EVIDENCE LINKING THE STRUCTURE AND FUNCTION OF THE INTERNAL PUDENDAL ARTERY TO ERECTILE FUNCTION:
IMPACT OF AGING, HYPERTENSION, ANTIHYPERTENSIVE TREATMENTS AND LIFESTYLE MODIFICATIONS

by

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Abstract

Erectile dysfunction and cardiovascular disease share etiologies, and commonly coexist. One unifying concept is that the arterial insufficiency in hypertension is also the primary basis for blunted sexual responses. The objective of these studies was to characterize the age-related changes in the structure and function of the pudendal artery (the main resistance vessel) in young and old normotensive and hypertensive animals in relation to erectile function. In addition, we assessed the impact of antihypertensive treatments and lifestyle modifications, such as exercise and/or caloric restriction, on erectile responses and the structure and function of the pudendal artery.

In 30 week old hypertensive rats or following re-challenges at 50 and 70 weeks, antihypertensive treatment (enalapril or hydralazine) did not prevent the age-related decline in erectile function. Experiments involving cross-over kidney transplantations between treated and untreated young hypertensive rats revealed that changes in penile vasculature and not the level of arterial pressure were important for normalizing erectile responses. In addition, intervention with exercise and caloric restriction showed that these treatments substantially improved erectile responses in normotensive and hypertensive rats.

The pudendal artery in young normotensive rats was found to have a thick medial layer but a relatively small lumen. With age, the pudendal lumen didn’t change, but all components of the medial layer were markedly increased. Of interest, the smooth muscle cells within the pudendal medial layer became more disorganized with aging, although
contractions were similar. In contrast, endothelium-dependent relaxation decreased with age.

Young hypertensive rats also had an increased wall thickness, but not lumen diameter or extracellular matrix. Antihypertensive therapy significantly decreased the pudendal wall thickness. In aging hypertensive rats, the pudendal artery walls were even thicker, lumen decreased and extracellular matrix greatly enhanced compared to younger rats. In addition, there were numerous regions of intimal thickening associated with marked disruptions of the internal elastic lamina. Moreover, pudendal smooth muscle cells bordering the intima and in the neointima were round in shape, and electron microscopy confirmed their synthetic state.

Taken together, these findings provide key evidence of the importance of the structure and function of the pudendal artery in facilitating erectile responses.
Co-Authorship

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<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>APO</td>
<td>Apomorphine</td>
</tr>
<tr>
<td>AT-1</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine-3',5'-monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine-3',5'-monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
</tr>
<tr>
<td>CR</td>
<td>Caloric restriction</td>
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<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DAPI</td>
<td>Diamidino-phenylindole dihydrochloride</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ED</td>
<td>Erectile dysfunction</td>
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<tr>
<td>ELS</td>
<td>Enalapril low salt</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
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<td>ETₐ</td>
<td>Endothelin A</td>
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<td>GPCRs</td>
<td>G-protein coupled receptors</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>ICP</td>
<td>Intracavernosal pressure</td>
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<tr>
<td>IIEF</td>
<td>International Index of Erectile Function</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
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<td>K⁺</td>
<td>Potassium ion</td>
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<td>LV/BW</td>
<td>Left ventricle to body weight ratio</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<tr>
<td>MPOA</td>
<td>Medial preoptic area</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NANC</td>
<td>Non-adrenergic, non-cholinergic</td>
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<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>PE</td>
<td>Phenylephrine</td>
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<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
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<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>sGC</td>
<td>Soluble guanylate cyclase</td>
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<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>WKY</td>
<td>Wistar-Kyoto rat</td>
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Chapter 1:

General introduction
1.1 Male sexual and erectile dysfunction

Male sexual dysfunction is a group of conditions that significantly affect the self-esteem, self-confidence, and quality life for both the men suffering from this affliction, as well as their partners \(^1\)\(^-\)\(^6\). This disorder includes problems associated with libido or desire, arousal, ejaculation, orgasm, sexual pain, penile malformations such as Peyronie’s disease and priapism, but the most common complaint is erectile dysfunction (ED) \(^7\)\(^-\)\(^9\). ED, a particular focus of this thesis, has been defined as the persistent inability to achieve and/or maintain a penile erection sufficient for satisfactory sexual intercourse \(^10\).

Epidemiological studies have shown that ED is highly prevalent, and occurs in more than 20% of men between the ages of 18 and 59, and in 60% of men between the ages of 40 and 70 in the United States \(^11\). Thus, there is no doubt that there is an age-related aspect to the condition.

Although aging is considered to be one of the most significant contributing risk factors, ED is not necessarily an inevitable outcome of the normal aging process. Other identified risk factors for ED include many that are common to cardiovascular diseases (CVD) in general, such as hypertension, hyperlipidemia, diabetes, physical inactivity, obesity and smoking \(^12\)\(^-\)\(^18\). However, the specific mechanisms by which these risk factors negatively impact erectile function have not been fully characterized. Due to the widespread nature of this disease and the multi-factorial underlying basis, it is not surprising that 152 million men worldwide report suffering from ED, and that projections show that the worldwide prevalence of ED could more than double by 2025 \(^19\).
Over the past decade, substantial progress has been made in the diagnosis and management of ED. Only a decade ago, sexual problems were believed to be mainly psychological in nature, whereas today they are clearly understood to have origins in pathophysiological conditions. Thus, ED has been categorized as having a psychogenic cause (central mechanisms), an organic cause (peripheral mechanisms), or a combination of the two. Some examples of the psychogenic aspects include depression, stress, performance anxiety, relationship issues, and sexual arousal difficulties. However, the majority of ED is deemed to be organic and has been subdivided into categories such as: neurogenic, hormonal (e.g. testosterone deficiency), associated with penile injury (e.g. trauma, priapism) or disease (e.g. Peyronie’s), arteriogenic (e.g. atherosclerosis, hypertension) and drug-induced. Neurogenic ED can result from nerve injury during surgery, as well as from general neurogenic deficits associated with spinal cord injuries, multiple sclerosis, strokes, Alzheimer’s disease, and Parkinson’s disease.

As indicated above, it is well documented that ED and CVD share many common risk factors, such that ED, particularly of arteriogenic origin, may actually be a sentinel of underlying or future systemic vascular disease. Arteriogenic ED is often called arterial insufficiency-induced ED, and leads to inadequate perfusion pressure or lower arterial flow into the penis and the inability to produce an erection. Dysfunctional veno-occlusive mechanisms, as well as abnormal structural changes in the penile vascular smooth muscle and endothelium have all been thought to contribute to this form of ED. Thus, among the primary organic causes of ED, 40% can be attributed to vascular
problems. Further complicating the diagnosis and management of ED is the issue of the drug-induced or iatrogenic ED. In particular, pharmacological agents such as antihypertensive drugs, antidepressants, antipsychotics and many other widely prescribed medications have been found to have a negative impact on the neuroendocrine and/or neurovascular control of erectile function. Thus, ED in a given patient can have a complicated, multi-factorial basis. A common example would be a pharmacologically-treated, hypertensive patient who is experiencing erectile difficulties. In the generation of ED in this patient, the hypertensive state of this patient could have induced vascular structural changes or endothelial dysfunction of the supplying vasculature or of the intrapenile vascular tissue itself. The problem could be further exacerbated by an antihypertensive treatment that has ED as an adverse effect. A possible outcome in these cases is that the patient does not comply with his medication and as a consequence both the hypertension and ED worsen.

The importance of sexual health to an individual’s quality of life was finally emphasized in 2000 by the World Health Organization (WHO), when they formally recognized sexual dysfunction as an important medical condition and classified normal sexual function as a basic human right. Evidence for this conclusion came from studies, such as those from the United States, showing that men suffering from ED have low levels of physical and emotional satisfaction and low general happiness. More recently, studies have also stressed that sexual dysfunction not only impacts negatively on the quality of life of the individuals with ED, but the presence of the condition also affects their
partners $^{6,50,51}$. Social awareness and acceptability has increased drastically, in part, due to these statements made by the WHO as well by the development of phosphodiesterase-5 (PDE5) inhibitor drugs such as Levitra®, Viagra® and Cialis®, which have become part of the lexicon. Despite the advent of these treatments, it is important to realize that they are not always effective and even when they are successful; they only temporarily restore sexual responses $^{52-54}$. Thus, more research is required to further elucidate the physiology and pathophysiology of erectile function to develop treatment options that will potentially cure or at least attenuate the progression of the underlying problem.

1.2 Anatomy and physiology of an erection

The penis is made up of three bodies of erectile tissue; the two corpora cavernosa, the primary erectile bodies, and the corpus spongiosum, which envelops the urethra (Figure 1.1). Functioning as the primary erectile end organs, each corpus cavernosum is comprised of a complex network of innervated vascular sinuses lined with endothelium, and separated by trabecular smooth muscle and fibroblast containing connective tissue $^{55,56}$. The two corpora are anchored to the ischiopubic ramus and are separated by an incomplete septum. In addition, the paired corpora are surrounded by multiple circular and longitudinal layers of collagen and elastin fibers which make up the tunica albuginea. This connective tissue layer provides strength (collagen) and compliance (elastin) required for the corpora to expand during an erection $^{57}$, but also to facilitate the veno-occlusive mechanism (see below). The corpus spongiosum is generally similar to the corpus cavernosum, although it has larger sinusoids and there is less intravascular
Figure 1.1 Anatomy of the penis and cavernosum in erect and flaccid states. During an erection, relaxation of the trabecular smooth muscle and vasodilatation of the arterioles results in increased blood flow, which expands the sinusoidal spaces and compresses the subtunical venular plexus against the tunica albuginea. In addition, the stretched tunica compresses the emissary veins to prevent the outflow of blood. In the flaccid state, inflow through the constricted and tortuous helicine arteries is minimal, and there is free outflow via the subtunical venular plexus. Modified from TF Lue NEJM 2000.
pressure than in the cavernosum. The corpus spongiosum originates from the bulbus spongiosum and terminates in the glans penis, an area rich in sensory nerve endings. The arterial blood supply to all three of these erectile tissues comes via the bilateral internal pudendal arteries which arise from the internal iliac arteries. Close to the penis, the pudendal artery branches into the bulbourethral, cavernous and dorsal arteries. The glans and the urethra are supplied by the bulbourethral artery. The cavernous artery travels down the middle of each corpus cavernosum, branching into the helicine arteries which then directly supply the sinusoidal spaces (Figure 1.1). The dorsal arteries extend the length of the penis, running parallel with the dorsal nerve, to supply the superficial structures of the penis as well as other parts of the cavernous tissue via the circumflex arteries. The venous drainage of the trabeculae and sinusoids form the subtunical venous plexus, which merges to become the emissary veins which empty into the cavernous vein (Figure 1.1). Other veins which contribute to the outflow of the penis include the deep dorsal, superficial dorsal and circumflex veins, all of which drain into the internal pudendal veins.

An erectile response results from a complex interplay between psychological, neuronal (cerebral and spinal), hormonal, vascular, and cavernous smooth muscle systems (Figure 1.2). An erection is initiated by a stimulus in the central nervous system, particularly involving activation of oxytocinergic neurons in the medial preoptic area (MPOA) and paraventricular nucleus (PVN) of the hypothalamus. Much of the pro-erectile initiation occurs through dopaminergic and adrenergic signalling and is terminated, at
Mechanism of an erection

CNS initiation (memory, fantasy, stimuli)

↓

Neural activation (adrenergic, cholinergic, NANC, etc)

↓

Vasodilation of the penile arterial blood vessels

↓

Rapid increase in arterial inflow into the corpora cavernosa

↓

Occlusion of sub-tunical veins

↓

Maintenance full rigidity

(ERECITION)

Figure 1.2 Flow chart of the mechanisms required for an erectile response. NANC, non-adrenergic non-cholinergic.
least in part, via inhibitory serotonergic signalling. In the periphery, the erectile, ejaculatory and orgasmic responses involve an interplay between autonomic (sympathetic, cholinergic and non-adrenergic, non-cholinergic; NANC) and somatic (sensory and motor) innervation. The dorsal penile nerve and the pudendal nerves are somatic nerves which are primarily responsible for mediating sensory signalling as well as the contraction of the bulbocavernosus and ischiocavernosus muscles. Autonomic innervation controls penile tumescence, detumescence and ejaculation. Sympathetic and parasympathetic pre-ganglionic neurons converge in the major pelvic ganglion and continue towards the corpora cavernosa and corpus spongiosum of the penis together with the bilateral cavernous nerves. Activation of the sympathetic nervous system maintains the penis in a flaccid state (i.e. detumescence) via the contractile effects of norepinephrine acting on \( \alpha_1 \)-adrenoceptors expressed on corporal and arteriole smooth muscle cells (SMC). During the initiation of an erection, the parasympathetic nervous system is dominant and initiates pre-penile and cavernosal SMC relaxation via the increased firing of cholinergic nerves. These nerves from the pelvic plexus contain neuronal nitric oxide synthase which synthesizes and releases nitric oxide into the corpus cavernosum to relax SMC. In addition, the release of acetylcholine acts on endothelial cells that produce NO and cause further cavernous SMC relaxation. Overall, the net effect is a decrease in the pre-cavernosal and pre-spongiosal vascular resistance as well as relaxation of the smooth muscle in the sinusoidal tissue, leading to a rapid increase in blood flow into the trabecular spaces of the corpora cavernosa and corpus spongiosum. When there is sufficient inflow, the rapid filling of the corpora causes
expansion sufficient to compress the plexus of subtunical veins against the more rigid, tunica albuginea. This veno-occlusive mechanism impedes the venous outflow and thereby facilitates further filling and increased intracavernosal pressure, a change which generates the rigidity required for a full erectile response \(^{72,73}\). Thus, penile smooth muscle tone is regulated by a coordinated balance between contractile and relaxation factors and pathways (Figure 1.3).

The key vasoactive factors have already been mentioned but other factors have also been reported to play a role in the erectile response. That is, factors such as endothelin, angiotensin II (Ang II) and various prostaglandins (thromboxane A\(_2\), prostaglandin H\(_2\) and F\(_2\alpha\)) have all been shown to have the capacity to stimulate contractile responses in penile vascular tissue. Endothelin, synthesized predominantly by endothelial cells, can evoke contractions via the endothelin A (ET\(_A\)) receptor \(^{74-76}\). Thromboxane is produced in cavernous tissues as a product of the arachidonic acid-cyclooxygenase pathway and binds to the thromboxane receptors to mediate contraction \(^{77,78}\). Interestingly, it has been suggested that the contractile prostaglandins are produced to balance the effects of vasodilating prostaglandins, such as prostacyclin (PGE\(_1\)). The renin-angiotensin system (RAS) also been suggested to contribute to penile smooth muscle tone. Ang II, a potent vasoconstrictor that can bind to angiotensin type 1 (AT-1) receptors, has been shown to have activity in the penile vasculature and in trabecular smooth muscle \(^{79}\). These contractile events occur as a result of activation of G protein coupled receptors (GPCRs), which link to pathways leading to the activation of phospholipase C (PLC), generation of
Figure 1.3  Schematic representation of the processes of relaxation and contraction in cavernous smooth muscle cells. The tone of penile smooth muscle is regulated by a balance between relaxant and contractile factors and pathways. ACh, acetylcholine; BK, bradykinin; NO, nitric oxide; O2, oxygen; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; VIP, vasoactive intestinal peptide; VIP-R, vasoactive intestinal peptide receptor; EP-R, prostaglandin receptor; NE, norepinephrine; α1, α1 adrenoceptor; ET, endothelin; ETA, endothelin type A receptor; AngII, angiotensin II; AT-1, angiotensin type 1 receptor; TP, thromboxane receptor; sGC, soluble guanylyl cyclase; cGMP, guanosine-3′,5′-monophosphate; cAMP, adenosine-3′,5′-monophosphate; DAG, diacylglycerol; IP3, inositol triphosphate; PDE, phosphodiesterase; Ca2+, calcium ion; AC, adenylate cyclase.
inositol triphosphate (IP₃), and increases in intracellular calcium. Activation of calcium-calmodulin dependent myosin light chain kinase (MLCK) mediates phosphorylation of the myosin light chain and the resulting cross-linking of the contractile proteins to cause a contraction. This process also involves changes in the calcium sensitizing pathways. That is, RhoA, a small monomeric G protein, activates Rho-kinase to facilitate maintenance of the contractile state by preventing the dephosphorylation of the myosin light chain filaments⁸⁰-⁸². In fact, both RhoA and Rho-kinase have a 17-fold higher expression in penile tissue compared to aortic vascular smooth muscle and inhibition of these has been shown to elicit cavernosal relaxation and penile erections⁸³-⁸⁵.

As indicated above, the full relaxation of corporal smooth muscle and penile arteries is critical in the development of a full erectile response. Thus, to account for the full relaxation signal, the contribution of inhibitory vasoactive substances released into the circulation, from endothelial cells, from within the smooth muscle cells and those from adjacent nerves need to be understood. The role of endothelial cells in establishing an appropriate milieu is well established⁸⁶-⁸⁹. The endothelium is made of flat epithelioid cells which line both the pre-penile and intra-penile blood vessels as well as the trabecular sinusoids. Endothelium-dependent vasorelaxation can occur in response to factors such as acetylcholine and bradykinin, binding to muscarinic and bradykinin receptors, respectively. In general, this signal increases the intracellular levels of calcium in the endothelial cells, which activates nitric oxide synthase (NOS), and results in the increased production of nitric oxide from molecular oxygen and L-arginine. Of the three
different isoforms of NOS, the two constitutive forms are neuronal NOS (nNOS) and endothelial NOS (eNOS) which are mainly present in the nervous system and the vasculature, respectively. There is also an inducible form of NOS (iNOS) which has increased expression following injury or inflammation. Regardless of its source, once NO is released from nerve terminals, or is produced in endothelial cells, it can readily diffuse into smooth muscle cells to activate the enzyme soluble guanylate cyclase (sGC). Activated sGC catalyzes the formation of guanosine 3’, 5’ cyclic monophosphate (cGMP) from guanosine-5’-triphosphate (GTP). Increased intracellular cGMP can modulate various processes including inhibition of the cAMP degrading enzyme, PDE3, as well as downstream activation of cGMP-dependent protein kinases (PKG) 90-92. These processes would induce a loss of contractile tone by decreasing the intracellular calcium concentrations.

Other vasodilator signalling involves activation of the other cyclic nucleotide pathway. In this process, adenylate cyclase (AC) is activated by a GPCR-linked event to catalyze the formation of a different vasodilating second messenger, adenosine 3’, 5’ cyclic monophosphate (cAMP). Substances such as vasoactive intestinal protein (VIP) and calcitonin-gene related peptide (CGRP) can be released from nerve terminals, and certain prostaglandins (e.g. prostacyclin) can be secreted in a paracrine or autocrine manner from smooth muscle and endothelial cells 93-95. It is generally agreed that although cAMP can initiate relaxation in a similar manner to cGMP, overall the cAMP pathway appears to be a lesser contributor to the physiology of an erectile response 96.
As described above, the levels of cGMP and cAMP are both rendered inactive by phosphodiesterases (PDE), albeit different isoforms. The initiation and maintenance phase of an erection are generally thought to involve high concentration of cGMP. It is not surprising that penile tissue was found to have substantial expression of PDE5, given its essential role in terminal hydrolysis of cGMP. However, it was the remarkable effectiveness of PDE5 inhibitors, such as sildenafil, tadalafil and vardenafil, in men with ED that confirmed the importance of the NO-cGMP pathway, the erectile response pathway.

There is no doubt that an imbalance in the contractile-relaxation equilibrium within the penile tissue will impair the ability to achieve an erection. In many disease states or health conditions (e.g. hypertension, diabetes, depression, stress) there is evidence for increased levels of contractile substances and/or their receptors. In one study, experimental evidence revealed increased adrenergic sensitivity and increased RhoA/Rho-kinase activity were the underlying cause that made it very difficult to generate an erectile response. From the other side of the vasoactive balance, other studies have revealed that endothelial dysfunction associated with various diseases and conditions, can lead to a sufficient decrease in NO production to impair or even prevent the generation of penile erection.
1.3 Cardiovascular disease and sexual dysfunction

Cardiovascular diseases (CVD) such as stroke, congestive heart failure, coronary artery and peripheral vascular disease, are disorders of the heart and blood vessels that are the number one cause of death globally \(^{102}\). Although the rates of CVD have declined in Canada by 50% over the last 20 years, more than 72,000 deaths in 2004 were attributable to CVD \(^{103}\). Annually, heart disease and stroke costs the Canadian economy over $18 billion in physician services, hospital costs, lost wages and decreased productivity \(^{104}\). Often, there are no recognizable warning signs or symptoms of the underlying disease until there is a heart attack or stroke.

However, despite the early stages of CVD often being thought of as asymptomatic, over the last decade a number of studies have now shown that ED may actually be a sentinel manifestation of CVD \(^{34, 38, 105, 106}\). In a study by Schouten et al, a group of men aged 50-75 years old suffering from ED, but who appeared to be free of CVD, were followed for 8 years \(^{107}\). Their findings revealed that the severity of ED increased in a manner associated with the incidence of myocardial infarction, stroke or sudden death.

Moreover, other studies have confirmed that cardiovascular symptoms, in general, become evident with 3-5 years following the development of ED \(^{108, 109}\). Of interest, traditional cardiovascular screening such as cholesterol, blood pressure or exercise stress testing do not always identify the silent progression of CVD, such that the onset of ED could be used as a harbinger of impending CVD and thereby mark the time point where intervention for CVD risk reduction should be aggressively instituted \(^{110}\). Although the
specific link between ED and CVD has not been fully established, the commonality of risk factors suggests a remarkable similarity in the likely mechanisms (Table 1.1)\textsuperscript{107,111-113}. In the present thesis, the risk factors that are most relevant to consider include aging, hypertension, obesity and physical inactivity.

As indicated earlier, aging is one of the most significant risk factors of ED, with epidemiological studies showing the occurrence of severe ED increasing 3-fold between the ages of 40 and 70 years or older\textsuperscript{114}. Importantly, prospective clinical and experimental evidence has now corroborated these findings of the age-related decline in erectile function\textsuperscript{115-117}. Although a number of mechanisms have been proposed to explain the deleterious effect of age on erectile function, it has been difficult to distinguish the impact of aging \textit{per se} on erectile function, from the effect of the other co-morbidities that occur frequently in an aging population (e.g. poor lifestyle habits, increased use of pharmacotherapy)\textsuperscript{118}. Thus, more clinical and experimental studies are needed in order to characterize quantitatively the specific age-related influence of these confounders.

In hypertensive men, the prevalence of ED is approximately twice that seen in the age-matched normotensive males\textsuperscript{119}. Experimentally, although there is an age-related decline in erectile function in normotensive animals, we and others have previously shown that ED occurs earlier and to a greater extent in hypertensive rats\textsuperscript{115,120}. Although the specific etiology of this linkage has not been established, a potential cause for the exacerbated ED in hypertensive subjects has been suggested to result from pathological
Table 1.1 Relationship between risk factors of cardiovascular diseases and erectile dysfunction.

<table>
<thead>
<tr>
<th>Common Risk Factors of CVD and ED</th>
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</thead>
<tbody>
<tr>
<td>• Age</td>
</tr>
<tr>
<td>• Dyslipidemia</td>
</tr>
<tr>
<td>• Hypertension</td>
</tr>
<tr>
<td>• Diabetes</td>
</tr>
<tr>
<td>• Kidney Disease</td>
</tr>
<tr>
<td>• Smoking</td>
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<tr>
<td>• Sedentary lifestyle</td>
</tr>
<tr>
<td>• Obesity</td>
</tr>
<tr>
<td>• Depression</td>
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<td>• Alcohol</td>
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<table>
<thead>
<tr>
<th>CVD as Risk Factors of ED</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Coronary artery disease</td>
</tr>
<tr>
<td>• Peripheral vascular disease</td>
</tr>
<tr>
<td>• Congestive heart failure</td>
</tr>
<tr>
<td>• Stroke</td>
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structural changes in the corporal and penile vascular tissue. The changes that have been suggested include increased media-to-lumen ratio, decreased number of smooth muscle cells and increased collagen of both the cavernosal and arterial vasculature 121-123.

The current prevalence of obesity is already high and yet the incidence is still increasing globally. It is estimated worldwide that approximately 1.6 billion adults are overweight and 400 million adults are obese 124. A number of epidemiological studies indicate that obesity is a significant, independent risk factor for ED 125-128. The Health Professionals Follow-up Study found that men with a BMI greater than 28.7 carry a 30% higher risk for ED than those with a normal BMI (≤25) 129. However, despite the lack of knowledge of fundamental mechanisms, the deleterious impact on the vasculature is most often proposed to be a key predisposing factor linking obesity and the pathogenesis of ED.

1.4 Vascular structure and sexual dysfunction

Appropriate regulation of regional vascular resistance is critical to ensure tissues have sufficient blood flow to meet their metabolic needs and/or functions. Arterial blood pressure is controlled acutely by changes in cardiac output and vascular tone while long-term control is achieved by renal mechanism regulating blood volume and/or vascular structure 130-133. Short term “steady-state” regulation of arterial pressure is maintained on a second-to-second, minute-to-minute, and day-to-day basis via changes in neural and local factors which can rapidly increase or decrease vascular resistance and cardiac output as required. In the long term, the kidney is particularly dominant in establishing
the level of arterial pressure by maintaining fluid and electrolyte balance, in part, via the pressure-natriuresis mechanism\textsuperscript{134-137}. In particular, the structural basis of vascular resistance of renal vessels appears to be important in establishing the level of arterial pressure\textsuperscript{137-139}. In hypertension, adaptive structural changes involving increased wall thickness and decreased lumen diameter occur to allow vessels to withstand the increased wall stress. Poiseuille’s law states that resistance is proportional to the inverse of the radius raised to the fourth power (R $\propto 1/r^4$). Thus, a 7% decrease in the average lumen radius would result in a 34% increase in vascular resistance\textsuperscript{140}. Accordingly, these small changes in lumen size significantly impact vascular resistance and result in an increased blood pressure.

Both experimentally and clinically, many studies have demonstrated that a hypertensive state results in an increased vascular structure throughout the body\textsuperscript{141-145}. Furthermore, there is considerable evidence that arterial insufficiency is an important underlying cause of ED in many patients with hypertension and/or atherosclerosis\textsuperscript{33, 106, 112, 146-148}. Experimentally, we and others have shown in spontaneously hypertensive rats (SHR) that there is altered vascular structure, in both conduit and resistance arteries, compared to the normotensive counterparts, the Wistar Kyoto (WKY) rats\textsuperscript{149-151}. In fact, in SHR, changes in vessel structure have been shown to occur before the full development of hypertension and have been considered to contribute to the initiation and maintenance of hypertension. In hypertensive humans and animals, there is an increase in the vascular wall-to-lumen ratio, in part, due to a thickening of the medial smooth muscle layer with
or without a narrowing of the vessel’s lumen. The enlarged medial layer is thought to occur as a result of smooth muscle cell (SMC) hyperplasia, hypertrophy, or rearrangement of SMC around a smaller lumen \(^{151-153}\). The types of changes are specific to different vascular beds and are generally acknowledged to have the greatest impact on resistance vessels, which are believed to play a larger role in overall vascular resistance \(^{140, 154, 154}\). The consequence of these changes, to ensure normal blood flow, is the necessity for an increase in perfusion pressure even under conditions of maximum dilation (i.e. minimal level of vasoconstrictor tone) \(^{140, 154, 155}\). Despite the evidence, it is still widely disputed whether the structural changes are causally-linked to the development of hypertension/circulatory disorder or are a compensatory response of the increased hemodynamic load \(^{156, 157}\).

Regardless of the cause, structural changes throughout the vasculature can impact beyond the level of arterial pressure, for example, in the development of erectile dysfunction. In particular, as previously mentioned, the bilateral internal pudendal arteries are the primary vessels supplying the penile tissue. Furthermore, the pudendal artery is responsible for over 70% of the resistance to blood flow during flaccidity as well as throughout an erectile response \(^{158}\). Previous studies from our laboratory have demonstrated that the penile vasculature is not protected in hypertension and is structurally increased similar to other vascular beds \(^{121}\). These hypertension-associated structural changes suggest that basal flow to penile tissue would be reduced and produce a lesser ability to initiate and/or maintain an erectile response. In addition, chronic
insufficiency of arterial inflow into the cavernous spaces could lead to ischemia-related changes in cavernosal tissue, promoting fibrosis, and thereby potentially worsen erectile difficulties \cite{159, 160}.

### 1.5 Antihypertensive therapy and erectile dysfunction

Understanding the link between ED and hypertension is further complicated by evidence indicating that erectile function can be compromised in patients receiving certain antihypertensive treatments \cite{161-163}. That is, drug-induced ED has been reported with specific classes of antihypertensive agents, an adverse event that might also contribute to the low patient compliance with antihypertensive treatment \cite{164, 165}. In particular, clinical and experimental studies have demonstrated that central acting sympatholytics (e.g. \(\alpha_2\)-receptor agonist, clonidine), diuretics (thiazides) and \(\beta\)-adrenoceptor antagonists are all linked to a high incidence of ED \cite{165, 166}. Although the link between diuretics and \(\beta\)-blockers, in particular, and the exacerbation of ED in hypertensive patients is widely known, the pathophysiological mechanisms involved remain unresolved. Thiazide and \(\beta\)-blockers were once the most commonly prescribed treatments for hypertension, but with the advent of the newer drugs with improved tolerability profiles these older drugs have been replaced in primary therapy. Similarly, centrally acting sympatholytics, such as clonidine, have also fallen even further out of favor, in part, because of the high incidence of adverse effects including ED. In contrast, calcium channel antagonists, angiotensin converting enzyme (ACE) inhibitors and AT-1 receptor antagonists have better treatment compliance and lower frequency of ED \cite{167-170, 171}.
The frequency of ED appears to be the lowest with agents that inhibit the RAS\textsuperscript{45,46,163}. In fact, many studies have suggested that inhibitors of the RAS might actually benefit erectile function. For example, separate studies by Fogari and Llistterri have shown improved sexual function and satisfaction in hypertensive patients after treatment with AT-1 receptor antagonists, valsartan and losartan or the ACE inhibitor lisinopril\textsuperscript{169-171}. Recently, Baumhakel \textit{et al} has also shown that treatment with irbesartan alone, as well as in combination with hydrochlorothiazide was associated with improved sexual desire, frequency of sexual contacts and erectile function in hypertensive patients with the metabolic syndrome\textsuperscript{172}. Experimentally, our lab and others have shown that treatments with RAS antagonists improve erectile responses in different animal models of hypertension and diabetes\textsuperscript{173-175}.

The improvement in erectile function from RAS inhibition does not appear to be related to the magnitude of the decrease in blood pressure \textit{per se} as Fogari has shown β-blockers that equivalently lowered blood pressures did not improve erectile function\textsuperscript{169,170}. Previous experimental studies have shown that after only two weeks of treatment with the ACE inhibitor enalapril there is a persistent decrease in arterial pressure and a 12% regression in penile vascular structure in the previously treated SHR\textsuperscript{173}. In addition, this RAS-based intervention was also found to decrease α-adrenoceptor sensitivity of penile vasculature. These findings of improved erectile and vascular function have been confirmed by studies from Toblli \textit{et al}, who showed that treatment involving RAS
inhibition decreased intra-penile collagen deposition and cavernous vascular smooth muscle content, and improved corporal endothelial function \(^{176,177}\). Taken together, the evidence suggests that there might be a multi-factorial impact on penile vascular structure and function, although to date these findings have related primarily to the end tissue.

### 1.6 Lifestyle modifications and erectile function

Recently, a number of studies have shown that lifestyle modifications, such as increasing physical activity and implementing a healthy diet, can have beneficial effects on both cardiovascular and ED risk factors, as well as on erectile function itself \(^{178-184}\). This is not surprising given the underlying concept that ED and CVD have similar risk factor profiles and therefore common underlying etiologies (Table 1.1). However, one aspect that remains to be elucidated is whether both conditions are equally amenable to these preventative measures.

Several epidemiological studies have shown that there is an inverse association between physical activity and erectile dysfunction \(^{17,125,129,185}\). In one study, frequent vigorous exercise was associated with 30% lower risk for ED \(^{129}\). Despite the ample epidemiological evidence for this beneficial impact of exercise on cardiovascular health, only one lifestyle intervention study to-date by White and colleagues (1990) prospectively assessed the effect of exercise alone on some aspects of sexual function in men \(^{178}\). Specifically, they found that a 9 month exercise intervention (60 min/day, \(\sim\)3.5 days/week at 75-80% maximum aerobic capacity) significantly enhanced the frequency
of intercourse, orgasms and maintained erections as recorded in sexuality diaries of the exercising group, compared to the control group prescribed a low-intensity walking program. However, the optimal level of exercise intensity, duration, and frequency required to maintain or improve erectile function still remains to be established. Regardless of the parameters of the exercise program, the mechanism(s) involved in the improvement in erectile function also remain to be elucidated. In this regard, short and long term changes in the vasculature (e.g. improved endothelial function) and long term changes related to body composition (e.g. weight-loss, decrease in visceral adipose tissue (VAT)) have been proposed to be part of the process. This evidence, coupled with the findings that physical activity improves other risk factors of ED (e.g. hypertension, insulin resistance) suggests that exercise may be an ideal strategy for the treatment of ED, as well as for overall vascular health.

The combined effects of changing diet, caloric intake and physical activity on body composition and overall cardiovascular health have now been discussed in a number of studies. Esposito et al. (2006) assessed the dietary intake of men with and without ED both qualitatively and quantitatively. Specifically, they assessed the impact of a Mediterranean-style diet (i.e. rich in whole grain, fruits, vegetables, legumes, walnut, and olive oil) versus a Western-diet (i.e. meat, poultry, dairy products, refined grains). It was determined that Mediterranean-style dietary patterns were more prevalent in subjects without ED compared to men with ED (based on the International Index of Erectile Function, IIEF-5). Furthermore, in a 2 year follow-up study on men with both
ED and metabolic syndrome, Esposito et al. further determined that switching to a Mediterranean-style diet produced marked improvement in erectile responses (IIEF-5) and endothelial function, as well as a significant reduction in systemic markers of inflammation. In addition, the positive effects of caloric restriction (CR) alone on overall health have also been known for quite some time, in that chronic CR has been associated with overall enhanced health and prolongation of life-span. Taken together, although the mechanisms remain unresolved, the findings to date reveal that a healthy diet can benefit erectile function even though a number of key issues (i.e. the optimal dietary composition and quantity) still need to be determined.

Based on these studies it may be that combining the effects of increased physical activity, changes in dietary composition and/or CR would further improve erectile function. Indeed, one study performed a two year intervention involving both, and showed an improvement in existing erectile function among obese men, but also a third of men with severe ED regained sexual function as measured by the IIEF-5. Similar to the later studies by this group, the improvements were suggested to be linked to enhanced endothelial function and a reduction in markers of systemic vascular inflammation.

1.7 Current treatment of erectile dysfunction

The current oral pharmacotherapy of ED involves an intervention that facilitates erectile responses in an acute scenario, but does not treat the underlying disease. In the past, before the advent of oral therapy, the primary approach involved cavernosal injections of
vasoactive substances (e.g. alprostadil, papaverine, phentolamine, yohimbine) or urethral suppositories (e.g. alprostadil). These treatments would induce vasodilation directly (e.g. prostaglandins, nitric oxide donors), via decreasing the breakdown of cAMP and cGMP (e.g. papaverine) or by blocking α-adrenoceptors (e.g. phentolamine, yohimbine)\textsuperscript{194-196}.

In the last decade a number of oral agents have become available. The current, most widely prescribed drugs are the PDE5 inhibitors. As previously mentioned, the NO/cGMP pathway is an essential mechanism in an erectile response\textsuperscript{197}. During sexual arousal, the NO released from nerves and endothelial cells binds to guanylate cyclase in corporal SMC to produce cGMP which activates cGMP-dependent kinase (PKG) (Figure 1.4). PKG in turn proceeds to phosphorylate several proteins which can result in decreased intracellular calcium levels, corporal smooth muscle relaxation, venous occlusion and a penile erection. PDE5 is the predominant phosphodiesterase in the penis and normally inhibits penile erection through the degradation of cGMP\textsuperscript{198}. PDE5 can also become phosphorylated by PKG which increases its level of enzymatic activity and its affinity for cGMP\textsuperscript{199}. The PDE5 inhibitors work by competitively binding the catalytic site of PDE5 to prevent the inactivation of cGMP, thereby, leading to greater relaxation of the corporal smooth muscle and penile feeder arteries. Sexual arousal is required to initiate the cGMP production and as a result of this response being localized in the penile tissue, PDE5 inhibitors have little effect in other smooth muscle tissues. There are currently three different PDE5 inhibitors on the market (sildenafil, vardenafil, tadalafil) which differ in the time of onset of action and half-life.
Figure 1.4  Nitric oxide-cGMP pathway for relaxation and the mechanism of phosphodiesterase type 5 (PDE5) inhibition in cavernosal smooth muscle. NO, nitric oxide; GTP, guanosine triphosphate; cGMP, guanosine-3’,5’-monophosphate; 5’-GMP, 5’-guanosine monophosphate; PKG, cGMP-dependent protein kinase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Ca^{2+}, calcium ion; PDE5I, phosphodiesterase 5 inhibition. Modified from JD Corbin IJIR 2004.
Another orally available drug that was marketed until 2006 was the dopaminergic agonist apomorphine. This substance has been presumed to act within the MPOA and PVN of the hypothalamus, by stimulating dopamine (D2, D4) receptors on oxytocinergic neurons, to centrally initiate the autonomic nervous system cascade involved in the erectile response \(^{63, 200}\). This drug was effective in a sub-group of ED patients but was taken off of the market as the PDE5 inhibitors began to dominate the sales for this indication. If the oral therapies fail and/or the injection of vasoactive substances is not desired, the alternatives that remain include the use of vacuum constriction devices, or more invasive and terminal measures such as the implantation of a penile prosthesis.

Unfortunately, patients suffering from ED with severe arterial dysfunction as the primary cause have the poorest response to pharmacotherapy. That is, as the severity of vascular dysfunction progresses in an ED patient, they often become unresponsive to any erectile specific therapies \(^{201}\). Thus, since both sexual dysfunction and CVD have common risk factors and often co-exist, a treatment that targets the underlying vascular abnormality may be beneficial to both conditions. Further studies are required to elucidate the damaging mechanism of the risk factors behind these diseases and find a better prevention and treatment strategy.

1.8 General hypothesis and objectives

The overall working hypothesis of the research presented in this thesis is that ED associated with aging, hypertension, and obesity, is due to pathological changes in the
structure of the pudendal vasculature; and that the ability of antihypertensive treatments or lifestyle modifications to prevent or reverse ED is based on the ability to improve pudendal vascular structure and function.

The specific research hypotheses were as follows:

1. Hypertension, aging and obesity will produce greater pathological changes in the structure and function of the pudendal artery than in other non–penile vessels.

2. Benefits to erectile function from antihypertensive treatment or lifestyle modification will result from improved structure and/or function in the vasculature supplying the penile tissue.

To test these hypotheses, the studies were designed to determine:

1. The age-related changes in the internal pudendal artery in young and old normotensive and hypertensive rats.

2. Whether antihypertensive drug treatment could impact on erectile function in aging animals.

3. Whether improved erectile function from antihypertensive treatment or lifestyle modifications correlates with improved pudendal vascular structure and function.

1.9 Rationale and approach

To date ED research has focused primarily on the changes in the corpus cavernosum in animal models of different pathological states with or without treatment interventions. A
previous study from our laboratory has shown that the majority of the vascular resistance
during an erectile response is not found within the penile tissue but lies upstream in the
vasculature supplying it. We have previously demonstrated using isolated penile
perfusion methodologies that the penile vasculature is structurally altered in hypertension
and aggressive antihypertensive treatment can decrease the overall vascular resistance of
this bed\textsuperscript{158}. This hemodynamic approach provided an index of the overall changes in the
penile vascular bed but did not allow for the determination of the location of specific
structural changes in these vessels. Thus, the main objective of the present work was to
examine in detail the predominant resistance vasculature supplying the penile tissue, the
internal pudendal artery.

Previous investigations have shown that antihypertensive treatments can recover erectile
function in aged hypertensive rats; however no one has examined the ability of these
treatments to delay the onset of erectile dysfunction. In Chapter 2, using a conscious rat
model of erectile function, we determined if antihypertensive therapy could prevent the
onset of erectile dysfunction in 30 week old SHR. Multiple treatments which did or did
not inhibit RAS were also assessed at different ages to determine when treatment should
be administered to provide the most benefit to erectile function. In order to elucidate if
the benefit to erectile function after antihypertensive therapy is due to lower arterial
pressure or structural changes, cross-over kidney transplantations were performed
between treated and untreated SHR. These transplants resulted in one group of SHR with
lower blood pressure due to their donor treated kidney with untreated vasculature and
another group who underwent treatment but operate at a high blood pressure from their untreated donor kidney. Continuous blood pressure recordings were performed by radio-telemetry and apomorphine (APO)-induced erectile responses were assessed.

To expand on the studies by Hale et al., the pudendal artery’s morphology was characterized in Chapters 3 and 4. Using light microscopy, immunohistochemistry and electron microscopy, proximal, middle and distal segments of the internal pudendal artery were assessed and compared to sections of thoracic aorta, renal and first order mesenteric arteries. Young and old Sprague-Dawley rats and SHR were assessed in Chapters 3 and 4, respectively. The physiological activity of the pudendal artery from young and old Sprague-Dawley rats was also examined with a wire myograph in Chapter 3. The impact of antihypertensive treatment on remodelling the pudendal artery was assessed in young SHR in Chapter 4.

The final chapter takes a different approach to treating ED and examines the impact of exercise and reducing caloric intake on improving erectile function. In 2004, Esposito et al. showed that modifications in exercise and diet improved erectile function and overall sexual satisfaction in men suffering from the metabolic syndrome. Using a similar approach, normotensive and hypertensive rats were monitored as their erectile function declined and an intervention of exercise and decreased caloric intake attempted to recover erectile function. The findings from these studies will provide further understanding of
the vascular aspect of ED and shed light on the mechanisms in which treatment interventions improve erectile function.
Chapter 2:

Impact of antihypertensive treatments on erectile responses in aging spontaneously hypertensive rats

2.1 Introduction

Over 30 million men in North America between the ages of 40 and 70 are affected by erectile dysfunction (ED)\textsuperscript{114}, with a higher proportion also having some form of cardiovascular disease\textsuperscript{106,200}. In many cases, sexual dysfunction appears to precede the conventional signs of cardiovascular disease and thereby acts as a diagnostic indicator\textsuperscript{113}. In the hypertensive male population, the prevalence of erectile dysfunction is approximately twice that seen in the normotensive group, with the main cause found to be arterial dysfunction in 89\% of these patients\textsuperscript{119,202}. The link between ED and cardiovascular disease is complicated by the understanding that some antihypertensive agents induce sexual dysfunction. Specifically, although antihypertensive drugs such as beta-blocking agents, diuretics and central sympatholytics are very effective at lowering blood pressure, all have been shown to deleteriously impact on sexual function\textsuperscript{166,203,204}.

The magnitude of the reduction in blood pressure does not appear to account for the antihypertensive drug-induced ED, as effective agents such as calcium channel blockers and inhibitors of the renin–angiotensin system (RAS) do not appear to impair sexual function\textsuperscript{170,174,205}. In fact, more recently, results from clinical and experimental studies using RAS-inhibiting drugs show improvements in sexual function. Specifically, treatment of hypertensive men with ED using AT-1 receptor antagonists resulted in increased sexual function, satisfaction and frequency of sexual activity\textsuperscript{169,171}. Similarly, brief, aggressive treatment with an ACE inhibitor in spontaneously hypertensive rats (SHR) was able to recover erectile function\textsuperscript{205}. 

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Treatment of SHR with inhibitors of the renin–angiotensin system also persistently reduces arterial pressure and regresses vascular structure long after treatment is stopped. More relevant to the effect on sexual function is the finding that short-term antihypertensive treatment also induces structural remodelling of the penile vasculature even in aged SHR.

In the present study, the time course of changes in ED was characterized in aging SHR (older than 30 weeks) with and without antihypertensive treatment. The antihypertensive treatment strategies used targeted both the prevention and the recovery of erectile function using various agents. An additional complication of aging is that there is diminished activity within the hypothalamic–pituitary–gonadal axis, resulting in decreased testosterone. Androgen supplementation has been found effective in improving sexual function both clinically in aging males and following experimental castration in rats. To address this issue, supplementation with testosterone was performed in the aged SHR to assess whether hormonal deficiencies also contributed to the ED. Finally, using a kidney transplantation approach, involving a cross-over between treated and untreated rats, we determined whether antihypertensive drug-induced improvement in sexual function was dependent on changes in arterial pressure or on effects specific to the penile vasculature.
2.2 Methods

2.2.1 Animals

Twenty-six male SHR were purchased at 13 weeks of age (Charles River Laboratories, Montreal, Quebec, Canada) and housed individually at a temperature of 22–24°C with a 12-h light/dark cycle. All rats were provided with free access to regular rodent chow (0.4% Na+) and tap water. Procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 Antihypertensive treatments

At 30 weeks of age, rats were randomly divided into three groups. The control animals received tap water and regular rodent chow (n=12). The other groups received a 2-week treatment with enalapril maleate (30mg/kg per day, n=7) or hydralazine (45mg/kg per day, n=7) and regular rodent chow. All drugs were supplied by Sigma Chemical Co. (St Louis, Missouri, USA) and administered in the drinking water. Aspartame (100mg/l) was added to the hydralazine solution to improve palatability. Drug concentrations were adjusted bi-weekly to account for fluctuations in body weight or fluid intake. Based on the results of the initial treatment cycle, a second 2-week antihypertensive treatment was performed at 50 weeks of age using the same drugs in a further attempt to restore erectile function.
Subsequent to the second treatment, all rats still had demonstrable signs of ED and therefore underwent a third, 2-week antihypertensive treatment at 68 weeks of age, in which rats were re-allocated into four new treatment groups (Table 2.1). The previously untreated rats (n=12) received enalapril (30mg/kg per day, n=6) or losartan (LOS) (30mg/kg per day, n=6; Merck Frosst Canada Inc., Point-Claire, Quebec, Canada) with a low-sodium diet (0.04% Na+). The rats that had previously been treated with two cycles of either enalapril or hydralazine were also subdivided for the third round of treatment. Table 2.1 shows that groups 2A and 3A received enalapril with a low-sodium diet (n=7), whereas groups 2B and 3B received a triple treatment (n=7) combining hydralazine (45mg/kg per day), nifedipine (200mg/day) and hydrochlorothiazide (100mg/l) (all drugs supplied by Sigma Chemical Co.). In the case of the animals receiving a low-salt diet, on day 6 of treatment the rats were given daily 4 hour access to regular chow (0.4% Na+) containing normal levels of salt to stabilize the depressor response as previously described. Drugs were mixed into the drinking water, excluding nifedipine that was mixed into ground chow. Aspartame (100mg/l) was added to the hydralazine solution to improve palatability.

2.2.3 Testosterone treatment

At 47 and 67 weeks of age, each rat received a single dose of testosterone (480µg/kg) administered via subcutaneous injection 36 hours prior to erectile testing according to a previously optimized protocol. Testosterone was prepared from a stock solution of testosterone propionate (Taro Pharmaceuticals, Bramalea, Ontario, Canada) in peanut oil.
### Table 2.1 Summary of antihypertensive treatments throughout study

<table>
<thead>
<tr>
<th>Group 1A</th>
<th>Treatment #1</th>
<th>Treatment #2</th>
<th>Treatment #3</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enalapril LS</td>
<td>6</td>
</tr>
<tr>
<td>Group 1B</td>
<td></td>
<td></td>
<td>Losartan LS</td>
<td>6</td>
</tr>
<tr>
<td>Group 2A</td>
<td>Enalapril</td>
<td>Enalapril</td>
<td>Enalapril LS</td>
<td>4</td>
</tr>
<tr>
<td>Group 2B</td>
<td>Enalapril</td>
<td>Enalapril</td>
<td>Triple Therapy</td>
<td>3</td>
</tr>
<tr>
<td>Group 3A</td>
<td>Hydralazine</td>
<td>Hydralazine</td>
<td>Enalapril LS</td>
<td>3</td>
</tr>
<tr>
<td>Group 3B</td>
<td>Hydralazine</td>
<td>Hydralazine</td>
<td>Triple Therapy</td>
<td>4</td>
</tr>
</tbody>
</table>

n is the number of animals in each group for Treatment #3.
2.2.4 Assessment of erectile response

Erectile responses are assessed at numerous time points before, during and after treatments using the well-established bio-assay rat model of centrally-induced erections using the dopaminergic agonist, apomorphine (APO)\textsuperscript{205,206}. The rats were placed in separate, hanging wire cages with clear Plexiglas bottoms in a dimly lit, quiet room and allowed to acclimatize for 20 min. Each rat received subcutaneous injection of APO in saline (80μg/kg with 100μg/ml ascorbic acid, 1ml/kg) in the loose skin of the neck or back. Erections and yawns were counted over a 30 minute period (scored in 5-min intervals) via videomonitoring in an adjacent room. Erectile responses were characterized from the overall physical and behavioural changes, including concave arching of the back, pelvic thrusts followed by the emergence of the engorged glans penis and the distal shaft and immediate grooming of the genital area. APO-induced yawns are recorded to confirm drug delivery to the central nervous system\textsuperscript{207}.

2.2.5 Assessment of blood pressure and heart weights

Based on previously established protocols, surgeries were performed to examine conscious blood pressure in randomly selected rats at 79 weeks of age\textsuperscript{208}. Briefly, single lumen catheters were implanted into the abdominal aorta distal to the kidneys and secured in place with cyanoacrylate glue. Catheters were filled with heparinized saline (50U/ml), tunneled subcutaneously, exteriorized and sutured into place at the nape of the neck. The rats were allowed 3 days recovery prior to blood pressure assessment. The catheter was connected to a pressure transducer for arterial pressure recording, which was
continuously recorded for 1 hour on a data acquisition system (MacLab; AD Instruments, Houston, Texas, USA). Following the blood pressure measurements, rats were anesthetized with pentobarbital (65mg/kg, intraperitoneal). Hearts were removed and blotted dry, and the right and left ventricle plus septum were then separated and weighed. Analysis of the left-ventricle-to-body-weight (LV/BW) ratio was used as an index of change in cardiac structure.

2.2.6 Kidney transplantation

Cross-over kidney transplantations were performed in a separate group of male SHR (n=16) using established methods described by Smallegange. At 15 weeks of age, SHR were treated for 2 weeks with losartan (LOS, 30mg/kg per day; Merck Frosst) and a parallel control group (CON) that did not receive treatment. Transplants were performed at 19 weeks. SHR previously treated with losartan received a control kidney (LOS\textsubscript{K}=CON, n=4x2 donor/recipient) whereas control animals were given a previously treated kidney (CON\textsubscript{K}=LOS, n=4x2 donor/recipient). All animals were implanted with radio-telemetric pressure transducers and were uninephrectomized 1 week after transplantations (i.e. the only remaining kidney comes from the donor). Mean arterial pressures (MAP) were determined from data collected for each animal (15s every 5min, at 150Hz). APO-induced erectile responses were assessed in all rats starting at 8 weeks post-transplantation. All animals underwent another 2-week losartan treatment at 29 or 38 weeks of age and erectile responses were assessed before, during and after treatment was
withdrawn. At the study’s end, the hearts were removed and the right and left ventricles were separated and weighed.

2.2.7 Statistical analysis

Erectile function data are expressed as the erection response rate (i.e. the proportion of animals responding per test period) and the average number of erections per test period (±SD). Statistical analysis of erectile responses and yawns between and within treatment groups was performed using the Mann–Whitney rank sum test and one-way analysis of variance, with a comparison of means using a Newman–Keuls post-hoc test (p<0.05). Differences in erectile responses and yawns during testosterone treatment were determined using Student’s t test (p<0.05). Arterial pressure and heart weight data are presented as the mean±SD and were assessed by analysis of variance followed by a Newman–Keuls post-hoc test (p<0.05).

2.3 Results

2.3.1 Impact of age on apomorphine-induced sexual responses

To demonstrate the changes in erectile function with increasing age, the data are presented as the average number of erections per group, the percentage of animals responding and the time course of drug-induced erectile responses (Figure 2.1). Between 30 and 50 weeks of age, the number of erectile responses had diminished by 85%. As would be expected, the proportion of animals with severe ED was also found to increase with age. Whereas a small proportion of rats had mild dysfunction at 30 weeks, two-
Figure 2.1  (A) The age-related decrease in erectile responses was examined in untreated animals.  (B) The severity of erectile dysfunction was classified as no dysfunction ($\geq 2.0$ erections), mild ($\geq 1.0$ erection), moderate ($0.5 < 1.0$ erection) or severe ($<0.5$ erection) dysfunction.  (C) There was a significant age-related delay in the timing of the apomorphine induced erectile response whereas aging minimally altered (D) the timing of the yawning response.  Data are expressed as total number of responses per time interval.  Prop.=proportion.  *P<0.05 versus 30-32 weeks of age.  †P<0.05 versus 35-41 weeks of age.  NR = no response.
thirds of the rats had severe dysfunction by 65 weeks (Figure 2.1B). Degree of erectile dysfunction was defined as: no dysfunction (≥2.0 erections), mild dysfunction (≥1.0 erection), moderate dysfunction (0.5 to <1.0 erection) or severe dysfunction (<0.5 erection) (Figure 2.1B). In addition, the timing of the APO-induced responses was significantly altered with increasing age (Figure 2.1C). At 30 weeks the peak response period occurred within the first 10 min of the test period, whereas by 50 weeks the peak responses were more delayed and shifted to the 10–20 min time interval. Overall, throughout all three treatments, yawns did not change with age (Figure 2.1D). Although in all groups there appeared to be fewer yawns at the 30-week time point, the majority of the yawns occurred after the 10-min mark in the 30-min testing period.

2.3.2 Impact of antihypertensive therapy

In contrast to our previous findings in which brief aggressive antihypertensive therapy was shown to recover erectile responses in 40-week-old SHR\textsuperscript{205}, in the present study the initial, more conventional, treatments were found to not be as effective. Specifically, in 30-week-old SHR treatment for 2 weeks with enalapril or hydralazine did not prevent or attenuate the fall in erectile responses (Figure 2.2A). In addition, the treatments did not alter the proportion of animals responding (i.e. the number of animals responding with one or more erections did not change). In contrast, yawns were not affected by treatment or aging and remained at an average 14.3±7.5 throughout all test periods within all three groups. The yawning response rate was always 100%.
Figure 2.2  (A) Treatments with enalapril or hydralazine at 30 weeks of age did not prevent the age-related decline in erectile function seen in untreated (CON) animals.  (B) A rechallenge with identical treatments at 49 weeks of age significantly increased erectile responses in comparison to untreated animals of the same age.  Erectile responses were determined before (Pre), during (On) and after (Off) withdrawal of treatment.  Data are presented as mean ± SD.  *P<0.05 versus 30 and 32 weeks of age.  †P<0.05 versus 35-41 weeks of age.  ‡P<0.05 versus CON 52-53 weeks of age.  #P=0.06 versus CON 52-53 weeks of age.
2.3.3 Impact of second treatment

Although the initial treatment did not prevent the age-associated fall in erectile responses, a second treatment was found to be effective in recovering erectile function (Figure 2.2B). In fact, overall the erectile responses in previously treated animals were nearly 3-fold greater compared with age-matched controls. Consistent with this finding, the erectile response rate in treated SHR was determined to be 65%, compared with only 25% of the control animals responding. The treatments again had no significant impact on yawns.

2.3.4 Aggressive antihypertensive treatment

Based on the incomplete effect of the previous two treatments, at 68 weeks of age the impact of a third more aggressive antihypertensive treatment was determined (Figure 2.3). In the previously untreated rats, the brief aggressive therapy involving enalapril or losartan combined with a low-salt diet had only a small effect on erectile responses (i.e. there was a 2-fold increase in erectile responses, which was significant only in the enalapril plus low-salt diet group, going from 0.26 to 0.58 post-treatment). In the previously treated animals, the effect of the aggressive treatment was dependent on the drugs used. There were no statistically significant treatment-induced changes in the temporal pattern of erectile responses during the test period. Although there was a significant improvement overall in erectile responses in the post-treatment period, the degree of erectile dysfunction was not found to be associated with increased MAP or
Figure 2.3  (A) Apomorphine induced erectile responses were examined before (Pre), during (On) and after (Off) withdrawal of treatment in the aged SHR (treatment at 68 weeks of age). Overall, there was a significant increase in the number of erections after treatment withdrawal (all treated animals pooled). (B) The severity of erectile dysfunction was determined before and after treatment by classifying animals as having no dysfunction (≥2.0 erections), mild (≥1.0 erection, white), moderate (0.5-<1.0 erection, grey) or severe (<0.5 erection, black) dysfunction. After treatment, there was a small decrease in those in the severe category and a corresponding increase in the number of rats with mild dysfunction. (C) In the individual group assessments of the previously untreated rats (top 2 graphs) and the previously treated rats (bottom 2; ELS¹, Triple¹), there was significant increase (post-treatment) in erectile responses in the triple therapy group. LLS is losartan plus a low salt diet, ELS is enalapril plus a low salt diet. *P<0.05 versus 65-66 weeks of age.
elevated LV/BW ratio (Figure 2.4). That is, across all three classifications of erectile dysfunction, similar values for the MAP and LV/BW ratio were found (see below).

2.3.5 Testosterone supplementation in aging SHR

Overall, the erectile responses were not found to be improved following testosterone supplementation at 47 and 67 weeks of age (Table 2.2). Only at one time point (47 weeks) in enalapril-treated rats was there any evidence of increased numbers of erections (there was no effect of testosterone treatment in these same rats at 67 weeks). Similarly, there was no change in the number of yawns, and the yawning response rate remained 100% in all animals.

2.3.6 Mean arterial pressure and heart weights

Conscious arterial pressure and LV/BW ratios were determined in some of the SHR at 79 weeks. The control rats that were aggressively treated with enalapril low-salt or losartan low-salt, during weeks 69 and 70, had similar MAP (154±27.2 and 152±0.1 mmHg) and LV/BW ratios (2.76±0.12 and 2.77±0.14 mg/g). The SHR that had previously received treatments at both 30 and 50 weeks, followed by a third treatment at week 69 (enalapril–low-salt or triple therapy), had slightly increased MAP (167±27.3 and 179±17.3 mmHg) but similar LV/BW ratios (2.78±0.34 and 2.77±0.21 mg/g) compared with the other groups.
Figure 2.4 The severity of erectile dysfunction (top) did not correlate with the level of mean arterial pressure (middle) or left ventricular mass (bottom). Animals were classified as having no dysfunction (≥2.0 erections), mild (≥1.0 erection), moderate (0.5<1.0 erection) or severe (<0.5 erection) dysfunction. Data are presented as mean ± SD.
Table 2.2  Impact of Testosterone Supplementation on Erections and Yawns

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Enalapril</th>
<th>Hydralazine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Erections</td>
<td>Yawns</td>
<td>Erections</td>
</tr>
<tr>
<td><strong>44-48 weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Testosterone</td>
<td>0.5±0.72</td>
<td>15.8±8.22</td>
<td>0.3±0.49</td>
</tr>
<tr>
<td>On Testosterone</td>
<td>0.5±0.53</td>
<td>15.3±7.54</td>
<td>1.0±0.63*</td>
</tr>
<tr>
<td>Off Testosterone</td>
<td>0.3±0.46</td>
<td>19.4±7.48</td>
<td>0.3±0.52</td>
</tr>
<tr>
<td><strong>65-68 weeks</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pre-Testosterone</td>
<td>0.3±0.56</td>
<td>9.2±4.76</td>
<td>0.2±0.38</td>
</tr>
<tr>
<td>On Testosterone</td>
<td>0.4±0.67</td>
<td>11.1±10.87</td>
<td>0.3±0.49</td>
</tr>
<tr>
<td>Off Testosterone</td>
<td>0.2±0.39</td>
<td>8.1±7.12</td>
<td>0.4±0.79</td>
</tr>
</tbody>
</table>

Erections and yawns were examined before (Pre), during (On) and after (Off) testosterone supplementation. Data are expressed as mean ± SD. *P<0.05 vs. pre-testosterone.
2.3.7 Kidney transplantation

The initial 2-week losartan treatment induced a significant, persistent decrease in arterial pressure (-12 mmHg; Figure 2.5A). Following kidney transplantation, this persistent lowering of MAP was completely transferred to untreated SHR (i.e. pressure in untreated control rats was decreased following kidney transplantation from a previously treated rat). Furthermore, treatment-induced decreases were completely reversed by transferring an untreated kidney from a control SHR into a previously treated rat. Regardless of the source of the kidney received during transplantation, SHR previously treated with losartan had significantly increased erections compared with untreated SHR (Figure 2.5B). The positive impact of losartan on erectile responses, specifically, was confirmed when the final losartan treatment increased erections (2-fold) in the previously untreated SHR (with a treated kidney) (Figure 2.5C). Re-treatment of SHR that had already received losartan treatment did not further improve erectile responses (i.e. both groups were now at the same level of erectile function).

2.4 Discussion

Taken together with our previous studies, a main finding of the present study is that although age-related progression of ED in SHR can be attenuated, in part, by relatively brief antihypertensive therapy, a more aggressive or longer therapy is needed in older animals. As previously established, the present data confirmed that the antihypertensive treatments induced improvements in erectile responses that persisted after therapy was
Figure 2.5  (A) Cross-over of mean arterial pressure (MAP) after cross-transplantation of kidneys between animals with AT-1 receptor antagonist-induced persistent lowering of pressure and control animals. LOS<sub>K=CON</sub> indicates previously treated animals that received a control kidney (CON). CON<sub>K=LOS</sub> indicates control animals that received a treated kidney (LOS). Day 0 is at 19 weeks of age and is the time point at which transplantation and uninephrectomy occurs. The pressures prior to transplantation are from uninephrectomized control and treated animals. (B) In the LOS<sub>K=CON</sub> animals there was a significant increase in the number of APO-induced erections still occurred at least 8 weeks after transplantations versus the CON<sub>K=LOS</sub> animals (left). (C) Erectile responses were determined before (Pre), during (On) and after (Off) withdrawal of the second round of treatment (right). There was a significant increase in erectile function in the CON<sub>K=LOS</sub> animals after treatment cessation which was similar to pre-treatment levels of the LOS<sub>K=CON</sub> animals. A second treatment only marginally increased the number of erections of the LOS<sub>K=CON</sub> animals. **P<0.01 overall versus control. †P<0.01 overall versus CON<sub>K=LOS</sub>. ‡P<0.05 versus CON<sub>K=LOS</sub>. ψP<0.05 vs Pre and On CON<sub>K=LOS</sub>. 
stopped. In contrast, short-term testosterone administration was not found to be particularly efficacious in these aging, hypertensive rats. It is somewhat surprising that the improvements in erectile responses were found to occur even though arterial pressures were at hypertensive levels in most of the animals. No correlation was thus found between the magnitude of the hypertension (or cardiac hypertrophy) and the severity of ED. Consistent with this finding, the results of the kidney cross-over transplantation study revealed that the drug-induced benefits to erections were not related to changes in arterial pressure, but more likely to the direct pharmacological targeting of the penile tissue.

The present finding of an age-related decrease in erectile responses confirms the results of previous studies that have shown that erectile responses are diminished in adult SHR. Common to both hypertension and ED is the development of abnormalities in vascular structure and function. For example, numerous studies have established that resistance vessels in aged hypertensive patients and in SHR have a structurally-based increase in the media-to-lumen ratio, an associated encroachment on the vascular lumen and accelerated collagen deposition in small arteries. In the penile vascular bed, specifically, aging-induced changes in the extracellular matrix and vascular smooth muscle function could promote fibrous proliferation of the intima, medial fibrosis and calcification. In addition, it may be that in the aging rat the markedly diminished erectile function is linked to degeneration of elastic fibres resulting in abnormal corporal compliance as well as damage to the innervation traversing the interstitium.
Although in the present study an initial single, 2-week antihypertensive treatment did not prevent the development of ED in SHR, a subsequent re-challenge did provide some incremental benefit. These data appear to contrast with previous findings in which 40-week-old SHR were successfully recovered, at least in part, following a brief, aggressive treatment. Some of the difference in the impact of the early treatments probably results from using a less intense treatment than in the prior investigation. The improvements in older rats may also reflect either cumulative impact of the second cycle of treatment or age-associated change in susceptibility of the penile tissue to drug-induced effects. The partial success using the more aggressive treatment strategy at 68 weeks emphasizes that improvement is still possible even when erectile function has markedly declined. Defining the optimal treatment duration and whether any particular agent will be found to have specific benefit remains to be established.

Agents that inhibit the RAS have been found to be very efficacious in inducing a reduction in arterial pressure and regression of vascular structure in SHR that persist long after cessation of treatment. Evidence regarding the efficacy of other single agents in inducing these persistent changes has been equivocal. For example, although monotherapy treatment with hydralazine or an angiotensin-converting enzyme inhibitor attenuates hypertension in SHR, following withdrawal of treatment only the angiotensin-converting enzyme-inhibitor groups are found to have persistently downregulated arterial pressure and vascular structure. Our own studies using hydralazine confirm that there is a lesser effect on penile vascular structure than with an
ACE inhibitor\textsuperscript{173}. Previous studies have indicated that the rank order for the effectiveness of antihypertensive agents for inducing persistent lowering of arterial pressure is enalapril combined with a low-salt diet, enalapril alone and then triple therapy\textsuperscript{173}. However, the substantial effectiveness of triple therapy given in 68 week old SHR suggests that other mechanisms may require assessment.

The data revealed that although there were some improvements in erectile responses, the SHR remained quite hypertensive, regardless of treatment, at the end of the study. It was thus important to investigate whether the effect was mediated via changes in arterial pressure or by drug-induced improvements in the penile vascular function. To accomplish this we determined whether receiving, via transplantation, a kidney from a previously treated animal similarly impacts on the recipient SHR by lowering the level of arterial pressure and improving erectile function. The opposite was in fact found. That is, SHR that were previously treated with losartan and then received an untreated kidney had higher arterial pressure but twice the number of erections in comparison with the other SHR with lower arterial pressure. Taken together with the finding that there was no correlation between blood pressure and erectile responses, these data suggest that the treatment-induced improvement in erectile responses is tissue specific, and not based on changes in blood pressure \textit{per se}. This concept was reinforced when the second cycle of treatment doubled the number of erections only in the SHR with a previously untreated penile vasculature and had no further impact on erectile responses in SHR that had been previously treated.
Testosterone deficiency as a potentially important contributor to the aging-induced ED was largely ruled out due to the lack of overall improvement following testosterone administration. This finding is somewhat counter to evidence indicating that there is commonly a progressive decline in androgen production in aging men, which is often accompanied by decreased libido and sexual dysfunction. Numerous studies have demonstrated the androgen dependence of penile erections in rats, since castration is widely known to markedly decrease erections and testosterone administration will recover them. The effects of antihypertensive agents on testosterone levels are equivocal. In hypertensive males, the β-blocker atenolol, but not the angiotensin receptor antagonist valsartan, has been found to decrease plasma testosterone levels and reduce sexual activity. Furthermore, although the angiotensin-converting enzyme inhibitor lisinopril has been found to decrease levels of free testosterone, no measure of sexual function is reported.

The present findings suggest that aggressive antihypertensive treatments may be more beneficial in the persistent improvement in erectile function in aged SHR. Although a more conventional treatment may help to minimize the progression of ED, it appears that with the severe changes evident in animals older than 60 weeks of age, a very strong antihypertensive challenge is needed. Taken together with previous studies it appears that the antihypertensive treatment-induced improvement in erectile responses is tissue specific, and not based on changes in blood pressure. Whether the improvements are causally linked to changes such as regression of penile vascular structure, improved
endothelial function and/or decreased inhibitory sympathetic tone remains to be established. The potential dual benefits of a therapeutic approach involving antihypertensive agents in patients with both hypertension and ED warrants further investigation.
Chapter 3:

Evidence that morphological and functional changes in the pudendal artery contribute to aging-induced erectile dysfunction

3.1 Introduction

Age is the most significant risk factor for erectile dysfunction (ED) as the prevalence and severity of ED increases 3-fold from 40 to 70 years of age \(^{114, 239}\). With the expanding aging population it has been projected that by 2025 the number of men with ED will more than double to 322 million men \(^{19}\). Further complicating the etiological basis of ED in the aging population is the concomitant increase in cardiovascular and metabolic conditions in this group including hypertension, coronary artery disease, chronic kidney disease, diabetes, hyperlipidemia and obesity \(^{17, 20, 36, 106, 147, 240-242}\).

During aging, impaired neural signalling, decreased androgen levels and pathogenic vascular remodelling have all been shown to occur \(^{214, 243-246}\). Since an erection is dependent upon the appropriate integration of neurological, hormonal and vascular pathways, alterations in one or more of these can lead to ED. Specifically, relevant to the current study, systemic vascular changes involving calcification, fibrosis, degeneration of elastic fibers, endothelial dysfunction and changes in the content of smooth muscle and extracellular matrix (ECM) have been found with aging, although most of this work involves non-penile tissues \(^{247-250}\). Even small changes in vascular structure and function can produce dramatic changes in the capacity for arterial inflow, alterations that could negatively impact erectile responses \(^{140}\).

Although many studies have focused on the pathological changes within the penile vascular tissue, particularly alterations in the corpus cavernosum, an earlier study
demonstrated that the critical location for control of resistance properties was external to the penis\textsuperscript{158}. Specifically, they found that 70\% of the total resistance of the penile vasculature was located in the bilateral internal pudendal arteries.

Previously, the internal pudendal artery in the rat was described solely from an anatomical perspective\textsuperscript{251-253}. Akyurek \textit{et al}, described its origin from the dorsomedial surface of the internal iliac artery (hypogastric trunk), from which it passed between the flexor cauda brevis and the abductor cauda internus muscles and travels along the medial surface of the pelvis\textsuperscript{252}. For much of this region the vessel runs parallel to the internal pudendal vein and the pudendal nerve, emerging from the ischiorectal fossa to supply the urethra, corpus spongiosum and the ischiocavernosus and bulbocavernosus muscles before dividing into the dorsal and deep penile arteries supplying the penis\textsuperscript{252}. Despite this previous gross anatomy characterization, the morphology and physiological aspects of this vessel have not been well characterized.

In the present study, pharmacologically-induced erectile responses were assessed in young (15 week old) and aged (77 week old) normotensive rats prior to tissues being taken for histological assessment. The left and right internal pudendal arteries, first order branches of the mesenteric bed (similar lumen diameter as pudendal artery), both renal arteries (similar wall thickness as pudendal artery) and the aorta were excised and processed for morphological characterization. In addition, the contractile and relaxation
properties of the pudendal artery from young and old rats were assessed using a wire myograph.

3.2 Methods

3.2.1 Animals

Male Sprague-Dawley rats (n=9) were purchased at 12 weeks of age (Charles River Laboratories, Montreal, Quebec, Canada) and housed individually in room set at 22–24°C with a 12-h light/dark cycle. All rats were provided with regular rodent chow (LabDiet® 5001, Ren’s Feed and Supply Ltd, Oakville, Ontario, Canada) and tap water ad libitum. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and the project was approved by the Queen’s University Animal Care Committee.

3.2.2 Assessment of erectile response

Prior to sacrifice, erectile function was assessed. This procedure involves monitoring erectile responses (and yawns) using a well-established procedure of pharmacologically initiating responses using the centrally acting dopaminergic agonist, apomorphine (APO) \(^{205, 220, 254}\). Specifically, rats were placed into one of four individual, hanging cages (with clear Plexiglas inserts used to replace the wire floor) in an isolated, quiet, dimly lit room adjacent to the holding room. After a 20 minute period of acclimation, each rat received subcutaneous injection of APO in saline (80 μg/kg with 100 μg/ml ascorbic acid, 1 ml/kg) in the loose skin of the neck or back. Erectile responses and yawns were counted
over a 30-min period via videomonitoring in an adjacent room. A full erectile response was recorded following identification of characteristic physical and behavioural responses including concave arching of the back, pelvic thrusts followed by the full emergence of the engorged glans penis and shaft, and immediate oral grooming of the genital area. APO-induced yawns were also recorded as an index of delivery and to confirm bioavailability of the drug within the central nervous system. Administration of saline does not induce these responses.

3.2.3 Tissue preparation and morphometry

At 15 and 77 weeks of age, animals were anaesthetized with sodium pentobarbital (65mg/kg, intraperitoneally) followed by intravenous doses of the ganglionic blocker hexamethonium and heparin (30mg/kg and 1000 units/kg, Sigma Aldrich Chemical Co., St Louis, Missouri, USA) to allow for perfusion under maximally relaxed conditions. After the heart was excised, the animals were perfused at 70mmHg through the thoracic aorta with saline (0.9% Na+, Baxter Corp., Mississauga, On, Canada) containing the vasodilator sodium nitroprusside (300mg/L, Sigma Aldrich Co.) to facilitate flushing out the blood. The animals were then perfusion fixed with 2% paraformaldehyde, 2% gluteraldehyde, 4% sucrose, and 0.05% calcium chloride in a 0.1M cacodylate buffer. Segments of the thoracic aorta, first-order mesenteric branches, right and left renal and pudendal arteries were excised and stored in fixative overnight. Figure 3.1 demonstrates the specific sample sites from which the pudendal arteries were obtained.
Figure 3.1 Illustration of the bilateral internal pudendal arteries (IPA) in the male rat. The IPA branches off the internal iliac (Int Il) and travels along the inside of the pelvic medial fossa towards the penis. Arrows indicate where proximal (A), middle (B) and distal (C) pudendal segments were sampled. Drawing in collaboration with Lauren Oldfield.
Subsequently, the vessels were rinsed several times with buffer, post-fixed in cacodylate buffered 1% osmium tetroxide, and stained en bloc with aqueous 4% uranyl acetate for 1 hour. The vessels were further dehydrated through a graded series of alcohols, infiltrated with propylene oxide, and embedded in Epon 812. From each tissue bloc, 1µm thick sections were cut with glass knives, subsequently stained with 0.1% azure methylene blue, and catalogued according to orientation.

In a separate group of 15 week old male Sprague-Dawley rats (n=6), tissues were embedded in paraffin for further morphological and histological analysis. Specifically, in this part of the study, animals were anaesthetized and perfused as before, but using 4% paraformaldehyde (Sigma, Aldrich Co.) in 10X phosphate buffered saline (PBS). Pudendal arteries were excised, stored overnight in fixative and processed routinely for paraffin embedding (1 hr each in graded ethanols [70% x 2, 95% x 2, 100% x 3], Slidebrite x 2 [xylene substitute; Jones Scientific Products, Kitchener, ON, Canada], paraffin x 3) [257]. Vessels were cut into 5 µm sections and placed on glass slides. The pudendal artery sections were stained with hematoxylin and eosin stain (H&E stain). Immunohistochemistry was carried out using the Vector® M.O.M. immunodetection kit (Vector Laboratories, Inc., Burlingame, CA) to stain for smooth muscle α-actin and visualize cell nuclei. To assess alpha (α-) smooth muscle actin in the vascular sections a Vector FITC-linked Immunodetection Kit for monoclonal antibody (Cedarlane Laboratories, Canada) was used. Briefly, 5 µm thick paraffin sections of rat pudendal arteries were de-waxed in toluene and rehydrated through reverse graded series of ethanol.
solutions. Sections were then washed in PBS prior to blocking with avidin/biotin blocking solution to reduce background staining. Immunostaining was achieved by incubating sections with a monoclonal antibody against α-smooth muscle actin (1:800 dilution) as the primary antibody at 4°C overnight, washed in PBS, incubated with biotinylated horse anti-mouse secondary antibody (1:200 dilution) for 10 min, washed in PBS, stained with FITC-linked avidin for 5 min, washed in PBS, mounted with VectaShield 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) mounting medium. Photomicrographs were viewed and captured using a Zeiss fluorescence microscope (Axio Imager M1, Canada).

3.2.4 Morphological quantification
Quantification of blood vessels was performed with Image-Pro Plus 6.0 (Media Cybernetics). Wall thickness and lumen diameter of each vessel was measured for every octant and quadrant, respectively. Total wall cross-sectional area (CSA) was measured by manual and automatic edge detection in Image-Pro to produce an area of interest around the vessel wall, and total CSA was calculated. Determination of the proportion of smooth muscle within the CSA of the vessel was performed with an automated algorithm developed for use with Image-Pro which detected smooth muscle cell (SMC) profiles versus extracellular matrix (ECM) using automatic threshold to detect dark and bright objects within the area of interest.
3.2.5 Myograph experiments

In a separate group of 15 (n=12) and 75-80 week old (n=9) male Sprague-Dawley rats, contractile and relaxation properties, and vasomotion, in two distinct areas of the pudendal artery were assessed. Rats were anaesthetized with sodium pentobarbital (60 mg/kg), and pudendal arteries were removed and were cut into 2 mm rings taken from the B segment. These rings were mounted in a wire myograph (Danish Myograph Technology 610M) with 25 µm tungsten wire and bathed in physiological Krebs solution (118mM NaCl, 4.74mM KCl, 2.5mM CaCl₂, 1.18mM MgSO₄, 1.18mM KH₂PO₄, 25mM NaHCO₃, 10mM dextrose) maintained at 37°C and aerated with a mixture of 95% O₂ and 5% CO₂. Vessels were then stretched to an optimal resting tension based on a passive length tension curve of approximately 1.2-1.5mN and equilibrated at the chosen tension for 30 minutes. Vessels were then submaximally contracted with phenylephrine (PE, 0.1 µM) to achieve a stable contractile response (5-10 min). The average generation of force was determined from the integration of all oscillatory contractions over the final 7 minutes of each response. Concentration response curves to PE and acetylcholine (ACh) were performed in order to assess adrenergic contraction and endothelium-dependent relaxation. All recordings were carried out with a DMT myograph, using Chart 5 software and Powerlab acquisition hardware (Model ML866, ADI Instruments, Colorado Springs, CO, USA) sampling at 10 Hz. All compounds and drugs were obtained from Sigma Chemical Co.
3.2.6 Statistical analysis

Erectile response data are expressed as the number of erectile responses per test period ± standard deviation (SD). Statistical analysis of erectile responses between and within treatment groups was performed using the Mann–Whitney rank sum test (P<0.05). Body weight and heart weight are shown as mean ± SD and were assessed by an unpaired Student’s t test (P<0.05). All morphological measurements are presented as the mean ± standard error of the mean (SEM) and analyzed using unpaired Student’s t test (P<0.05). Contractions to PE were examined by measuring the maximum response to each dose. Tissue relaxation by ACh was assessed as the percent relaxation from that originally induced by PE contraction. EC50 values were calculated by non-linear regression analysis in Graph Pad Prism 5. Reported values are mean ± SD, and were compared using Student’s unpaired t-test.

3.3 Results

3.3.1 Overall effects of aging in Sprague-Dawleys rats

As described previously, there was a significant decrease in APO-induced erectile responses in the 77 week old rats compared to the 15 week old rats (Figure 3.2). There were also fewer yawns per testing period in the aged rats. There was a 1.6-fold increase in body weight over time (461g±11.1 to 728g±119.2), and yet there was only a minimal change in the left ventricle in proportion to body weight (LV/BW: young 1.87±0.16 vs old 1.68±0.09).
Figure 3.2  Age-related decrease in apomorphine-induced erectile responses (A) and yawns (B), per 30 min observation period, in 77 week old Sprague-Dawley rats (n=6) compared to their younger counterparts (15 weeks). Data are presented as mean ± standard deviation. *P<0.001 vs 15-16 weeks.
3.3.2 Characterization of the internal pudendal artery

The internal pudendal artery, which branches off from the internal iliac artery, is approximately 3 cm in length with very few branches (3-5), as it is the major vessel supplying all of the erectile tissues (Figures 3.1, 3.3, 3.4). The pudendal artery was found to have an equivalent lumen diameter to a first order branch of the mesenteric bed in young Sprague-Dawley rats, but with a considerably thicker wall (Table 3.1). Thus, it was also determined to have a significantly increased medial CSA. Both vessels had similar proportional content of ECM. Furthermore, there were 7-9 layers of smooth muscle cells (SMC) in the media of the pudendal artery whereas the mesenteric arteries only had 3-5 SMC layers. The orientation and shape of the SMC in the pudendal artery were also different compared to those in the aorta, mesenteric and renal arteries in that they were quite irregular: with elongated SMC as well as many smaller, round cells (Figure 3.5). Immunostaining for smooth muscle α-actin confirmed that the round cells were also SMC (Figure 3.5A). Supporting this finding, nuclear staining with DAPI revealed both elongated and round nuclei indicating that the SMC orientation may be complex, and greatly differ from the medial layer of the other vessels with a predominant circumferential arrangement (Figure 3.5B).

3.3.3 Age-induced morphological changes

The lumen diameter and wall thickness significantly increased with age in the aorta (1.2-fold) and renal arteries (1.6 to 1.9-fold) (Table 3.2), reflecting, in part, the developmental structural changes linked to the 1.6-fold increase in body weight. The age-related
Figure 3.3  Lateral view of the internal pudendal artery (IPA) branching to supply the urethra, corpus spongiosum and the ischiocavernosus and bulbocavernosus muscles and finally dividing to become the dorsal and deep penile arteries. Drawing in collaboration with Lauren Oldfield.
Figure 3.4 Ventral view of the internal pudendal artery (IPA), vein (IPV) and nerve (PN) and their insertions into the penis in the rat. Drawing in collaboration with Lauren Oldfield.
Table 3.1 Morphometric comparison of mesenteric artery and internal pudendal artery of young Sprague-Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>Mesentery</th>
<th>Pudendal</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen (μm)</td>
<td>329±30.7</td>
<td>303±13.8</td>
<td>0.92</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>27.1±3.9</td>
<td>46.9±2.2†</td>
<td>1.73</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>8.2±0.9</td>
<td>15.8±1.1†</td>
<td>1.92</td>
</tr>
<tr>
<td>CSA (x10^3 μm²)</td>
<td>32±5.9</td>
<td>15.8±1.10†</td>
<td>1.64</td>
</tr>
<tr>
<td>ECM (x10^3 μm²)</td>
<td>8.7±1.8</td>
<td>13.4±0.9*</td>
<td>1.53</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>27±1.4</td>
<td>26±0.5</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Values are mean±SEM. CSA=cross-sectional area. ECM=extracellular matrix. n=5 young Sprague-Dawley rats. *p<0.05, †p<0.01 and ‡p<0.001 versus mesentery.
Figure 3.5  Smooth muscle $\alpha$-actin (A, green) and DAPI (B, blue) immunofluorescence in pudendal arteries of a young Sprague-Dawley rat. Light micrographs of glutaraldehyde-fixed, azure methylene blue stained, pudendal arteries in young (C, E; 15 weeks) and old (D,F; 77 weeks) Sprague-Dawley rats. Scale bar is 40µm in length.
Table 3.2  Morphometric analysis of aorta, left and right renal arteries of young and old Sprague-Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>Aorta</th>
<th>Right Renal</th>
<th>Left Renal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td>Fold Change</td>
</tr>
<tr>
<td>Lumen (µm)</td>
<td>1566±40.3</td>
<td>1953±32.7†</td>
<td>1.25</td>
</tr>
<tr>
<td>Wall thickness (µm)</td>
<td>80.6±5.2</td>
<td>96.7±1.6*</td>
<td>1.20</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>5.2±0.4</td>
<td>5.1±0.2</td>
<td>0.98</td>
</tr>
<tr>
<td>CSA (x10³ µm²)</td>
<td>405±26.2</td>
<td>668±34.4‡</td>
<td>1.65</td>
</tr>
<tr>
<td>ECM (x10³ µm²)</td>
<td>8.0±1.1</td>
<td>44.0±8.9*</td>
<td>5.50</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>12±3.5</td>
<td>32±2.6*</td>
<td>2.67</td>
</tr>
</tbody>
</table>

Values are mean±SEM. CSA=cross sectional area. ECM=extracellular matrix. n=5 young Sprague-Dawley rats, 4 old Sprague-Dawley rats. *p<0.05, †p<0.01, ‡p<0.001 versus young Sprague-Dawley rats.
changes in the renal arteries in old Sprague-Dawley rats were significantly greater than those in the aorta. Interestingly, with age, both the wall thickness and the lumen diameter increase proportionately (2-10% change) such that there was no change in the wall-to-lumen ratio (Table 3.2, Figure 3.6A). As expected with these changes, there was also a substantial increase the medial CSA in the aged Sprague-Dawley rats. Further analysis of the percent medial ECM content of the renal arteries, revealed that there was a 2 to 2.5-fold increase with age (Figure 3.6B).

There were no morphological differences detected between the left and right pudendal arteries at either age. Thus, the data were grouped within their respective age groups for further analysis. Although outward vascular remodelling (increase in lumen diameter and cross-sectional area) was evident in both the aorta and renal arteries of the older Sprague-Dawley rats, this type of change did not occur in the pudendal arteries (Table 3.3). That is, since the lumen diameter and wall thickness did not increase proportionately with age in the pudendal arteries, there was a much more marked increase in the wall-to-lumen ratio (1.5-fold) in these vessels than in the others (no change). In fact, the relationship between lumen diameter and wall thickness was 4-fold steeper in the pudendal arteries compared to the other vessels (Figure 3.6A).

Assessment of changes in the amount of ECM in renal and pudendal vessel segments from the young and old rats revealed, in general, that with increasing CSA there is also an
Figure 3.6  A) Comparison of the overall relationship (line of best fit) between wall thickness and lumen diameter in pudendal arteries (squares) and the other vessels (aorta, renal and mesenteric arteries, triangles) in young (15 weeks, open) and old (77 weeks, solid) Sprague-Dawley rats.  B) Relationship between medial extracellular matrix (ECM) and cross-sectional area (CSA) in the pudendal arteries of young (open squares) and old (solid diamonds) Sprague-Dawley rats. The individual symbols represent data from each artery.
### Table 3.3 Morphometric analysis of the internal pudendal artery of young and old Sprague-Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>Segment A</th>
<th>Segment B</th>
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<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td>Fold change</td>
</tr>
<tr>
<td>Lumen (μm)</td>
<td>272±14.7</td>
<td>284±15.9</td>
<td>1.04</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>36.6±2.6</td>
<td>55.5±3.9‡</td>
<td>1.52</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>13.6±0.9</td>
<td>19.7±1.5†</td>
<td>1.45</td>
</tr>
<tr>
<td>CSA (x10³ μm²)</td>
<td>35±3.7</td>
<td>62±6.2</td>
<td>1.77</td>
</tr>
<tr>
<td>ECM (x10³ μm²)</td>
<td>11±1.0</td>
<td>20±1.8‡</td>
<td>1.82</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>30±1.5</td>
<td>32±0.7</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Values are mean±SEM. CSA=cross-sectional area. ECM=extracellular matrix. n=5 young Sprague-Dawley rats, 4 old Sprague-Dawley rats. *p<0.05, †p<0.01, ‡p<0.001 vs young Sprague-Dawley rats.
increase in the amount ECM (Figure 3.6B, Tables 3.2 and 3.3). However, whereas in the pudendal arteries the percent ECM increased proportionally to the change in CSA with age, in the renal tissue there was much less ECM relative CSA to start with, but then was increased (6 to 9-fold) in the older rats. That is, although there was an increase in the CSA in all vessels with age, in the renal arteries, there was a nearly 3-fold increase in the relative amount of ECM per CSA (∼13% up to ∼30%). In other words, the renal vessel tissue composition in the older rats had “caught up” to the tissue composition of the pudendal arteries. That is, the amount of ECM per cross-sectional area in the pudendal arteries was already close to 30% in the segments from the young animals.

A general examination of the photomicrographs indicated that in the media of the pudendal arteries from old rats there were a greater number of SMC that were round rather than elongated (Figure 3.5). Further, these round cells were found to be distributed closer to the medial-intimal border whereas the elongated SMC appeared to be located closer to the advential layer. A detailed analysis of this phenotype was considered to be beyond the scope of the present study, but could be of interest.

3.3.4 Physiological assessment of young and old pudendal arteries

Assessment of the PE concentration response relationship in 15 and 75-80 week old Sprague-Dawley rats revealed that there was a rightward shift in the curve indicating the older animals were less sensitive to the $\alpha_1$ agonist (EC50: Young 1.4±1.2 µM; Old 3.8±1.8 µM, p<0.001) but no differences in maximum contractile responses
(19.7±6.4 mN vs 23.1±5.7 mN) (Figure 3.7A). An ACh concentration response relationship was obtained in only some of the preparations because of the interfering oscillatory activity.

Thus, the characterization of this relationship could only be performed in preparations in which the onset of oscillatory activity was delayed (i.e. 15 week old rats) or did not occur. In vascular segments from 75-80 week old rats, oscillations occurred immediately following contraction (therefore no ACh response could be obtained) or were absent. These relaxation responses were markedly decreased in vessels from old compared to young animals (Figure 3.7B). In non-oscillating vessels from the 15 week old rats (n=8), the average ACh relaxation response was 70% whereas in 75-80 week old rats the pudendal arteries relaxed only 30% of the PE contractile response. There were no differences in the EC50 of the acetylcholine-mediated relaxations between the young and old vessels (2.4±3.0 µM vs 7.2±0.2 µM, p=0.4). Under PE-induced contraction, the pudendal artery begins to spontaneously oscillate in very slow, high amplitude waves (Figure 3.8). In young animals high amplitude activity was evident and the waves returned to baseline in all preparations. In contrast, the oscillations were more heterogeneous in aged animals as only 4 of the 9 animals examined had high amplitude, slow frequency waves present in left and right pudendal arteries (Figure 3.9). The amplitude of the oscillations varied between preparations from 3 to 30mM of tension and the waves did not return back to baseline.
Figure 3.7  (A) Phenylephrine (PE) and (B) acetylcholine (ACh) concentration response curves in young and old pudendal arteries from Sprague-Dawley rats. *P<0.05 vs young pudendal artery.
Figure 3.8 Representative tracings of phenylephrine-induced oscillations in the middle segment of the pudendal artery in young Sprague-Dawley rats. The line represents 3 minutes.
Figure 3.9 Representative tracings of phenylephrine-induced oscillations in the middle segment of the pudendal artery in old Sprague-Dawley rats. The line represents 3 minutes.
3.4 Discussion

The present study is the first to characterize the internal pudendal arteries in the male rat anatomically, histologically, morphometrically and functionally. Based on previous assessments of vascular resistance in this bed, these vessels are critical to the blood supply of all of the erectile tissues. The present study confirmed an age-related decrease in erectile responses but provided novel findings that potentially linked this decline in function with extensive morphological changes in this key artery. Specifically, there were marked increases in wall thickness, CSA and ECM as well as phenotypic changes in the SMC population from an elongated to a more rounded shape. The ECM in renal arteries in young and old animals was found to increase from 12 to 32% of the CSA. On the contrary, smaller increases (26-34%) were apparent in the pudendal artery, as these vessels already had a higher percentage of ECM compared to renal arteries at a young age. The increases in wall thickness, CSA and ECM in aged aorta and renal arteries, impact structurally-based vascular resistance since the lumen diameter expanded proportionately with body weight gains in the aged rats. In contrast, pudendal artery function was markedly reduced during aging. That is, along with the morphological changes and a lack of an increase in the lumen diameter, significant physiological changes in the oscillatory activity and endothelium-dependent relaxation were evident in aged pudendal vessels.

The gross anatomy of the pudendal artery in the rat was confirmed in the present study, as previously described. In fact, the arterial supply of penile tissue in the rat and
the human via the internal pudendal artery were found to be quite similar\textsuperscript{259,260}. In both humans and rats, the internal pudendal artery branches off the internal iliac (hypogastric) artery and travels towards the penis along the lateral wall of the ischiorectal fossa. There is significant variation in the branching of the pudendal artery from the internal iliac artery in the rat and human. Specifically in humans, the inferior gluteal artery can branch independently (50\% of cases) or directly from the pudendal artery while in rats the inferior gluteal artery branches off the pudendal artery as it passes between the flexor cauda brevis and abductor cauda internus muscles\textsuperscript{251,259}. The artery travels along the ischiorectal fossa with the accompanying nerve in a protective fascia and the pudendal vein runs adjacent to them. Similarly in rats and humans, the pudendal artery branches to supply the urethra and the urethral bulb, the ischiocavernosus and bulbocavernosus muscles, and then becomes the deep penile artery\textsuperscript{251,259}. The extent of these similarities indicates that the rat can be considered a useful model in the study of erectile function and dysfunction.

Apart from the anatomical evidence regarding the primacy of the pudendal artery, there is also significant physiological evidence. As previously mentioned, this artery is responsible for more than 70\% of the total penile vascular resistance\textsuperscript{158}. Clinically, in a subgroup of patients with vasculogenic ED, evidence of arterial lesions in the pudendal arteries was present in 53\% of men\textsuperscript{261}. Unilateral or bilateral occlusion of the internal iliac or pudendal arteries in dogs and rats generates arteriogenic ED. In the canine model, acute bilateral occlusion of the pudendal arteries caused a 50\% decrease in blood
flow to the cavernosal arteries and a 60% decrease in intracavernosal pressures 159. Similarly, in arteriogenic rat models, the maximum intracavernosal pressures induced by nerve stimulation after chronic unilateral ligation of the internal iliac artery significantly decreased 50% and bilateral ligation caused a further 75% decrease in intracavernosal pressure 160, 262. Histological examination of the cavernous tissue in these rats showed collapse of sinusoids, increased cell debris, intracellular deposition of fat and collagen, and decreased nNOS and smooth muscle cell α-actin 160, 262. Thus, not only is the blood flow carried by the pudendal artery important in eliciting an erectile response, it is also critical in maintaining the integrity of the cavernosal tissue. Although these studies examined the consequences of internal iliac artery occlusion on erectile responses, they did not assess structure or function of the downstream segments of the pudendal artery.

Morphological examination of the pudendal artery revealed a vessel with a small lumen but with a very thick smooth muscle wall. Although the pudendal artery was found to have a lumen diameter equivalent to a first order mesenteric branch, the wall thickness was found to be more comparable to a renal artery that has a 2-fold larger lumen. Functionally, the pudendal artery is normally under low blood flow conditions until an erectile response is initiated; for example, in dogs, blood flow during an erection increases up to 8-times the basal rate 159. This considerable increase in blood flow is required to allow for rapid filling of the cavernous spaces, thereby creating venocclusion and a rigid erection. In rats implanted with intracavernosal telemetric devices, intracavernosal pressures recorded during copulation have been recorded above 600-700
mmHg during intromissions and ejaculations. As the primary feeder vessel, our novel findings reveal that the pudendal artery’s thick smooth muscle medial layer is able to withstand these marked increases in wall tension that occur during an erectile response.

Similar to previous studies, severely impaired erectile function was evident in aged animals, a finding that was consistent with the marked changes in pudendal vascular structure. Age-related structural changes are well documented and the current study confirms that with age there is an increase in lumen diameter, media thickness and CSA of most vessels. Although the mechanistic basis of the increased medial area has not been elucidated, it has been well documented that, with age, there is a greater number of smooth muscle cell layers, an increase in collagen content and deposition, and hypertrophy of SMC. In the penile vascular bed, aging has been associated previously with significant changes in the penile ultrastructure, with reductions in the elastic fibers of the tunica albuginea, increasing collagen and decreasing SMC, increasing sinusoidal spaces of the corpus cavernosum. Similar results are apparent in the dorsal penile artery, in that aging leads to a decrease in the SMC to collagen ratio, an increase in reactive oxygen species, and apoptosis, although morphometric changes in overall vascular structure have not been described. The vascular structure of the pudendal artery was found to be markedly altered with age. Paralleling trends in the aorta and renal arteries, there was an increase in wall thickness, CSA and ECM content in the aging normotensive Sprague-Dawley rats, but there was no change in lumen diameter. The lack of increase in pudendal lumen diameter, while other vessels have expanded
appropriately, suggests that problems in distribution of blood flow to this high resistance bed are escalating with advancing age. In addition, the expansion of the medial layer has important implications to the control of vessel tone, as the level of vasoconstrictor stimulus would cause greater functional encroachment and thereby impact on delivery of blood to the penile tissue. As previously discussed, decreased blood flow alone could lead to ED due to insufficient perfusion pressure of tissue such that these would be progressive pathological changes to the cavernous tissue 159, 273, 274.

The present findings have revealed there is unequivocal structural reorganization of the medial layer of the pudendal artery, whereas in young animals, the SMC appear phenotypically homogeneous with a spindle-shaped appearance and fewer small round-shaped cells. In contrast, in the aged animals, there appears to be considerably more small rounded cells. The majority of these round SMC are located towards the intimal side of the media. Similar phenotypic cell shifts in SMC sizes have previously been observed in carotid arteries that have undergone balloon injury 275, 276. The SMC in the innermost part of the media assume a round-shaped synthetic phenotype with a loss of myofilaments and increased endoplasmic reticulum and Golgi complex. These cells have been shown to be capable of migrating through fine openings in the internal elastic lamina to create a growing neointima by proliferating and secreting ECM components 276. The spindle-shaped cells are in the contractile state with a higher amount of α-actin, do not have migratory or proliferation properties and are able to contract and relax 275. Aging has been shown to enhance neointimal formation after arterial injury 277, 278. Thus,
it may be that the round cells seen in the pudendal artery of aged animals are synthetic
cells that are migrating (or are ready to migrate) toward the intima, ready to proliferate
and generate a new ECM. The changes to this critical vessel would significantly impact
its ability to deliver blood to the penile tissue. Although the phenotype of these cells
needs to be confirmed via electron microscopy studies, this study would be novel in
demonstrating age-related occurrence of this type of SMC, i.e. arising spontaneously and
not due to mechanical or pharmacological insult.

Many pathological changes take place during aging which contribute to structural and
physiological alterations to the vasculature which can impact erectile function.
Interestingly, although the pudendal artery in the aged rats has marked increases in wall
thickness there were no differences in overall contractile ability induced by the $\alpha_1$
adrenoceptor agonist PE. Further studies are required to see if age alters the contractile
response to vasoactive substances such as endothelin-1, angiotensin II and
catecholamines, for example, acting via Rho-kinase and other signalling systems. This is
likely as the activation of vasoconstrictor responses has been shown to be altered with
age, and thereby play a role altering the erectile response $^{117,266}$. In addition, many
studies have demonstrated that aging leads to endothelial dysfunction, in the systemic
vasculature and in penile vascular tissue $^{279,280}$. Endothelial nitric oxide (NO) production
is a critical factor in erectile physiology of the penis and is known to be reduced during
aging $^{69,279,281}$. Likely as a compensatory mechanism, endothelial NO synthase (NOS)
has been found to be expressed to a greater extent in aged vascular tissues $^{282}$. However,
despite the upregulation, NOS is often found to be less effective due to decreased phosphorylation at its regulatory site, via uncoupling of the enzyme as a result of elevated concentrations of reactive oxygen species (ROS), and from decreased NO bioavailability. Further, inducible NOS has been shown to be upregulated, possibly as an adaptive response in attempt to counteract the fibrosis in cavernous SMC. One proposed mechanism is that increased NO production will scavenge reactive oxygen species and thereby inhibit excessive collagen deposition. A shift induced during aging on this regulatory pathway controlling vascular development could further impair blood flow within the pudendal artery.

The pudendal artery was found to exhibit distinctive spontaneous oscillatory activity (vasomotion) when pre-contracted with a $\alpha_1$ agonist. These contractile waves were similar to those previously seen in the tail arteries of stroke prone SHR, although the pudendal artery exhibited oscillations with a greater amplitude and slower frequency. It has been proposed that spontaneous oscillations occur as a consequence of the coordinated activity of the smooth muscle cells in the vessel wall. Some proposed mechanisms for these coordinated oscillations include entrainment of the smooth muscle cells via gap junction coupling, as well as regulation by Na+, K+-ATPase, at the level of the membrane potential. Other studies have also shown that vasomotion has an endothelium dependent component, driven by the activity of the endothelial cell sarcoplasmic reticulum Ca\(^{2+}\) pump. Some of the hypothesized benefits of vasomotion include avoidance of the “latch state” to provide increased vascular
responsiveness and greater conductance of blood flow for a given average lumen diameter. Maintaining a dynamic state of vascular control allows for a system that responds quickly to change and ensures optimal tissue oxygenation, a factor which might be particularly important since overall perfusion is limited in the pudendal artery.

The present studies showed that the oscillatory activity in the pudendal arteries of the old rats was more irregular with respect to amplitude and frequency compared to vessel segments from the younger counterparts. Given the finding of decreased endothelial function in the vessels from the older rats, it may be that this aspect of vascular dysfunction plays an important role in the altered vasomotion properties. Whether, these age-related changes are linked to other aspects of vessel phenotype will require further study. Regardless, the aged pudendal artery clearly has an altered phenotype both structurally and functionally such that it would no longer be as dynamic in reacting to the large changes in blood flow and wall tension during an erectile response.

Finally, it appears that the vasculature supplying the penis and the penile bed are more susceptible to injury and show pathological changes prior to other vascular beds. This is evident in the significant amount of literature indicating that ED is often a precursor to underlying cardiovascular disease and a predictor of its severity. As found in this study, aging caused a greater impact on this portion of the vasculature compared to the aorta and renal arteries. Thus, it appears the pudendal artery undergoes “rapid aging” compared to other vessels, likely as a consequence of the episodic hemodynamic load.
that these small diameter vessels have to withstand. There is no doubt that this vascular
bed is very distinctive because of the episodic nature of its circulation; that is, this tissue
normally receives minimal blood flow, has minimal metabolic autoregulation, and only
has increased perfusion during erectile responses. In contrast, the aorta, renal arteries and
others are subjected to a much more consistent load. As described earlier, penile tissue
experiences wide ranging flow and pressure conditions, and these attributes are likely to
underpin the increased susceptibility of this vascular bed to damage.

In summary, the histological, morphometric and functional findings regarding the
puberal artery demonstrate that it is a distinctive vessel structured to withstand the
episodic increases in blood flow and pressure that occur during an erectile response. It
appears that aging significantly alters the puberal structure in a manner that would
impact negatively on the vascular responses. The data show unequivocally that
physiological function and morphological phenotype of this vessel are compromised with
age, such that the outcome of impaired ability to vasodilate would be anticipated, and
thereby would significantly impinge on the delivery of blood to the penile tissue.
Chapter 4:

Impact of hypertension, aging and antihypertensive treatment on the morphology of the pudendal artery

4.1 Introduction

Hypertension is a multifactorial disease that has a number of confounders including genetic inheritance, stress as well as environmental and lifestyle factors.\textsuperscript{288-290} It is a leading global health risk and an independent risk factor for coronary artery disease, diabetes, stroke, cardiac and renal failure and erectile dysfunction (ED)\textsuperscript{291-295}. Erectile dysfunction impacts over 30 million men in North America between the ages of 40-70 and significantly affects the quality of life of men and their partners\textsuperscript{4,239}. Recent studies have suggested that 67-68\% of hypertensive males have some degree of ED and hypertension is one of the most common co-morbidities in patients with ED\textsuperscript{3,112,296,297}.

It is not surprising that hypertension and ED are closely linked as they share many common risk factors such as age, smoking, alcohol, physical inactivity, and obesity\textsuperscript{106,296,298}. Further, it is well known that a common cause of ED is arterial insufficiency. In hypertension, alterations in vascular structure occur as a result of medial thickening and a decrease in lumen diameter. These changes are known to decrease the blood flow required for erectile function at a given arterial pressure\textsuperscript{115,120,121}. In fact, recent studies have shown that symptoms of ED are evident 3-5 years prior to the first diagnosis of other cardiovascular disease\textsuperscript{33,37}. Thus, the incidence of ED is a harbinger of future events in an otherwise asymptomatic individual.

As previously mentioned, the majority of previous studies have focused on the pathological changes within the penile tissue, particularly alterations in the corpus
cavernosum. With hypertension, it has been shown that there is increased collagen deposition, endothelial dysfunction and altered structure of the corpus cavernosum and the intrapenile feeder vessels 89, 120, 122. However, the intrapenile vasculature contributes less than 25% of the total vascular resistance of this bed, whereas the bilateral internal pudendal arteries contribute 70% 158. Previous hemodynamic studies from our lab, using an approach which isolates and perfuses the arteries supplying the erectile tissue, have demonstrated in hypertensive animals that the penile vasculature is structurally upregulated in a similar manner to other vascular beds 121. Despite this finding, direct morphological evidence specific to the pudendal artery has not yet been reported. One hypothesis is that in aged hypertensive rats additional accumulative damage to the pudendal vasculature will accelerate the deterioration in erectile function.

The actions of certain antihypertensive agents have complicated the understanding of the link between hypertension and erectile function; that is, some medications induce ED 46, 165, 299. Even so, other studies have revealed that the mechanism of action is important, since some antihypertensive treatments have been found to improve erectile function in hypertensive rats and in humans 170, 171, 174, 203, 205. In particular, drugs that antagonize the renin-angiotensin system are associated with improved endothelial function and decreased type III collagen in corpora cavernosum, as well as improved erectile function in hypertensive male rats and vasocongestive arousal responses in female rats 176, 177, 205, 300. For example, treatment with the ACE inhibitor, enalapril combined with a low salt diet, produced a decrease in vascular resistance of the isolated, perfused penile
vasculature by greater than 20% in SHR. Despite this functional evidence, the impact of these changes on the morphology of the pudendal artery remains to be determined.

In the previous chapter, erectile function and morphometric properties of the internal pudendal artery were examined in young and old Sprague-Dawley rats. Morphological and physiological assessment revealed a resistance artery with a thick medial layer and distinctive spontaneous, oscillatory contractile properties. Further examination of the pudendal vasculature in aged animals (with ED) also showed that there were marked structural changes that would very likely impact on vascular resistance and thereby impair blood flow. The present investigation used a similar methodology to assess the impact of aging and hypertension on the structure of the pudendal artery. The frequency of erectile responses was assessed by administration of the centrally acting dopaminergic agonist, apomorphine, and the left and right internal pudendal arteries, first-order branches of the mesenteric bed, renal arteries and aorta were characterized morphologically in 15 week old WKY and SHR and in 77 week old SHR. In addition, the impact of brief, aggressive antihypertensive treatment on the vascular structure of the pudendal artery in young SHR was also examined.

4.2 Methods

4.2.1 Animals

Male Wistar Kyoto (WKY, n=5) and spontaneously hypertensive rats (SHR, n=12) were purchased at 12 weeks of age (Charles River Laboratories, Montreal, Quebec, Canada)
and housed individually at a temperature of 22–24°C with a 12-h light/dark cycle. All rats were provided with regular rodent chow (LabDiet® 5001, Ren’s Feed and Supply Ltd, Oakville, Ontario, Canada) and tap water *ad libitum*. All procedures were approved by the Queen’s University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

4.2.2 Assessment of erectile response

Erectile function was assessed with the rat bio-assay of centrally-induced erections which uses the dopaminergic agonist, apomorphine (APO) 220. Specifically, prior to (20 min acclimatization period) and during testing, rats were placed in one of four individual, hanging cages (with clear Plexiglas inserts used to replace the wire floor) in an isolated, quiet, dimly lit room adjacent to the holding room. Each rat received subcutaneous injection of APO in saline (80 µg/kg with 100 µg/ml ascorbic acid, 1 ml/kg) in the loose skin of the neck or back. Erections and yawns were counted over a 30-min period via videomonitoring in an adjacent room. Erectile responses were recorded following identification of characteristic physical and behavioural responses including concave arching of the back, pelvic thrusts followed by the emergence of the engorged glans penis and shaft, and immediate oral grooming of the genital area. APO-induced yawns were recorded as an index of drug delivery and bioavailability within the central nervous system 222.
4.2.3 Tissue preparation and morphometry

At 15 and 77 weeks of age, animals were anaesthetized with sodium pentobarbital (65mg/kg, intraperitoneally) followed by intravenous doses of the ganglionic blocker, hexamethonium, and heparin (30mg/kg and 1000 units/kg, Sigma Aldrich Chemical Co., St Louis, Missouri, USA) to allow for perfusion under maximally relaxed conditions. The heart was excised and animals were perfused at 70mmHg via the thoracic aorta with saline (0.9% Na+, Baxter Corp., Mississauga, On, Canada) containing sodium nitroprusside (300mg/L, Sigma Aldrich Co.) to flush out the blood. The animals were then perfusion fixed with 2% paraformaldehyde, 2% gluteraldehyde, 4% sucrose, and 0.05% calcium chloride in a 0.1M cacodylate buffer. Segments of the thoracic aorta, first-order mesenteric branches, right and left renal and pudendal arteries (specific sample sites shown in Figure 3.1) were excised and stored in fixative overnight.

Subsequently, tissues were rinsed in 5.4% sucrose in 0.1 M cacodylate buffer for 1 hr, post-fixed 0.1% osmium tetroxide in cacodylate buffer for 1 hr, rinsed in cacodylate buffer, stained en bloc with aqueous 4% uranyl acetate for 1 hour, dehydrated through a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon 812 for sectioning. Sections were cut at 1 μm thick with a glass knife and stained with 0.1% azure methylene blue and catalogued according to orientation. Photomicrographs were captured using a Zeiss microscope (Axio Imager M1, Canada). For electron microscopy, ultrathin (70-90 nm) sections were cut with diamond knife using LKB ultramicrotome (LKB Instruments Inc., Gaithersburg, MD), placed on formvar-carbon
coated slotted copper, stained en face with uranyl acetate and lead citrate, viewed under electron microscope (Hitachi EM-7000), and photographed.

4.2.4 Antihypertensive treatment intervention
The antihypertensive treatments were performed in a group of young male SHR with telemetric recording (n=5) and a separate group for morphological assessment (n=7). At 12 weeks of age, SHR were implanted with radio-telemetric devices and allowed to recover for 2 weeks. Both groups of rats were separated into control (CON) and treated (TX) groups (telemetry rats: CON n=2, TX n=3; morphology rats: CON n=4, TX n=3). At 15 weeks of age, TX SHR were treated for 2 weeks with enalapril and hydrochlorothiazide (30mg/kg per day, Sigma Aldrich Co.) in their drinking water and a parallel control group that did not receive treatment. Changes in mean arterial pressure (MAP, mmHg) were determined from collected data (15 sec every 5 minutes, at 150 Hz). Two weeks after treatment was withdrawn, the morphological group of SHR were anaesthetized, perfused, and aorta, renal, mesenteric and pudendal arteries were excised and processed for Epon embedding as previously described. Again, sections were cut at 1 µm thick, stained with 1% methylene blue and azure and photographed. Prior to perfusion, the hearts were removed and the right and left ventricles were separated and weighed.
4.2.5 Morphological quantification

Quantification of blood vessels was performed with Image-Pro Plus 6.0 (Media Cybernetics). Wall thickness and lumen diameter of the vessels were measured for every octant and quadrant respectively. Total wall cross-sectional area (CSA) was measured by manual and automatic edge detection in Image-Pro to produce an area of interest that circumscribed both inner and outer aspects of the vessel wall, and total CSA was calculated. Determination of the proportion of smooth muscle within the CSA of the vessel was performed with an automated algorithm using Image-Pro tools which allowed for detection of individual smooth muscle cell (SMC) profiles versus extracellular matrix (ECM) using automatic threshold to detect dark and bright objects within the area of interest.

4.2.6 Statistical analysis

Erectile response data are expressed as the number of erectile responses per test period ± standard deviation (SD). Statistical analysis of erectile responses between and within treatment groups was performed using both the Mann–Whitney rank sum test and unpaired Student’s t test (P<0.05). Body and heart weight are shown as mean ± SD and were assessed by an unpaired Student’s t test (P<0.05). All morphological measurements are presented as the mean ± standard error of the mean (SEM) and analyzed using unpaired Student’s t test (P<0.05).
4.3 Results

4.3.1 Impact of aging and hypertension on erectile function

There were no differences in the number of apomorphine-induced erections and yawns between young WKY and young SHR (Figure 4.1). In the older SHR there was a significant decrease in erectile responses and yawns compared to their younger counterparts similar to the results in older normotensive rats in Chapter 2. In old SHR, body weight was increased compared to the young WKY and SHR (Table 4.1). The young SHR had an 18% larger LV/BW compared to the young WKY and in the older SHR the magnitude of the left ventricular hypertrophy was 24% greater than in young SHR. In contrast, there were no between strain or age differences in the RV/BW ratio.

4.3.2 Impact of hypertension on the internal pudendal artery

Comparison of the middle (B) segment of the internal pudendal arteries in young WKY and SHR revealed no significant differences in the lumen diameters, but the wall thickness was increased more than 20% in the SHR (Table 4.2). Similar to the findings in young Sprague-Dawley rats, in general, the pudendal arteries in the WKY had a media consisted of 6-8 layers of smooth muscle cells. In contrast, the medial layer in the sections from young SHR contained 8-10 layers of smooth muscle cells. These alterations in the hypertensive rats increased the wall-to-lumen ratio by 17% and the cross sectional area of the vessels by 32%. Assessment of the ECM composition indicated that the changes in CSA were not due to increased ECM content, as this component was proportionally similar in the young WKY and SHR.
Figure 4.1  No difference in apomorphine-induced erectile responses (A) or yawns (B), per 30 min observation period, between young Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). With age, there is a decrease in erections (A) and yawns (B) in 77 week old SHRs (n=6) compared to their younger counterparts (15 weeks). Data are presented as mean ± standard deviation. *P<0.01 vs Young WKY, Young SHR.
Table 4.1  Body and heart weights of young and old WKY and SHR.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>LV/BW (g/kg)</th>
<th>RV/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young WKY</td>
<td>312±9.1</td>
<td>2.26±0.10</td>
<td>0.61±0.08</td>
</tr>
<tr>
<td>Young SHR</td>
<td>325±23.4</td>
<td>2.68±0.09†</td>
<td>0.65±0.13</td>
</tr>
<tr>
<td>Old SHR</td>
<td>380±7.5*†</td>
<td>3.34±0.10*†</td>
<td>0.71±0.14</td>
</tr>
</tbody>
</table>

Values are mean±SD.  n=3 young WKY, 5 young SHR, 3 old SHR. *p<0.01 versus young SHR, †p<0.001 versus young WKY.
Table 4.2  Morphometric comparison of internal pudendal artery of young WKY and SHR.

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen (μm)</td>
<td>294±8.5</td>
<td>277±6.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>30.9±1.2</td>
<td>37.8±1.1‡</td>
<td>1.23</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>10.5±0.3</td>
<td>13.8±0.5*</td>
<td>1.32</td>
</tr>
<tr>
<td>CSA (x10^3 μm^2)</td>
<td>32±1.9</td>
<td>37±1.4‡</td>
<td>1.17</td>
</tr>
<tr>
<td>ECM (x10^3 μm^2)</td>
<td>8.8±0.8</td>
<td>9.1±0.4</td>
<td>1.04</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>26.4±0.1</td>
<td>25.1±0.1</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Values are mean±SEM.  CSA=cross sectional area.  ECM=extracellular matrix.  n=3 young WKY, 5 young SHR.  *p<0.0, ‡p<0.001 versus young WKY.
Further studies are required to determine whether the increased smooth muscle content is due to hyperplasia or cellular hypertrophy.

The differences between the pudendal artery and the first order branch of the mesenteric bed in the SHR were analogous to those described in the Sprague-Dawley rats in Chapter 3. That is, the pudendal artery had only a slightly smaller lumen diameter (-10%) compared to the mesenteric arterial bed in young SHR, but a markedly increased (+74%) wall thickness (Table 4.3). The CSA was also greater in the pudendal artery compared to the mesenteric vessel, although there was a similar proportional amount of ECM.

4.3.3 Morphometric changes in the vasculature with age

In SHR, there was an age-related increase in the lumen diameter and wall thickness of the aorta and renal arteries (Table 4.4). The magnitude of these age-related changes in lumen diameter were less (-45% in aorta, -70-85% in renal arteries) than those seen in the older Sprague-Dawley rats (Chapter 3). With age, the sedentary Sprague-Dawley rats had a marked increase in body weight of ~1.7-fold. In contrast, the SHR are a smaller strain of rat and over a similar age span the increase their body weight was less than 20% (Table 4.1). As previously established, in older Sprague-Dawley rats, wall thickness (1.2 to 1.9-fold increase) and lumen diameter (1.2 to 1.8-fold increase) of the aorta and renal vessels increased proportionately in both vessels, such that there was no change in the wall-to-lumen ratio (Figure 3.6A). In contrast, but consistent with the smaller overall increase in body weight in the older SHR (Table 4.5), there were much smaller increases in lumen
Table 4.3  Morphometric comparison of mesenteric artery and internal pudendal artery of young SHR.

<table>
<thead>
<tr>
<th></th>
<th>Mesentery</th>
<th>Pudendal</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen (μm)</td>
<td>314±14.5</td>
<td>282±7.0*</td>
<td>0.90</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>22.0±0.9</td>
<td>38.8±1.5‡</td>
<td>1.74</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>7.0±0.1</td>
<td>13.9±0.7‡</td>
<td>1.99</td>
</tr>
<tr>
<td>CSA (x10³ μm²)</td>
<td>23±1.7</td>
<td>39±1.7‡</td>
<td>1.70</td>
</tr>
<tr>
<td>ECM (x10³ μm²)</td>
<td>5.5±1.0</td>
<td>13.4±0.9†</td>
<td>2.44</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>24.2±0.1</td>
<td>25.1±0.1</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Values are mean±SEM. CSA=cross sectional area. ECM=extracellular matrix. n=5 young SHR. *p<0.05, †p<0.01 and ‡p<0.001 versus mesentery.
Table 4.4 Morphometric analysis of aorta, left and right renal arteries of young and old SHR.

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta</td>
<td></td>
<td>Right Renal</td>
<td></td>
<td>Left Renal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td>Fold Change</td>
<td>Young</td>
<td>Old</td>
<td>Fold Change</td>
<td>Young</td>
<td>Old</td>
</tr>
<tr>
<td>Lumen (μm)</td>
<td>1609±47.9</td>
<td>1830±32.7*</td>
<td>1.14</td>
<td>688±34.3</td>
<td>842±112</td>
<td>1.22</td>
<td>641±13.7</td>
<td>710±81.4</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>76.95±2.2</td>
<td>110.3±0.7†</td>
<td>1.43</td>
<td>36.4±1.8</td>
<td>65.1±5.6‡</td>
<td>1.79</td>
<td>35.1±1.9</td>
<td>58.7±4.9†</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>4.8±0.2</td>
<td>6.0±0.1†</td>
<td>1.25</td>
<td>5.3±0.1</td>
<td>8.0±1.4*</td>
<td>1.51</td>
<td>5.5±0.3</td>
<td>8.3±0.7†</td>
</tr>
<tr>
<td>CSA (x10³ μm²)</td>
<td>382±15.6</td>
<td>734±48.2†</td>
<td>1.92</td>
<td>84±7.5</td>
<td>193±21.8‡</td>
<td>2.30</td>
<td>77±4.8</td>
<td>136±22.1†</td>
</tr>
<tr>
<td>ECM (x10³ μm²)</td>
<td>12±9.3</td>
<td>74±13.9‡</td>
<td>6.17</td>
<td>18±1.7</td>
<td>39±14.0†</td>
<td>2.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>13.7±0.1</td>
<td>35.5±0.1*</td>
<td>2.59</td>
<td>23.5±0.1</td>
<td>25.6±0.1</td>
<td>1.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM. CSA=cross-sectional area. ECM=extracellular matrix. n=5 young SHR, 3 old SHR. *p<0.05, †p<0.01, ‡p<0.001 vs young SHR.
Table 4.5  Morphometric analysis of sections of the internal pudendal artery of young and old SHR.

<table>
<thead>
<tr>
<th></th>
<th>Proximal</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
</tr>
<tr>
<td>Lumen (μm)</td>
<td>272±11.3</td>
<td>228±17.6</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>36.9±1.7</td>
<td>77.3±7.1‡</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>13.7±0.8</td>
<td>37.2±7.8‡</td>
</tr>
<tr>
<td>CSA (x10³ μm²)</td>
<td>36±2.3</td>
<td>73±5.5‡</td>
</tr>
<tr>
<td>ECM (x10³ μm²)</td>
<td>8.5±1.6</td>
<td>25.9±1.9‡</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>24.2±1.7</td>
<td>34.8±2.2†</td>
</tr>
</tbody>
</table>

Values are mean±SEM.  CSA=cross sectional area.  ECM=extra cellular matrix.  n=5 young SHR, 3 old SHR. *p<0.05, †p<0.01, ‡p<0.001 versus young SHR.
diameter (1.1 to 1.2 fold increases) and greater increase in the medial wall thickness (1.4 to 1.8 fold increases) in all vessels assessed. These structural changes generated marked increases (1.3 to 1.5-fold) in the wall-to-lumen ratio of these older vessels in SHR. Similar to the results in the aging Sprague-Dawley rats, assessment of the relative SMC and ECM content in the medial layers of the renal arteries revealed increases in the proportion of ECM versus CSA with age in the renal artery up to 2.6-fold (Table 4.4).

There were no morphological differences between the left and right pudendal arteries of young or old SHR; therefore, they were grouped for further analysis. The overall orientation and shape of SMC in young SHR did not appear to differ from those in the young Sprague-Dawley rats (Figure 3.5 & 4.2). However, in the pudendal artery sections from the aged SHR, the SMC were disorganized similarly to the older normotensive vessels previously examined. An additional difference was that the pudendal vessels from the old SHR also had marked intimal thickening (Figure 4.2). The consequence of this luminal encroachment was that a 10-15% decrease in lumen diameter was evident in both A and B segments of the pudendal artery (Table 4.5). Furthermore, there was a doubling in wall thickness and CSA of the pudendal artery sections in old SHR. Thus, unlike the aorta, renal and mesenteric arteries, the lumen diameter of the pudendal arteries in SHR did not grow proportionally with age (Figure 4.3A). This altered relationship is reflected in the extreme increase in the wall-to-lumen ratio and the steep and negative slope of the relationship found for the pudendal arteries.
Figure 4.2  Light micrographs of gluteraldehyde-fixed, azure methylene blue stained, pudendal arteries in young (15 weeks) and old (77 weeks) spontaneously hypertensive rats. Scale bar is 40µm in length.
Figure 4.3 A) Comparison of the overall relationship (line of best fit) between wall thickness and lumen diameter in pudendal arteries (squares, $R^2=0.41$) and the other vessels (aorta, renal and mesenteric arteries, triangles, $R^2=0.75$) in young (15 weeks, open) and old (77 weeks, solid) SHR. B) Relationship between medial extracellular matrix (ECM) and cross sectional area (CSA) in pudendal arteries of young (open diamonds, $R^2=0.38$) and old (solid squares, $R^2=0.89$) SHR. The individual symbols represent data from each artery.
Assessment of the relationship between CSA and ECM for young versus old SHR (Figure 4.3B) demonstrated that in the old vessels ECM accumulated more (2.5-fold) for a given CSA than it did in the younger tissues. In addition, similar to the change with age in pudendal arteries from Sprague-Dawley rats, in older tissues there were more rounded SMC as opposed to the elongated SMC in old SHR. Similar to the sections from the Sprague-Dawley rats (Chapter 3), these round cells were orientated close to the medial-intimal border and within the intimal layer whereas the elongated SMC were closer to the adventitial layer.

4.3.4 Electron microscopy

The characteristics of SMC from young and old pudendal arteries were markedly different. In young SHR, the SMC in the media had a definitive contractile phenotype, with elongated nuclei and overall shape, and with filaments running parallel with the longitudinal axis of the cell (Figure 4.4). In these differentiated vascular SMC, the mitochondria, Golgi complex and endoplasmic reticulum were located near the nucleus and were a minor overall component of the cell compared to the dominant proportion occupied by filaments (Figure 4.4D). In contrast, many of the vascular SMC in both the media and the neointima of the pudendal sections from the older SHR were both round and showed clear signs of a more synthetic phenotype. In particular, the round cells had a noticeable increase in endoplasmic reticulum, mitochondria, Golgi complexes and well developed vesicles, and had fewer actin filaments near the cell’s edges (Figures 4.5 & 4.6).
Figure 4.4  Electron micrographs of typical smooth muscle cells in the contractile state located near the luminal border of the pudendal artery in young SHR. (A=x4000, B=x3000, C=x6000, D=x15000) N, nucleus; IEL, internal elastic lamina; EC, endothelial cell; F, filaments; M, mitochondria; ER, endoplasmic reticulum; G, Golgi complex.
Figure 4.5  Electron micrographs of smooth muscle cells in the medial and intimal layers of the pudendal artery in old SHR.  (A=x1000, B=x3000, C=x7000, D=x15000) N, nucleus; IEL, internal elastic lamina; EC, endothelial cell; F, filaments; M, mitochondria; G, Golgi complex; C, collagen filaments.
Figure 4.6  Electron micrographs of smooth muscle cells in the intimal layer of the pudendal artery in old SHR. (A=x1500, B=x12000, C=x30000)  N, nucleus; IEL, internal elastic lamina; EC, endothelial cell; F, filaments; M, mitochondria; C, collagen filaments; V= vesicles.
4.3.5 Effects of antihypertensive treatment in young SHR

The two-week treatment with enalapril and hydrochlorothiazide markedly decreased arterial pressure during treatment (-47% versus pre-treatment and controls) and produced persistent lowering of arterial pressure after treatment was withdrawn (-21% vs pre-treatment and controls) (Figure 4.7). Two weeks after treatment was stopped, there were no significant differences in body weight between the control and treated SHR (374±16.4g vs 352±27.8g). In addition, the treatment induced a regression of both left and right ventricular mass compared to controls (LV/BW: 2.71±0.11 g/kg vs 2.30±0.02 g/kg, p<0.005; RV/BW: 0.60±0.02 g/kg vs 0.52±0.02 g/kg, p<0.005).

Two weeks post-treatment, the predicted changes in vascular structure (i.e. wall-to-lumen ratio) in the aorta or renal arteries of the previously treated SHR had not achieved significance (Table 4.6). Morphometric analysis of the first order mesenteric and pudendal arteries demonstrated there were no alterations in lumen diameter, whereas, in contrast, there was a 15-25% reduction in the wall thickness, wall-to-lumen ratio and CSA (Table 4.7). The amount of ECM remained proportional to the total CSA in the mesenteric and pudendal segments despite the significant level of remodelling in both tissues.

4.4 Discussion

The current study characterizes both the age-related structural changes in the internal pudendal artery in an experimental hypertensive condition and the persistent effects of a
Figure 4.7  Treatment with enalapril and hydrochlorothiazide (closed circles) induced a significant reduction in mean arterial pressure (MAP) during treatment compared to controls (open circles) that persisted when treatment was withdrawn. The black bar represents the 2-week treatment period. Data is presented as 24-hour mean ± standard deviation.
Table 4.6 Morphometric analysis of aorta, left and right renal arteries of control and treated SHR.

<table>
<thead>
<tr>
<th></th>
<th>Aorta</th>
<th></th>
<th></th>
<th>Right Renal</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Left Renal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Fold change</td>
<td>Control</td>
<td>Treated</td>
<td>Fold change</td>
<td>Control</td>
<td>Treated</td>
<td>Fold change</td>
<td>Control</td>
</tr>
<tr>
<td>Lumen (μm)</td>
<td>1337±124.9</td>
<td>1184±46.4</td>
<td>0.83</td>
<td>668±15.2</td>
<td>673±34.3</td>
<td>1.01</td>
<td>641±5.8</td>
<td>710±9.9</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>69.8±7.77</td>
<td>56.0±3.0</td>
<td>0.80</td>
<td>38.3±1.2</td>
<td>34.7±3.1</td>
<td>0.91</td>
<td>32.0±0.6</td>
<td>29.7±1.3</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>5.2±0.10</td>
<td>5.0±0.2</td>
<td>0.98</td>
<td>5.7±0.1</td>
<td>5.2±0.5</td>
<td>0.91</td>
<td>5.3±0.2</td>
<td>4.8±0.3</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM. n=4 control SHR, 3 treated SHR
Table 4.7  Morphometric analysis of first order mesenteric arteries and sections of the internal pudendal arteries of control and treated SHR.

<table>
<thead>
<tr>
<th></th>
<th>Mesenteric Artery</th>
<th>Proximal Pudendal</th>
<th>Middle Pudendal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Fold Change</td>
</tr>
<tr>
<td>Lumen (μm)</td>
<td>266±4.0</td>
<td>252±13.6</td>
<td>0.95</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>21.9±0.5</td>
<td>17.8±0.1‡</td>
<td>0.81</td>
</tr>
<tr>
<td>Wall:Lumen (%)</td>
<td>8.2±0.1</td>
<td>7.1±0.4*</td>
<td>0.86</td>
</tr>
<tr>
<td>CSA (x10³ μm²)</td>
<td>19±12.7</td>
<td>14±6.7*</td>
<td>0.76</td>
</tr>
<tr>
<td>ECM (x10³ μm²)</td>
<td>5.2±0.3</td>
<td>4.0±0.6</td>
<td>0.77</td>
</tr>
<tr>
<td>ECM/CSA (%)</td>
<td>27.7±0.1</td>
<td>27.6±0.1</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Values are mean±SEM. CSA=cross-sectional area. ECM=extracellular matrix. n=4 control SHR, 3 treated SHR. *p<0.05, †p<0.01, ‡p<0.001 vs control SHR.
drug-treatment previously known to decrease the vascular resistance properties in the penile vascular bed. With age, there was severe reduction in APO-induced erectile responses in the SHR that was comparable to the decrease seen in aged Sprague-Dawley rats which were significantly more overweight. That is, although body weight increased only 1.2-fold over the 62 week age span in the SHR, there was clear evidence of end organ damage (25% increase in LV:BW ratio) resulting from the prolonged untreated hypertension. In addition, signs of pathological changes in the vasculature were also manifest based on the significant intimal proliferation-induced encroachment on the lumen, increased wall thickness, CSA and wall-to-lumen ratio, as well as the ECM. Similar to the findings in aged normotensives, there appeared to be even more round-shaped SMC in the pudendal artery of old SHR which proved to be SMC in the synthetic state upon examination via electron microscopy. These remarkable intimal formations were only present in the pudendal arteries and not in the renal arteries or the aorta although they also underwent significant vascular remodelling. Treatment in young SHR was able to normalize the pudendal arteries to dimensions similar to the young WKY.

Many studies have examined intracavernosal pressure (ICP) differences in young WKY and SHR, and shown SHR to average approximately 30% lower ICP than an age-matched young WKY. Interestingly in the present study, there are no differences in APO-induced erectile responses. This differs from previous results presented by Jiang et al. showing young SHR to be severely dysfunctional compared to WKY however does correspond with the young SHR values seen in previous studies from our lab. The
majority of studies have focused on the differences in cavernous tissue in WKY and SHR and have shown that there is an increase in vascular SMC proliferation in corporal tissue and cavernous arteries, increased collagen and fibrosis, decreased elastic fibers, damaged mitochondria in endothelial cells and SMC and thinning of the tunica albuginea \(^{122,301}\). The present study reinforces the isolated penile perfusion studies which suggested that there is a thickening of the vascular wall and a narrowing of the lumen in the penile vasculature \(^{121}\). Although these structural changes will impede inflow of blood to the penis, the SHR’s preserved erectile function could be due to its elevated arterial pressure helping it to overcome the additional resistance to permit an erectile response to occur. As well, at this age, there are no differences in \(\alpha_1\)-adrenoceptor tone in the penile circulation and the cavernosal tissue is still able to relax normally \(^{120,121}\).

As demonstrated in Chapter 3, aging significantly impairs erectile function and upregulates vascular structure. Hypertension further exacerbates these pathological effects. Clinically, there is evidence of altered vascular structure in hypertensive men with ED. Increases in common carotid intima-media thickness, low flow-mediated dilation of the brachial artery and increased inflammatory markers such as C-reactive protein and interleukin-6 are common in aged hypertensive men suffering from ED \(^{302}\). In a slightly older group of men, the presence and severity of ED was associated with the severity of small-vessel lower extremity arterial disease \(^{303}\). Thus, in men where the vascular structure is increased in small resistance vessels evidence of impaired erectile function implies that the penile vasculature is also affected.
The present findings confirm previous studies, in that with age there is a greater increase in lumen diameter, media thickness and CSA in aorta and renal arteries in SHR.\textsuperscript{235, 248} Compared to age-matched Sprague-Dawley rats, there is a smaller increase in lumen diameter and a greater increase in wall thickness resulting in a significant impact on wall-to-lumen ratio in aged SHR. The greater changes in lumen diameter seen in the old Sprague-Dawley rats compared to the SHR could be accounted for by their larger increases in body weight (1.63 vs 1.14-fold increase). With a smaller increase in body mass, the SHR are not required to increase their lumen diameters as much to ensure that there is sufficient blood flow to meet the demand of the kidneys. In contrast, SHR have equivalent increases in wall thickness due to the compensatory mechanisms to allow the blood vessels to sustain the increasing arterial pressures seen with age. In aged SHR, it has been well documented that there is a greater number of SMC which contributes to the development of increased vessel contractility as well as evidence of increase in collagen content and hypertrophy of SMC.\textsuperscript{150, 151, 304, 305} In the aging SHR, erectile function has been shown to be severely diminished in both intracavernosal pressure values and APO-induced erectile responses.\textsuperscript{115, 120, 205} As previously mentioned, ED has been shown to be associated with further changes in penile ultrastructure, with reductions in the elastic fibers of the tunica albuginea, increasing collagen and decreasing SMC, increasing sinusoidal spaces of the corpus cavernosum in aging normotensive animals and young SHR.\textsuperscript{264, 270-272} To date, cavernous tissue in SHR older than 36 weeks of age has not been assessed, but we can presume that these changes would be further intensified in older SHR.
Changes in the pudendal artery’s vascular structure are amplified in aged hypertensive rats. Similar to the aged Sprague-Dawley rats, there are significant increases in wall thickness, CSA and ECM content in the aging SHR however there was a considerable decrease in lumen diameter. These changes to lumen diameter are due to substantial intimal formation. To our knowledge, this is the first study to show natural intimal formation that is not induced from injury (denudation, balloon injury), pharmacological intervention or a high fat diet in small arteries in the rat. Separate studies by Scebat and Hoover, have shown spontaneous aortic lesions in aged rabbits and cebus and squirrel monkeys, respectively although they did not examine smaller arteries $^{306,307}$. The pudendal artery’s wall-to-lumen ratio increases a striking 2.3 to 2.7-fold with age in SHR compared to modest 1.5 to 1.6-fold increases in old Sprague-Dawley rats. These intimal changes would significantly impact vessel tone and the ability to adequately supply blood to the penis.

There appears to be an increase in the number of rounded SMC in the intimal and medial-intimal layer of pudendal arteries in aged SHR. As hypothesized in Chapter 3, examination of these SMC via electron microscopy proved that they were of synthetic phenotype. They display a loss in contractile filaments and large amounts of rough endoplasmic reticulum, Golgi complexes and other cytoplasmic organelles $^{275,276,308-310}$. Synthetic smooth muscle cells are able migrate into the intima via small pores in the internal elastic lamina where they begin to divide and deposit ECM components furthering the growth of the neointima $^{276,309}$. The intimal formation evident in the
pudendal arteries of aged SHR is very similar to that seen in the carotid arteries of Sprague-Dawley rats after balloon injury. In both cases, the luminal surface of the arteries is completely covered and the SMC are surrounded extensively by ECM. Chemical and mechanical damage from elevated arterial pressure and sheer stress in SHR can lead to endothelial damage in pudendal arteries and lead the SMC to change from a contractile to synthetic state. The mechanism behind this change in SMC phenotype remains unknown.

Both age and hypertension have been shown to impact on the progression of intimal thickening after balloon injury. Aged animals have been shown to develop more extensive and severe aortic and carotid atherosclerotic plaques over a shorter period of time compared to younger animals when placed on a hypercholesterolemic diet. Isolated SMC from aged rats have a higher proliferative rate than young rats and in the absence of serum aged rat SMC doubled while young rat SMC remained quiescent. Similarly in hypertension, neointimal formation after balloon injury is increased compared to normotensive rats. It is believed that the increased activity of the RAS in the SHR contributes to the susceptibility of neointimal formation. Angiotensin II infused animals that underwent balloon injury showed more marked neointimal SMC. Treatment with antihypertensive agents antagonizing RAS was able to prevent the neointimal formation after balloon injury in both normotensive and hypertensive rats.
The beneficial impact of some antihypertensive treatments on the recovery of erectile function in hypertensive rats is well known. Two previous studies by Hale et al. have shown that treatment with enalapril alone or with a low salt diet was able to decrease resistance in the penile vasculature 12-24% in both young and 40 week old SHR. The present morphological findings confirm the hemodynamic data showing that treatment is able to regress the wall thickness in pudendal arteries of young SHR thereby decreasing vascular resistance. Small changes in the aorta and renal arteries were also apparent however not significant. The first-order mesenteric arteries had comparable changes in wall thickness, wall-to-lumen ratio and CSA to pudendal arteries after treatment was withdrawn. The young SHR have not accumulated as much ECM in the medial layers as their older counterparts therefore it is not surprising that there are minimal decreases in ECM with treatment. We can hypothesize that the decreases in CSA are due to a decrease in SMC hypertrophy as described in previous studies. Further experiments examining SMC numbers are required to confirm this theory. The penile cavernosal tissue has also been shown to be altered after antihypertensive treatments. Decreases in cavernous SMC, fibrosis and collagen type III content and increases in sinusoidal spaces were demonstrated. Recent studies have also examined the impact of combining antihypertensives with phosphodiesterase inhibitors; a combination often used in impotent hypertensive males. Treatment with losartan and sildenafil showed significantly lower cavernous smooth muscle and collagen values and higher eNOS expression in sinusoidal endothelium compared to SHR undergoing...
monotherapy with losartan. Whether this combination treatment would further regress the pudendal vasculature remains to be elucidated.

The impact of hypertension on the pudendal vasculature is evident in the structural changes seen in young SHR. Aging further exacerbates the damage in hypertensive animals causing marked intimal formation and decrease in the wall-to-lumen ratio. Antihypertensive therapy was able to normalize young SHR penile vasculature to similar values of age-matched WKY. Further studies are required to determine if antihypertensive treatments are able to induce equivalent remodelling effects in the pudendal arteries of aged hypertensive animals. A greater understanding of the mechanisms that cause the pathological changes such as intimal formation and the beneficial vascular remodelling may help provide therapeutic targets to treat the underlying cause of both hypertension and ED.
Chapter 5:

Recovery of erectile function in aging hypertensive and normotensive rats using exercise and caloric restriction

5.1 Introduction

Erectile dysfunction (ED) is a pathophysiological condition that is based on multifactorial etiologies. This is not surprising, as the generation of an erection requires the integrated contribution of multiple regulatory systems including psychological, hormonal, neurological, and vascular components. The progression to ED is linked to a long list of risk factors such as aging, smoking, alcohol, obesity, abnormal cholesterol, altered endocrine function and is commonly linked with co-morbidities such as hypertension, coronary artery disease, stroke, renal failure, diabetes, neurogenic disorders and mental illness. In particular, cardiovascular conditions, such as hypertension and coronary artery disease, appear to share common underlying pathophysiological processes with ED including endothelial dysfunction, vasculopathies and abnormal lipid metabolism. Of particular interest regarding the progression of these conditions is the evidence emphasizing that ED may be a harbinger for underlying cardiovascular disease. Findings from studies such as the Massachusetts Male Aging Study have revealed that aging is an independent risk factor in the development of ED, with the prevalence and severity increasing 3-fold between 40 and 70 years of age. Finally, obesity has become a co-morbidity of epidemic proportions across North America, with the incidence of ED in overweight or obese men reaching nearly 80%. Mechanistically, the initial onset and the subsequent progression of ED is recognized to be a consequence of the composite of insults to neurological, hormonal, and vascular pathways involved in the physiology of an erection. For example, events, procedures or
conditions that disrupt central neural networks involving dopaminergic or oxytocinergic pathways, particularly in the paraventricular nucleus (PVN) and medial pre-optic area (MPOA) of the hypothalamus, or the cascade of signalling in peripheral autonomic pathways involved in sexual responses, all can result in ED. Similarly, an appropriate androgen milieu appears to be necessary for normal sexual desire, erectile responses, and ejaculation. Based on this understanding, both clinically and experimentally, these aspects of sexual function have been shown to improve with androgen supplementation, in conditions in which there is depressed testosterone bioavailability. Finally, vascular insufficiency, specifically in vessels supplying blood to the penis and in the intrapenile erectile tissue, has also been strongly linked with decrements in erectile function.

As previously stated, men suffering from hypertension are twice as likely to experience ED compared to age-matched normotensive men. Experimental studies using the spontaneously hypertensive rat (SHR) have corroborated this finding and have further pointed to pathological structural changes in the penile vasculature as a potential mechanism of the dysfunction. Further, reversal of these structural abnormalities in SHR using aggressive antihypertensive treatments has been linked with improvements in erectile function even after withdrawal of treatment. In the various animal studies, the occurrence of diminished erectile status has been characterized by fewer centrally-initiated erections, blunted nerve-stimulated cavernous responses, decreased smooth muscle contractility, elevated adrenergic sensitivity and altered morphology of the corpus
A number of studies have compared the age-related decline in erectile function in SHR versus normotensive Wistar-Kyoto (WKY), although the pathophysiological mechanisms remain substantially unresolved. In addition, although there have been several aging-related studies of outbred, normotensive strains, the focus has not been on the impact of caloric intake or changes in body composition, but more on effects involving conditions such as castration, experimental diabetes, secondary hypertension, hormone supplementation or pharmacologic treatments.

Clinically, obesity and physical inactivity have been established as risk factors for cardiovascular disease and are further linked to an increased prevalence of ED, just as exercise and weight reduction have been shown to be beneficial. One recent study found that increased exercise and caloric restriction (CR) improved erectile function in a third of obese patients. Experimentally, obesity has usually been modeled using genetically modified animals or by giving animals abnormal diets. Interestingly, normal Sprague-Dawley and Wistar rats, but not SHR, who individually housed and allowed to eat regular chow ad libitum will progressively gain weight such that their body composition reflects the accumulation of abdominal adipose tissue to a much greater extent than in young adult normotensive animals or age-matched SHR. In fact, CR in normotensive rodents has previously been found to extend the maximum life span as well in primates.
In the present study, one objective was to determine whether the time course of the age-related decline in erectile function in hypertensive rats (SHR) compared to outbred normotensive rats (Wistar and Sprague-Dawley) was different. This characterization was performed in contrast to the conventional SHR versus WKY comparison to emphasize that age-related changes can be quite different depending on the initial phenotype and genotype. Further, once a significant age and/or obesity associated decline in erectile function was found, an additional objective was to assess whether a regimen of combined CR and exercise, followed by CR alone could recover and then maintain erectile function. Finally, short term testosterone supplementation was given to assess whether the deficits in erectile responses in the aging animals was, at least in part, due to decreased levels of androgens, although we did not address the implications of chronic androgen deficiency as this would have required separate treatment groups for all strains.

5.2 Methods

5.2.1 Animals

Male Wistar (n=16), Sprague-Dawley (n=18) and SHR (n=6) rats were purchased at 12 weeks of age (Charles River Laboratories, Montreal, Quebec, Canada) and housed individually at a temperature of 22–24°C with a 12-h light/dark cycle. All rats were provided with free access to regular rodent chow (LabDiet® 5001, Ren’s Feed and Supply Ltd, Oakville, Ontario, Canada) and tap water. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care as approved by the Queen’s University Animal Care Committee.
5.2.2 Assessment of erectile response

From 15 weeks of age onward, erectile function and body weight were measured weekly. Erectile responses were monitored via the well-established rat bio-assay of centrally-induced erections using the dopaminergic agonist, apomorphine (APO) \(^{220}\). Prior to (20 min acclimatization period) and during testing (30 min), rats were placed in one of four individual, hanging cages (with clear Plexiglas inserts used to replace the wire floor) in an isolated, quiet, dimly lit room adjacent to the holding room. Each rat received subcutaneous injection of APO in saline (80 \(\mu\)g/kg with 100 \(\mu\)g/ml ascorbic acid, 1 ml/kg) in the loose skin of the neck or back. Erections and yawns were counted over a 30-min period via videomonitoring connected to monitors in an adjacent room. Erectile responses were recorded following identification of characteristic physical and behavioral responses including concave arching of the back, pelvic thrusts followed by the emergence of the engorged glans penis and shaft, and immediate oral grooming of the genital area. APO-induced yawns were recorded as an index of delivery and to confirm bioavailability of the drug within the central nervous system \(^{222}\).

5.2.3 Caloric restriction and exercise

Based on the marked decline in erectile responses, when all animals had reached 30 weeks of age, daily food intake was then progressively restricted in all rats (decrement of 10% per week from intake at 29-30 weeks) up to a maximum of 40%, or until they had lost 10% of their body weight, according to a previously established protocol \(^{338}\). Upon
losing 10% of their body weight, food intake was then reduced by only 10-20% from initial intake levels in order to maintain body weight. In addition, throughout this period (30-40 weeks of age) rats were exercised for 30 minutes, five days per week using a modified, full-size treadmill fitted to rotate rodent exercise balls at a speed of 24m/min. At 40 weeks of age, exercise was stopped but the food intake remained restricted by 10-20% to maintain their body weight. *Ad libitum* access to food was reinstated when the animals reached 50 weeks of age. Erectile responses were always assessed prior to daily exercise. During the implementation of the CR protocol in the SHR, a change in the approach had to occur to maintain their healthy status. Specifically, SHR are much leaner and have a high metabolic rate such that the protocol could not be as prolonged. The protocol was adjusted to so that the objective became to limit the increases in body weight rather than to cause decreases in body weight. This approach was approved and validated by the health assessments done by the animal care staff.

5.2.4 Testosterone treatment

At 44 weeks of age, each rat from the three initial groups (Wistar, Sprague-Dawley and SHR) received a single dose of testosterone (480μg/kg, s.c.), administered 36 hours prior to erectile testing, according to a previously established protocol for normalizing erections in previously castrated rats. Testosterone was prepared from a stock solution of testosterone propionate (Taro Pharmaceuticals, Bramalea, Ontario, Canada) diluted in peanut oil.
5.2.5 Assessment of heart weights

Rats were anesthetized with pentobarbital (65 mg/kg, intraperitoneally) and hearts were removed and placed in cold saline. The right and left ventricle plus septum were then separated, blotted dry and weighed. Analysis of the left ventricle-to-body weight (LV/BW) and right ventricle-to-body weight ratios was used as an index of changes in cardiac structure.

5.2.6 Caloric restriction and exercise in old rats

In two separate groups of older rats the impact of exercise and caloric restriction on erectile function was assessed at a later age. Aged male Wistars (56 weeks, n=10) and Sprague-Dawley rats (67 weeks, n=12) were separated into two treatment groups which underwent a 10% CR or a 10% incremental weekly decrease to 40% and low impact exercise on a treadmill (7m/min, 30 minutes per day, five days a week). Erectile responses and body weight were recorded weekly as previously described.

5.2.7 Statistical analysis

Erectile response data are expressed as the number of erections per test period ± standard deviation. Body weight is expressed as mean ± standard deviation (SD). Statistical analysis of erectile responses between and within treatment groups was performed using both the Mann–Whitney rank sum test and one-way analysis of variance, with a comparison of means using a Newman–Keuls post-hoc test (P<0.05). This unpaired analysis was used because of the nature of the experimental testing protocols.
Specifically, the testing on the 3 groups of animals normally involved trials when the three strains were tested at the same time to account for the experimental error specifically associated with that trial (i.e. the testing error occurred independently in each trial). Strict pairing of animal responses in a repeated measures design is not possible because of the technical limitations of having to directly monitor all animals in a given test in real time. Differences in erectile responses during testosterone treatment were determined using Student’s t test ($P<0.05$). Heart weight data are presented as the mean±SD and were assessed by analysis of variance followed by a Newman–Keuls post-hoc test ($P<0.05$).

5.3 Results

5.3.1 Overall effects of exercise and caloric restriction on body weight

Despite being adult, there was a substantial increase in body weight between 15 and 30 weeks of age in the normotensive (34% in Sprague-Dawley rats, 45% in Wistars) and hypertensive (25% in SHR) rats fed ad libitum (Figure 5.1). Following the initiation of the 10 week combined exercise and CR and 10 week CR alone, overall the weight of the Wistar and Sprague-Dawley rats was decreased by 10% and 8.5% respectively compared to 30 weeks of age. In the SHR, a different approach had to be taken since this strain of rat is a much leaner strain, with a higher metabolic rate, and can not be subjected to
Figure 5.1 Overall profile of body weight gain throughout study in Sprague-Dawley (n=6), Wistar (n=6) and SHR (n=6). Periods in which rats underwent exercise and CR (CR+EX) and CR alone (CR) are outlined. Data is shown as mean.
prolonged or severe caloric restriction. That is, to maintain a healthy status of this strain the protocol was adjusted to attenuate body weight increase in SHR (to 3% increase over 20 weeks), rather than to produce progressive reductions in body weight.

Regardless of the approach, in all three strains, after ad libitum feeding was re-introduced at 50 weeks, body weight again progressively increased over the next 10 weeks (Wistar and Sprague-Dawley 16%, SHR 8%), an effect which was particularly evident in the first two after stopping CR. At the end of the study, there was no significant left ventricular hypertrophy in the heart weights (LV/BW) of Wistar or Sprague-Dawley (1.7±0.3 and 1.7±0.1 mg/g body weight). Conversely, as expected, in SHR after the various treatments, the left ventricle remained significantly enlarged (3.3±0.1 mg/g body weight).

5.3.2 Effect of aging and/or associated weight gain on sexual function

As seen in previous studies, there was a significant decline in erectile responses with age in all three strains of rats (Figure 5.2 left), such that by 29 weeks of age the normotensive rats averaged only 0.2±0.4 erections and the SHR were at 0.6±0.8 erections per test period. In general, the increase in body weight paralleled the decline in erectile function in all rats. Paralleling the decrease in erectile responses was a progressive increase in the proportion of animals suffering from severe erectile dysfunction (Figure 5.2 right). Specifically, by 29 weeks of age, all animals had at least some level of erectile dysfunction according to the pre-defined severity scale (i.e. no dysfunction ≥2.0 erections; mild ≥1.0 erection; moderate 0.5<1.0 erection or severe <0.5 erection).
Figure 5.2 An age-related decrease in erectile responses was seen in age-matched Sprague-Dawley (top left, n=6), Wistar (middle left, n=6), and SHR (bottom left, n=6). A significant parallel increase in body weight was also evident with age. The severity of erectile dysfunction was classified as no dysfunction (≥2.0 erections), mild (≥1.0 erection), moderate (0.5<1.0 erection) or severe (<0.5 erection) dysfunction (right). Data is presented as mean ± standard deviation. *P<0.001 vs 15-16 weeks. †P<0.05 vs 15-16 weeks. ‡P<0.05 vs 17-18 weeks.
5.3.3 Effect of exercise and caloric restriction on sexual function

The average number of erectile responses increased progressively during the combined protocol (Figure 5.3 left) reaching 1.8±0.7 and 1.7±1.4 erections in the SHR and Sprague-Dawley rats, respectively. In fact, the improvement was such that none of these animals had severe dysfunction by the end of this combined treatment period (Figure 5.3, right). In contrast, a complication arose in the Wistar rats at 34 weeks of age when they began to develop hyper-responsiveness to the APO administrations. Specifically, these rats became progressively more hyperactive (i.e. circling of the test cage during the entire trial period) with each APO dose such that at 40 weeks of age all Wistar rats had severe dysfunction (most had no responses). Accordingly, Wistars were not included in any further erectile function testing. The effects of withdrawing exercise but maintaining the CR (Figure 5.4) from 40 weeks of age onward included a slight increase in body weight but minimal impact on erectile responses. That is, as a result of these interventions in all strains, the rats still had only mild to moderate erectile dysfunction and none had severe dysfunction at 49 weeks of age; values which were similar to those prior to the withdrawal of exercise. The transient decrease in erectile responses at 46 weeks of age corresponded with construction noise within the animal housing facility.

5.3.4 Effect of reinstatement of ad libitum feeding

After CR was stopped, body weight increased within the first 2 weeks by approximately 10%. In particular, the time course of weight gain (62 g, 11.5%) corresponded well with the decrease in erections in Sprague-Dawley rats (Figure 5.5).
Figure 5.3  With exercise and CR, erectile function improved significantly in all three strains (left, n=6 per strain). A decrease in body weight was also seen in Sprague-Dawley and Wistar. The severity of erectile dysfunction decreased in both Sprague-Dawley and SHR, which was classified as no dysfunction ($\geq 2.0$ erections), mild ($\geq 1.0$ erection), moderate ($0.5<1.0$ erection) or severe ($<0.5$ erection) dysfunction (right). Data is presented as mean ± SD. *P<0.05 vs 28-29 weeks.
Figure 5.4 Upon withdrawal of exercise, CR alone appears to maintain body weight and erectile function (left). Overall there is minimal change in the severity of erectile dysfunction (right). The severity of erectile dysfunction was classified as no dysfunction (≥2.0 erections), mild (≥1.0 erection), moderate (0.5<1.0 erection) or severe (<0.5 erection) dysfunction (right). Data are presented as mean SD. *P<0.05 vs 40-41 weeks.
Figure 5.5 After CR is stopped, a decrease in erections and an increase in body weight are apparent in both Sprague-Dawley and SHR (left, n=6 per strain). A corresponding increase in the severity of erectile dysfunction was also seen in both strains. The severity of erectile dysfunction was classified as no dysfunction (≥2.0 erections), mild (≥1.0 erection), moderate (0.5<1.0 erection) or severe (<0.5 erection) dysfunction (right). Data is presented as mean ± SD. *P<0.05 vs 48-50 weeks. †P<0.001 vs 48-50 weeks.
Although the trend was similar in SHR, the rate of change in body weight (30 g, 8.5%) was more gradual and corresponded with a lesser impact on erections. Correspondingly, the proportion of animals with severe ED is higher in Sprague-Dawley rats in comparison to SHR (Figure 5.5 right).

5.3.5 Testosterone administration
At 44 weeks of age, all rats were administered testosterone according to a previous protocol. There were no significant changes in erectile responses observed when animals were tested 36 hours after this treatment (data not shown).

5.3.6 Exercise and caloric restriction in aged normotensive rats
The aged Wistar and Sprague-Dawley rats which underwent 40% CR and exercise rapidly lost approximately 17% body weight which was maintained with 20% CR (Figure 5.6 top). The Wistar and Sprague-Dawley rats which had food intake restricted 10% only lost 2-5% body weight and this loss was maintained throughout the 12 week treatment period. Minimal erectile function was sustained in the Wistar rats undergoing 10% CR whereas, the older Sprague-Dawley rats were not responding from 71 weeks of age onwards (Figure 6 bottom). A significant improvement in erectile function was observed in all rats undergoing 40% CR and exercise. A greater improvement in erectile function was seen in the Wistars who were 11 weeks younger than the Sprague-Dawleys.
Figure 5.6  Body weight profiles in Wistar (n=10) and Sprague-Dawley (n=12) that underwent exercise and CR (EX+CR 10-40%) are significantly lower than rats that only underwent CR of 10% (CR 10% only). The treatment time-course of the EX+CR 10-40% is outlined. Erectile function improved significantly in both Wistar and Sprague-Dawley rats undergoing EX+CR 10-40% versus the rats receiving CR 10% only. Data is represented as mean ± SD. NR=no response. *P<0.05 vs CR 10% only. †P<0.01 vs CR 10% only.
5.4 Discussion

The major findings in this study are that (i) there is comparable, age-related decline in erectile responses in three different strains of rats with different genetic backgrounds, (ii) erectile responses were partially recovered with a chronic intervention that includes exercise and CR, resulting in a blunting or a prevention of excessive weight accumulation, (iii) the beneficial impact on erectile responses of this intervention, in aged animals, can be maintained after the exercise regimen is stopped if CR is maintained (in association with continued attenuation of body weight gain), and (iv) re-introduction of ad libitum feeding increased body weight in all animals and was associated with a negative effect on erectile responses within 10 weeks, an effect which was not prevented by short term testosterone treatment.

Confirming previous findings, the age-related decline in apomorphine-induced erectile responses occurred as expected. Interestingly, although the decline in the quantity of erectile responses in all strains occurred at an earlier age than in previous reports, the profile of the deterioration clearly followed the body weight increases, at least in the normotensives. Of interest, however, is that previous studies in aging normotensives have characterized the decay in erectile responses predominantly using the anesthetized, cavernous nerve stimulation model. Although this experimental approach is very useful in assessing quantitatively the peripheral neurovascular coupling, the APO-induced model also incorporates both central and spinal mechanism in the generation of the response. Future studies using both approaches at the same time will provide very
useful data to enable comparison of the mechanisms involved. Regardless of the specific
timing of the decline in function, the current study provides strong experimental evidence
reinforcing that aging *per se*, as well as genetic hypertension, are important independent
risk factors for ED 341. This concept is emphasized by the finding that with age a decline
in erectile responses occurs in both SHR and normotensive rats, despite the marked
differences in the changes that are occurring (i.e. body composition and metabolic
changes in the normotensive rats and pathological changes associated with the prolonged
hypertension in the SHR). Although the particular mechanism(s) of the age-related
decrease in erections were not assessed in this study, previous investigators have revealed
that over time there are a number of morphological, structural and functional changes in
the erectile tissue as well as in the innervation which explain, at least in part, this
downward progression. Specifically, morphological examinations have shown that in
aging penile tissue there are marked changes in the elastin and collagen makeup of the
tunica albuginea and corpus cavernosum, as well as a decrease in cavernous smooth
muscle 342, 343. In addition, a number of studies have demonstrated age-dependent
reductions in the nitric oxide-cGMP-mediated vasodilatory capacity of cavernous tissue
in both rabbits and rats 281, 344. Previously, these kinds of pathophysiological changes
have been found to occur earlier and to a greater extent when hypertension is present in
young animals. In particular, even at the earliest stages of experimental hypertension,
abnormalities in penile vascular structure and function, including an increase in media-to-
lumen ratio and luminal encroachment become evident 121, 123. Although it is likely that
many of these pathological changes play a role in the age-associated decline in SHR seen
in the present study, a detailed analysis of the entire penile vasculature, not just the
corpus cavernosum needs to be performed to fully elucidate the magnitude of changes
during aging, as well as with respect to hypertension. Furthermore, the role of abnormal
changes in peripheral neurovascular coupling, spinal signalling and central nervous
system mechanisms needs to be fully characterized in future studies. In addition, given
the comparable time course of deterioration of erections in both the hypertensive and
normotensive future studies will need to assess all these putative mechanisms in various
animal models to appropriately elucidate their quantitative mechanistic role in the decay
of function.

Although there have been only a few experimental studies assessing the impact of
exercise on erectile function, the relationship to cardiovascular health has been
thoroughly characterised 179,345. The present results provide strong evidence in three
strains of rats that moderate exercise is also beneficial to erectile function, particularly
when it involves a reduction in body weight. Although the approach taken in this study
was to combine exercise with CR, there was a clear, progressive improvement in erectile
responses only during the exercise phase, whereas during CR alone, erectile responses
were, at best, maintained. Previous studies have established that the intensity of exercise
is important, such that when exercise intensity is too low there is little benefit and when
exercise is excessive there can be problems with generation of oxidative stress 346. Thus,
the clinical and experimental evidence suggests that moderate exercise is likely the most
beneficial in improving endothelium-dependent vasodilation, lowering oxidative stress,
attenuating adrenergic sensitivity and elevating the levels of nitric oxide synthase (NOS) and bioavailable NO \(^{347-350}\). It is likely that many of these changes contribute to the benefits observed in the present study, although tissue specific assessments of vascular mechanism will need to be performed to determine the quantitative involvement across the different ages.

The effects of CR have been known for a long time. For example, in 1935 McCay et al. demonstrated that chronic caloric restriction could even prolong species-specific maximum lifespan \(^{192}\). It is important in all studies that animals are restricted carefully so as not to induce a state of malnutrition. For rodents, this is accomplished by using restriction ranges that are less than 40% of caloric intake for short periods of time, and 10-20% for longer periods \(^{338}\). Interventions involving exercise and CR have been shown to produce similar beneficial changes in cardiovascular health and sexual function, although the data is more limited in scope for ED. For example, even short term CR has been shown to increase endothelial-dependent vasodilation in obese hypertensives, lower blood pressure and sympathetic activity in rats \(^{351-353}\), effects which were found to be reversible upon cessation of the restriction. In the present study, a similar trend was evident as ongoing CR, without exercise, maintained erectile function and reintroduction of \textit{ad libitum} feeding was associated with a marked decrease in erectile function in both the aged hypertensive and normotensive rats.
Similar to the aforementioned cardiovascular improvements with exercise and CR, neurological status has also been shown to be improved. Specifically, exercise has been demonstrated to improve dopamine synthesis and inhibit sympathetic nerve activity\textsuperscript{354}, as well as elevate oxytocin signalling in the hypothalamus in both SHR and WKY\textsuperscript{355}. Similarly, CR augments dopaminergic function and enhances the neuroprotective status of brain tissue by decreasing oxidative stress\textsuperscript{356,357}. These changes are important in erectile function since dopaminergic and oxytocinergic neurons, in the PVN and MPOA of the hypothalamus, are key components of the neural cascade leading to penile erection\textsuperscript{58}. Relevant to the present study, aging and hypertension have both been shown to produce deficits in these neurotransmitter systems\textsuperscript{355,358}. The overall beneficial effects on erectile responses found in both the normotensive and hypertensive animals with the prolonged, combined intervention and as well as with the CR alone, without a requirement for substantial reduction in body weight, suggests that an improved neurological status may, at least in part, be the basis of the augmentation of erectile function. Given that the improvements in erectile responses were found using the APO-bioassay of erections, it may be that the benefits involve central, spinal and peripheral nervous system modifications as well as changes in penile vascular function. Assessments of each of these component mechanisms will be required to decipher the source of the overall benefit observed in this study.

A recent clinical study investigated, prospectively, the effects of exercise and CR on erectile function\textsuperscript{184}. In their protocol, obese men, with no underlying diabetes,
hypertension, or hyperlipidemia, but suffering from ED, were prescribed exercise regimens and a decrease in caloric intake in order to reach a target of 10% reduction in body weight. The findings were that sexual function improved in one third of obese men within two years after lifestyle changes were initiated. Although the specific cause of the benefit was not determined, the improvement was associated with significant decrease in body weight, blood pressure, levels of glucose, insulin, C-reactive protein, total cholesterol and triglycerides as well as a significant increase in high-density lipoprotein cholesterol and vascular responses to L-arginine. The magnitude of the benefit found in the present studies appears to provide strong evidence for advancing the current experimental model in further investigations of the pathophysiological mechanisms involved.

Previous experimental and clinical studies have established that there is an age-related decline in testosterone levels, which can negatively impact on sexual function, obesity and cardiovascular status and that chronic testosterone supplementation was able to reverse some of these changes. In the present study, short term administration of testosterone, at doses sufficient to normalize castrated rats, did not recover erectile responses in 44 week old rats. This lack of effect likely reveals that the age-related ED was not due to non-genomic effects of testosterone deficiency, but more likely to the longer term reduction in genomic effects of testosterone on cardiovascular and neural tissues that are corrected during chronic testosterone administration.
Overall, these findings demonstrate that exercise and CR can recover erectile responses in both normotensive and hypertensive animals. Furthermore, while the combination treatment clearly provides substantial benefit, it appears that CR alone can also blunt the age-related decline in erectile function. Whether changes such as remodelling of the penile vasculature, improved endothelial function, decreased inhibitory sympathetic tone or improved neural pro-erectile signalling are the mechanisms by which erectile function is improved still needs to be elucidated. Even in conditions involving hypertension, obesity and aging, modifying lifestyle factors such as exercise and CR appears to provide a non-drug intervention that can effectively modify the progression of sexual dysfunction.
Chapter 6:

General discussion and future directions

6.1 General discussion

There has been a marked increase in the scope and depth of studies in the field of sexual medicine over the last two decades. As a result, erectile function has been assessed in many different animal models ranging from horses and dogs to rabbits, rats and mice and in a variety of diseased states such as hypertension, diabetes, hyperlipidemia, obesity and atherosclerosis. Experimentally, the *in vivo* methods used to measure erectile function in these different models have involved approaches that directly measure intracavernosal pressure or those which characterize the sexual behavior responses to centrally acting drugs. To date, studies in the various models and diseased states have focused predominantly on the changes in the penile cavernous tissue, including morphological, physiological and molecular approaches. More recently, studies have also begun to examine the physiological properties of SMC of deep penile arteries from horses and rats. However, in general, the main focus of ED research has remained on the penile tissue, whereas the upstream vasculature responsible for supplying blood from the systemic circulation has been largely ignored.

In this regard, a key objective of this thesis was to characterize the structure and function of a pair of key upstream vessels, the internal pudendal arteries. As previously described, the bilateral pudendal arteries are the primary feeder vessels supplying all erectile tissue. We have previously demonstrated the importance of this vessel’s contribution to the resistance of blood flow during an erectile response. In fact, evidence revealed, under both conditions of maximal constriction and dilatation, that approximately 70% of the
vascular resistance is located in the pre-penile arteries while the small arteries within the penis contribute less than 20% of the total resistance (Figure 6.1). With this understanding it became clear that structural damage or alteration to this vessel would clearly compromise the ability to generate blood flow during an erectile response and also impact negatively on blood flow to the penile tissue during non-erectile periods thereby potentially leading to further progressive tissue dysfunction and damage.

In the present studies, the anatomical location of the pudendal artery in the rat was confirmed from numerous dissections. The results of this examination have been provided and illustrated in Chapter 3 (Figures 3.1, 3.3, 3.4). Our careful examination demonstrated in the rat, that both the left and right pudendal arteries are roughly 3 cm long, bifurcating from the internal iliac artery, passed between the flexor cauda brevis and the abductor cauda internus muscles, traveling along the medial surface of the pelvis, and emerging from the ischiorectal fossa to supply all erectile tissue including both cavernosal bodies as well as the spongiosum. This vessel was found to be extraordinarily muscular for its size; with a lumen diameter similar to a first-order mesenteric artery (~300 µm) and yet a wall thickness greater than that of a renal artery (~45µm). One explanation for this finding is that during the initiation of an erectile response, the walls of the pudendal artery experience marked changes in both blood flow and intralumenal pressures, with the latter rising to and above the level of aortic pressure \(^{263,363}\).
Figure 6.1  The relative proportion (%) of each vascular segment to total resistance in maximal vasodilation induced by sodium nitroprusside in Sprague-Dawley rats (n=7). Taken from Manabe, et al. Int J Impot Res. 2000.
Interestingly, recent preliminary studies have demonstrated noteworthy structural and physiological changes along the length of the pudendal artery that might reflect differences in the localized interaction of structure and function. That is, comparison of the middle (B) segment, which travels adjacent to the pelvis, and the proximal (C) segment (between the branch of the inferior gluteal and its origin from the internal iliac) distinctive morphological differences were evident (Figure 3.1). In particular, the middle segment of the pudendal artery was determined to have a substantially thicker wall (↑40%) and a smaller lumen diameter (↓20%, Figure 6.2) than the upstream segment. As a result, the wall-to-lumen ratio of middle segment was almost 2-fold greater than that of the proximal segment (Figure 6.2). Furthermore, the responses of these pudendal artery segments in myograph studies were distinctly different. The middle segment initially contracted to 20mN of tension (similar to the tension generated by an aortic ring) prior to the generation of spontaneous oscillations in tone consisting of very slow, high amplitude waves of up to 3 minutes duration (Figure 6.3A). In contrast, the proximal segment displayed higher frequency and lower amplitude oscillations and consistently reached only 10mN of tension (Figure 6.3B). Thus, the overall average tension of these two segments assessed over a 7 minute interval was significantly higher in the middle segment versus the proximal segment (Figure 6.3C). Phenylephrine (PE) concentration-response curves confirmed the increased contractility of the middle segment (B:22 mN vs C:17 mN, P=0.01; Figure 6.4A). In addition, the middle segment was found to be more sensitive to PE-induced contractile responses (EC50s: B:3.21x10⁻⁷ M vs C:7.74x10⁻⁷ M,
Figure 6.2  Morphological differences in wall thickness (A), lumen diameter (B) and wall:lumen (C) in segments from the middle and proximal portions of the pudendal artery in young male Sprague-Dawley rats. *P<0.01 vs middle segment.
Figure 6.3 Representative tracings of phenylephrine-induced oscillations in middle (A) and proximal (B) segments of the pudendal artery. The line represents 2.5 minutes. The middle segment has a higher overall tension compared to the proximal segment (C). *P<0.01 vs middle segment.
Figure 6.4 Phenylephrine (PE) (A) and acetylcholine (ACh) (B) concentration-response curves (CRC) in middle and proximal segments of the pudendal artery. *P<0.05 vs middle segment. CRC to ACh was only plotted to (10µM) in proximal segments of the pudendal because at higher concentrations contractions were seen. Contractions to ACh were not seen at any concentration in the middle segment.
P=0.01) despite the lack of difference in the magnitude of the endothelium-dependent relaxation (Figure 6.4B).

The distinctive, large magnitude oscillations in the pre-contracted downstream segment of the pudendal artery, and not in the upstream segment, provide strong evidence that this vessel likely has unique capabilities. We have hypothesized that the anatomical location of this segment requires it to have a thick wall in order for it to withstand both the large increase in blood flow and intra-arterial pressure during an erectile response. The proximal segment, which is surrounded solely by skeletal muscle and is much farther from the penis, will not experience as great of an increase in pressure and thus would not require as thick of a smooth muscle layer to normalize wall stress. Further studies are required to determine whether alterations in adrenergic tone or populations of $\alpha_1$ receptors along the length of the pudendal artery play a role in regulating the different responses of this key vessel.

Many studies have demonstrated that aging can drastically impair erectile function in a variety of rodent strains including normotensive (Sprague-Dawley and Wistar) and hypertensive (SHR) rats. Thus, in Chapters 3 and 4, the pudendal artery was further characterized in aged rats when erectile function was severely impaired. Aging contributed to significant morphological and physiological changes in the pudendal artery that were different from those found in other vessels. That is, whereas the aorta and renal artery displayed increased wall thickness and cross-sectional...
area, there was no change in the wall-to-lumen ratio; reflecting the proportional change required in the older, larger animal (Table 3.2). Conversely, in the pudendal artery, during aging there was a very marked increase in the wall-to-lumen ratio in both normotensive and to an even greater extent in hypertensive rats. Similar to previous studies examining the cavernous tissue in aged rats, our findings revealed that there was a significant aged-related increase in the amount of extracellular matrix in the smooth muscle layer of the pudendal arteries. Additionally, with age, there was significant intimal proliferation in the pudendal arteries of hypertensive rats that was not evident in the other vascular beds examined or in age-matched normotensive animals.

As demonstrated in Chapter 2, the timing of treatment is critical in the prevention or recovery of erectile function. Our lab has previously shown that a two week treatment of enalapril combined with a low salt diet recovered erectile function in 40 week old SHR. In Chapter 2, an attempt to prevent the age-related decline in erectile function in aging SHR with conventional antihypertensive agents such as enalapril and hydralazine did not prevent or delay the onset of ED when animals were first treated at 30 weeks of age (Figure 2.2A). In the same group of animals, the treatment was repeated at 50 weeks of age and erectile function was still not improved (Figure 2.2B). A more aggressive treatment approach was taken at 70 weeks of age in which animals received enalapril or losartan and a low salt diet or a triple therapy of hydralazine, nifedipine and hydrochlorothiazide. Overall, only a slight improvement in erectile function was demonstrated, despite the aggressiveness of the treatment, with the greatest benefit seen
in the animals treated with triple therapy (Figure 2.3). Having characterized the pudendal artery in similarly aged SHR, we now know there is significant structural and functional damage to the pudendal artery of these rats and it is possible that these pathological changes cannot be overcome with antihypertensive treatments.

Other studies have shown that erectile function can be improved in aged normotensive animals using different therapeutic strategies. For example, various gene therapies have successfully improved erectile function in aged Sprague-Dawley rats. Intracavernosal injection of cDNA for the hSlo gene, which encodes the α or pore forming subunit of the large conductance, Ca\(^{2+}\) activated K\(^{+}\) (maxi-K) channel gene, ameliorated the age-related decrease in erectile function in 9 month old Sprague-Dawley rats\(^{332}\). Similarly, an adenoviral gene transfer of pre-pro-calcitonin gene-related peptide (CGRP) improved erectile function in the aged rat\(^ {373}\). Another approach that recovered erectile function in 20 month old Sprague-Dawley rats was a 45-day treatment with the PDE5 inhibitor sildenafil\(^ {271}\). Further studies are required to elucidate the mechanisms behind these improvements to determine the best time course and type of treatment required to permanently reverse ED.

Based on the pudendal artery perfusion studies\(^ {173, 205}\) and the work presented in this thesis, the improved erectile function after antihypertensive treatment can likely be attributed to both structural and functional changes in the vasculature. In Chapter 2, the cross-over kidney transplant study between treated and untreated SHR, demonstrated that
improved erectile function was dependent on changes to the penile vasculature and not to
general changes in the circulation associated with altering arterial pressure. That is, the
SHR that had previously received antihypertensive treatment and had a higher arterial
pressure due to their transplanted control kidney showed improved erectile function
compared to the untreated SHR who were operating at a lower arterial pressure due to the
receipt of a kidney from a previously treated littermate (Figure 2.5B). Specifically, the
improved vascular function of the previously treated animals clearly had a greater impact
on erectile responses than did the increase in arterial pressure that resulted from receipt of
a kidney from an untreated littermate. Moreover, when the untreated SHR, after
transplantation, were then treated with losartan their erectile function improved to similar
levels of the previously treated SHR (Figure 2.5C). This evidence pointed to penile
vascular specific changes as being the causal link to the improvement in erectile function.

Further evidence supporting the importance of the penile vasculature was demonstrated
in previous studies which examined the perfusion pressure in isolated penile vascular
beds. Following a two-week antihypertensive treatment in both young and aged SHR,
there was a decrease in the structurally based vascular resistance suggesting that a
normalization of the penile vasculature had occurred\textsuperscript{173,205}. In Chapter 4, these findings
were confirmed through morphological assessment of the pudendal arteries in young
SHR after a two-week treatment of enalapril and hydrochlorothiazide. Although no
changes were evident in lumen diameter, there was a 15-20\% reduction in wall thickness,
and the wall to lumen ratio in the pudendal arteries (Table 4.7). Many studies have
shown the impact of antihypertensive therapy on different vascular beds, including cavernosal tissues \(^{176, 177, 325, 326}\), however this study was the first of its kind to show direct evidence of remodelling of the vasculature supplying the penis.

To extend clinical findings, in Chapter 5 we took another therapeutic approach involving exercise and dietary adjustments to address the issue of the age-related decline in erectile function. Thus, by combining exercise and caloric restriction (CR) we assessed whether this intervention, at least in part, could attenuate the age-related decline in APO-induced erectile function in both normotensive and hypertensive rats. The results indicated that the decrease in the number of pharmacologically-induced erections appeared to correlate, at least in part, with increased body weight. Although it was not specifically examined, one hypothesis that was generated was that this age-associated weight gain involved an accumulation of visceral adipose tissue (VAT). Therefore, by initiating the combination intervention at 30 weeks of age, it was found that exercise and CR caused a progressive improvement in erectile function in both normotensive and hypertensive rats. This occurred even though significant decreases in body weights were evident only in the normotensive strain (Figure 5.3). These results provided strong evidence that experimental modification of lifestyle factors can improve or maintain erectile function in normotensive and hypertensive rats, without necessarily having a marked impact on body weight. That is, it may be that body composition is the more appropriate link.
Further analysis of this intervention, comparing the subsequent effects following removal of the CR regimen on body weight and ED, have now been performed (Figure 6.5).

Specifically, our new analysis has assessed the impact of returning the animals to the sedentary and ad libitum feeding condition at two stages of life (i.e. young versus old). The new findings show that the rate of decline in erections in the untreated animals (no CR or exercise) for a given accumulation of body weight is much greater in old animals than in young, and is even more severe in older hypertensive rats. That is, similar to epidemiological findings, the presence of hypertension appears to greatly exacerbate the decline in erectile responses at a given age, such that severe ED is found in older hypertensive rats when only small increases in body weight occur. Thus, these data suggest that the presence of hypertension and ED should be a sentinel for initiating more aggressive treatment strategies targeting body weight gain, or more likely inappropriate changes in body composition leading to accumulation of VAT.

Following this investigation, we determined the effects of instituting CR at an even earlier stage of development to prevent rather than reverse the pathology. That is, using Sprague-Dawley rats, we calorically restricted animals in early adulthood by up to 20% of their normal intake for a 4 month period in order to blunt body weight gain. Assessment of APO-induced erectile responses after this period revealed that the CR was very effective in preventing the decline in erections seen in the overweight animals, an
Figure 6.5  Relationship (line of best fit) between erectile responses and the change in body weight (%) in A) young spontaneously hypertensive rats (triangles) and Sprague-Dawley rats (circles) untreated (from 4-8 months) and B) old (12-18 months) spontaneously hypertensive rats (triangles) and Sprague-Dawley rats (circles) after exercise and caloric restriction intervention is stopped. C) Comparison of the relationship in young (solid and dotted lines on right side) and old (solid and dotted lines on left side) demonstrates that body weight gain impacts more negatively on erectile responses in older animals than in younger ones, and this effect is further amplified by the presence of hypertension. The individual symbols represent data from each animal according to a 3 week moving average for a given body weight gain over that time period. HT = hypertensive, NT = normotensive.
effect which was most pronounced in the animals with the least body weight gain (Figure 6.6). The impact of this intervention of CR on the vascular reactivity and function of the mesenteric and pudendal arteries was also assessed. The vascular oscillations in the mesenteric arteries were not found to be different between the lean and overweight animals (Figure 6.7). In contrast, the pudendal arteries in the overweight animals displayed very fast, high frequency oscillations whereas the leaner animals had the slow, high amplitude waves found in younger animals (Figure 6.8). Overall in both the mesenteric and pudendal arteries, no differences in tension were seen with increasing concentration of phenylephrine within the lean and overweight rats (Figure 6.9). No evidence of changes in endothelial function, assessed by acetylcholine-induced vasodilation, was apparent in mesenteric arteries of the heavier rats; but there was an obvious improvement in endothelial function, shown by the acetylcholine vasorelaxant response, in the pudendal arteries of aggressively CR compared to the moderately restricted and the overweight animals (Figure 6.10). It was noteworthy that morphological assessment of the pudendal arteries did not reveal any obvious differences in vascular structure between the three groups (data not shown). These results suggest that endothelial dysfunction may precede the pathological structural changes in the pudendal artery. Consistent with the clinical findings, these data provide further evidence that the pudendal arterial bed is more susceptible to injury than other vascular beds; i.e. reinforcing clinical data which suggests that the onset of ED precedes the onset of cardiovascular disease 33, 34, 37, 38, 105, 106.
Figure 6.6 Body weights (A) and average number of erections (B) of male Sprague-Dawley rats that underwent 20 weeks of moderate (10%) or aggressive (20%) caloric restriction (CR) or fed ad libitum (control). *P<0.05 vs controls.
Figure 6.7  Representative tracings of phenylephrine-induced oscillations in first-order mesenteric arteries from control (A), moderately (B) and aggressively (C) caloric restricted male Sprague-Dawley rats.
Figure 6.8 Representative tracings of phenylephrine-induced oscillations in the pudendal artery from control (A), moderately (B) and aggressively (C) caloric restricted male Sprague-Dawley rats.
Figure 6.9  Phenylephrine (PE) concentration-response curves in first-order mesenteric (A) and pudendal (B) arteries from *ad libitum* fed, moderately (10%) or aggressively (20%) caloric restricted (CR) male Sprague-Dawley rats.
Figure 6.10  Acetylcholine (ACh) concentration-response curves in first-order mesenteric (A) and pudendal (B) arteries from *ad libitum* fed, moderately (10%) or aggressively (20%) caloric restricted (CR) male Sprague-Dawley rats. *P<0.05 vs controls.
6.2 Future directions

The major findings of this thesis provide further evidence for the importance of vascular structure in the role of erectile dysfunction. Overall, the focus of these studies has been to characterize the pudendal artery from morphological and physiological perspectives in young and old normotensive and hypertensive animals. The impact of different pharmacological treatments and lifestyle modifications on erectile function and the pudendal vasculature was also assessed. Further studies are required to expand on these findings and determine the mechanisms responsible for the changes seen in the pudendal artery.

These studies have only begun to characterize the pudendal artery. Additional studies are required to further describe the proximal section of the pudendal artery and determine why this vessel differs structurally as it descends towards the penis. Current work in our laboratory is investigating the structural changes by creating vascular casts of the pudendal vasculature which will allow us to determine where these changes take place. Further elucidation of the distinctive mechanisms related to the smooth muscle in the pudendal artery will be necessary to fully understand the distinctive properties and functions of this vessel. For example, determining the innervation of this vessel and the location, density and function of various receptors and signaling pathways in the regulation of contraction and relaxation of the pudendal artery will be critical to fully determine its functionality. Furthermore, understanding whether these mechanistic attributes change in pathological states or with different treatments may help to suggest
improved treatment strategies that can produce persistent or at least long-term changes to the vasculature and thereby benefit erectile function.

Although the focus of these studies has been on ED, sexual dysfunction is also present in aging women and those suffering from cardiovascular disease. One study showed that hypertensive women, in particular, demonstrated decreased vaginal lubrication, decreased orgasm, and increased pain compared to non-hypertensives. Previous studies in our laboratory have experimentally assessed sexual responses in aged normotensive female rats and found a marked age-associated dysfunction compared to young rats. Similar to the studies in males, the diminished sexual response in 40 week old normotensive females was improved by brief, aggressive antihypertensive treatment and for up to 10 weeks after the withdrawal of treatment. The genital tissue in females has an analogous blood supply to the males and work on characterizing the female pudendal artery is underway. To date, we have anatomically mapped the artery in female rats and morphological assessments are currently being conducted. Unfortunately, attempts to address female sexual dysfunction, such as arousal disorders, therapeutically have not succeeded. Thus, determining the vascular mechanisms of the female pudendal artery could provide new therapeutic targets.

6.3 Conclusions

This thesis research has substantially contributed to the characterization of the pudendal artery, a key blood vessel in the erectile response. Using different therapeutic approaches
such as antihypertensive drug treatment or lifestyle modifications, improvements in erectile function and pudendal arterial structure and function were demonstrated. However, future studies are still needed to provide greater understanding of the mechanisms. Finally, these therapies not only have the potential to benefit erectile function, but can be applied to the management of cardiovascular disease.
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