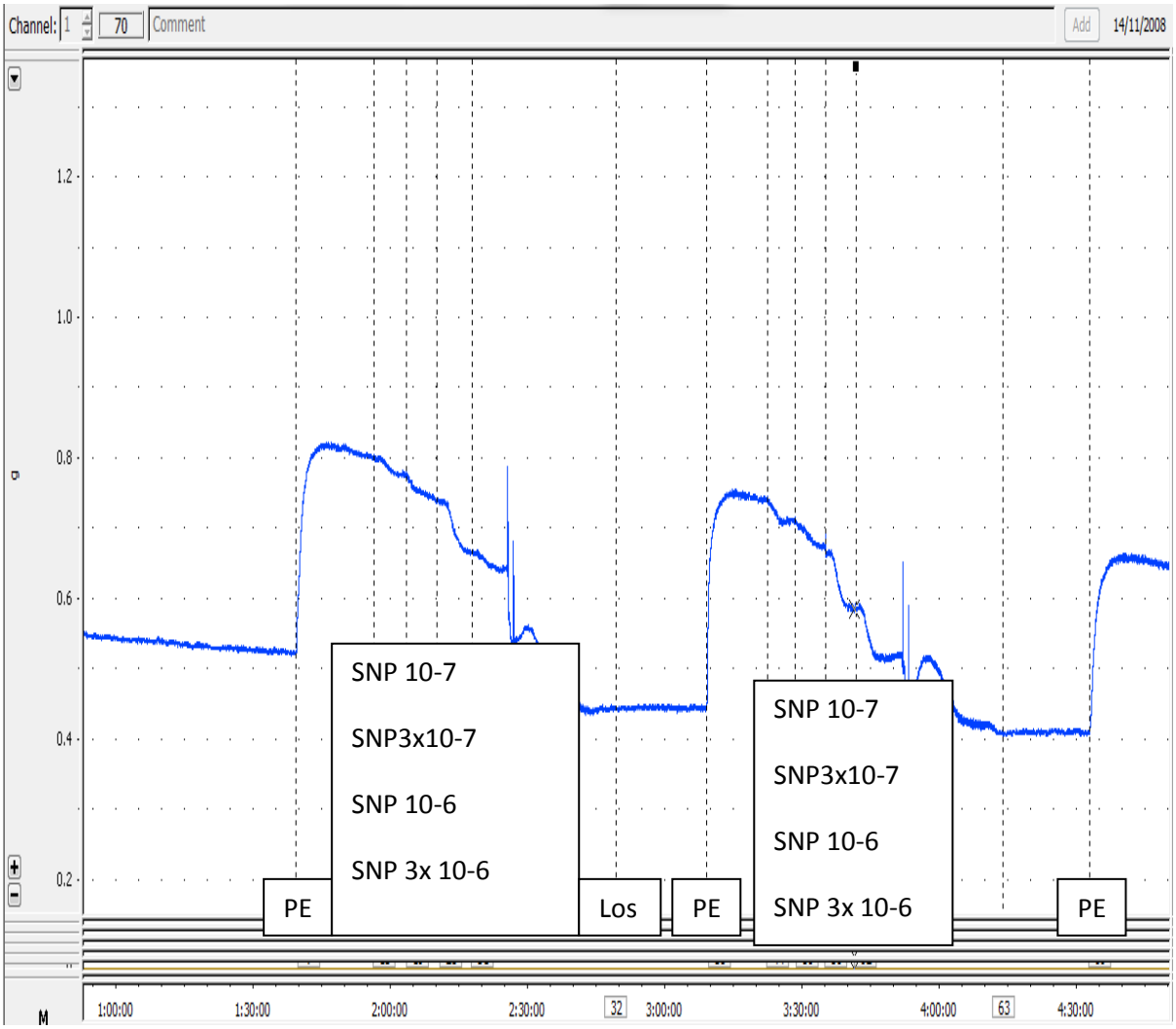


**Figure 8:** Representative tracing of SNP-induced relaxation of a corpus cavernosal strip pre- and post- losartan.

**Figure 8a**

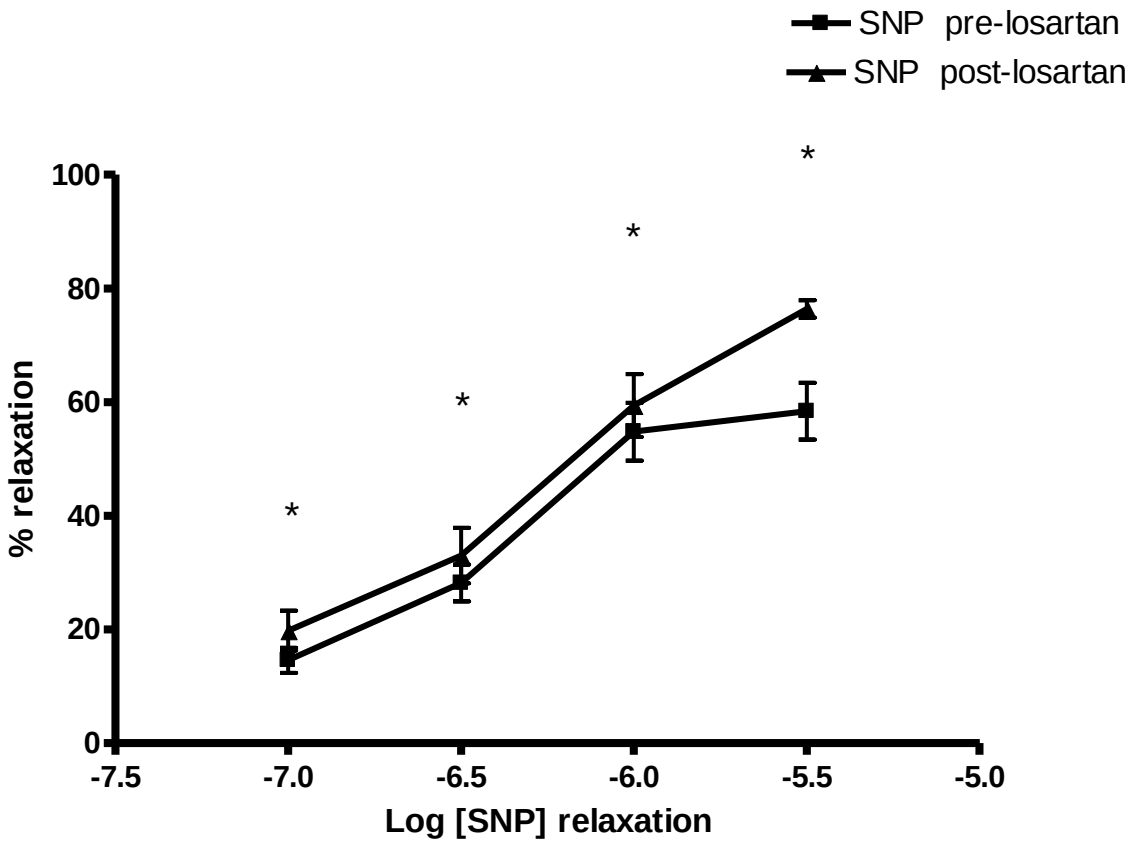


**Figure 8a.** SNP-induced relaxation of corpus cavernosal strips pre- (■) and post- (▲) losartan. * Denotes the data points where there was a significant difference in SNP-induced relaxation pre- and post-losartan (SNP (M) $10^{-6}$, $p<0.024$; $3x10^{-6}$, $p<0.018$, paired Student's t-test, n = 5).

**Figure 9**

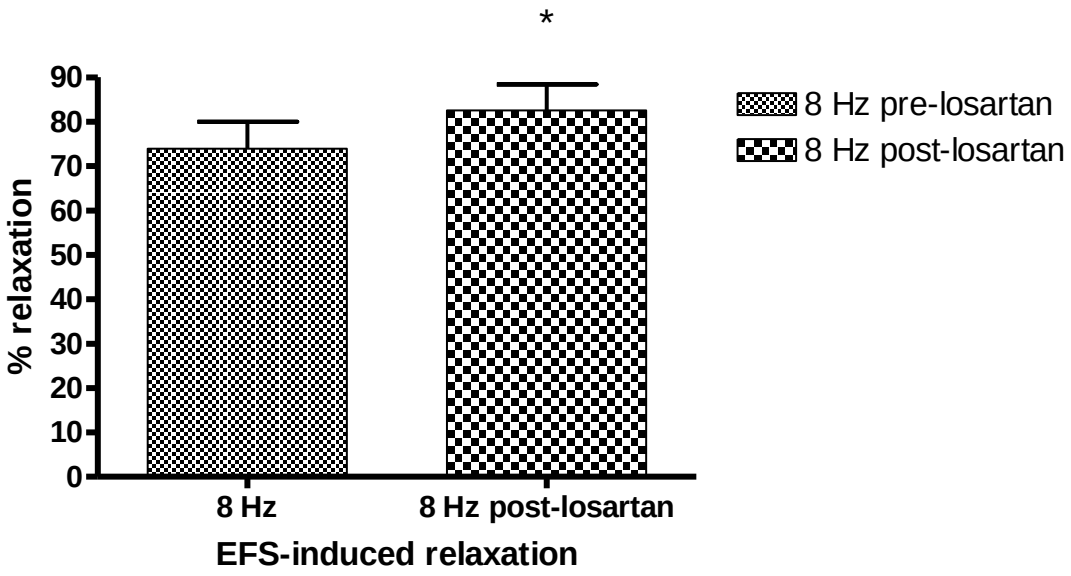


**Figure 9.** EFS-induced relaxation of corpus cavernosal strips at 8 Hz following the addition of guanethidine, atropine and indomethacin, pre- and post-losartan. * Denotes a significant difference in EFS-induced relaxation pre- and post- losartan ($p < 0.007$, paired Student's t-test, $n = 8$).

### 3.3.5 The effect of oxidative stress on Ang II contraction

**3.3.5.1 Contraction:** DPI significantly reduced the contractile effect of Ang II (68.4+/-6.0 vs 23.00+/-6.0; P = 0.0006, Fig 10). Although, SOD diminished the contractile effect of Ang II by 15 %, this was not statistically significant (Fig 11).

**Figure 10**

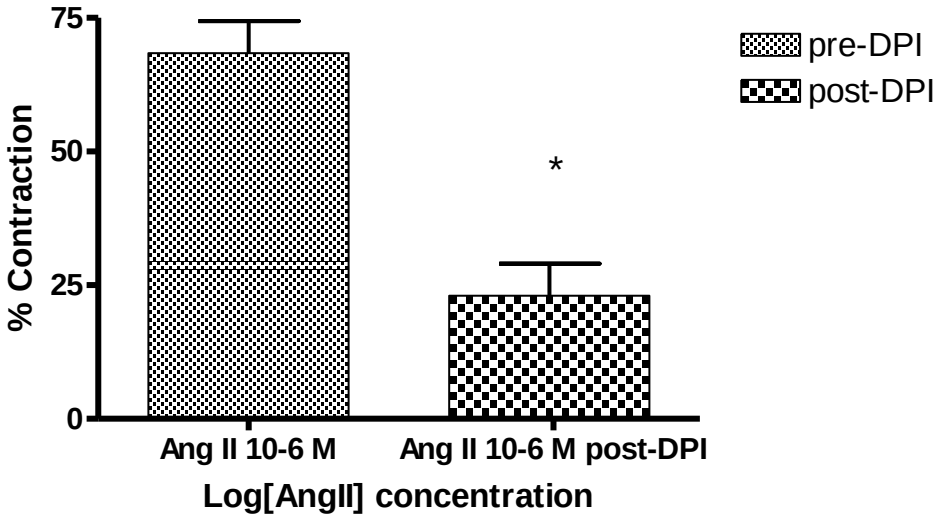


**Figure 10.** Ang II-induced contraction ($10^{-6}$ M) of corpus cavernosal strips pre- and post-DPI ($10^{-4}$ M).*Denotes a significant difference in Ang II-induced contraction pre- and post-DPI (P = 0.0006, paired Student's t-test, n = 7).

**Figure 11**

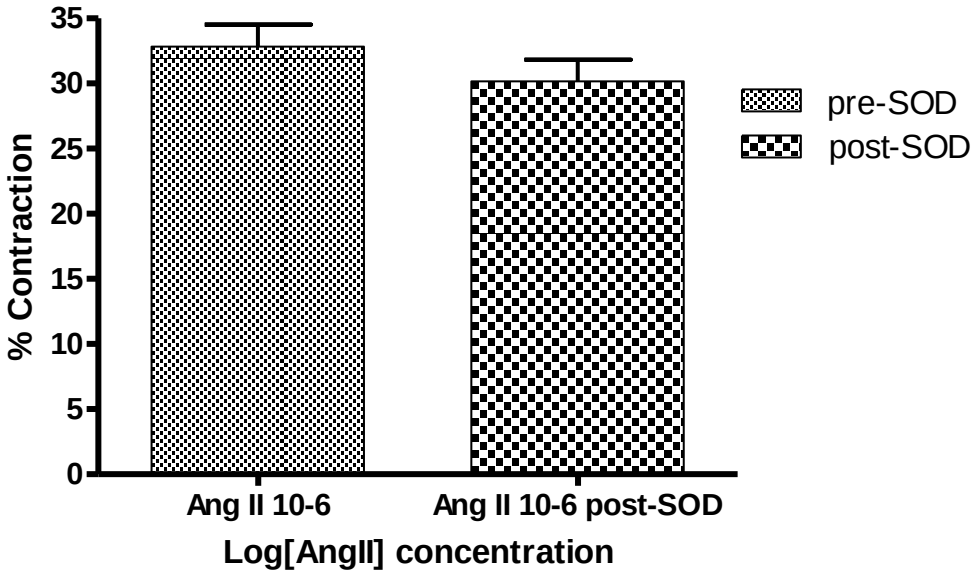


**Figure 11.** Ang II-induced contraction ($10^{-6}$ M) of corpus cavernosal strips pre- and post-SOD (200 IU/ml). No significant difference in Ang II-induced contraction pre- and post-SOD (P = 0.137, paired Student's t-test, n=7).

**3.3.5.2 Relaxation:** The SNP dose response curve was significantly (ANOVA; P<0.0001) enhanced following the addition of DPI (Fig 12).

Following exposure to guanethidine, atropine and indomethacin the EFS-induced relaxation of CCSM was significantly enhanced by 36% after the addition of DPI and losartan (39.2+/-6.4 vs 61.0+/-4.3; P< 0.007, Fig 13)

**Figure 12**

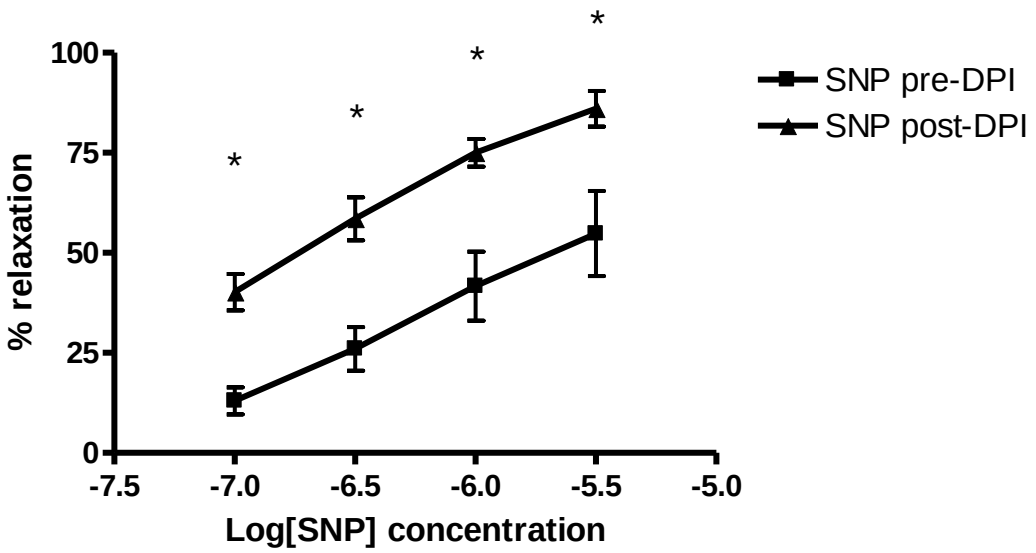


**Figure 12.** SNP-induced relaxation of corpus cavernosal strips pre- (■) and post- (▲) DPI. * Denotes the data points where there was a significant difference in SNP-induced relaxation pre- and post-losartan (SNP, M), $10^{-7}$ p<0.0096; $3x10^{-7}$ p<0.01;$10^{-6}$ p<0.043; $3x10^{-6}$ p<0.018, student paired *t* test).

**Figure 13**

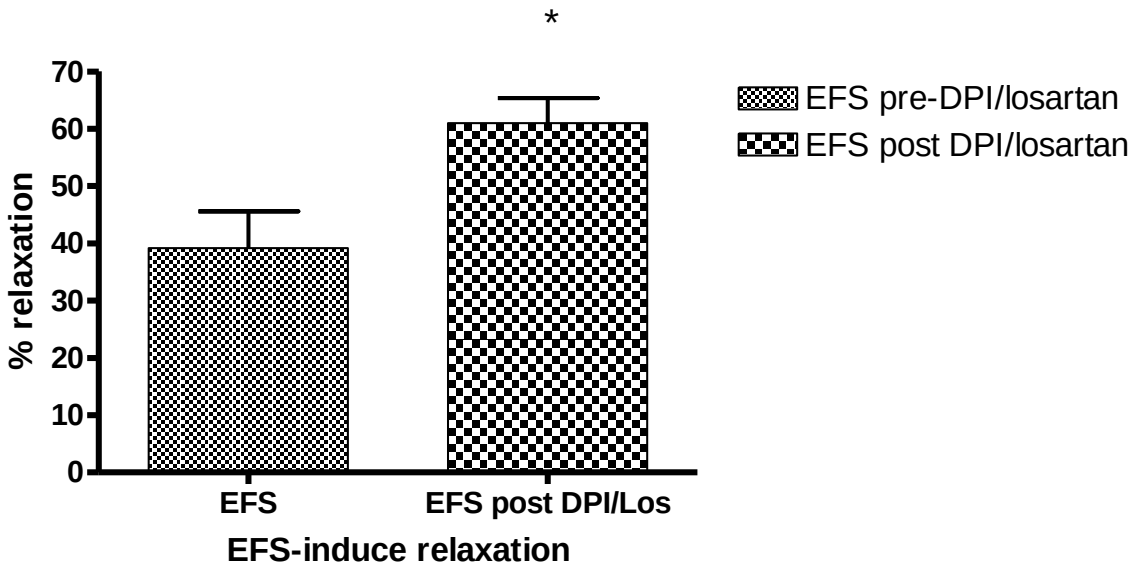


**Figure 13.** EFS-induced relaxation of corpus cavernosal strips at 8 Hz following the addition of guanethidine, atropine and indomethacin, pre- and post-losartan and DPI. * Denotes a significant difference in EFS-induced relaxation pre- and post- losartan and DPI ($p < 0.007$, paired Student's t-test).

## 3.4 Discussion

The role of the renin-angiotensin-aldosterone system (RAAS) in the pathophysiology of CVD and renal disease is well established (Dzau, 2001). Ang II is a key mediator of this system, with actions beyond its hemodynamic and renal activity, influencing the function and structure of various tissues in a manner that can promote CVD (Dzau, 2001).These include vasoconstriction, vascular and cardiac growth and tissue remodeling , OS and inhibition of NO activity (Nickenig & Harrison, 2002).

Several clinical trials have provided additional evidence of the pathophysiological role played by Ang II in the development of CVD (see Chapter 1).

In fact, drugs that reduced the influence of Ang II have been successfully used to treat CVD. For example, ACE inhibitors and AT1 receptors antagonist are beneficial therapeutic options for treating hypertension, post myocardial infarction, heart failure, and renal dysfunction (Chobanian *et al*, 2003).

As the corpus cavernosum consists mainly of endothelial and smooth muscle cells and is regarded as a specialized vascular tissue (Becker *et al,* 2001), it is likely that it is equally vulnerable to the pathological actions of Ang II.

The possible role of Ang II in ED has previously been explored both in clinical and laboratory studies. Ang II levels in the cavernous blood of men with organogenic ED was found to be higher than men with psychogenic ED or healthy subjects (Becker *et al*, 2001). While, chronic infusion of exogenous Ang II for 4 weeks induced ED in Sprague-Dawley rats (Jin *et al*, 2008). In addition, intracavernosal injection of Ang II terminated spontaneous erection in anesthetised dogs, while the administration of losartan induced CCSM relaxation, resulting in penile erection (Kifor *et al*, 1997).

The role of NO and Ang II in penile physiology has been discussed in Chapter 1. Interaction between these two important mediators on CCSM function has been previously investigated (Schulman *et al*, 2006). Ang II acts on AT1 and AT2 receptors to regulate NOS activity and NO production; NO opposes Ang II signalling pathways at multiple levels, creating a fine physiological balance between these two vasoactive mediators.

Our finding that Ang II caused a dose dependent contraction of CCSM strips that is blocked by losartan but not PD123,319, confirms a previous study also using rabbit CCSM tissue, which reported that Ang II acts mainly through AT1 receptor activation (Park *et al* ,1997). Further evidence of the functional interaction between NO and Ang II is provided by the findings that blocking the NO pathway with L-NAME, potentiated the contractile effect of Ang II, while the NO donor SNP caused a dose dependent relaxation which was enhanced with losartan. Also important was the finding that AT1 receptor blockade by losartan enhanced EFS-induced CCSM relaxation following inhibition of the adrenergic, cholinergic and cyclo-oxyegnase pathways. This clearly demonstrates the effect of Ang II on NANC neurotransmission.

Although, aspects of these finding have been reported using canine CCSM tissue (Comiter et al, 1996), that study lacked clarity. They did not use the triple blockers during their EFS experiments, which left these important contractile pathways unopposed, which would undoubtedly influence the results. Furthermore, the SNP-induced relaxation was tested by adding the NO donor at the peak of the Ang II contraction and abolishing the contractile effect of Ang II. From this they concluded that there was interaction between NO and Ang II, however, the disappearance of the contraction may simply reflect the SNP-induced relaxation and not interplay

between NO and Ang II. Here, the evidence that losartan induced an enhancement of SNP-mediated CCSM relaxation is scientifically more compelling evidence of the NO/Ang II interaction.

Ang II is an important modulator of vascular smooth muscle cell biology and many of its actions are mediated through ROS generation, in particular $O_2^-$ (Touyz and Schiffrin, 2000). As stated earlier, vascular $^{\bullet}O_2^-$ interacts with NO to reduce NO bioavailability and generates the potent oxidative $ONOO^-$ radical, which plays an important role in vascular pathophysiology (Cai and Harrison, 2000; White *et al*, 1994). The cellular source of vascular $^{\bullet}O_2^-$ vary in different vessel types, most studies have shown a membrane-associated oxidase that is stimulated by NADH or NADPH, which is consistent with NAD(P)H oxidase being an important source of $^{\bullet}O_2^-$ production in human, as well as animal models (West *et al*, 2001).

Not surprisingly, Ang II is a potent stimulator of NAD(P)H oxidase, whereby short term exposure to Ang II enhance NAD(P)H oxidase activity (Cruzado *et al*, 2005). DPI the NAD(P)H oxidase inhibitor was found to markedly decreased Ang II-mediated contraction of human arteries. The same study showed an increase in ROS generation after 40 minutes exposure to Ang II using fluorescent microscopy. Taken together, these findings confirm that the contractile effect of Ang II, at least in part, is due to ROS/$^{\bullet}O_2^-$ generation (Puntmann *et al*, 2005).

It appears that tunica media and adventitia are the predominant vascular areas for basal $^{\bullet}O_2^-$ production, however, in disease states such as DM, endothelial production is significantly increased (Guzik *et al*, 2002; Mollanau *et al*, 2002). This maybe a feature of diabetic ED and explain why Khan *et al*, (2001b) found that the impaired

relaxation of CCSM from diabetic rabbits was improved by SOD (the enzyme that accelerates that break down of $O_2^-$ to $H_2O$) .

We have shown a marked reduction in Ang II-induced CCSM contraction after adding DPI, due to inhibition of NAD(P)H oxidase activity and ultimately OS. A similar finding has previously been reported (Puntmann *et al*, 2005). This inhibitory effect on OS is further strengthened by the observation that the addition of SOD also reduced the Ang II contractile response, albeit less profoundly than DPI.

DPI-induced inhibition of the Ang II contractile response and OS also influenced NANC- and NO-mediated CCSM relaxation, since EFS and SNP-induced relaxations were significantly enhanced by this inhibitor. The fact that Ang II stimulates the ROS pathway through the induction of NAD(P)H oxidase is a clear indication of the importance of the Ang II/ROS pathway in penile pathophysiology.

The findings reported here on; the interaction between Ang II and NO, inhibition of Ang II-induced CCSM contraction by losartan and the effect of OS on this process have important clinical significance/implications. Relaxation of corporal smooth muscle is a salient feature of the erectile process, as it allows the expansion of the lacunar spaces and reduces cavernosal venous outflow by compression of venules against the tunica albuginea, the surrounding fibrous structure (Azadozi *et al*,1992). Conversely, to maintain the penis in the flaccid state, the smooth muscle of penile arteries and trabeculae are kept contracted by the noradrenergic system, however other contractile mediators, such as Ang II also play an important role. This is supported by the finding that losartan had a significant protective role against structural changes in the vessels and cavernosal spaces of erectile tissue caused by

arterial hypertension, which was not the case for amlodipine, despite the fact that both drugs achieved systemic blood pressure control (Tobil *et al*, 2004).

Spontaneously hypertensive rats (SHR) treated with losartan alone or losartan combined with sildenafil for 6 months, was found to have functional and structural improvement to the erectile tissue when compared to non-treated animals (Tobil *et al,* 2007).

Heme oxygenase (HO)-1 has been shown to be expressed in vascular smooth muscle cells (VSMCs), including those found in cavernous tissues (Abdel Aziz *et al*, 2007). HO-1 gene expression exerts antioxidant actions, which participates in defense mechanisms against agent that induce oxidative injury (Abraham *et al*, 2004). Ishizaka *et al*, (1997) stated that Ang II causes HO-1 gene down regulation in rat VSMCs, which might augment *in vivo* vasoconstriction, which was blocked by losartan. In addition, HO-1 gene expression, cGMP assay, HO enzymatic activity were significantly reduced in cavernous tissue from diabetic rats, due to diabetic OS but were significantly improved by losartan (Abdel Aziz *et al*, 2009). Taken together, these findings highlight the antioxidant action of losartan, reinforcing the concept that Ang II-induced OS is an integral part of its physiological action.

### 3.5 Conclusion

Ang II caused a dose dependent contraction of CCSM strips, an effect that was reversed by losartan the AT1 receptor antagonist. The interaction between Ang II and NO on CCSM function was also demonstrated. It is clear from the results that blocking NAD(P)H oxidase reduced OS and the contractile effect of Ang II, whilst enhancing NANC- and NO-mediated CCSM relaxation. Clearly, Ang II is a major contractile vasoactive peptide that contributes to the tonic contraction of CCSM and

blocking this pathway could be of significant importance in the understanding of the treatment of ED (see Chapter 5).

# Chapter 4

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# Effect of angiotensin II and its receptor antagonists on human corpus cavernosal contractility.

## 4.1. Introduction

The important interactive role Ang II, its receptor antagonists, NO and OS play in modulating rabbit CCSM tone has been discussed in Chapter 3. However, this has not been confirmed using human CCSM tissue. This confirmation is vitally important, since it has been reported that the penile contractile response to Ang II is species dependent (Klinge & Sjostrand, 1977).

It is assumed that Ang II is involved in modulating the tone of human penile arteries and trabecular smooth muscle (Comiter *et al,* 1997; Becker *et al,* 2001) and that its regulation is governed by a balance with NO (Comiter *et al.*1997;Yan *et al.* 2003).

Here, the effect Ang II and its receptor antagonists have on human corpus cavernosum contractility; together with their modulation of NO-mediated relaxation has been determined. The presence and distribution of Ang II in human corpus cavernosal tissue was also determined using immunohistochemistry. The results from this study provide an important insight in to the physiological role of Ang II during human penile erection.

## 4.2. Materials and Methods

### 4.2.1Tissue acquisition

See Chapter 2 for details of tissue acquisition and experimental procedure.

### 4.2.2 Effect of Ang II

The effect of Ang II ($10^{-8}$M – $10^{-5}$M) on CCSM function was investigated. As previously mentioned in the control rabbit experiments conducted in Chapter 3,

repeated applications of Ang II caused a marked desensitisation of the functional response leading to tachyphylaxis, making it difficult to construct a cumulative dose response curve, for this reason and to avoid tachyphylaxis a single dose of Ang II / individual human tissue was used in a dose range of $10^{-8}$M – $10^{-5}$M.

### 4.2.3 Effect of Ang II receptor antagonists and L-NAME

The effect of Ang II on tissue strips was determined; the tissue was then washed over a 30 min period and exposed to either losartan ($10^{-5}$ M; AT1 antagonist) or PD123,319 ($10^{-5}$ M; AT2 antagonist) for 20 min before repeating the Ang II response. The effect of the vehicle (distilled water for 20 min) on the Ang II response was also determined. In other experiments L-NAME ($10^{-4}$ M) was added to the bathing solution for 20 min and the effect on the Ang II ($10^{-6}$ M) response determined.

### 4. 2.4 Electrical field stimulation (NANC neurotransmission)

In a series of experiments, guanethidine (5 x $10^{-6}$ M), atropine ($10^{-5}$ M) and indomethacin ($10^{-6}$ M) were added to the bathing solution and left for 20 min to inhibit the adrenergic, cholinergic and cyclo-oxygenase pathways, respectively, leaving the NANC pathway intact. Tissues were then pre-contracted with phenylephrine (PE; $10^{-4}$ M) followed by EFS of penile nerves with a Grass S88 (Astro-med Grass, Slough UK) stimulator. The stimulator delivered single square waves (duration 0.4 ms; 20V) at a frequency of 8.0 Hz in 5 s trains. In another experiment guanethidine was omitted from the cocktail of inhibitors, leaving atropine ($10^{-5}$ M) and indomethacin

($10^{-6}$ M) in the bathing solution, which was left for 20 min. EFS measurements were again made at 8 Hz, followed by the addition of losartan and the EFS repeated.

### 4.2.5 Electrical field stimulation (contractile response)

Guanethidine (5 x $10^{-6}$ M), atropine ($10^{-5}$ M), indomethacin ($10^{-6}$ M) and L-NAME ($10^{-4}$ M) were added to the bathing solution and left for 20 min to inhibit the adrenergic, cholinergic, cyclo-oxygenase and NANC pathways, respectively. EFS measurements were performed with a Grass S88 stimulator, which delivered single square waves (duration 0.8 ms; 100V) at a frequency of 8.0 Hz for 5 s. Losartan was then added to the bathing solution for 20 min and the EFS measurements repeated.

Tetrodotoxin was added at the end of each experiment to determine the component of the response that was due to direct muscle fibre stimulation.

### 4.2.6 Effect of SNP

Tissue strips were pre-contracted with PE and cumulative response curves were constructed for SNP ($3x10^{-7}$ - $10^{-6}$ M). The tissues were then washed several times followed by the addition of losartan to the organ bath for 20 min. The tissues were re-contracted with PE and cumulative response curves were again constructed for SNP.

### 4.2.7 Effect of NAD(P)H oxidase inhibition on Ang II and EFS-mediated responses

The effect of apocynin, the NAD(P)H oxidase inhibitor ($10^{-4}$M) on the Ang II ($10^{-6}$M) response was determined. The effect of apocynin and losartan on EFS-mediated CCSM relaxation was also determined.

### 4.2.8 Immunohistochemistry

See Chapter 2 for the experimental procedure

### 4.2.9 Statistical analysis

The raw data from Ang II pre- and post- losartan and PD123,319 were expressed as % contraction of the PE contractile response. EFS and SNP tissue responses determined pre- and post-losartan were expressed as % relaxation of PE-induced tone. These were analysed and expressed as mean ± SEM using Graph Pad Prism 4.0 software (see examples, Tables 5 & 6). Comparisons of the resultant Ang II dose response curves and SNP cumulative dose response curves were made using analysis of variance (2 way ANOVA, $p < 0.05$). Student's unpaired and paired t-test statistical analysis was also determined on the raw data by the software package with statistical significance accepted at $p < 0.05$. The individual number of tissue strips used is included in the figure Legends. Strips from at least 5 animals were used in each experiment.

## 4.3 Results

### 4.3.1 Effect of Ang II on corpus cavernosal smooth muscle contraction

Ang II caused a dose dependent contraction ($10^{-8}$M – $10^{-5}$M) of corpus cavernosal strips, which was significantly (ANOVA; P = 0.0006) reduced by losartan (Table 5, Figs 14 & 14a).

In contrast, the addition of PD123319 had no significant effect on Ang II-mediated contraction (Fig 15). The addition of L-NAME significantly enhanced the Ang II-induced contraction of corpus cavernosal strips by 32% (p=0.04). The addition of the vehicle (distilled water) did not significantly influence the Ang II response.

**Table 5 :** Ang II–induced contraction of corpus cavernosal strips pre- and post- losartan

| Strip No. | Ang II $10^{-8}$M % PE contraction | Ang II $10^{-8}$M post-los % PE contraction | Ang II $10^{-7}$M % PE contraction | Ang II $10^{-7}$M post-los % PE contraction | Ang II $10^{-6}$M % PE contraction | Ang II $10^{-6}$M post-los % PE contraction | Ang II $10^{-5}$M % PE contraction | Ang II $10^{-5}$M post-los % PE contraction |
|---|---|---|---|---|---|---|---|---|
| 1 | 29. | 4.1 | 11.0 | 0.5 | 10.0 | 1.0 | 20.0 | 3.0 |
| 2 | 37.0 | 4.0 | 23.0 | 7.0 | 36.0 | 1.0 | 6.0 | 1.0 |
| 3 | 27.0 | 7.0 | 11.0 | 6.0 | 5.0 | 2.0 | 3.0 | 0.9 |
| 4 | 60.0 | 44.0 | 7.0 | 1.3 | 13.0 | 1.0 | 2.0 | 0.5 |
| 5 | 22.0 | 1.0 | 15.0 | 4.0 | 9.0 | 3.0 | 4.0 | 0.5 |
| 6 | 2.8 | 0.8 | | | 8.0 | 1.0 | 1.6 | 7.0 |
| 7 | | | | | 19.0 | 8.0 | 12.0 | 1.2 |
| 8 | | | | | | | 3.0 | |
| Mean | 29.6 | 10.6 | 13.4 | 3.8 | 14.3 | 2.5 | 6.6 | 2.0 |
| Std. Deviation | 18.8 | 16.7 | 6.0 | 2.8 | 10.6 | 2.5 | 6.3 | 2.6 |
| Std. Error | 7.7 | 6.8 | 2.7 | 1.3 | 4.0 | 0.9 | 2.2 | 0.9 |

**Figure 14**

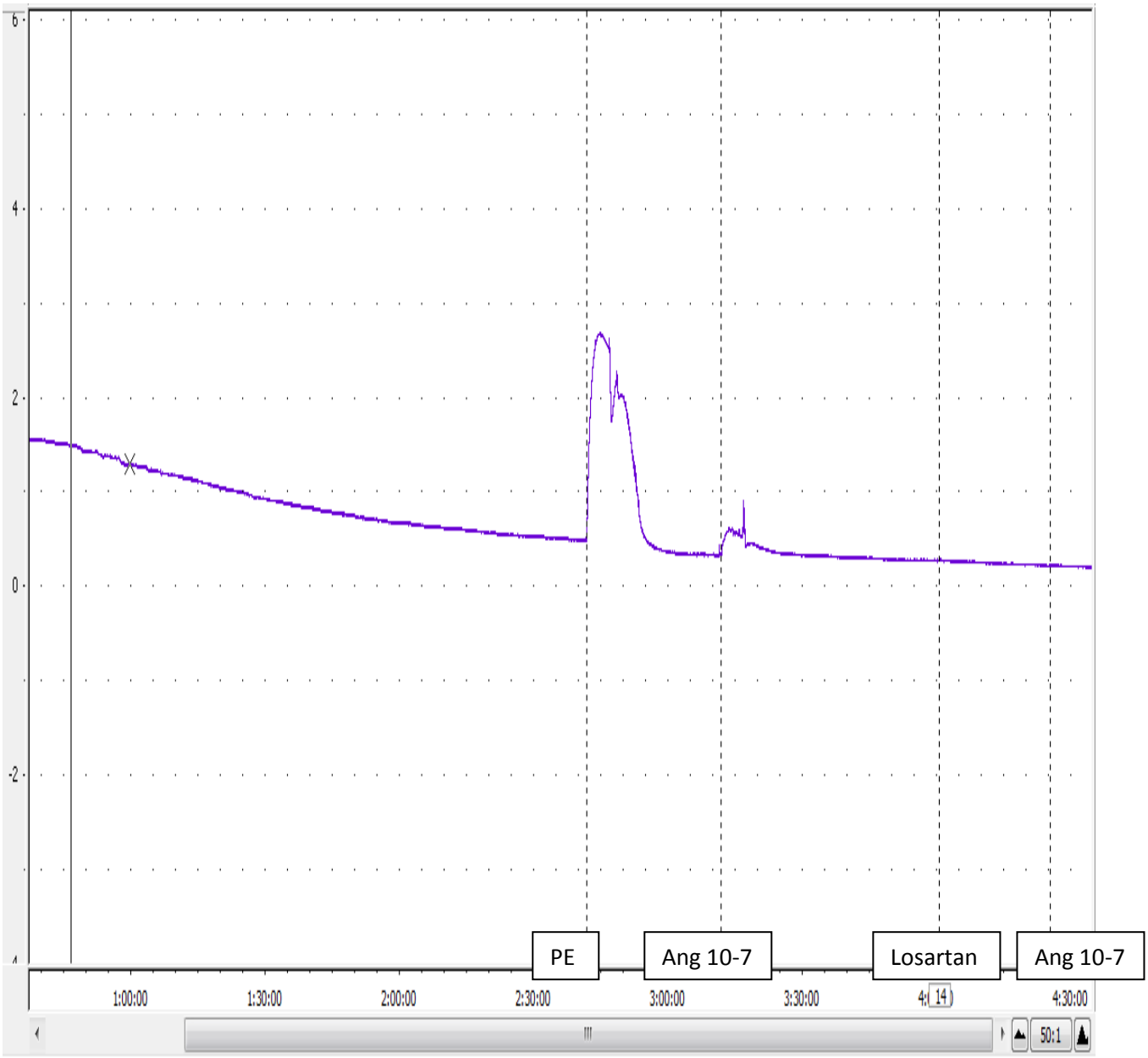


**Figure 14:** Representative tracing of Ang II ($10^{-7}$ M)–induced contraction of a corpus cavernosal strip pre- and post- losartan.

**Figure 14a**

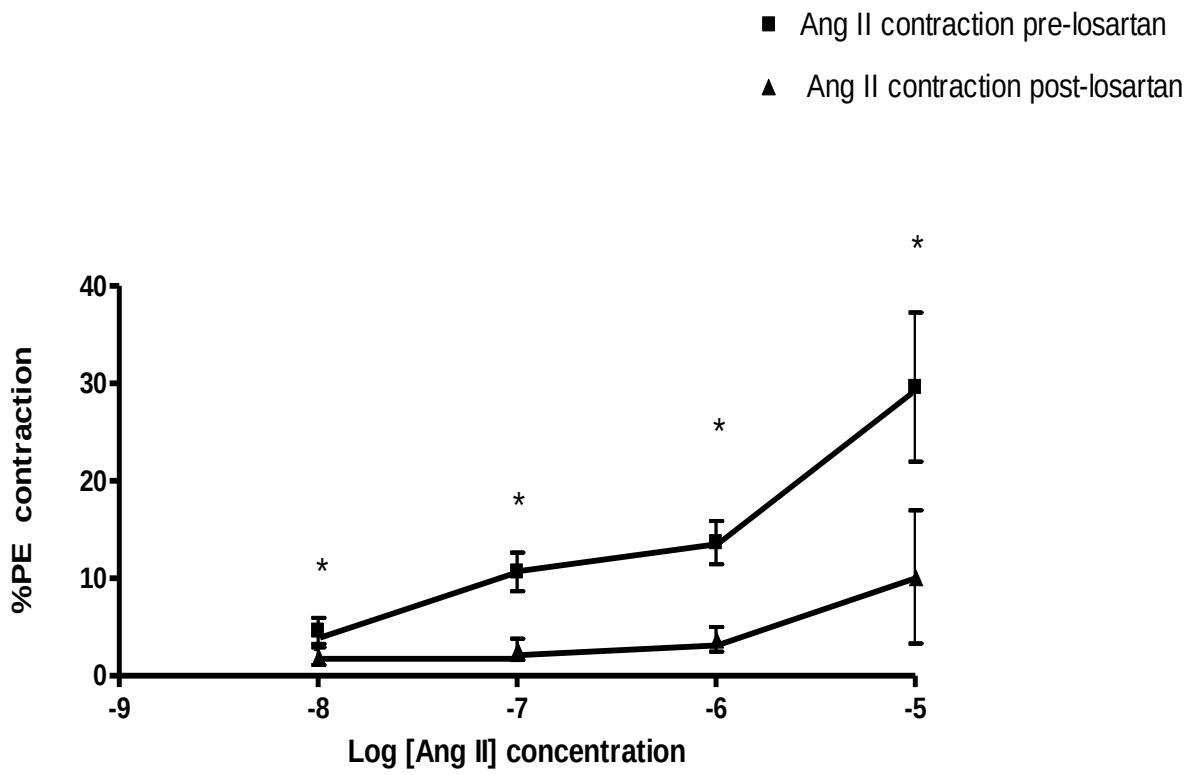


**Figure 14a.** Ang II-induced contraction of human corpus cavernosal strips pre- (■) and post- (▲) losartan. *Denotes the data points where there was a significant difference in Ang II-induced contraction pre- and post- losartan (Ang II (M): $10^{-8}$, $p < 0.002$; $10^{-7}$, $p < 0.03$; $10^{-6}$, $p < 0.009$; $10^{-5}$, $p < 0.006$, paired Student's t-test, n =5).

**Figure 15:**

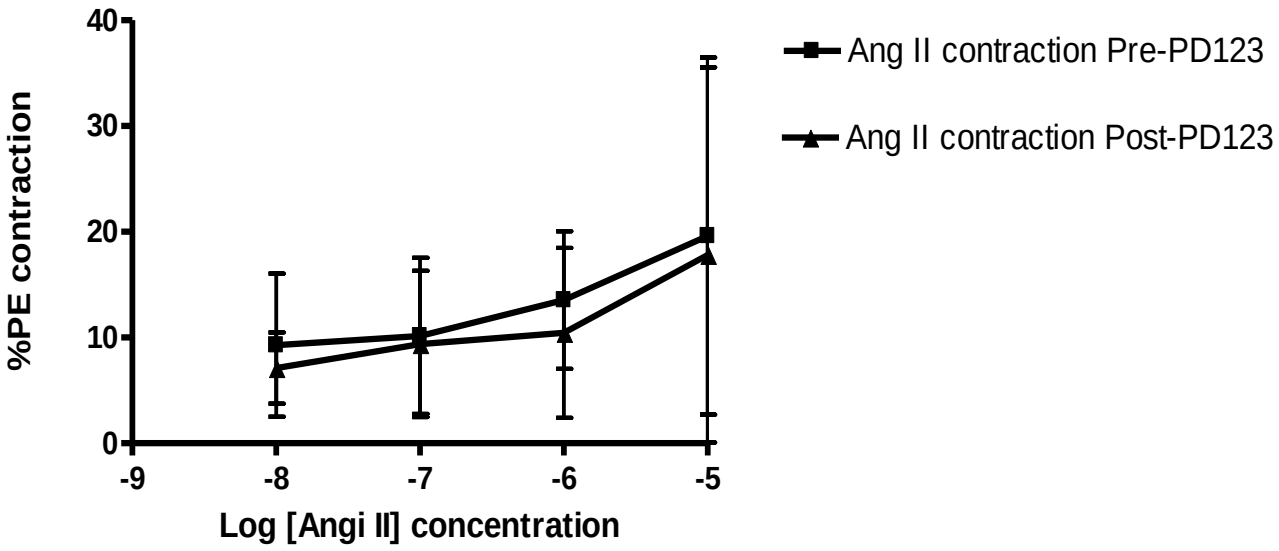


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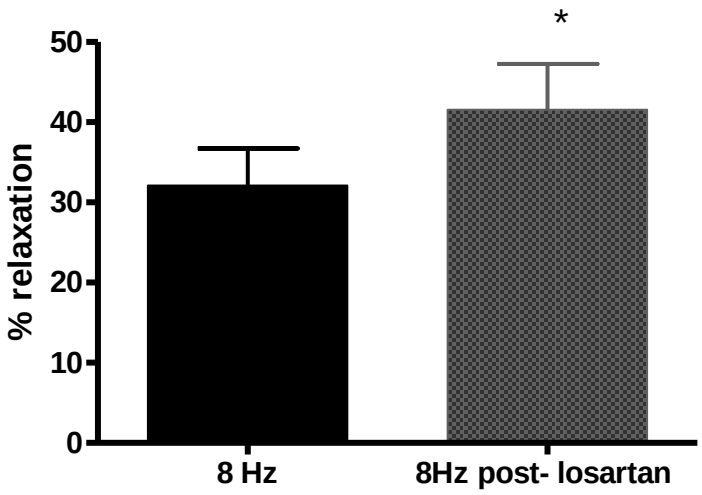
**Figure 15.** Ang II-induced contraction of human corpus cavernosal strips pre- (■) and post- (▲) PD123,319 (n = 6).

### 4.3.2 EFS-induced cavernosal smooth muscle relaxation

EFS-induced relaxation of corpus cavernosal strips after exposure to guanethidine, atropine and indomethacin was increased by 29.4% following the addition of losartan (Fig 16).

The losartan-induced increase in EFS relaxation of corpus cavernosal strips was greater (140%) when guanethidine was omitted from the cocktail of inhibitors (Fig 17).

**Figure 16**




Effect of Losartan on EFS-induced relaxation (with guanethidine)

**Figure 16.** EFS-induced relaxation of human corpus cavernosal strips at 8 Hz following the addition of guanethidine, atropine and indomethacin, pre- and post-losartan. * Denotes a significant difference in EFS-induced relaxation pre- and post- losartan ($p < 0.0009$, n=9, paired Student's t-test).

**Figure 17**

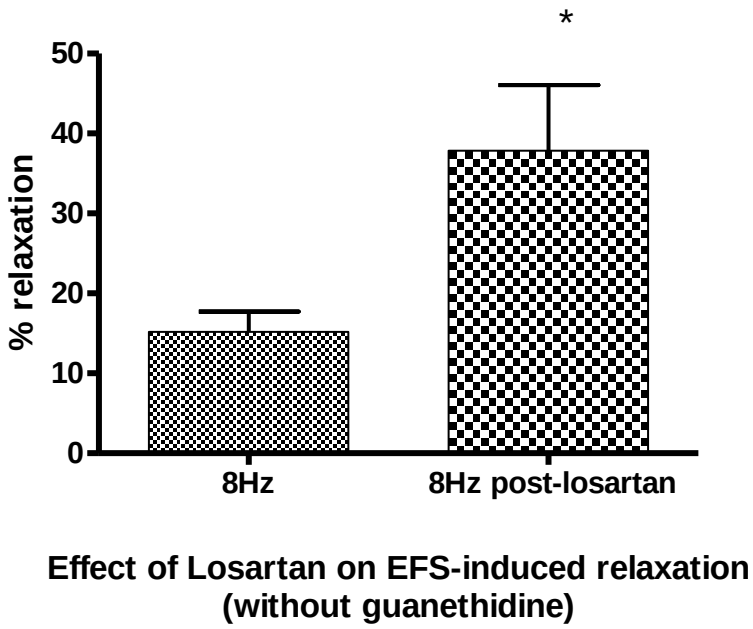



**Figure 17.** EFS- induced relaxation of human corpus cavernosal strips at 8 Hz following the omission of guanethidine from the cocktail of inhibitors, pre- and post-losartan. * Denotes a significant difference in EFS-induced relaxation pre- and post- losartan ($p = 0.027$, $n=7$, paired Student's t-test).

### 4.3.3 EFS-induced cavernosal smooth muscle contraction

EFS-induced contraction of corpus cavernosal strips following exposure to L-NAME, guanethidine, atropine and indomethacin was significantly reduced following the addition of losartan ($p < 0.02$, Fig 18).

**Figure 18**

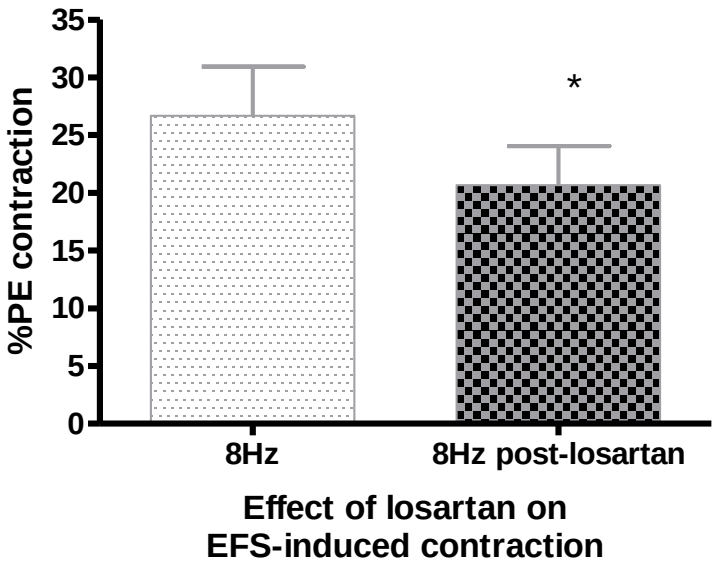



**Figure 18.** EFS-induced contraction of human corpus cavernosal strips at 8 Hz following the addition of guanethidine, atropine, indomethacin and L- NAME, pre- and post-losartan. * Denotes a significant difference in EFS-induced contraction pre- and post-losartan ($p < 0.02$, n=7, paired Student's t-test).

### 4.3.4 SNP-induced cavernosal smooth muscle relaxation

Cumulative response curves constructed for SNP showed that the relaxation of tissue strips was significantly enhanced by losartan (ANOVA; P<0.0001; Table 6, Figs 19 & 19a).

**Table 6:** SNP-induced relaxation of corpus cavernosal strips pre- and post- losartan.

| Strip No. | SNP $10^{-7}$ M<br>% PE relaxation | SNP $10^{-7}$ M post –los<br>% PE relaxation | SNP $3x10^{-}$ M<br>% PE relaxation | SNP $3x10^{-7}$ M post-los<br>% PE relaxation | SNP $10^{-6}$M<br>% PE relaxation | SNP $10^{-6}$M post -los<br>% PE relaxation | SNP $3X10^{-6}$ M<br>% PE relaxation | SNP $3X10^{-6}$ M post-los<br>% PE relaxation |
|---|---|---|---|---|---|---|---|---|
| 1 | 19.0 | 49.0 | 31.0 | 49.0 | 49.0 | 51.0 | 50.0 | 53.0 |
| 2 | 14.0 | 23.0 | 24.0 | 25.0 | 36.0 | 53.0 | 34.0 | 41.0 |
| 3 | 13.0 | 15.0 | 4.0 | 13.5 | 19.0 | 40.0 | 57.0 | 82.0 |
| 4 | 1.5 | 13.0 | 30.0 | 74.0 | 45.0 | 81.0 | 56.0 | 89.0 |
| 5 | 14.0 | 16.0 | 30.0 | 75.0 | 45.0 | 89.0 | 56.0 | 93.0 |
| 6 | 17.0 | 35.0 | 38.0 | 79.0 | 48.0 | 88.0 | 72.0 | 68 |
| 7 | 24.0 | 70.0 | 39.0 | 41.0 | 37.0 | 37.0 | 73.0 | 77.0 |
| 8 | 23.0 | 24.0 | 29.0 | 48.0 | 63.0 | 70.0 | 79.0 | 86.0 |
| 9 | 24.0 | 35.0 | 47.0 | 48.0 | 70.0 | 71.0 | | |
| Mean | 16.6 | 31.1 | 30.2 | 50.3 | 45.8 | 64.4 | 59.6 | 73.6 |
| Std. Deviation | 7.1 | 18.7 | 12.0 | 22.6 | 15.0 | 19.9 | 14.6 | 18.4 |
| Std. Error | 2.4 | 6.2 | 4.0 | 7.5 | 5.0 | 6.6 | 5.2 | 6.5 |

**Figure 19**

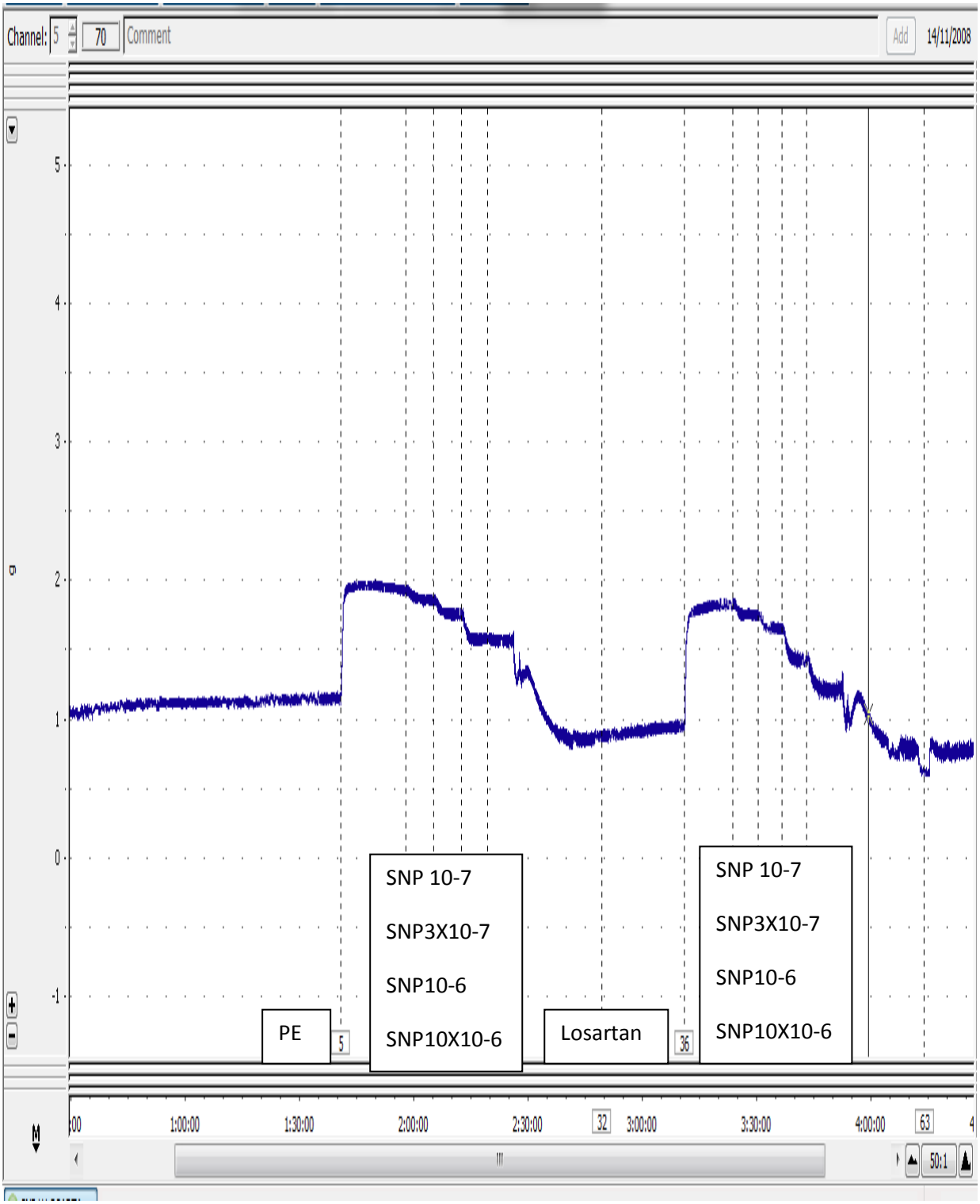



**Figure 19:** Representative tracing of SNP-induced relaxation of a corpus cavernosal strip pre- and post- losartan.

**Figure 19a**

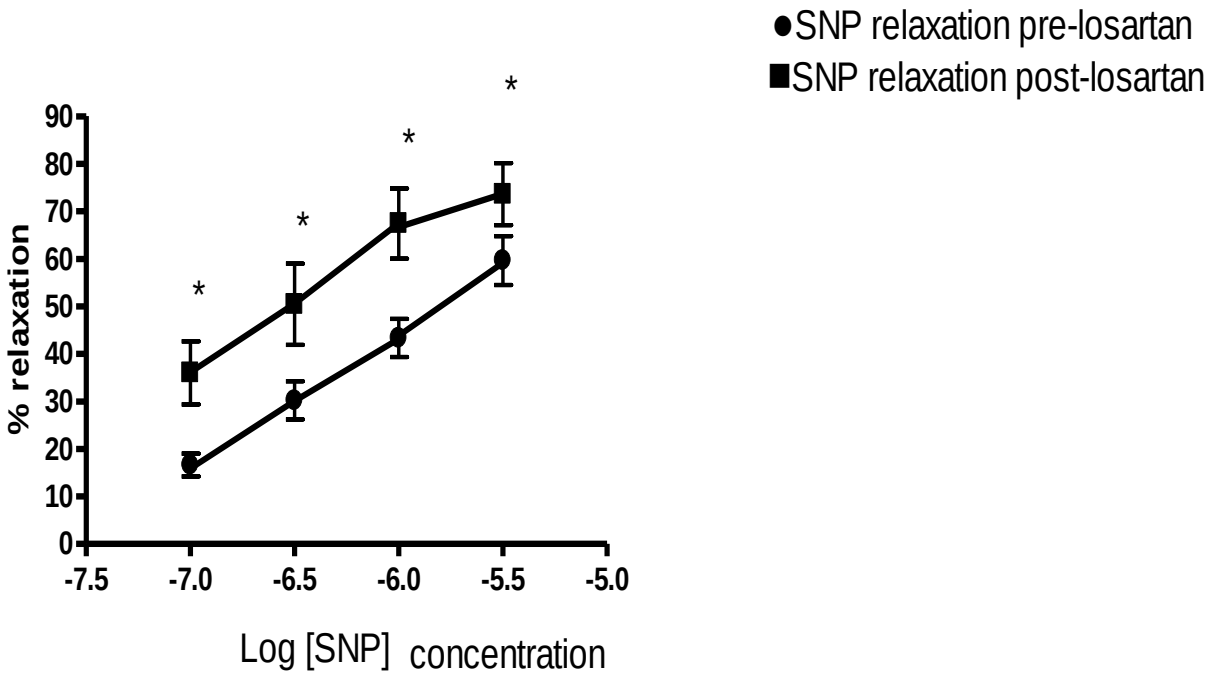
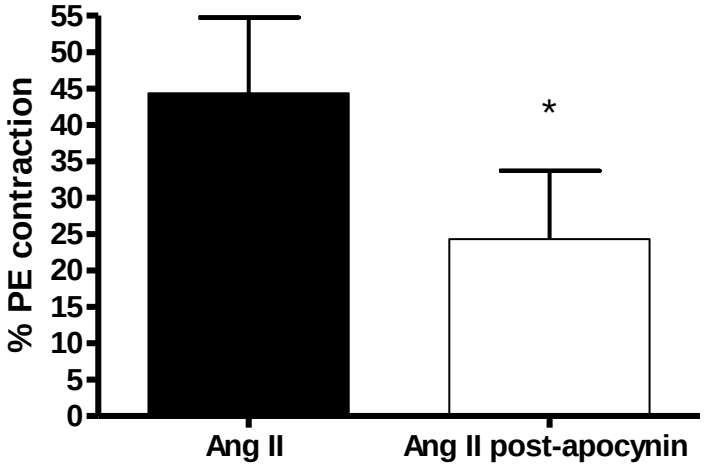



**Figure 19a.** SNP-induced relaxation of human corpus cavernosal strips pre- (●) and post- (■) losartan. * Denotes the data points where there was a significant difference in SNP-induced relaxation pre- and post-losartan (SNP (M): $10^{-7}$, $p < 0.02$; $3x10^{-7}$, $p < 0.013$; $10^{-6}$, $p = 0.013$; $3x10^{-6}$, $p < 0.04$, paired Student's t-test, $n = 8$).

### 4.3.5 NAD(P)H oxidase inhibition on Ang II and EFS-mediated responses

The NAD(P)H oxidase inhibitor apocynin significantly reduced the Ang II-induced contraction of corpus cavernosal strips, ( P = 0.006, Fig 20). Apocynin also potentiated EFS-induced relaxation when given with losartan by 33% (P=0.029).

**Figure 20**




**Effect of apocynin on Ang II-induced contraction**

**Figure 20.** Ang II-induced contraction ($10^{-6}$ M) of human corpus cavernosal strips pre- and post-apocynin ($10^{-4}$ M).*Denotes a significant difference in Ang II-induced contraction pre- and post-apocynin (P = 0.006, n=6, paired Student's t-test).

### 4.3.6 Immunohistochemistry

Control sections showed no positive staining (Fig. 21a). Ang II was detected mainly in the endothelium of arterioles, with less immunoreactivity in smooth muscle bundles of the corpus cavernosum, and the endothelium lining sinusoids (Fig. 21b). CD34 immunostaining showed an extensive network of endothelium (Fig. 21c). Immunostaining for smooth muscle actin showed the bundles of smooth muscle in the corpus cavernosum (Fig. 21d).

**Figure 21**

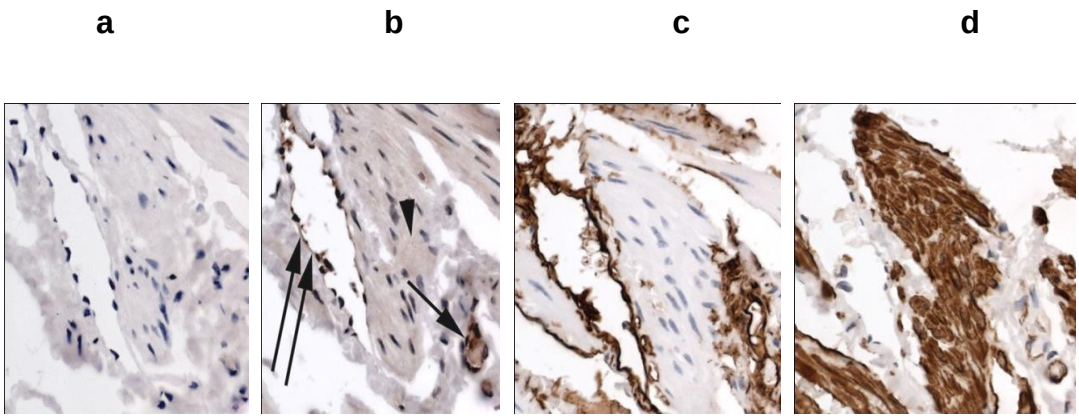



**Figure 21.** Immunostaining of the corpus cavernosum on adjacent sections. (a) Negative control section. (b) Ang II is detected in the endothelium of arterioles (*single arrow*) and to a lesser extent in the endothelium of sinusoids (*double arrow*) and in smooth muscle bundles (*arrowhead*). (c) CD34 immunostaining shows a widespread network of endothelium. (d) Smooth muscle bundles are shown by smooth muscle actin immunostaining.

## 4.4 Discussion

This is the first study to comprehensively examine the effect of Ang II on human corpus cavernosal function and to outline the possible mechanism of action. Organ bath studies showed that Ang II caused a dose dependent contraction of cavernosal tissue through the activation of AT1 and not AT2 receptors. Although the penile contractile response to Ang II is species dependent (Klinge *et al*,1977) the present finding supports previous studies using canine and rabbit cavernosal tissue (Comiter *et al*,1997;Park *et al* 1997). It also supports work presented earlier in this Thesis (see Chapter 3).

In addition, immunohistochemical studies revealed, for the first time, Ang II-containing cells primarily distributed in human corpus cavernosal arteriolar endothelium, the interface between the sinusoidal cavities and to a lesser extent in smooth muscle. A similar distribution of Ang II immunohistochemical staining has also been reported in animal studies (Kifor *et al*, 1997).

As previously stated, the role of Ang II in the control of vascular tone and in the pathophysiology of CVD is well known (Oliver *et al*, 1984; Cohn ,2006). The Large scope HOPE study demonstrated that the ACE inhibitor ramipril significantly reduced rates of cardiovascular death, myocardial infarction and stroke compared to placebo among patients with high risk of CVD (The heart out come prevention and evaluation study, 2000).The role of Ang II in other systems have also been demonstrated, for example, ACE inhibition is known to slow the progress of renal disease in patients with Type I DM (Lewis *et al* ,1993). Several randomized controlled trails have also shown that Ang II receptor blockers slow renal disease progression in patients with

Type II DM and early or late stage renal dysfunction independent of blood pressure reduction (Lewis *et al*, 1993; Karalliedde *et al*, 2006).

Here, using human tissue we confirm the findings of the rabbit study that Ang II, acting in a paracrine manner, is also a mediator of CCSM tone and contractility (Comiter *et al*,1997;Becker *et al*, 2001), providing a physiological role for this octapeptide in the human erectile process. This would also explain, at least in part, why Ang II is produced and secreted by the human corpus cavernosum in physiological amounts (Kifor *et al*,1997) and is higher in the cavernous blood during penile detumescence than in the tumescence phase (Becker *et al*, 2001).

As previously mentioned, the deleterious effect Ang II has on penile erection is evident from *in vivo* experiments using anesthetised dogs where intracavernosal injections of the octapeptide terminated spontaneous erections. In contrast, the injection of losartan relaxed CCSM and caused penile erections by affecting tone and contractility of vascular smooth muscle within the blood vessels embedded in the corporal bodies, as well as the tone of the smooth muscles of the corpus cavernosum itself (Kifor *et al*,1997).

More recent work has shown that bilateral cavernosal nerve injury in a rat model caused a decrease in the erectile response, which was associated with up regulation of fibrotic activation in the penis, an effect that can be reversed with losartan, suggesting a role for Ang II in penile fibrosis following nerve injury. This may have clinical importance as this type of injury is common following pelvic surgery e.g. radical prostatectomy (Canguven *et al*, 2009).

The Ang II-containing cells identified by immunohistochemistry presumably secrete their Ang II content on adrenergic stimulation, similar to vascular tissue (Kifor *et al*, 1997). Our functional studies using losartan and PD123319 verify that the secreted Ang II acts on AT1 and not AT2 receptors to elicit the human corpus cavernosal contractile response, as reported in other species (Comiter *et al*, 1997;Becker *et al*, 2001).

The balance/functional interplay between Ang II and NO/cGMP have previously been reported in the vascular system (Sigmon *et al*, 1992; de Gasparo *et al*, 2002), as well as here using rabbit CCSM (Chapter 3). Several studies have suggested that NO might be a direct modulator of ACE activity (Higashi *et al*, 1995) and that Ang II may also have receptor-mediated effects on NOS activity and NO generation (Yan *et al*, 2003). Thus, an imbalance between these mediators is thought to be an important factor in the development of CVD (Yan C *et al*, 2003). Interestingly, impaired CCSM relaxation to ACh, diminished eNOS expression and increased VSMCs has been found in cavernous arteries taken from spontaneously hypertensive rats. All these parameters were improved by the combined treatment of losartan with sildenafil, implying that the enhancement of the NO/cyclic cGMP pathways by sildenafil supplements the actions of losartan by inhibiting the Ang II response through AT1 receptor blockade ( Tobil *et al*, 2007).

NANC neurotransmission is an important component of penile erection, since stimulating the cavernous nerve leads to smooth muscle relaxation (Burnett *et al,*1997). Losartan significantly enhanced the EFS-induced relaxation of corpus cavernosal strips following the addition of guanethidine, atropine and indomethacin, inhibitors of the adrenergic, cholinergic and prostaglandin pathways, respectively. This finding is significant, since Ang II released via adrenergic stimulation should

have been blocked by guanethidine-induced chemical sympathectomy (Kempinas W,1998; Villanueva et al ,2003) making losartan ineffective. However, the data implies that guanethidine did not fully inhibit the adrenergic pathway. This is supported by previous studies; daily injections of guanethidine only destroyed 60-70% of sympathetic nerves in adult rats (Robin *et al* ,2006), while guanethidine added to the bathing solution of rabbit thoracic aortic rings attenuated EFS-evoked noradrenaline release by 50% (Nap *et al*, 2001). Thus, EFS may be recruiting the remaining sympathetic neurones unaffected by guanethidine, causing the release of Ang II, which counteracts NANC neurotransmission. This would also explain why losartan had a significantly greater effect on EFS-induced relaxations when guanethidine was omitted from the bathing solution.

The addition of L-NAME to the cocktail of inhibitors blocked the NO pathway resulting in EFS-induced contraction of corpus cavernosal strips. This contractile response is probably due to the recruiting of sympathetic neurones not affected by guanethidine causing Ang II-mediated contraction, which losartan reduced. As previously discussed, the release of NO from endothelial cells is also a vital step in the erectile process (Burnett *et al*, 1997). Our group previously showed that NO and the NO donor SNP causes relaxation of CCSM tissue taken from control rabbits (Khan *et al* ,2001a ;Thompson *et al* ,2001). Here, we found that SNP caused a dose dependent relaxation of human corpus cavernosal strips, which was significantly enhanced following the addition of losartan.

The interaction between Ang II and NO provides an insight into the possible mechanism of action of Ang II on corpus cavernosal contractility. As in the case of the rabbit experiments, the effect of Ang II is due to the physiological development of OS. In fact, in human blood vessels the membrane associated NAD(P)H oxidase is

thought to be the principle source of basal and Ang II-induced $^{\bullet}O_2^-$, although other enzyme pathways are thought to play a role (West *et al*, 2001; Puntmann *et al*, 2005).

We found the selective NAD(P)H oxidase inhibitor, apocynin reduced corpus cavernosal contraction and potentiated EFS-induced relaxation when given with losartan, both responses are probably due to the abolishment of $^{\bullet}O_2^-$ generation, a similar conclusion was made in a study using vascular tissue (Lu *et al*, 2008). Further evidence of the role of OS in the action of Ang II is provided by the findings that showed the $^{\bullet}O_2^-$ scavenger superoxide dismutase significantly attenuated vascular smooth muscle contraction (Kawazoe *et al*, 2000). While pyrogallol a generator of $^{\bullet}O_2^-$ mimicked the Ang II enhancement (Lu *et al*, 2008).

## 4.5 Conclusion

Ang II-induced contraction of human CCSM plays a major role in the termination of penile erection; this has greater significance since the penis is usually in a flaccid/ detumescence state. The use of Ang II antagonists provides a vehicle to identify the underlying mechanistic interactions that involve the paracrine actions of Ang II. For example, losartan potentiated SNP- and EFS-mediated CCSM relaxation, providing important evidence of the interplay between Ang II and NO/cGMP pathways in the regulation of human corpus cavernosal tone. Evidence presented in this Thesis also supports the concept that OS plays a significant role in Ang II –induced contraction of corpus cavernosal tissue an effect mediated by the production of $^{\bullet}O_2^-$. It is, therefore, conceivable that a disruption in the delicate interaction between these mediators may play a significant role in the development of ED.

# Chapter 5

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# The effect of angiotensin II on corpus cavernosal function from partial bladder outlet obstructed rabbits.

## 5.1 Introduction

It is now recognised that benign prostatic hyperplasia (BPH) can cause partial bladder outlet obstruction (PBOO) (Berry *et al*, 1984), resulting in structural and functional changes to the bladder that can influence the storage and emptying of urine, with many patients developing detrusor overactivity (Eckhardt *et al*, 2001). The clinical consequences of PBOO associated with BPH (Mauroy, 2008), including urodynamics and structural changes in bladder pathophysiology can be reproduced in animal models, by tying a ligature around the proximal urethra at the base of the bladder neck (Beamon *et al*, 2008; Calvert *et al*, 2001a). An increase in bladder mass, as well as hypertrophy and hyperplasia of bladder smooth muscle, with thickening of the outer serosal layer have been reported in rabbits following this procedure (Calvert *et al*, 2001a). Functional studies have also revealed a reduction in electrical field stimulation (EFS) and chemical-induced bladder smooth muscle contraction (Lin *et al*, 2007), supporting the clinical findings of impaired bladder function following PBOO.

There has been a continuing debate as to whether there is a link between BPH and ED. Although some clinical studies suggest an association, (Namasivayam *et al*,1998; Rosen *et al*, 2009), where sexual performance is related to the severity of BPH (Baniel *et al*, 2000), a literature based study could not identify this link (Vale, 2000). However, the use of the PBOO animal model has helped to investigate this association (Chang *et al*, 2000; Lin *et al*, 2008). Our group were the first to show increased collagen deposition in the corpus cavernosum (Khan *et al*, 1999) and preliminary evidence of impaired corpus cavernosal smooth muscle (CCSM) relaxation in a chronic PBOO rabbit model (Calvert *et al*, 2001b and c). This has been substantiated by the findings that endothelium-dependent (ACh-mediated) and

endothelium-independent (ATP/SNP-mediated) CCSM relaxation is impaired, with a marked reduction in smooth muscle cells in this model (Lin *et al*, 2008). These findings imply that PBOO animals exhibit many of the features of ED and offer a test bed to determine its influence on other components of the erectile process, for example pro-contractile mechanisms, which terminate penile erection by keeping the smooth muscle of the penile arteries and trabeculae contracted (Holmquist *et al*, 1992).This is of particular importance, since unlike most smooth muscle cells those of the corpus cavernosum spend the majority of the time contracted (Chang *et al*, 2002).

Ang II as previously determined is an important mediator of human CCSM contractility and tone and that its regulation is governed by a balance with NO (Chapter 4).

Here, we determined the effect Ang II, AT1 receptor inhibition and OS have on CCSM contractility, together with their modulation of NO-mediated relaxation in a chronic rabbit model of PBOO. The results from this study provide important information on the pathological role of Ang II in ED.

## 5.2 Materials and Methods

### 5.2.1 Induction of partial bladder outlet obstruction (PBOO) and tissue aquisition

See Chapter 2 for details of experimental procedure and tissue acquisition.

### 5.2.3 Organ bath studies

The tissue strips were mounted vertically in 10 ml organ baths, equipped with two parallel platinum electrodes for EFS (See Chapter 2 & 3 for details). Tension was applied to the suspended tissue strips and left for 1h to equilibrate (tension recorded on a Grass Polygraph, model 7D; Astro-med Grass, Slough, UK).

### 5.2.4 Effects

#### 5.2.4.1 Bladder weights:

The bladders were excised from sham-operated and PBOO rabbits after 8 weeks and weighed.

#### 5.2.4.2 Ang II

The effect of Ang II ($10^{-8}$M – $10^{-5}$M) on CCSM function was investigated using tissue strips from sham-operated and PBOO rabbits.

### 5.2.4.3 Ang II receptor antagonists

After the effect of Ang II on tissue strips taken from sham-operated and PBOO rabbits were determined; the tissues were washed over a 30 min period and exposed to losartan ($10^{-5}$ M) for 20 min before repeating the Ang II response. The effect of the vehicle (distilled water for 20 min) on the Ang II response was also determined.

### 5.2.4.4 Oxidative Stress

#### 5.2.4.4.1 DPI

The effect diphenylene iodonium chloride made up in DMSO (DPI, $10^{-4}$M, NAD(P)H oxidase inhibitor, which inhibits $^{\bullet}O_2^-$ production) has on the Ang II ($10^{-6}$M) response from sham-operated and PBOO tissue strips was determined.

#### 3.2.4.4.2 SOD

The effect superoxide dismutase (SOD, 200 IU/ml; the enzyme that accelerates the breakdown of $^{\bullet}O_2^-$) has on the Ang II ($10^{-6}$M) response from sham-operated and PBOO tissue strips was also determined.

### 5.2.4.5 Electrical Field Stimulation

#### NANC neurotransmission

In a series of experiments CCSM tissue from sham-operated and PBOO animals were exposed to guanethidine ($5 \times 10^{-6}$ M), atropine ($10^{-5}$ M) and indomethacin ($10^{-6}$ M), which were added to the bathing solution and left for 20 min to inhibit the adrenergic, cholinergic and cyclo-oxygenase pathways, respectively, leaving the

NANC pathway intact. The tissue strips were then pre-contracted with PE followed by EFS of penile nerves with a Grass S88 (Astro-med Grass, Slough, UK) stimulator. The stimulator delivered single square waves (duration 0.4 ms; 100V) at a frequency of 8.0 Hz in 5 s trains. Losartan and DPI was then added and the EFS repeated.

We choose 8 Hz since this stimulation frequency is ideal to evaluate the effect of losartan on NANC neurotransmission (Chapter 3 & 4).

#### 5.2.4.6 SNP

CCSM tissue strips from sham-operated and PBOO rabbits were pre-contracted with PE ($10^{-4}$M) and cumulative response curves were constructed for the NO donor SNP, $10^{-7}$ – $3x10^{-6}$ M).

#### 5.2.4.7 Losartan

In another series of experiments SNP cumulative response curves were again constructed using CCSM tissue from PBOO animals. The tissue was washed several times before the addition of losartan ($10^{-5}$ M) to the organ bath for 20 min and re-contracted with PE and a cumulative response curve constructed for SNP.

### 3.2.5 Statistical Method

The raw data from Ang II tissue responses were expressed as mg tension / mg tissue. EFS and SNP tissue responses were expressed as % relaxation of PE-induced tone. These were analysed and expressed as mean ± SEM using Graph

Pad Prism 4.0 software (see examples, Tables 7 & 8). Comparisons of the resultant Ang II dose response curves and SNP cumulative dose response curves were made using analysis of variance (2 way ANOVA, $p < 0.05$). Student's unpaired and paired t-test statistical analysis was also determined on the raw data by the software package with statistical significance accepted at $p < 0.05$. The individual number of tissue strips used is included in the figure Legends. Strips from at least 5 animals were used in each experiment.

## 5.3 Results

### 3.3.1 Bladder weight

There was a significant increase in rabbit bladder weights following 8 weeks PBOO when compared with sham-operated animals (sham-operated rabbits, median 2.0g, range 1.7 - 2.8g vs PBOO rabbits, median 20.0g, range10 - 42.0g; n =13 p<0.0001 unpaired Mann Whitney, Figure 22).

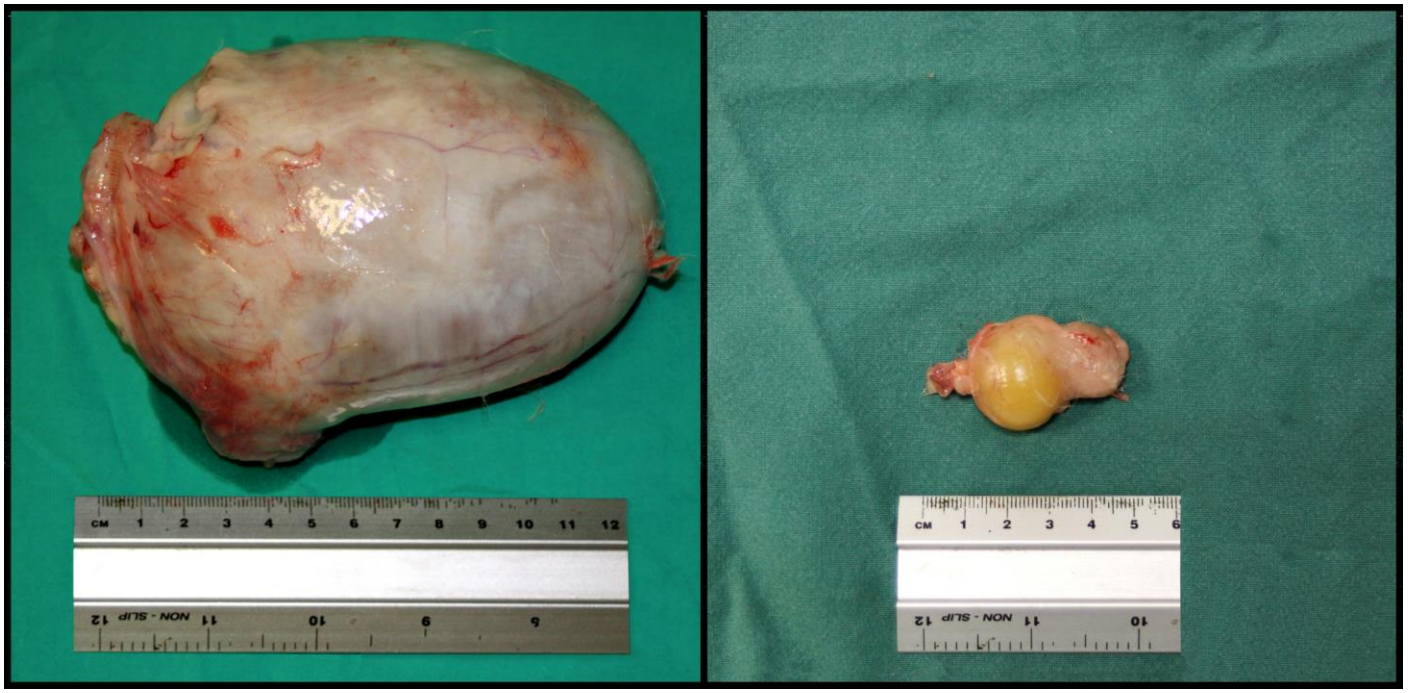

**Figure 22**. Representative urinary bladder from a PBOO rabbit (42g, left) and a sham-operated rabbit (2g, right) 8 weeks after surgery.

### 5.3.2 Ang II and CCSM contraction

The size and weight of cavernosal strips from sham-operated and PBOO rabbits were similar. Ang II caused a dose dependent contraction ($10^{-8}$M – $10^{-5}$M) of CCSM strips from sham-operated and PBOO rabbits, which was markedly increased in the PBOO group and reduced by losartan (Fig 23). The addition of the vehicle did not significantly influence the Ang II response in any experiment.

**Figure 23**

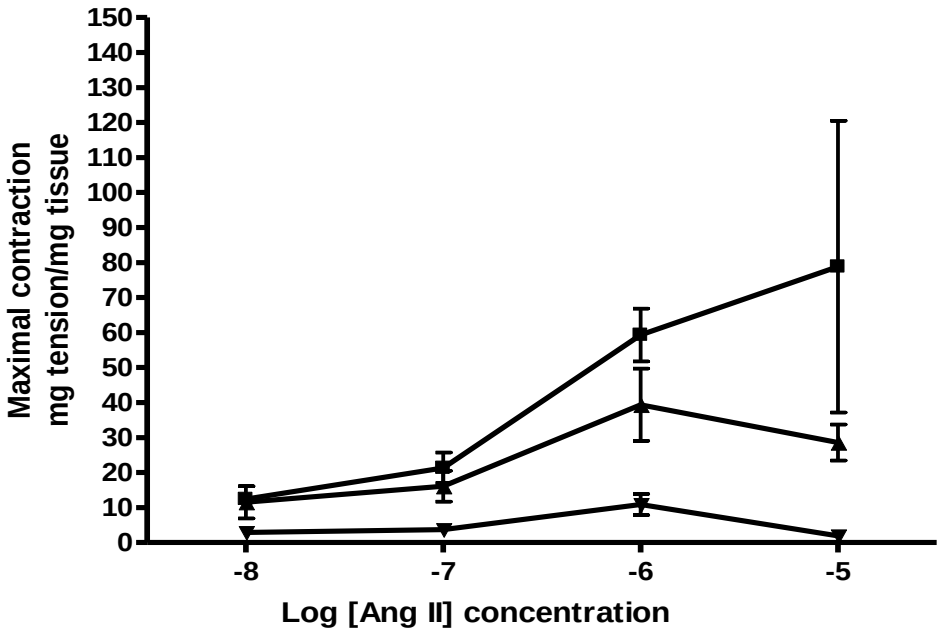


**Figure 23.** Ang II-induced contraction of CCSM strips taken from PBOO rabbits (■) was significantly ($P<0.01$) increased compared with sham-operated animals (▲). The PBOO-induced increase in CCSM contractility was significantly reduced post-losartan (▼), $P<0.0001$, n = at least 5 strips/concentration.

### 5.3.3 Oxidative Stress

**5.3.3.1 CCSM contraction.** DPI and SOD reduced the Ang II-induced contraction of CCSM strips from sham-operated and PBOO rabbits. (Table 7,Figs 24, 24a &24b ).

**Table 7:** Ang II–induced contraction of corpus cavernosal strips pre- and post- SOD

| Strip No. | Ang II $10^{-5}$M<br>%PE Contraction | Ang II $10^{-5}$M post-SOD<br>%PE Contraction |
|---|---|---|
| 1 | 68.0 | 57.0 |
| 2 | 56.0 | 33.0 |
| 3 | 46.0 | 35.0 |
| 4 | 74.0 | 55.0 |
| 5 | 73.0 | 63.0 |
| 6 | 51.0 | 51.0 |
| 7 | 43.0 | 39.0 |
| 8 | 29.6 | 10.6 |
| Mean | 58.7 | 47.6 |
| Std. Deviation | 12.9 | 11.8 |
| Std. Error | 4.9 | 4.4 |

**Figure 24**

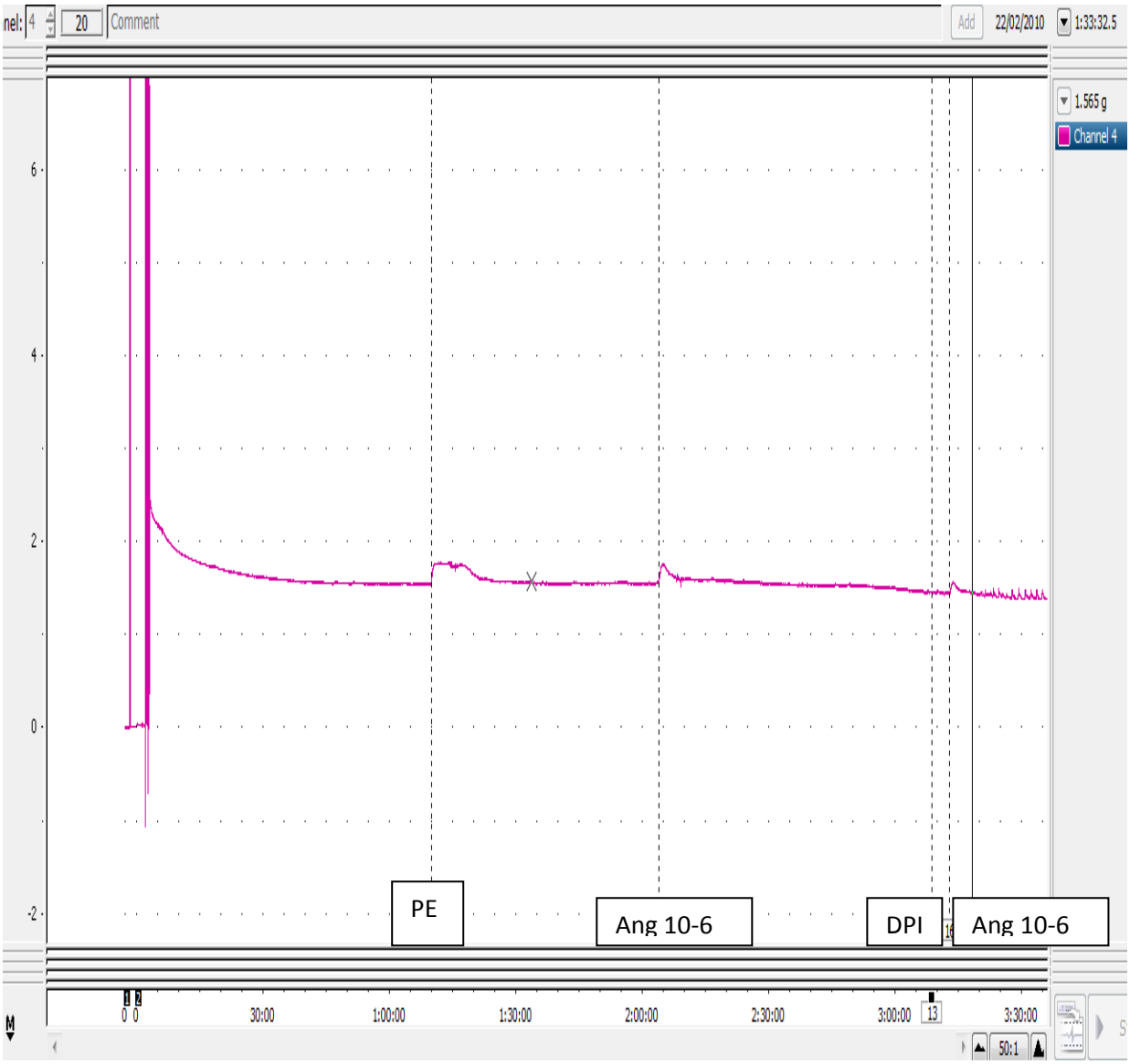


**Figure 24:** Representative tracing of Ang II ($10^{-6}$ M)–induced contraction of a corpus cavernosal strip pre- and post- DPI.

**Figure 24a**

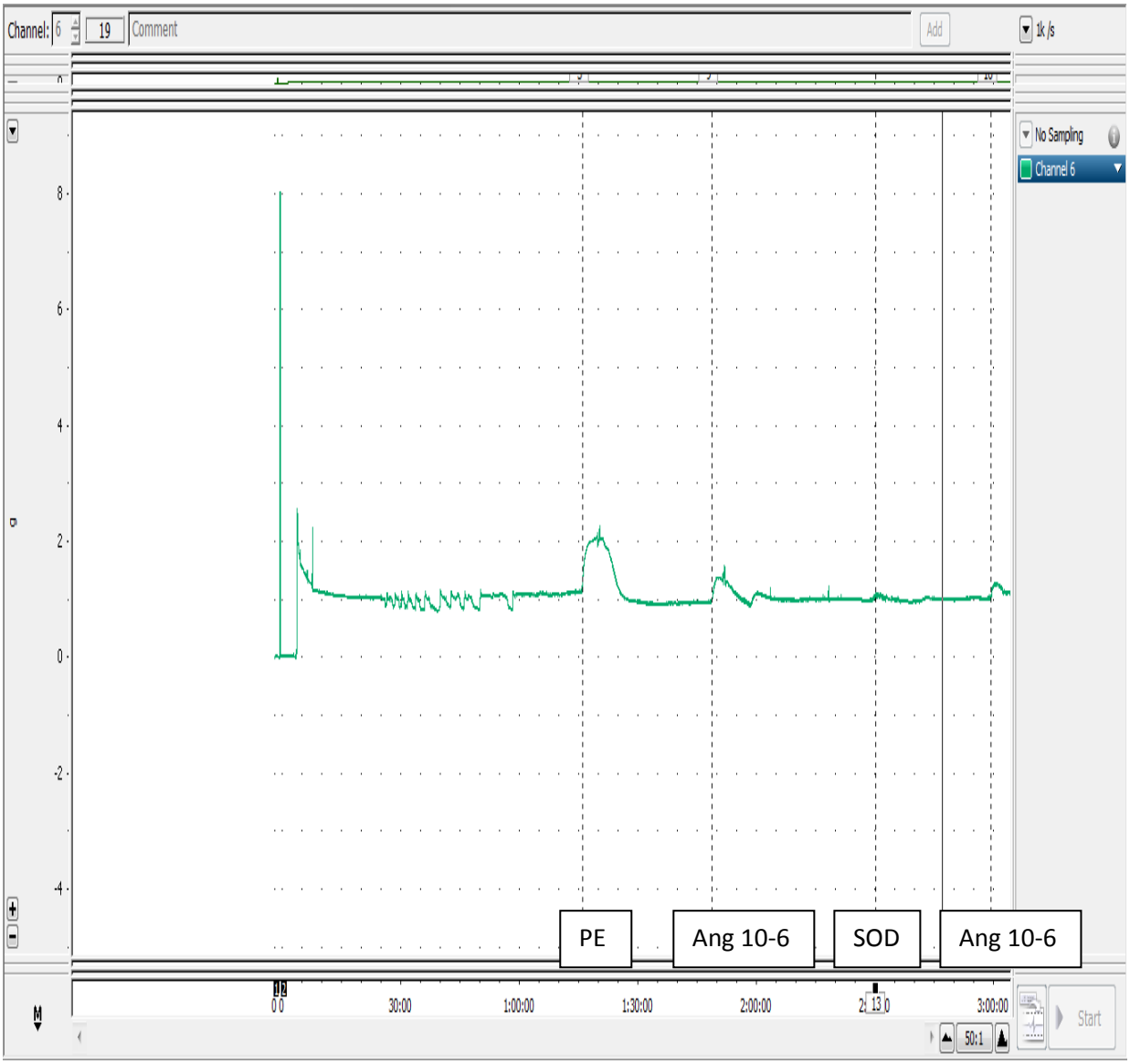


**Figure 24a:** Representative tracing of Ang II ($10^{-6}$ M)–induced contraction of a corpus cavernosal strip pre- and post- SOD.

**Figure 24b**

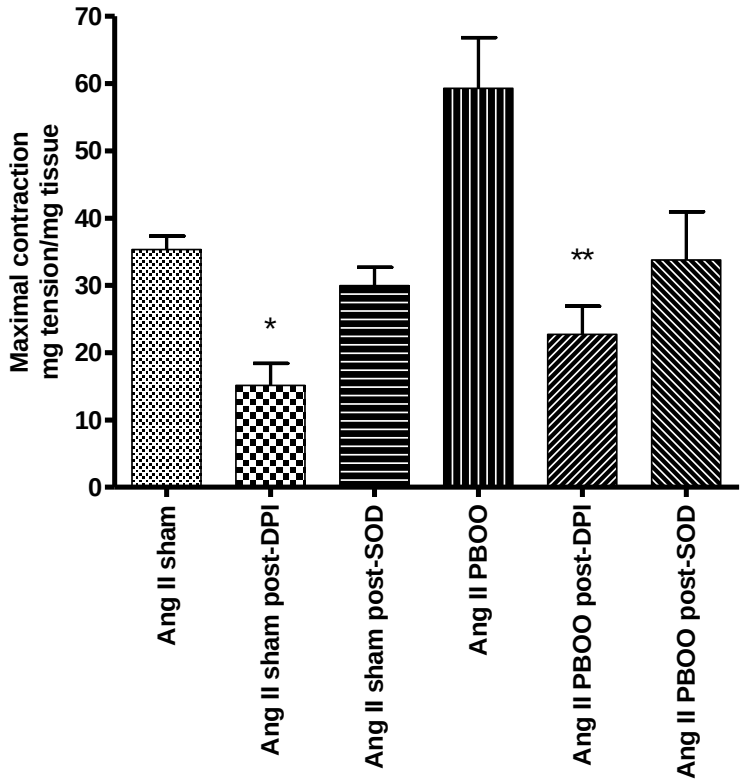


**Figure 24b.** Ang II-induced contraction ($10^{-6}$ M) of corpus cavernosal strips taken from sham-operated and PBOO rabbits was significantly decreased post-DPI ($10^{-4}$ M), * $p<0.03$, ** $p=0.001$ and not significant post-SOD (200UI/ml), n = at least 5 strips, unpaired Student's t-test.

### 5.3.3.2 CCSM relaxation

EFS-induced CCSM relaxation of sham-operated and PBOO strips, was increased following adrenergic, cholinergic and cyclo-oxygenase inhibition and in the presence of losartan (Fig 25). In addition, EFS-induced CCSM relaxation of PBOO strips was increased by 18.4% following addition of the triple inhibitors (guanethidine, atropine and indomethacin), and in the presence of DPI.

**Figure 25**

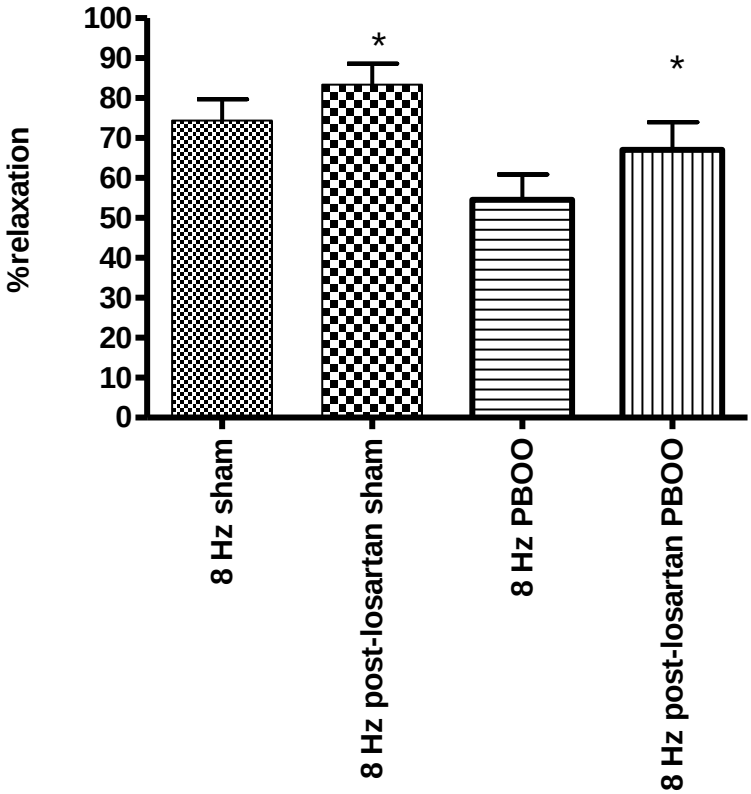


**Figure 25.** EFS-induced relaxation of corpus cavernosal strips taken from sham-operated and PBOO rabbits at 8 Hz (following the addition of guanethidine, atropine and indomethacin) was significantly increased post-losartan * $p<0.02$, n = at least 5 strips, paired Student's t-test.

### 3.3.4 SNP and CCSM relaxation

SNP-induced relaxation of CCSM strips taken from PBOO rabbits was impaired when compared with sham-operated animals and improved by losartan (Table 8, Figs 26, 26a ).

**Table 8 :** SNP-induced relaxation of corpus cavernosal strips pre- and post- losartan.

| Strip No. | SNP $10^{-7}$ M<br>% PE relaxation | SNP $10^{-7}$M post –los<br>% PE relaxation | SNP $3x10^{-7}$M<br>% PE relaxation | SNP $3x10^{-7}$ M post-los<br>% PE relaxation | SNP $10^{-6}$M<br>% PE relaxation | SNP $10^{-6}$M post -los<br>% PE relaxation | SNP3X$10^{-6}$ M<br>% PE relaxation | SNP $3x10^{-6}$ M post-los<br>% PE relaxation |
|---|---|---|---|---|---|---|---|---|
| 1 | 15.0 | 22.0 | 26.0 | 36.0 | 38.0 | 68.0 | 50.0 | 80.0 |
| 2 | 14.0 | 10.0 | 17.0 | 16.0 | 29.0 | 37.0 | 54.0 | 63.0 |
| 3 | 2.0 | 2.4 | 7.0 | 7.0 | 28.0 | 45.0 | 48.0 | 52.0 |
| 4 | 4.0 | 4.0 | 5.0 | 8.0 | 32.0 | 35.0 | 46.0 | 60.0 |
| 5 | 5.0 | 9.0 | 21.0 | 35.0 | 45.0 | 47.0 | 47.0 | 67.0 |
| 6 | | | | | 29.0 | 30.0 | | |
| 7 | | | | | 32.0 | 35.0 | | |
| Mean | 8.0 | 9.5 | 15.3 | 20.4 | 33.3 | 42.4 | 49.0 | 64.4 |
| Std. Deviation | 6.0 | 7.7 | 8.8 | 14.2 | 6.2 | 12.8 | 3.1 | 10.3 |
| Std. Error | 2.7 | 3.4 | 3.9 | 6.4 | 2.3 | 4.8 | 1.4 | 4.6 |

**Figure 26**

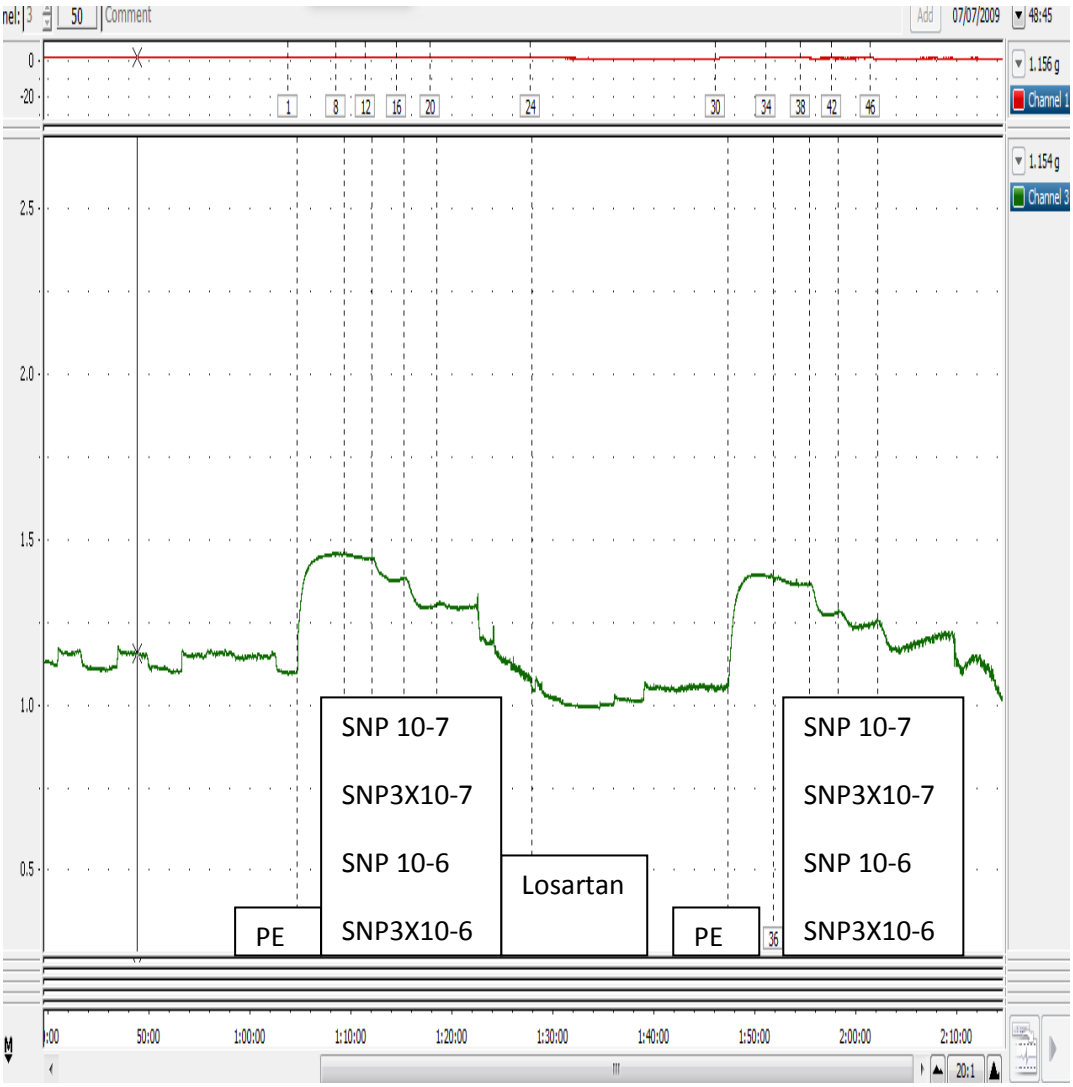


**Figure 26:** Representative tracing of SNP-induced relaxation of a corpus cavernosal strip pre- and post- losartan.

**Figure 26a**

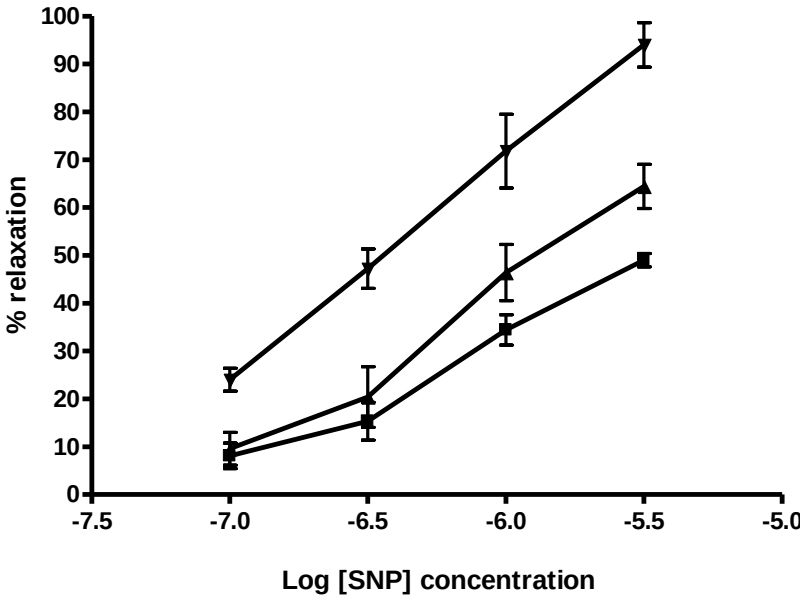


**Figure 26.** SNP-induced relaxation of CCSM strips taken from PBOO (■) rabbits was significantly (P <0.0001) impaired compared with sham-operated (▼) animals and was significantly improved post-losartan (▲), P<0.01, n = at least 5 strips.

## 5.4 Discussion

This study shows, for the first time, a dose dependent enhancement of the Ang II-induced contraction of CCSM tissue taken from PBOO rabbits when compared with sham-operated controls. It also supports previous findings using human and normal rabbit corpus CCSM tissue, which revealed the Ang II–mediated contractile response is due to AT1 receptor activation with the development of OS (Chapter 3 & 4)

The present findings demonstrate that Ang II increases CCSM contraction as a pathological consequence of PBOO. We have used only one concentration of Ang II on each individual tissue strip, since multiple application of Ang II to isolated human arteries results in a marked desensitisation of the functional response (i.e. tachyphylaxis) (Hidaka *et al*, 2005). This phenomenon may explain the large variation in the Ang II $10^{-5}$M error bar following PBOO (Fig 23).

Results from previous studies using PE as a mediator of CCSM contractility following PBOO in rabbits are inconclusive. Chang *et al*, 2002. noted a 50% increase in CCSM contractile force after 2 weeks PBOO, due to an increase in smooth muscle bundles and cellular alterations in the contractile myosin-isoform composition. This increased contractility was not evident in the studies of Demir *et al*, 2008. and Lin *et al*, 2008. at a similar time point. This is possibly due to post-operative inflammation in the sham-operated group and a reduction in CCSM cells, respectively. Results from chronic PBOO studies have revealed a time-dependent change in the contractile response. Demir et al., found an increase in CCSM contractility after 4 weeks PBOO, as the inflammatory response in the sham-operated group subsided. Whereas, Lin et al., found the contractile response was reduced at 8 weeks, due to a reduction in CCSM content and an increase in collagen deposition.

In an attempt to shed more light on the changes in corpus cavernosal function following chronic PBOO, we conducted our experiments using Ang II a known physiological mediator of human and rabbit CCSM contraction. Our data suggest that the contractile capacity of each individual smooth muscle cell is increased in this model, even though the overall numbers are reduced due to collagen deposition (Lin *et al*, 2008) a finding that may have clinical relevance in the development of ED. This is in keeping with a previous study that showed PE elicited an increased contractile response of CCSM strips taken from men with ED, suggesting an increase in corporal vascular smooth muscle contractility may contribute to the pathophysiology of ED in older men (Christ *et al,* 1991).

The PBOO-induced augmentation of the Ang II pathway could be due to an increase in Ang II release and/or AT1 receptor density, increased coupling efficiency of the agonist-receptor complex to the signal transduction machinery and/or increased amplification of second messenger formation subsequent to receptor activation. While the importance of each potential mechanism is uncertain, it is likely an increase in OS and excessive $^{\bullet}O_2^-$ production is involved. The role of OS in the Ang II-mediated contraction of human corpus cavernosum is known (Chapter 4). We found the selective NAD(P)H oxidase inhibitor DPI significantly attenuated Ang II-induced CCSM contraction in sham-operated and PBOO rabbits. Although, the reduction in the Ang II response induced by the $^{\bullet}O_2^-$ scavenger SOD was not significant, this trend particularly following PBOO suggests that SOD, similar to DPI is capable of reducing OS. The effectiveness of these drugs is probably due to the abolishment of $^{\bullet}O_2^-$ generation. Similar observations have been reported using vascular tissue (Kawazoe *et al*, 2000; Lu *et al*, 2008), while pyrogallol a generator of $^{\bullet}O_2^-$ mimicked the Ang II enhancement (Lu *et al*, 2008).

NANC neurotransmission is an important component of penile erection, since stimulating the cavernous nerve leads to smooth muscle relaxation (Burnett *et al*, 1997). This pathway is impaired following PBOO (Lin *et al*, 2008), providing further evidence of the development of ED in this model. Losartan significantly increased the EFS-induced relaxation of corpus cavernosal strips taken from PBOO rabbits following cholinergic, prostaglandin and adrenergic inhibition. However, as Ang II release is via adrenergic stimulation, guanethidine should have blocked this pathway making losartan ineffective. The data implies that guanethidine does not fully inhibit the adrenergic pathway during EFS-induced relaxation, a point previously discussed in Chapter 4.

Our findings also confirm that SNP-mediated relaxation of CCSM tissue is impaired in chronic PBOO rabbits as previously reported (Calvet *et al*, 2001b; Lin *et al*, 2008). This reinforces the concept that this model demonstrates the salient features of ED.

Losartan significantly improved the SNP-mediated relaxation of CCSM strips taken from PBOO rabbits, highlighting the interplay between Ang II and NO/cGMP, implying that an imbalance between these mediators is an important factor in the development of ED. Interestingly, an inverse correlation between Ang II responsiveness and endothelium-dependent relaxation has been demonstrated in isolated human arteries, which was related to the development of OS (Voors *et al*, 2005).

The actions of losartan may have clinical importance in ED management. For, although PDE 5 inhibitors have become extremely effective oral agents for the treatment of ED, it has become increasingly apparent not all patients respond to this form of therapy (Shabsigh *et al*, 2000), moreover, some who initially respond

develop tachyphylaxis or discontinue their use due to loss of efficacy (El-Galley *et al,* 2001), Thus, the use of an Ang II antagonist, which reduce CCSM contractility, in conjunction with a PDE5 inhibitor that increases CCSM relaxation, may be beneficial for ED patients; not only by reducing the percentage of none responders but also the concentration of PDE 5 inhibitor required to maintain erection. This could explain, at least in part, why long-term losartan and PDE5 combination therapy has a beneficial effect on the structure and function of CCSM tissue taken from SHR (Toblli *et al*, 2007).

### 5.5 Conclusion

Ang II causes a pathological enhancement of CCSM contraction from PBOO rabbits that was inhibited by losartan, probably due to a direct/indirect reduction in $^{\bullet}O_2^-$ production and OS. Losartan improved the impaired SNP/EFS-mediated relaxation, providing important evidence of the interplay between Ang II and NO/cGMP pathways in the regulation of CCSM tone. Elevated Ang II responsiveness, together with impaired relaxation of CCSM, may play a pivotal role in the development of ED. Further studies are required to determine the molecular events responsible for the cellular changes to CCSM in PBOO.

# Chapter 6

# GENERAL DISCUSSION

## 6.1 Correlation between PBOO and structural/functional changes to the corpus cavernosum.

Although PBOO is associated with direct changes to the CCSM, the mechanism(s) responsible for these changes are unclear. It seems unlikely that it is due to direct injury/trauma during surgery, since these changes were not evident in the sham-operated rabbits. It is, however, possible that constant compression of the nerves that travel through the bladder neck region and supply the corpus cavernosum caused by the partial ligation of the urethra, could have lead to a decrease in cavernosal innervation. This idea is supported by immunohistochemical staining of corpus cavernosal tissue taken from PBOO rabbits, which revealed decreased neuronal innervation (Chang *et al*, 2002).

Another explanation can be derived from the increased bladder mass and bladder overdistention associated with PBOO. In this scenario, stretching the nerves of the pelvic plexus or compressing small arteries in the base of the bladder would lead to ischemia of the corpus cavernosum, which in turn, could affect corpus cavernosal function (Chang *et al*, 2002).

## 6.2 Corpus cavernosal tone: balance between relaxing and contractile pathways.

Penile erection ultimately depends on the relationship between contractile and relaxation mechanisms acting on the smooth muscle cells of the corpus cavernosal arterioles and sinuses. CCSM relaxation and inhibition of contraction permits increased arteriolar blood inflow that fills and expands the corpus cavernosal

sinuses. This occurs in conjunction with a concomitant reduction in the rate of venous blood outflow due to veno-occlusion.

The balance/interplay between contracting and relaxant factors is paramount, since this phenomenon controls penile vasculature tone. The importance of NO in the erectile process is irrefutable, since it stimulates the intracellular production of NO/cGMP (Burnett, 1997), from both NANC nerves (Lue, 2000; Maggi et al, 2000) and the corpus cavernosal endothelial cells (Hedlund *et al*, 1985). This initiates CCSM relaxation and ultimately penile erection (Trigo-Rocha *et al*, 1993).

Importantly, it must be remembered that the penis remains in the flaccid state most of the time, thus, maintaining physiological CCSM basal tone. Although, the understanding of the various vasoconstrictor agents responsible for this functional phase remains incomplete, endothelin-1, serotonin, as well as adrenergic agonist have all been implicated/ investigated in this regard (Saenz De Tejada *et al*, 1991; Lau *et al*, 2006; Wingard *et al*, 2001). The role of Ang II is of particular interest, since it is produced by human CC tissue, as demonstrated in the immunohistochemical study and secreted in physiological amounts (Kifor *et al*, 1997), acting in a paracrine manner when causing CCSM contraction. This supposition is supported by the finding that the cavernosal blood level of Ang II is higher during flaccidity than when the penis is erect (Becker *et al*, 2001).

Both the rabbit and human studies presented in this Thesis (Chapter 3 & 4) using CCSM tissue from control animals and gender reassignment patients, respectively, highlight the important role Ang II plays in maintaining “normal” CCSM tone. A feature of these studies was the decision not to follow previous experimental protocols when determining the effect of Ang II on CCSM function. Other studies

have attempted to produce a cumulative dose response curve for Ang II-induced CCSM contraction. However, here it was recognised that such an approach might lead to erroneous results/invalid interpretation of the data. This is because multiple application of Ang II to isolated CCSM tissue results in a marked desensitisation of the functional response (i.e. tachyphylaxis). This is consistent with the findings from a previous study using isolated human arteries (Hidaka *et al,* 2005). Thus, to avoid this problem, only one concentration of Ang II was used on each individual tissue strip.

## 6.3 Ang II and NO interaction in CCSM

Previous work on the cardiovascular system has revealed the functional interaction between NO and Ang II (de Gasparo, 2002). Thus, NO antagonises the vasoconstrictor and pro-atherosclerotic effect of Ang II, whereas Ang II decreases the bioavailability of NO through OS (Chen *et al*, 2007). However, until now, little work has been carried out using CCSM tissue. In fact, the only study which demonstrated the interaction between Ang II and NO was carried out on canine CCSM tissue, which showed that modulation of Ang II could be achieved by changing the local NO environment (Comiter et al, 1997). However, that study did not mention the important role OS played in this interaction. With the use of a specific inhibitor of $^{\bullet}O_2^-$ production and the enzyme that accelerates the breakdown of $^{\bullet}O_2^-$, the precise involvement of OS has been determined in this Thesis. This information is particularly important for a clearer understanding of human penile physiology, since the effect of Ang II on CCSM function is species-dependent. The

data presented here using rabbit and human CCSM tissue confirms that the erectile process is similar in both species.

The finding that losartan the AT1 receptor antagonist blocked rabbit and human CCSM Ang II-induced contraction and potentiated EFS- and SNP-induce relaxation of CCSM strips, while L-NAME potentiated the Ang II induce contraction, as well as the EFS-induced contraction adds further credence to the notion of NO/Ang II interaction.

Based on these findings it is reasonable to assume that an imbalance between these mediators could be involved, at least in part, in the development of ED.

## 6.4 The role of Ang II and oxidative stress on CCSM function.

It is well described that Ang II increase the production of $^{\bullet}O_2^-$ through the activation of NAD(P)H oxidase (Lassegue *et al*, 2003) and the ROS produced by Ang II contributes to the pathogenesis of CVD by reducing NO, impairing endothelial function, and stimulating proatherogenesis (Cathcart, 2004).

Clearly, the finding that apocynin and DPI, the selective NAD(P)H oxidase inhibitors reduce human and rabbit CCSM contraction, reflects the involvement of OS due to the abolishment of $^{\bullet}O_2^-$ generation. Inhibiting OS also had an impact on NANC neurotransmission. EFS-induced relaxation was further improved by adding apocynin and losartan, when compared with losartan alone, suggesting that blocking the AT1 receptor alone does not fully inhibit OS. This is the first study to provide compelling experimental evidence that the development of OS is instrumental in human erectile physiology.

## 6.5 The role of Ang II/NO and oxidative stress in PBOO: a model of ED

A recent survey of patients above 50 years old demonstrated a strong link between ED and the severity of LUTS (Song *et al*, 2011). Experimental studied using animal models with PBOO have also demonstrated CCSM structural and functional changes in line with the development of ED. For example, CCSM tissue obtained from rabbits with PBOO showed increased deposition of collagen with a reduction in the density of smooth muscle cell/fibres (Khan *et al*, 1999, Lin *et al*, 2008). This was accompanied with impaired CCSM relaxation to SNP and EFS; a feature of ED. In the present study this model has been used to determine CCSM functional changes induced by Ang II and its relationship with NO and OS. It was found, for the first time, that Ang II caused a marked and significant increase in CCSM contraction in PBOO. The importance of this observation is even greater when it is considered in the context of a reduction in CCSM cells and impaired EFS- and SNP-mediated relaxation. The findings with Ang II imply that PBOO increases OS. This is supported by the DPI- and SOD-induced reduction in the Ang II-mediated CCSM contraction. Two further observations using this model may have profound clinical significance in the treatment of ED. Losartan significantly reduced the Ang II response and improved SNP-mediated CCSM relaxation. These findings further strengthen the concept of interplay between Ang II and NO/cGMP and that an imbalance between these mediators maybe a factor in the development of ED. In terms of ED management the use of a PDE 5 inhibitor in conjunction with an Ang II antagonist could have clinical benefit.

However, it is essential that these findings are confirmed using other experimental models, for example, DM rabbits. DM-induced impairment of CCSM relaxation is known to have a deleterious effect on penile erection, which explains why the incidence of ED is as high as 70% in diabetic men (Lerner, *et al* 1993; Ziegler, *et al* 2006) and why these patients are some times referred to as a "difficult-to-treat" ED group (Ishii, *et al* 2006). The use of the diabetic rabbit model is ideal, since these animals exhibit many features of human DM, including ED. Previously studies using diabetic rabbits have shown that impaired CCSM relaxation is improved by PDE 5 inhibition (Thompson *et al*, 2001; Lau *et al* 2009). It would be interesting to see if losartan-induced inhibition of Ang II resulted in a complimentary improvement in CCSM function. Other studies have highlighted the beneficial effect of losartan on the erectile process. The antioxidant quality of losartan was found to directly inhibit OS in diabetic rats (Abdel Aziz *et al*, 2009). It is also known to preserve the erectile function after bilateral cavernosal nerve injury in rats, by opposing the development fibrosis (Canguven *et al*, 2009). While, a clinical study that compared patients with hypertension alone against those with hypertension and ED found that losartan significant improved sexual satisfaction in the ED group (Llisterri *et al*, 2001).

## 6.6 Conclusion

In this thesis I have determined the role, at least in part; Ang II plays in corpus cavernosal erectile pathophysiology. To achieve this I have used CCSM tissues from three experimental models; control rabbits, gender reassignment men, as well as PBOO rabbits. The findings clearly demonstrate that Ang II works in a paracrine manner, since it is produced by and acts on the corpus cavernosum.

Using immunohistochemistry I have identified the presence of Ang II in the endothelium of human corpus cavernosum and blood vessels, as well as in the smooth muscle tissue. In addition, Ang II caused a dose-dependent contraction of CCSM in all three models, a response that was blocked by the AT1 receptor antagonist losartan but not by AT2 receptor inhibition.

I have also examined the interaction between Ang II and NO using NO donors and inhibitors of both vasoactive mediators, together with their effect on NANC neurotransmission. Evidence is provided that Ang II modulates NO-mediated CCSM relaxation, a response that is paramount in controlling CCSM tone. I have further expanded the work by looking at the role of OS on Ang II-induced CCSM contraction. Results indicate that inhibitors of OS significantly diminish the contractile effect of Ang II and improve the relaxation of the CCSM tissue.

In summary, I have found for the first time, Ang II-mediated CCSM contraction is enhanced in a rabbit PBOO model, (known to have impaired CCSM relaxation) and inhibited by losartan. These observations have clinical significance, since it implies the use of an AT1 receptor antagonist, in conjunction with a PDE5 inhibitor may provide a novel treatment option for ED.

## 6.7 Limitation of the study

During the course of this study I came across a few obstacles, which limited the scope of the work. The first relates to the inability to reproduce previous work, namely developing cumulative dose response curves for Ang II, in the presence and absence of its antagonist. I found the contractile effect of Ang II on CCSM strips was short lived with the peak response not sustained long enough to achieve a plateau. This resulted in an undulating curve that was not only difficult to interpret, but

potentially gave misleading results. In order to avoid this, I used a single Ang II dose on each tissue strip; this meant data generation was very slow and limited the amount of experiments I could do, making the study more expensive.

Developing the PBOO rabbit model was another challenging task, although this model has been previously described, the possibility of post-operative complication was a major concern, as unlike humans rabbit are highly likely to develop fibrosis and adhesions. One way to avoid this was to use liberal and constant saline irrigation during the surgical procedure and when closing the wound. Although, this approach was successful, it did not totally eliminate the problem; as a few animals did develop adhesions and were terminated before the end of the study.

Including a diabetic rabbit model in this study would have added valuable data, as this is a known model of ED and increased OS. Unfortunately, due to time constraints and the expensive of establishing and maintaining this model, it was not included.

## 6.8 Future work

There are several areas of study, which should be pursued as a result of the findings I have presented in this thesis. This work could provide further insight into the effect of Ang II on the pathophysiology of ED.

1. To confirm the Ang II findings using another model of experimental ED, such as the diabetic rabbit model.

2. To determine if the enhanced Ang II-mediated contraction of CCSM tissue taken from PBBO rabbits is reversible. This could be assessed by removing

the ligature around the base of the bladder at timed intervals after establishing PBOO, before carrying out the Ang II experiments.

3. The clinical relevance of the findings could be determined by repeating the experiments using cavernosal biopsies from men with PBOO and diabetes.

4. The work presented in this thesis was mainly a functional study, with some immunohistochemical data revealing the distribution of Ang II in the corpus cavernosum. More work is required to determine the cellular/molecular mechanisms that govern the Ang II response on the corpus cavernosum. This could be achieved by isolating CCSM cells from PBOO/ diabetic rabbits and determine the effect Ang II and its antagonist have on the cellular contractile apparatus using confocal microscopy, chemiluminescence and Western Blott analysis.

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