ROLE OF ANDROGENS IN BLOOD PRESSURE REGULATION: ANTI-HYPERTENSIVE EFFECTS IN NORMOTENSION AND BENEFICIAL VASCULAR EFFECTS IN PREGNANCY-INDUCED HYPERTENSION (PREECLAMPSIA)

A Dissertation

by

ANDREA ESTELLE HANSON

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	John N Stallone
Committee Members,	Christine L Heaps
	Michael C Golding
	Farida Sohrabji
	Mercedes Perusquia
Head of Department,	Larry J Suva

August 2020

Major Subject: Biomedical Science

ABSTRACT

The purpose of this dissertation was to elucidate the role of androgens in the regulation of systemic BP and uterine vascular function. There is a well-established sexual dimorphism in human cardiovascular disease (CVD), in which men are more likely to develop hypertension and coronary artery disease (CAD) compared to age-matched pre-menopausal women, which suggests that testosterone (TES) is deleterious to cardiovascular health. The findings of the present studies in this dissertation challenge this dogma, and instead, provide support that TES and its metabolites 5α- and 5β-dihydrotestosterone (DHT) are beneficial to blood pressure (BP) and vascular function. The first project utilized male Sprague-Dawley (SD) and testicular feminized male (Tfm) rats to evaluate the long-term effects of endogenous and exogenous androgens on systemic blood pressure (BP). This study provided new evidence and novel findings that androgens (TES and 5α-DHT) exert long-term anti-hypertensive effects on systemic BP in male rats. These anti-hypertensive mechanisms involve estrogen-independent, non-genomic, signaling pathways which reduce the renin angiotensin system (RAS) expression in the kidney. Thus, the antihypertensive effects of androgens appear to involve both rapid effects on systemic vasodilation and long-term effects on the kidney to promote fluid excretion. The present findings are important for future therapeutic applications of TES and/or DHT therapy to treat hypertension in hypogonadal men. The second project utilized female SD rats to investigate the vascular effects and signaling mechanisms of androgens at the uteroplacental interface during normal and preeclampsia (PE). In this study, the deoxycorticosterone (DOCA)-salt pregnant rat was validated as an acceptable animal

model of PE, with hallmark characteristic of the disease including hypertension, proteinuria, and fetal intrauterine growth restriction (IUGR). The results reveal that androgens elicit rapid vasorelaxation of isolated uterine arteries (UA) from normal pregnant (NP) and PE pregnant (PEP) rats and evoke mechanisms that appear to be highly dependent on endothelial nitric oxide synthase (eNOS) signaling and the activation of voltage-gated (K_V) and Ca^{2+}-activated (BK_{Ca}) K^+ channels. In PEP rats, UA sensitivity to TES decreased, while UA sensitivity to 5β-DHT increased compared to NP rats. Further, mRNA expression of 5β-reductase decreased in the uterine arteries and placenta of PEP rats compared to NP control rats. Thus, the vasodilatory effects of androgens may contribute to the regulation of uterine blood flow during normal pregnancy and reductions in androgen production may contribute to the pathogenesis of PE. The increased sensitivity of the UA to 5β-DHT and impaired production of 5β-DHT at the uteroplacental interface in the PEP rats suggest a possible therapeutic role for androgens, particularly 5β-DHT, in the treatment of reduced uterine blood flow and hypertension in women with PE.

ACKNOWLEDGEMENTS

I would like to personally thank the individuals who helped bring this dissertation to fruition by providing continuous advice, knowledge, support, and guidance throughout my tenure as a graduate student.

Thanks to my mentor, and the chair of my committee, Dr. John Stallone. I am truly indebted to the training, knowledge, and insight you graciously provided. My sincerest appreciation to Dr. Christine Heaps for helping to facilitate the vascular function studies in this dissertation and providing support and guidance along the way. Thanks to Dr. Michael Golding, whose enthusiasm for teaching, interest in reproductive physiology, and knowledge of molecular research was instrumental to this project. A heartfelt thank you to Dr. Farida Sohrabji, whose unwavering support and invaluable feedback was always encouraging. I would also like to thank Dr. Mercedes Perusquia, who continuously strives to further our understanding of sex steroid hormones. I would also like to thank Josh McKenna and Nico Garcia, the graduate students who worked diligently on these studies and provided continuous entertainment along the way and Tanya Herzog at Houston Methodist Hospital for her continued support and encouragement. I am also grateful to Texas A&M University and to the department of Veterinary Physiology and Pharmacology for the education and support during my time as a graduate student.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a dissertation committee consisting of Texas A&M University Professors John Stallone, Christine Heaps, and Michael Golding of the Department of Veterinary Physiology and Pharmacology Texas A&M Professor Farida Sohrabji of the Department of Neuroscience and Experimental Therapeutics, and Universidad Nacional Autonoma de Mexico Processor Mercedes Perusquia of the Department of Cellular Biology and Physiology.

Funding Sources

This graduate study was supported by the Michael E Debakey Institute, the National Institutes of Health National Heart, Lung, and Blood Institute, and the Texas A&M Veterinary Physiology and Pharmacology Department.

NOMENCLATURE

4-AP	4-aminopyridine
A_4	Androstenedione
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
Agt	Angiotensinogen
Akt	Protein kinase B
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of the variance
ANP	Atrial natriuretic peptide
ANS	Autonomic nervous system
AP	Action potential
AR	Androgen receptor
AT_1R	Angiotensin Type 1 Receptor
AT_2R	Angiotensin type 2 receptor
AU	Arbitrary unit
BH_4	Tetrahydrobiopterin
BK_{Ca}	Large conductance Ca^{2+}-activated K^+ channel
BNP	Brain natriuretic peptide
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAD	Coronary artery disease

CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CCC	Central control center
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CO	Cardiac output
Ct	Cycle threshold
CVD	Cardiovascular disease
DAG	Diacylglycerol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
DOCA	Deoxycorticosterone
EC_{50}	effective concentration 50
ECF	Extracellular fluid volume
EDHF	Endothelial derived hyperpolarizing factor
Ef-1	Elongation factor-1
E_{max}	Maximal response
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
GFR	Glomerular filtration rate
Gly	Glybenclamide
GPCR	G-protein-coupled receptors
GTP	Guanosine triphosphate

HR	Heart rate
HSP	Heat shock protein
IbTx	Iberiotoxin
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-trisphosphate
IUGR	Intrauterine growth restriction
JG	Juxtaglomerular
JGA	Juxtaglomerular apparatus
K^+	Potassium
K_{ATP}	ATP-dependent K^+ channels
KCl	Potassium Chloride
KDR	Kinase insert domain receptor
KHB	Krebs Henseleit solution
K_{ir}	Inward rectifying potassium channel
K_V	Voltage-gated K^+ channels
L-NMMA	L-NG -monomethyl Arginine citrate
LST	Losartan
MD	Macula densa
MI	Myocardial infarction
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
Na^+	Sodium
NaCl	Sodium Chloride

NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
nNOS	Neural nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Normal pregnant
NPA	NW-propyl-L-Arginine
NZGH	New Zealand genetically hypertensive
PE	Preeclampsia
PEP	Preeclampsia pregnant
PG	Prostaglandin
$PGF2_{\alpha}$	Prostaglandin $F2_{\alpha}$
PGI_2	Prostacyclin
PIGF	Placental growth factor
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PNS	Peripheral nervous system
RAS	Renin-angiotensin-system
ROCC	Receptor-operated Ca^{2+} channel
ROS	Reactive oxygen species
rt-PCR	Real-time polymerase chain reaction
RUPP	Reduced uterine perfusion pressure
SC	Subcutaneous

SCC	Side chain cleavage
SD	Sprague-Dawley
SEM	Standard error of the mean
sEng	Soluble endoglin
Ser	Serine
sFlt-1	Soluble fms-like tyrosine kinase-1
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rat
SMOC	Second messenger-operated Ca^{2+} channels
SNS	Sympathetic nervous system
SOCC	Store-operated Ca^{2+} channels
SSBG	Sex steroid binding globulin
SV	Stroke volume
TES	Testosterone
Tfm	Testicular feminized male
Thr	Threonine
TPR	Total peripheral resistance
TxA_2	Thromboxane A_2
UA	Uterine artery
UV	Uterine vein
VEGF	Vascular endothelial growth factor
VOCC	Voltage-operated Ca^{2+} channel
VSM	Vascular smooth muscle
WKY	Wistar Kyoto

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Systemic blood pressure

The mammalian cardiovascular system is a closed circulatory system that transports vital nutrients, oxygen, waste products, and various other homeostatic substances throughout the body. Structurally composed of blood, blood vessels, and the heart, the circulatory system generates a pressure gradient, which drives blood flow and tissue perfusion. Blood pressure (BP), the force that blood exerts against the vascular wall, is the measurable driving force for blood flow and is clinically used as a measure of cardiovascular function and health.

BP fluctuates continuously in response to changes in body temperature, exercise, fluid balance, posture, respiration, and other physical and physiological variables. These normal fluctuations elicit integrated responses from the cardiovascular, endocrine, neural, and renal systems to maintain adequate blood pressure, blood flow, and tissue perfusion. Adopted from Ohm's Law, *voltage = current x resistance*, the equation, *BP = cardiac output (CO) x total peripheral resistance (TPR)*, defines the simplified determinates of BP regulation. Systemically, a far more intricate process unfolds. Studies suggest that BP can vary by up to 30%, and these changes initiate dynamic, homeostatic, regulatory mechanisms that involve a combination of short-term and long-term compensatory adjustments in heart rate (HR), stroke volume (SV), total peripheral resistance (TPR), and extracellular fluid (ECF) volume (Figure 1). Disturbances to any of these regulatory or compensatory mechanisms may result in potentially dangerous physiological changes within the body. Understanding basic cardiovascular physiology and BP regulating

mechanisms and the roles that these mechanisms play in BP homeostasis may facilitate further understanding of the development of BP regulating abnormalities, particularly hypertension (Millar-Craig, Bishop et al. 1978, Joyner and Limberg 2014).

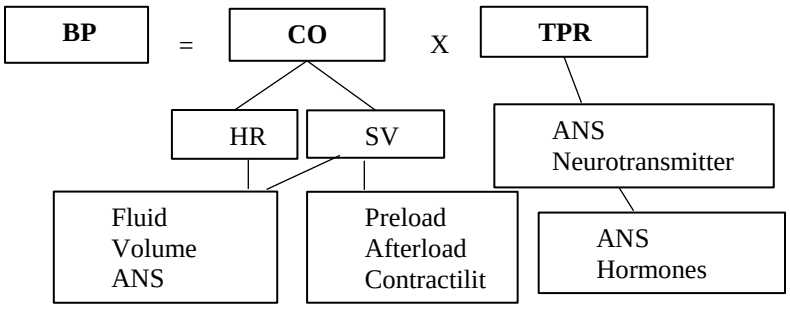

Figure 1. Regulation of blood pressure through the influences of cardiac output and total peripheral.

1.1.1 Cardiovascular physiology

1.1.1.1 Cardiac physiology

The amount of blood pumped by the heart per minute (CO) is directly proportional to the rate of cardiac contraction (HR) and the volume of blood ejected from the heart (SV) during each cardiac contraction. Specialized pacemaker cells within the heart, in addition to the autonomic nervous system (ANS), determine HR, while SV is dependent upon the volume of blood in the heart prior to contraction (preload), the pressure of blood in the vasculature working against the ejection of blood (afterload), and the force with which the cardiac muscle contracts (contractility). Both HR and SV are affected by systemic neural and hormonal activity, and work together to regulate CO (Young 2010).

1.1.1.2 Vascular physiology

As blood is pumped from the left ventricle of the heart into the systemic circulation, the structure and function of the vasculature facilitates a continuous flow of blood to ensure adequate tissue perfusion. Most blood vessels have three distinct tissue layers: the tunica intima, the tunica media, and the tunica adventitia. The inner most layer, the tunica intima, consists of a single layer of endothelial cells and an underlying layer of connective tissue, the internal elastic lamina. The endothelium provides a structural barrier between circulating blood and the vascular wall and regulates adhesion molecules, inflammatory responses, vascular permeability, and vascular tone. The middle vascular layer, the tunica media, is composed of connective tissue, both collagen and elastin, and vascular smooth muscle (VSM) cells. These cells contract and relax in response to neural and hormonal stimuli, which result in changes in the luminal diameter of the blood vessels, thereby altering the vascular resistance to blood flow. The outer most layer, the tunica adventitia, is mainly composed of collagen and elastin. This layer provides structural integrity to the vessels and supports the intravascular nutrient supply (Berne and Levy 2018).

Based on the size and function of the blood vessel, the tunic layers may vary in thickness and structural composition. Large conduit arteries (>10 mm luminal diameter) conduct blood away from the heart, and structurally, these arteries have the highest percentage of elastic fibers, which enable a vascular elastic accommodation and subsequent compression of blood within the vessel. This Windkessel effect allows for vascular distension during contraction of the heart (systole), as arterial pressure increases, and elastic recoil during relaxation of the heart (diastole), as the blood flows downstream and cardiac output declines (Mei, Zhang et al. 2018). This elastic recoil helps to maintain

BP between heart beats. Resistance vessels, or muscular arteries, are smaller arteries (0.1-10 mm) that have a greater percentage of VSM cells and a lower percentage of elastic tissue compared to the larger conduit arteries. The large number of VSM cells within the resistance vessels enable greater potential for change in arterial diameter for the regulation of vascular resistance. The resistance vessels constrict and dilate independent of upstream pressure changes and respond to various mechanical, neural, and hormonal stimuli. Thus, these resistance vessels play an important role in the regulation of TPR, downstream blood flow, and tissue perfusion (Young 2010).

1.1.1.2.1 Endothelial function

The vascular endothelium lines the inner surface of all blood vessels and provides a structural barrier between the circulating blood and the vascular wall. Additionally, the endothelium plays a role in the regulation of coagulation, immune response, and vascular tone. Endothelial cells secrete a variety of vasoactive mediators that maintain vascular health and alter the contractile state of underlying VSM cells. The common endothelium-derived vasodilators include nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factors (EDHF). The common endothelial-derived vasoconstrictors include endothelin (ET), thromboxane (TXA_2), and reactive oxygen species (ROS) (Kobayashi, Murata et al. 2011).

1.1.1.2.2 Nitric oxide

As the most potent endothelium-derived vasodilator, NO plays a central role in the maintenance of cardiovascular homeostasis, with effects on vascular tone, local blood flow, and systemic BP. NO is also a key modulator of vascular structure and function, and promotes angiogenesis and vascular remodeling, while inhibiting platelet aggregation and

monocyte adhesion (Landmesser, Hornig et al. 2004, Forstermann 2010). The bioavailability of NO is largely dependent upon its synthesis from L-arginine which is mediated by a family of three nitric oxide synthase (NOS) enzymes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). iNOS is an inducible isoform expressed in response to inflammatory parameters, whereas eNOS and nNOS are constitutively expressed in the endothelium and/or VSM cells. iNOS is a defense mechanism of the immune system while eNOS and nNOS regulate VSM structure and function (Forstermann, Closs et al. 1994).

The structure of each NOS isoform includes a multi-domain enzyme with a heme-containing N-terminal oxygenase and a C-terminal reductase domain. The oxygenase domain binds tetrahydrobiopterin (BH_4), oxygen (O_2), and L-arginine, while the reductase domain binds nicotinamide adenine dinucleotide phosphate (NADPH). The oxygenase and reductase domains are linked by a calmodulin (CaM)-recognition site, which binds calcium (Ca^{2+}). In the reductase domain, Ca^{2+}-CaM binding facilitates the transfer of electrons from NADPH to the oxygenase domain. The electron transfer facilitates the oxidation of L-arginine into L-citrulline, which results in the release of NO. Independent of Ca^{2+}-CaM binding, post-translational phosphorylation and dephosphorylation of serine (Ser) and threonine (Thr) residues also regulate eNOS activation. Simultaneous multi-site phosphorylation acts to balance the activation of NOS and subsequent production of NO (Mount, Kemp et al. 2007). There are five identified phosphorylation sites which either activate or deactivate production of NO by eNOS. While the effects of Ser^{114} and Ser^{615} phosphorylation have not been clearly established, phosphorylation of Ser^{633} and Ser^{1177} agonize eNOS production of NO, while phosphorylation of Thr^{495} antagonizes eNOS

function. eNOS phosphorylation stimulates a flux of electrons within the reductase domain, which increases the sensitivity of the Ca^{2+}-dependent pathways and activates the Ca^{2+}-independent protein kinase B (Akt) intracellular signaling mechanisms. In cultured endothelial cells, high basal levels of Thr^{495} phosphorylation contribute to NO synthesis, which may contribute to the regulation of vascular tone (Mount, Kemp et al. 2007). In addition to Ca^{2+}-CaM binding and NOS phosphorylation, other regulatory mechanisms such as heat shock proteins may also contribute to eNOS regulation (Forstermann, Closs et al. 1994, Weissman, Jones et al. 2002, Forstermann 2010).

The main source of endothelial NO is classically considered to be derived from constitutively expressed eNOS, yet accumulating evidence suggests that the expression of nNOS in endothelial and VSM cells may contribute to the regulation of blood flow and vascular tone (Buchwalow, Podzuweit et al. 2002, Costa, Rezende et al. 2016). For example, nNOS expressed in the coronary arterial endothelium has been observed to maintain blood flow via NO-activated cGMP vasodilation in eNOS knock-out mice (Huang, Sun et al. 2002). While nNOS expression may contribute to blood flow, the expression appears to vary among vascular beds. In the kidney, endothelial NO is derived from nNOS expressed in the endothelial lining of the efferent arterioles and from eNOS expressed in the afferent arterioles; together nNOS and eNOS appear to regulate renal blood flow and maintain microvascular function. In addition to the vasodilatory effects on the renal vasculature, nNOS-derived NO appears to reduce the vasopressor effects of angiotensin II (Ang II) in the kidney (Ichihara, Inscho et al. 1998).

The expression of the NOS isoforms is influenced by several physiological factors. Mechanical factors, such as sheer stress and exercise training significantly increase eNOS

expression and NO bioavailability in the aortic endothelium (Moncada and Higgs 1995, Nosarev, Smagliy et al. 2014). Hormonal factors, such as estradiol (E_2) increase both eNOS and nNOS gene expression in the skeletal muscle vasculature (Weiner, Lizasoain et al. 1994). Changes in isoform expression may be associated with the development of various vascular diseases. Decreases in eNOS expression have been observed in rodents predisposed to atherosclerosis (Won, Zhu et al. 2007) and variations in genomic NOS isoform expression appear to exacerbate endothelial dysfunction (Anea, Cheng et al. 2012).

1.1.1.2.3 Regulation of VSM cells

VSM cells of resistance vessels are maintained in a state of partial contraction known as basal vascular tone. This regulates blood flow and tissue perfusion, and contributes to the regulation of TPR and systolic BP. Changes in vascular tone depend upon factors that influence the contractile state of VSM cells, which is regulated by cytosolic Ca^{2+} concentration. A multitude of physiological factors, including humoral, neural, and mechanical stimuli modulate cell membrane ion permeability and alter cytosolic Ca^{2+} concentration.

Membrane permeability relies upon a resting membrane potential, which is an electrical potential gradient generated by the balance of electrolyte concentrations and permeabilities across the cell membrane. Within each cell, sodium-potassium ATP-ase (Na^+/K^+-ATP-ase) pumps are continuously extruding Na^+ ions out of the cell and transporting K^+ ions into the cell. K^+ channels regulate resting membrane potential as the intracellular K^+ ions move towards their equilibrium potential and cross the membrane to reestablish a chemical balance between intracellular and extracellular ion concentrations. K^+ channels are the most diverse group of membrane ion channels that regulate the

membrane potential and subsequently regulate vascular tone. There are four known classes of K^+ channels: voltage-gated potassium channels (K_V), Ca^{2+}-activated potassium channels (K_{Ca}), ATP-activated potassium channels (K_{ATP}), and inward rectifying potassium channels (K_{ir}). These channels share a common selectivity for K^+ ions and contribute to the overall electrical activity of the cell membrane. The basic K^+ channel structure consists of two, four, or six transmembrane helices which span the lipid bilayer and are divided into two parts: the pore-forming domain which transport K^+ ions and the regulatory domain which senses stimuli. All K^+ channels have pore-forming domains with two evenly spaced K^+ binding sites. Binding sites appear to operate under two configurations, with either an S1/S3 configuration or an S2/S4 configuration that transport K^+ ions. These channels rely on gaiting mechanisms to close channel accessibility and regulate ion transport across the membrane. As ions move across the membrane, changes in the equilibrium potential affect cell polarization and facilitate the opening and/or closing of ion channel function (Feletou 2011).

The contraction and relaxation of VSM cells is regulated by cytosolic Ca^{2+} concentration. Ca^{2+} release from the mitochondria and sarcoplasmic reticulum and the influx of extracellular Ca^{2+} through membrane-bound ion channels increase intracellular Ca^{2+} concentration. Ca^{2+} is transported across the membrane through Ca^{2+} ion channels that include voltage-operated Ca^{2+} channels (VOCC), receptor-operated Ca^{2+} channels (ROCC), second messenger-operated Ca^{2+} channels (SMOC), and store-operated Ca^{2+} channels (SOCC). Intracellular Ca^{2+} then binds to CaM and activates myosin light chain kinase (MLCK) phosphorylation of the myosin light chain (MLC) and activates myosin and actin cross-bridge cycling. Cross-bridge cycling shortens the VSM cells and

circumferentially reduces luminal diameter of the blood vessel. In contrast, VSM cell relaxation is induced by a reduction in cytosolic Ca^{2+} concentration, mediated by sarcoplasmic reticulum Ca^{2+} uptake and plasma membrane pump Ca^{2+} extrusion. Additionally, increases in MLC phosphatase (MLCP) reduces MLC phosphorylation and activates cyclic adenosine monophosphate (cAMP)-induce MLCK inhibition, which further facilitates MLC dephosphorylation (Korthuis 2011).

Vasoactive mediators modulate cytosolic Ca^{2+} concentration via several mechanisms including changes in membrane channel permeability and second messenger systems. For example, the endothelium-derived NO signaling transduction pathway regulates MLC phosphorylation and alters the contractile state of VSM cells via soluble guanylate cyclase (sGC)-mediated conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), which activates cGMP-dependent protein kinase G (PKG). The protein kinase signaling pathway reduces intracellular Ca^{2+} concentration and increases MLCP, which shift the VSM cell into a state of de-phosphorylated MLCK-induced vasorelaxation (Forstermann 2010). There are over one thousand identified G-protein-coupled receptors (GPCR) that activate Gs, Gi, and Gq proteins. G-proteins transduce extracellular signals into intracellular responses. Epinephrine, adenosine, and prostacyclin activate Gs-proteins and increase vasorelaxation via increases in cAMP, inhibition of MLCK, and reduction of MLC phosphorylation. Gi-protein-coupled receptors, which are activated by norepinephrine, inhibit cAMP and increase the activity of MLCK, which promotes VSM contraction. Gq-proteins promote vasoconstriction and are activated by several humoral and neural stimuli, which include norepinephrine, Ang II, and Et-1. These Gq-proteins activate intracellular phospholipase C (PLC) signaling

cascades and increase inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), and second messenger protein kinase C (PKC). IP_3 promotes sarcoplasmic reticulum Ca^{2+} release, while PKC phosphorylates regulatory proteins and affects plasma membrane channels to enhance VSM cell contraction (Thorneloe and Nelson 2005, Hill-Eubanks, Werner et al. 2011).

1.1.2 Renal physiology

Renal function is dependent upon an abundant and constant supply of blood. Under normal physiological conditions, the human kidneys receive roughly 25% of cardiac output and produce approximately 180 liters of filtrate each day. Blood enters the kidney via the renal artery, which continuously branches until the afferent arterioles deliver blood to each of the estimated 1 million nephrons in each kidney. Each nephron ultra-filters an estimated 20% of the circulating plasma through a highly intricate capillary network called the glomerulus. The glomerular capillaries are impermeable to red blood cells and larger solutes (e.g. proteins, larger peptides, etc.), but the filtered fluid that passes through the capillary bed collects in Bowman's capsule. As the filtrate collects, hydrostatic pressure within the capsule facilitates the flow of filtrate away from the glomerulus and into the renal tubule, where water, electrolytes, nutrients, and waste products are reabsorbed and/or secreted, and eventually excreted as urine. Unfiltered blood leaves the glomerulus through the efferent arteriole and enters the peritubular capillaries before leaving the renal circulation and returning to the systemic venous vasculature. The peritubular capillaries parallel the renal tubule and serve to support the process of reabsorption and/or secretion of fluids and solutes across the tubular membrane between the filtrate and circulating blood.

Filtrate successively flows through four functional sections of the renal tubule: the proximal tubule, the loop of Henle, the distal tubule, and a common collecting duct that is shared by numerous individual nephrons. The selective reabsorption of the filtrate begins in the proximal convoluted tubule, where approximately 67% of the filtered Na^+ and water are reabsorbed back into circulation. Filtrate reabsorption continues into the loop of Henle which consists of three major segments: thin descending limb, thin ascending limb, and thick ascending limb. Each segment is characterized by varying cell morphology, which functions to selectively allow the reabsorption of approximately 15-25% of filtered sodium chloride or water. The loop of Henle plays a central role in concentrating the urine as the differential movement of sodium chloride and water across the ascending and descending limb tubule membranes, respectively, establishes a hypertonic medullary interstitial osmotic gradient. As filtrate continues into the distal tubule and collecting duct, salt and water reabsorption is fine tuned in response to intrarenal and systemic endocrine signaling molecules, resulting in the formation of the final urine volume, that then flows to the ureter and to the bladder for storage and excretion (Berne and Levy 2018).

1.2 Blood pressure regulation

BP is a tightly regulated systemic variable aimed at providing optimal blood flow and tissue perfusion to all metabolically active tissues. The feedback mechanisms that detect changes in BP, blood volume, and tissue perfusion, work together to regulate BP. In the brain, heart, and kidney, baroreceptors and chemoreceptors activate neural and hormonal signaling mechanisms that mediate short-term and long-term effects on CO, TPR, and ECF volume. In addition, mechanical influences, such as shear stress and myogenic response, further influence blood flow and BP, respectively, at the local level.

The cardiovascular regulatory effects on HR, SV, and TPR mediate short-term BP homeostasis, while the renal effects contribute to long-term regulation of ECF volume and BP.

1.2.1 Shear Stress and Myogenic Response –Mechanical Regulation

BP and blood flow continuously exert internal physical stresses on the vascular wall. The frictional force, known as wall shear stress on the endothelium, and vascular stretch, known as circumferential distension of the vascular wall, influence biochemical reactions within the vessels that play an important role in the maintenance of blood flow and vascular health. Sustained shifts in local response mechanisms can result in deleterious changes in the vasculature and alter the form and function of the blood vessels.

Flow-mediated vasodilation occurs in response to acute increases in shear stress. The frictional force of blood flow against the vascular wall activates endothelial surface molecules and intracellular signaling pathways. As the primary bioactive chemical produced in response to increases in vascular shear stress, NO contributes to cGMP-induced vasodilation of VSM cells and endothelial oxidative balance. Other signaling molecules such as EDHF and PGI_2 are also activated during increases in flow, inducing flow-mediated vasodilation (Lu and Kassab 2011). Vasorelaxation of VSM cells increases luminal diameter, decreases flow-mediated sheer stress, and enhances downstream blood flow.

Circumferential stretch exerts perpendicular stress on the vascular wall in response to changes in arterial pressure. Circumferential increases in wall stress occur with increases in BP, which mechanically alters membrane bound, stretch-sensitive, Ca^{2+} channels in the vascular wall. This mechanical stretch opens Ca^{2+} channels and initiates

extracellular Ca^{2+} influx, which results in VSM cell contraction and the reduction of blood flow to downstream tissue beds. As the BP decreases, a decrease in vascular stretch reduces intracellular Ca^{2+} influx, and results in the subsequent relaxation of VSM, increasing downstream blood flow. This mechanical response regulates local blood flow to specific tissues and also contributes to changes in TPR (Meininger and Davis 1992). The applied loads of flow-mediated shear stress and pressure-mediated circumferential stress mediate the local homeostatic regulation of blood flow.

1.2.2 Autonomic Nervous System – Neural Regulation

The ANS consists of sympathetic and parasympathetic branches that innervate the cardiovascular and renal systems, in addition to many other organ systems in the body. Changes in systemic BP and ECF volume are detected by centrally located arterial baroreceptors in the aortic arch and carotid sinus, by volume receptors located in the heart and great veins, and through mechanical manipulation of membrane-bound stretch receptors. These signaling mechanisms propagate action potentials (AP) through afferent nerve fibers to the central control center (CCC) of the central nervous system (CNS). The CCC modulates ANS outflow to the cardiovascular system to reestablish setpoint BP in response to changes in BP and blood volume.

Systemic BP is regulated by a balance between the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) functions of the ANS. One function of the SNS is to mobilize the systemic fight-or-flight response, while both the SNS and PNS function to control homeostasis in a push-pull manner. The neurotransmitters of the ANS, norepinephrine (NE) and acetylcholine (ACh), initiate an intricate and dynamic balance of VSM contraction and relaxation, which regulate vascular tone. The SNS

adrenergic nerve endings innervate VSM cells, and NE release activates α_1- and α_2 -receptors, which initiate VSM cell contraction. Decreases in adrenergic activity result in a reduction of α_1-adernergic receptor-mediated VSM cell contraction, which promotes VSM cell relaxation. NE release also activates β_2-adrenoceptors and induces VSM cell relaxation, however, these β_2 receptors are specific to the vascular beds of the lungs and skeletal muscles. In the heart, the SNS neurotransmitters regulate CO through effects on B_1-adrenoreceptors to increase HR and cardiac contractility. Overall, the effects of the SNS result in an increase in CO and TPR and subsequently increase systemic BP, while the PNS modulates HR and CO during rest and opposes the stimulatory actions of the SNS, thereby decreasing systemic BP.

1.2.3 Endocrine System – Humoral Regulation

The endocrine system secretes chemical messengers to work with the nervous system to coordinate short- and long-term regulation of BP by cardiovascular and renal systems. These BP regulating hormones are derived from numerous endocrine organs, such as the brain, heart, and kidney, and act upon target tissues to elicit both short-term and long-term effects on the cardiovascular system and kidney. In the heart, cardiomyocytes secrete atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in response to atrial distension, an indicator of blood volume. ANP and BNP bind to target cells in the peripheral vasculature and induce acute increases in vasodilation and acute increases in vascular permeability, to promote the redistribution of fluid and decrease BP (Curry 2005). In the kidney, natriuretic peptides vasodilate the afferent arteriole and vasoconstrict the efferent arteriole, which increases GFR, eliciting a natriuretic action by

directly inhibiting sodium chloride reabsorption and increasing urinary sodium (Na^+) excretion (Ballermann and Brenner 1987).

In contrast to ANP and BNP, chemical messengers of the renin-angiotensin-system (RAS) increase systemic BP in response systemic hypotension and reductions in ECF volume. As the biologically active product of the RAS cascade, Ang II increases release of aldosterone from the adrenal cortex, and directly acts upon the cardiac, brain, vascular, and renal tissues to regulate BP. Continuously secreted by the liver, angiotensinogen (Agt) is cleaved in the circulation by renin. Renin, an aspartyl protease, plays a central role in BP regulation as the rate limiting step in the production of Ang II. Renin is primarily produced by renal juxtaglomerular (JG) cells, which are pressure-sensitive cells located in the walls of the renal afferent arterioles. Renin secretion increases in response to decreases in renal perfusion pressure and increases in sympathetic nervous activity. Renin and Agt catalyze to form angiotensin I (Ang I), which is then converted into the biologically active, Ang II, by angiotensin converting enzyme (ACE). Ang II binds to specific receptors in the adrenal gland, brain, cardiovascular, and renal systems, to increase TPR, CO, and increase Na^+ reabsorption (Wilcox, Subramanian et al. 1997). In the brain, Ang II influences hypothalamic and brainstem regulation of autonomic and neurosecretory centers to enhance sympathetic outflow (Young and Davisson 2015). In the cardiovascular system, Ang II stimulates cardiac contractility, enhances sympathetic signal propagation, and increases vasoconstriction. In the renal system, Ang II decreases glomerular filtration rate (GFR) and promotes tubule Na^+ reabsorption, while in the adrenal gland, Ang II stimulates the cortex to synthesize and secrete aldosterone and the adrenal medulla to produce catecholamines, which further enhance BP and renal tubule reabsorption of Na^+. Ang II

acts through two main receptors, the angiotensin type 1 receptor (AT_1R) and the angiotensin type 2 receptor (AT_2R). In the vasculature, AT_1R activation mediates arterial vasoconstriction and cardiovascular remodeling, while AT_2R activation functions to counterbalance AT_1R-mediated effects, by inducing vasodilation and increasing cardiomyocyte ANP production. Mas-receptors and angiotensin 1-7 receptors also act to offset AT_1R effects. ANP acts to suppress RAS function, despite evidence that Ang II stimulates ANP production (Kerkela et al. 2015), which suggests a push-pull balance exists between these two feedback mechanisms to maintains BP and ECF volume homeostasis under normal physiological conditions.

In the kidneys, a local RAS appears to influence renal function independent of the systemic RAS components. With all RAS components locally expressed within the kidney, local Ang II production offers a new perspective for the pathophysiology of hypertension and the renal regulation of BP. Specialized cells of the juxtaglomerular apparatus (JGA) respond to changes in renal perfusion pressure and renal tubule filtrate osmolarity. The JGA is comprised of the macula densa (MD), juxtaglomerular, and mesangial cells. The MD cells reside in a distinct portion of the early distal convoluted tubule, where they sense the delivery of solute from the loop of Henle to the distal tubule and communicate with the juxtaglomerular and mesangial cells to regulate glomerular filtration and Na^+ excretion. Elevated sodium chloride delivery to the distal tubule increases adenosine production from the MD cells. The increased adenosine production elicits vasoconstriction of the afferent arterioles and stimulates renin production and secretion from the juxtaglomerular cells. The afferent arteriolar vasoconstriction reduces glomerular blood flow and GFR, while renin production upregulates the local RAS cascade

and results in an increase of intrarenal Ang II production. Ang II receptors are widely distributed throughout the kidney and activation of AT_1Rs decrease renal blood flow, reduce glomerular filtration, and regulate renal Na^+ and water excretion. The AT_2Rs expressed in the proximal tubule and collecting ducts oppose the AT_1R effects on GFR and Na^+ excretion. In a number of experimental animal studies, upregulation of the intrarenal RAS promotes kidney disease and hypertension, while suppression mitigates these effects (Kobori, Nishiyama et al. 2003). Some studies suggest ACE expression regulates intrarenal Ang II production and protects against experimentally induced hypertension (Gonzalez-Montelongo, Marin et al. 2013, Yang and Xu 2017).

1.3 Hormonal Effects

There are many hormones that influence underlying vascular tone. The mineralcorticoid aldosterone, primarily synthesized in the zona glomerulosa of the adrenal gland, is also produced in both endothelial and vascular smooth muscle cells. Aldosterone acts through acute, non-genomic and/or chronic, genomic mechanisms that modulate vascular resistance and blood flow by altering NO synthesis and bioavailability (Toda, Nakanishi et al. 2013). The glucocorticoids, such as cortisol, influence vascular tone by increasing vascular sensitivity to catecholamines through cytosolic corticosteroid receptors such as type 1 mineralcorticoid receptors that bind aldosterone, deoxycorticosterone, and corticosterone and type II glucocorticoid receptors that bind cortisol and other glucocorticoids such as dexamethasone. Corticosteroids further potentiate the action of other vasoconstrictor hormones such as Ang II, vasopressin, endothelin, and thromboxane (Ullian 1999). Intravenous infusion of the androgen DHEA has vasodilatory effects *in vivo* (Perusquia, Hanson et al. 2018, Sharma, Coridon et al. 2018). Additional hormones

such as growth hormones, placental lactogen, and prolactin regulate vascular tone through NO and prostacyclin production in rats (Gonzalez, Rosas-Hernandez et al. 2015).

Androgens are a specific chemical group of steroid hormones that have long been recognized for their role in mediating the differentiation, development, and growth of the male reproductive system. Early experimental animal studies observed decreases in the sexual and aggressive behavior of castrated roosters that were attributed to chemical substances released from the testes. Testosterone (TES) and its metabolite dihydrotestosterone (DHT), were later identified as the testicular substances that enhanced secondary male sex characteristics, such as beard growth, voice deepening, and the male body configuration. Today, there is greater understanding of the roles of androgens in many other physiological functions besides reproduction, such as the cardiovascular, haemopoietic, immune, musculoskeletal, and neural systems (Navarro, Allard et al. 2015, Davey and Grossmann 2016). Androgen biosynthesis, summarized in Figure 2, is initiated with the enzymatic conversion of cholesterol to pregnenolone by cytochrome P450, a side-chain cleaving enzyme. This rate-limiting step occurs in the internal mitochondrial membrane of specific cell types in androgen-synthesizing tissues including the Leydig cells in the testis, theca interna and corpus luteum cells in the ovary, trophoblastic cells in the placenta, zona reticularis cells in the adrenal gland, and a few select cell types in the brain (Ghayee and Auchus 2007). Pregnenolone is then transferred to the endoplasmic reticulum and enzymatically converted into dehydroepiandrosterone (DHEA) or other precursors of TES. Approximately 10% of TES is reduced to the more potent metabolite 5α-DHT in peripheral target organs, while 0.3% is aromatized into estrogen in select cells

of the brain and peripheral tissues. The greatest source of aromatase in the brain resides in the hypothalamus; however, most systemic aromatase resides in adipose tissue.

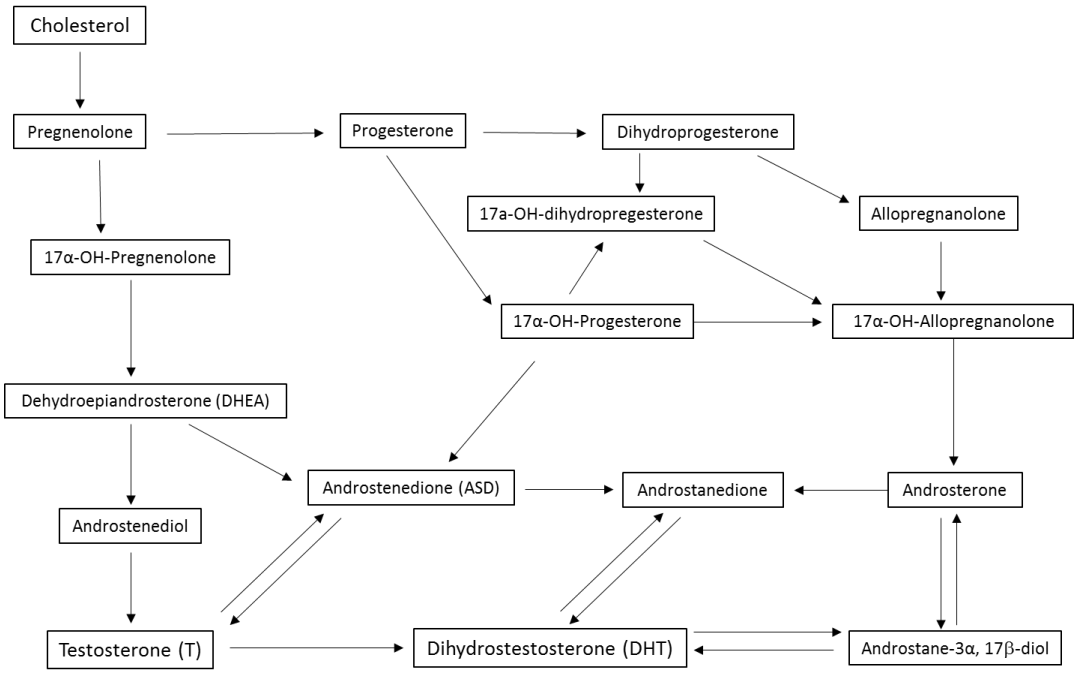

Figure 2. Biosynthesis of androgens

As a lipophilic steroid molecule, approximately 97% of circulating TES is bound to plasma proteins. Approximately 30-45% of TES is bound to a high-affinity sex steroid binding globulin (SSBG), while 50-70% is bound to lower-affinity plasma albumin, and 3% remains as a free unbound, bioavailable hormone. TES is metabolized in various target tissues and produces several interesting vasoactive metabolites, including its reduced metabolites 5α- and 5β-DHT. 5β-DHT, the stereoisomer to the genomically potent androgen 5α-DHT, is genomically inactive, but a highly efficacious vasodilator compared to TES and/or 5α-DHT (Perusquia, Navarrete et al. 2007, Perusquia and Stallone 2010).

5α-DHT is frequently employed as a tool to verify that the effects of TES are independent of its aromatization to estrogen, since 5α-DHT cannot be converted to estrogen (Chou, Sudhir et al. 1996, Deenadayalu, White et al. 2001).

Androgens are recognized as high lipophilic molecules that freely permeate the plasma membrane of target cells and bind to cytosolic androgen receptors (AR), a ligand-dependent nuclear transcription factor. This classic pathway results in the synthesis of proteins that mediate the biological effects of androgens. Independent of this classical pathway, there also appear to be rapid, non-genomic, transcription-independent pathways for TES and its metabolites that activate intracellular second messenger signaling mechanisms (Norman, Mizwicki et al. 2004).

1.3.1 Classical Genomic Pathway

TES and its major metabolite 5α-DHT have long been recognized as the most abundant and potent endogenous androgenic hormones, which operate through a classical genomic pathway by binding to cytosolic ARs found in multiple target tissues. Also known as nuclear receptor subfamily 3, group C, member 4, the AR is coded by a single gene found on the X chromosome at Cq11-12, which codes for the protein functional domains: N-terminal domain, DNA-binding domain, hinge region, and a ligand-biding domain. ARs are associated with a complex of heat shock proteins (HSPs), which aid in stability and function of unbound protein receptors and help facilitate ligand binding of TES or 5α-DHT. TES can directly bind to the cytosolic AR, or be reduced to 5α-DHT, and then bound as 5α-DHT to the same cytosolic ARs.

1.3.2 Non-Genomic Pathway

There is increasing evidence that androgens also act through non-DNA binding, nuclear receptor-independent, non-genomic mechanisms, which activate a variety of intracellular protein kinases, adenylyl cyclase, and voltage-gated and/or ligand-gated ion channels. These non-genomic effects of androgens can be demonstrated using androgens that are conjugated to bovine serum albumin (BSA), which prevents the steroids from crossing the cell membrane, or testicular feminized male (Tfm) rodents, which possess defective cytosolic ARs.

The non-genomic androgen signaling occurs in several target tissues, yet the signaling mechanisms remain poorly understood. Androgens induce rapid, non-genomic increases in intracellular Ca^{2+} concentration in skeletal muscle (Sinha-Hikim, Taylor et al. 2004), neuroblastoma cells (Estrada, Varshney et al. 2006), osteoblasts (Lieberherr and Grosse 1994), sertoli cells (Gorczynska and Handelsman 1995), T-cells, and macrophages (Benten, Lieberherr et al. 1997, Benten, Lieberherr et al. 1999, Wunderlich, Benten et al. 2002). In cardiomyocytes, TES increases intracellular Ca^{2+} release from the endoplasmic reticulum and mitochondria (Bennett, Gardiner et al. 2010). In the vasculature, TES exerts acute vasodilatory effects on large arteries such as the aorta and coronary and small resistance arteries such as the mesenteric, prostatic, pulmonary, and subcutaneous arteries in several species (Costarella, Stallone et al. 1996, English, Jones et al. 2000, Perusquia and Stallone 2010). Several studies suggest that TES induces endothelium-independent vasodilation (Deenadayalu, White et al. 2001, Ding and Stallone 2001), while other studies suggest that physiological concentrations of TES-produce endothelium-dependent vasorelaxation (Tep-areenan, Kendall et al. 2002). TES-induced vasorelaxation appears to

involve NO production as TES increases eNOS- or nNOS-derived NO availability through non-genomic signaling mechanisms. Non-genomic signaling pathways include cytosolic AR intracellular activation of Akt signaling-induced NOS phosphorylation (Yu, Akishita et al. 2012), VOCC inhibition (Crews and Khalil 1999, English, Jones et al. 2002, Jones, Pugh et al. 2003), or K^+ channel activation, more specifically K_v and/or BK_{Ca} channels (Deenadayalu, White et al. 2001, White, Owen et al. 2007, Deenadayalu, Puttabyatappa et al. 2012). There appears to be crosstalk between the genomic and non-genomic signaling mechanisms, which may influence the acute effects of androgens on vascular function. These effects of androgens on vascular function may provide valuable insight into the roles that androgens play in the progression of various vascular diseases, specifically hypertension and cardiovascular disease (CVD).

1.4 Hypertension

Hypertension is defined as chronically elevated arterial BP, and according to the American Heart Association, high blood pressure affects nearly 46% of American adults. Appropriately nicknamed the "silent killer", hypertension presents with few clinical symptoms and often remains undiagnosed. Left untreated, hypertension can cause irreversible organ damage (Yoon, Gu et al. 2015, Merai, Siegel et al. 2016) and uncontrolled hypertension can progress into various forms of CVD such as coronary artery disease (CAD), heart failure and stroke, and kidney failure. Uncontrolled hypertension also contributes to the development of vascular dementia and accelerates cognitive decline, and is linked to the development of Alzheimer's disease (Aronow, Fleg et al. 2011). Hypertension presents in approximately 69% of first time myocardial infarction (MI)

patients, 77% of first time stroke patients, 74% of patients with congestive heart failure, and 60% of patients with peripheral arterial disease (Aronow, Fleg et al. 2011).

BP measurements are used clinically as an indicator of cardiovascular function. The normal systolic and diastolic BP guidelines consider normal pressures to be less than 120/80 mmHg. Stage 1 hypertension is considered to fall within the systolic pressure range of 130 to 139 mmHg and diastolic pressure range of 80 to 89 mmHg, and any systolic BP measurement over 140 mmHg is considered Stage 2 hypertension (Putra, Balasooriya et al. 2019). Hypertension is diagnosed as either primary, also known as essential, or secondary hypertension. Primary hypertension, the most commonly diagnosed hypertensive disorder, is considered idiopathic hypertension with no identifiable cause, while secondary hypertension is associated with an underlying disease or medical condition such as renal artery stenosis. Medical disorders such as metabolic syndrome, sleep apnea, renal dysfunction, adrenal tumors, thyroid disorders, and vascular defects increase the risk for developing hypertension. Additionally, hypertension is associated with risk factors such as age, pregnancy, stress, and sex (Aronow, Fleg et al. 2011).

The observed sex disparities in hypertension that exist between men and women appear to reverse with age. In younger adults, 50 years of age or less, hypertension is more prevalent in men as compared to age-matched pre-menopausal women, whereas hypertension is more prevalent in post-menopausal women compared to age-matched men (Lloyd-Jones, Adams et al. 2010). Independent of sex disparities, hypertension progresses with age. It is estimated that 7.5% of adults between 18 and 39 years of age are hypertensive, while 63.1% of adults aged 60 and older are hypertensive (Fryar, Ostchega et al. 2017).

1.5 Cardiovascular Disease

Hypertension is an important predisposing factor in the development of CVD. As the leading cause of death in the United States, CVD varies with age, diet, race, and sex. Early clinical and epidemiological studies led to the dogma that TES exacerbates the development of hypertension and CVD in men. These studies imply that TES exerts deleterious effects on cardiovascular function and metabolism, leading to higher incidences of atherosclerosis, hypertension and CVD in males (Carson and Rosano 2012, Rezanezhad, Borgquist et al. 2018). This dogma gained further support from studies which demonstrated deleterious effects of exogenous androgen supplementation on CAD in athletes and on vascular complications in female-to-male transgender individuals (Death, McGrath et al. 2004). Some experimental animal studies also reveal that TES enhances vascular tone and exacerbates hypertension, which could result in higher incidence of CVD (Schror, Morinelli et al. 1994, Reckelhoff, Zhang et al. 1998). In contrast, more recent clinical and epidemiological studies challenge this dogma. Studies suggest a direct correlation between androgen deficiency and the increased prevalence of chronic diseases, such as atherosclerosis, chronic kidney disease, dyslipidemia, hypertension, insulin resistance, and metabolic syndrome. These studies further suggest that TES therapy improves these chronic conditions and provides cardio-protection (Liu, Death et al. 2003, Traish, Abdou et al. 2009, Carson and Rosano 2012, Kelly and Jones 2013, Kelly and Jones 2013, Traish, Guay et al. 2014, Wallis, Brotherhood et al. 2014, Traish and Zitzmann 2015, Shoskes, Tucky et al. 2016, Traish, Haider et al. 2017).

Analysis of the short-term and long-term effects of exogenous androgen therapy demonstrates marked improvements in the metabolic profile and vascular function of older

hypogonadal men, and reductions in the prevalence of CVD and CVD-associated mortality (Shoskes, Tucky et al. 2016, Traish, Haider et al. 2017). In women, controversial topics regarding hormone therapy and the selection of therapeutic strategies revolve around age-related differences in the systemic response to estrogen therapy. Experimental animal studies reveal that estrogen deficiency adversely affects the metabolic profile and vascular function of ovariectomized females and increases the risk for developing hypertension (Wong, Ma et al. 2007). However, there appears to be an age-related difference between the cardio-protective effects of estrogen in young, pre-menopausal vs. deleterious effects in aged, post-menopausal women (Yang and Reckelhoff 2011).

1.6 Animal models of hypertension and CVD

A wide range of animal models have been developed over the years to study the pathophysiology of primary and secondary hypertension. The first animal model used to study hypertension was a renal ischemia model in which the renal artery was surgically occluded to create a renin-dependent form of secondary hypertension (Goldblatt, Lynch et al. 1934). A variety of additional animal models have been developed to closely mimic the progression of secondary hypertension in several endocrine disorders such as aldosteronism, pheochromocytoma, Cushing's syndrome, hyperparathyroidism, and hypo- and hyper-thyroidism. While these animal models provide insight into secondary hypertension, they fail to mimic the complex and multifactorial progression of primary hypertension, which accounts for 90% of all clinically diagnosed hypertension (Velasco and Vongpatanasin 2014, Thomas, Ruel et al. 2015). The animal models that have been employed to study primary hypertension target specific theories regarding the pathophysiology of essential hypertension. The early experimental animal studies utilized

a canine model of hypertension, whereas the current experimental animal models more commonly employ the laboratory rat. Rats are a well-established and accepted animal models for CVD research (Miller, Kaplan et al. 2011). The experimentally-induced models of hypertension include the Ang II infusion model and deoxycorticosterone acetate (DOCA)-salt model, while the genetically induced animal models include the Dahl salt sensitive rat, the New Zealand Genetically Hypertensive (NZGH) rat, and the Spontaneously Hypertensive Rats (SHR) (Leong, Ng et al. 2015).

The two experimentally induced animal models increase ECF volume to induce hypertension. The Ang II infusion model uses Ang II infusion to vasoconstrict the vasculature, increase aldosterone secretion, and increase sympathetic outflow to the kidneys (Young and Davisson 2015). The DOCA-salt model represents a high-blood volume model of hypertension that uses a synthetic mineralcorticoid to expand ECF volume and increase CO and TPR. While uninephrectomy is often combined with this model to further induce volume overload (Selye, Hall et al. 1943), the model closely mimics the symptoms of hypertension in humans with hallmark cardiovascular remodeling, proteinuria, and glomerulosclerosis (Chamorro, Wangensteen et al. 2004).

The genetically-induced Dahl-salt sensitive rat is a high-blood volume animal model that was developed to induce hypertension when fed a high salt diet; however, their salt sensitivity appears to vary between animals, and in some instances the hypertension can rapidly progress into severe and fatal hypertension (Dahl, Heine et al. 1962). In this model, ACE and ANP receptor gene defects facilitate the development of hypertension, and these animals present with cardiac hypertrophy and cardiac failure around 4-5 months of age. Renal changes such as proteinuria and impairments in endothelium-dependent

relaxations are also prevalent in this model. RAS inhibitors, vasodilators, and ET-1 receptor antagonist decrease hypertension in Dahl-salt sensitive rats (Leong, Ng et al. 2015). The NZGH rat and SHR genetically-induced hypertensive animal models are high renin production models of hypertension. The NZGH rat has been genetically altered to have a BP quantitative trait that affects ATP-ase production and the vascular response to vasopressin (Barnard, Kelly et al. 2001). The SHR is the most widely used model to study hypertension and screen for antihypertensive agents (Trippodo and Frohlich 1981), as this model is derived from the Winstar-Kyoto rat lines and begin to show signs of increased BP around 5-6 weeks of age and reach hypertensive BP measurements by 10 weeks of age (Leong, Ng et al. 2015). This model is one of the most commonly used models to study essential hypertension in humans, as these rats presents with human-like hypertensive disorders, such as cardiac hypertrophy, cardiac failure, and renal dysfunction (Pinto, Paul et al. 1998).

1.7 Dissertation scope

The projects in this dissertation aim to investigate the genomic and non-genomic effects of TES and its metabolites on the regulation of BP in both normotensive and hypogonadal hypertensive male rats, and on the mechanisms of androgen-induced vasorelaxation in the uterine arteries of both normal-pregnant and hypertensive-pregnant (preeclamptic) female rats.

2. PROJECT RATIONALE

2.1 Project 1- Anti-hypertensive effects of androgens on BP regulation

This project utilized a normotensive male Sprague-Dawley (SD) rat model to investigate the antihypertensive effects of androgens on systemic blood pressure, and the role of the RAS in hypogonadal hypertension.

2.1.1 Hypertension and cardiovascular disease

2.1.1.1 Epidemiology

As one of the most common worldwide diseases, hypertension affects nearly 30% of American adults aged 40-59 and over 60% of adults over the age of 60. Associated with many environmental, demographic, and genetic risk factors, hypertension is a challenging public health concern. Left uncontrolled, hypertension can readily progress into various forms of CVD such as atrial fibrillation, CAD, MI, and stroke. As the leading cause of morbidity and mortality in the United States, CVD accounts for more deaths than cancer, accidental injuries, and lower respiratory diseases each year (Lloyd-Jones, Adams et al. 2010, Arnett, Blumenthal et al. 2019).

The increased prevalence of CVD in men compared to age-matched pre-menopausal women has led to the firmly entrenched dogma that androgens, more specifically TES, exacerbate the development of hypertension and CVD. In contrast, more recent clinical and experimental animals studies dispute the deleterious effects of TES and reveal beneficial and even cardio-protective effect of TES on cardiovascular health (Liu, Death et al. 2003, Wu and von Eckardstein 2003, McGrath, McRobb et al. 2008, Tsang, Wu et al. 2008, Traish, Haider et al. 2017).

2.1.1.2 Androgen Therapy

In healthy adult males, TES levels can range from 12 to 30 nmol/L (Burger 2002). TES production peaks during adolescence and then progressively declines approximately 0.4-2% annually after the age of 30 (Harman, Metter et al. 2001, Wu, Tajar et al. 2008). In the United States, low TES occurs in approximately 40% of men aged 45 years or older (Tenover, Matsumoto et al. 1987), and it is estimated that only 9% of these hypogonadal men undergo TES therapy to restore physiological concentrations (Harman, Metter et al. 2001, Shores, Sloan et al. 2004, Araujo, Esche et al. 2007).

Clinical studies from the 1940s first documented the beneficial effects of androgens on the cardiovascular system. In these studies, intramuscular injections of TES were used to treat angina pectoris and peripheral vascular disease (Hamm 1942, Walker 1942, Lesser 1946), and the results from these studies suggested that therapeutic TES improved systemic BP, peripheral blood flow, and coronary blood flow (Walker 1942). During this time, TES therapy was also clinically used to treat endocrine disorders involving pituitary tumors, brain resections, testicular-atrophy or testicular-absence, and prominent genetic syndromes of hypogonadism such as Klinefelter syndrome (Nieschlag and Nieschlag 2019). In 1941, the work of Charles B. Huggins and Clarence V. Hodges suggested that TES exerts deleterious effects on metastatic prostate cancer. This conclusion convinced urologists that castration suppressed prostate cancer while TES therapy exacerbated the disease (Huggins 1941). Charles B. Huggins was then awarded the Nobel Prize in Medicine or Physiology in 1966 for his "fundamental discoveries concerning the hormone dependency of normal and neoplastic cells". These publications sparked the beginning of a long-standing controversy between the deleterious vs. beneficial effects of androgens; a debate which

continues today in both human clinical and experimental animal studies (Huggins 1941, Reckelhoff, Zhang et al. 1998, Tsang, Wu et al. 2008, Yassin and Doros 2013, Hansson, Moll et al. 2016, Morgentaler and Traish 2018).

In 2013, support for the deleterious effects of TES gained momentum when a clinical study was prematurely terminated after an interim report suggested that TES therapy increased the incidence of MI, stroke, and death in hypogonadal men (Vigen, O'Donnell et al. 2013). Upon further investigation into this clinical study, significant shortcomings were revealed in the methodology and study design. Additionally, there were indications that the primary results were misreported and that women were included within the alleged all-male study (Traish, Guay et al. 2014, Morgentaler, Miner et al. 2015). In contrast to the results of this controversial study, more recent clinical studies suggest a direct association between TES deficiency and the development of hypertension. Further, these studies suggest that androgens prevent, and possibly reverse, both hypertension and metabolic syndrome (Traish, Haider et al. 2017), and long-term TES therapies reduce the incidence of CVD and prostate cancer (Sharma, Oni et al. 2015, Wallis, Lo et al. 2016).

Most experimental animal studies on hypertension fail to explain the beneficial effects of TES reported by human clinical studies, and support the dogma that TES is deleterious to cardiovascular health. These studies are dominated by genetic and induced animal models of hypertension, such as the widely used Spontaneously Hypertensive Rat (SHR). While TES does exacerbate hypertension in SHR males (Reckelhoff, Zhang et al. 1998, Honda, Unemoto et al. 1999, Reckelhoff, Zhang et al. 2000), these animal are hypertensive irrespective of androgen status, thus, the pathogenic actions of androgens are difficult to decipher. Further, the short-term nature of these experiments (often 1-4 weeks)

is problematic to understanding the longer-term effects of androgens on BP regulation. This may explain why findings from these established models of hypertension are so incongruous with the recent human clinical findings in which hypogonadism is associated with cardiovascular and metabolic dysfunctions, and TES therapy is associate with improved cardiovascular health.

2.1.2 Effect of androgens on the cardiovascular system

2.1.2.1 Genomic vs. non-genomic effects of androgens

Androgens are known to exert biological effects through both genomic and non-genomic pathways. The classical, genomic pathway involves gene transcription and translation, with subsequent changes in protein synthesis. In target tissues, steroid molecules, such as TES, freely permeate the plasma membrane and bind to AR within the cytosol. The hormone-receptor complex then undergoes translocation to the nucleus and functions as a transcription factor for protein synthesis. This process of androgen-induced gene transcription typically peaks several hours to days after androgen exposure (Cato, Skroch et al. 1988). In contrast, androgens have also been observed to initiate rapid, non-genomic effects independent of gene transcription. Many studies suggest that the rapid, non-genomic mechanisms include activation of second messenger signaling mechanisms and/or mitogen-activated protein kinase pathways (Heinlein and Chang 2002, Berg, Rice et al. 2014).

In the vascular system, TES and its metabolites 5α- and 5β-DHT, initiate rapid non-genomic vasodilation, which appears to play a role in the maintenance of vascular health and local blood flow (Perusquia, Herrera et al. 2017, Isidoro, Ferrer et al. 2018). In humans, TES vasodilates the pulmonary (Rowell, Hall et al. 2009) and umbilical arteries

(Saldanha, Cairrao et al. 2013), and acute intravenous infusion of TES into coronary arteries increases vasodilation and local blood flow in men with CAD (Webb, McNeill et al. 1999). Similarly, TES acts as a coronary artery vasodilator in a variety of animal species, including rabbits, dogs, pigs, and rats (Yue, Chatterjee et al. 1995, Chou, Sudhir et al. 1996, Murphy and Khalil 1999, English, Jones et al. 2000, Deenadayalu, White et al. 2001), with vasodilatory effects in various other vascular beds including the rat aorta (Perusquia, Hernandez et al. 1996, Ding and Stallone 2001), mesenteric arteries (Tep-areenan, Kendall et al. 2002), and pulmonary vasculature (Jones, English et al. 2002). Furthermore, acute bolus intravenous injections of TES relax the peripheral vasculature and reduces arterial BP in conscious normotensive and hypertensive male rats (Perusquia, Herrera et al. 2017, Perusquia, Contreras et al. 2019).

Several experimental methods, including the use of flutamide, Tfm rats, or bovine serum albumin (BSA), have been developed to validate and elucidate the non-genomic vs. genomic effects of androgens. Studies utilizing flutamide, an AR-antagonist with potent anti-androgenic effects, block classical AR binding and inhibit the genomic effects of male sex steroid hormones (Yue, Chatterjee et al. 1995). Inhibition of the genomic effects of androgens similarly can be studied by use of the Tfm rat, which exhibits an X-linked recessive defect in cytosolic AR function. The affected Tfm males are similar to the human testicular feminization syndrome, and although they are genotypically male (XY), the affected male rats exhibit a female phenotype and serve as a natural "knockout" model to study AR-mediated genomic effects of TES (Jones, English et al. 2002). TES covalently bound to BSA can also be used to prevent the diffusion of TES into target tissues and prevent genomic effects of the androgens. Each of these methods have been employed to

investigate the non-genomic effects of androgens on vascular function with similar conclusions that TES is able to activate VSM cells independent of the classical genomic AR pathway (Falkenstein, Tillmann et al. 2000, Perusquia and Stallone 2010).

2.1.2.2 Non-genomic effects on vascular endothelium

The non-genomic effects of androgens have been observed in a number of tissue beds (Montano, Espinoza et al. 2014, Lucas-Herald, Alves-Lopes et al. 2017), yet the underlying mechanisms of these non-genomic effects of androgens remain poorly understood. Several studies suggest that there is an interaction between androgens and endothelial function (Hotta, Kataoka et al. 2019), and that TES-induced vasodilation is dependent on the production of endothelial NO (Chou, Sudhir et al. 1996, Deenadayalu, White et al. 2001). In human endothelial cells, TES initiates a dose-dependent increase in eNOS activity (Yu, Akishita et al. 2010) and acute intravenous infusions of TES in the brachial arteries of men with CAD improve endothelial function and flow-mediated NO release (Webb, McNeill et al. 1999, Ong, Patrizi et al. 2000). Additionally, several experimental animal studies demonstrate reductions in androgen-induced vasodilation of resistance vessels pre-treated with NOS inhibitors (Chou, Sudhir et al. 1996). Interestingly, some studies suggest that nNOS may also contribute to androgen-induced endothelial NO (Goglia, Tosi et al. 2010, Perusquia and Stallone 2010). In rats pretreated with S-methyl-l-thiocitrulline (SMTC), an nNOS-inhibitor, the acute systemic hypotensive effects of TES were abolished (Perusquia, Greenway et al. 2015). Similar effects have been observed in humans, in which intravenous administration of SMTC produced dose-dependent increases in systemic vascular resistance and diastolic BP (Seddon, Chowienczyk et al. 2008, Shabeeh 2017).

2.1.2.3 Non-genomic activation of ion channel function

Some experimental animal studies suggest that the non-genomic mechanism of TES-induced vasodilation is preserved in endothelial denuded vessels, and is therefore, not dependent upon endothelial NO production (Yue, Chatterjee et al. 1995, Perusquia, Hernandez et al. 1996, Honda, Unemoto et al. 1999). In rabbits and male sheep, TES-induced vasodilation was abolished in the absence of endothelium (Marrachelli, Miranda et al. 2010), while in female sheep, TES-induced vasodilation was only partially inhibited in endothelium-denuded blood vessels (Yildirim and Erol 2011). While endothelial NO appears to contribute to TES-induced vasorelaxation, the signaling mechanism also relies upon changes in VSM cell ion channel permeability, specifically either Ca^{2+} and/or K^{+} ion channel function. In the rat mesentery, TES activates BK_{Ca} channels in VSM cells (Tep-areenan, Kendall et al. 2002). In the rat thoracic aorta, TES-induces acute vasodilation via endothelial-derived prostanoid production which activates VSM cell BK_{Ca} channels and inhibits Ca^{2+} influx (Ma, Jiang et al. 2009). In canine basilar arteries, TES activates VSM cell K_v and BK_{Ca} channels and inhibits voltage-dependent Ca^{2+} channels (Ramirez-Rosas, Cobos-Puc et al. 2011). These variations in ion channel function may be influenced by differences in ion channel expression. In male rats, castration decreases K_v channel expression in thoracic aorta VSM cells (Zhou, Fu et al. 2008). Taken together, the data on non-genomic mechanisms of TES-induced vasodilation remain unclear, yet these mechanisms appear to involve the activation of endothelial-derived vasoactive mediators and/or the modulation of VSM cell ion channel activity through a combination of extracellular and intracellular signaling mechanisms.

2.1.3 Role of the kidneys

While the vasculature plays a central role in the acute regulation of BP, the kidneys play a central role in the long-term regulation of ECF volume, which directly affects systemic BP. There is accumulating evidence that the function of an intrarenal RAS may contribute to the pathophysiology of hypertension (Yim and Yoo 2008). In humans, kidney transplants from a normotensive donor into a hypertensive recipient ameliorate the pre-transplantation hypertension (Curtis, Luke et al. 2000). Similar observations have been reported in experimental animal studies in which transplantation of a hypertensive SHR kidney into a normotensive WKY rat increases the post-transplantation arterial BP in the WKY rat (Rettig, Folberth et al. 1990, Rettig, Folberth et al. 1990).

The kidney is capable of locally producing all the RAS components; Agt is produced in the proximal tubules, renin is secreted from JG cells, and the AT_1R is expressed throughout the nephron. These components contribute to local Ang II production and effects independent of circulating concentrations. Studies suggest that a progressive increase in intrarenal Ang II occurs during the development of hypertension (Zou, Imig et al. 1996, Navar, Mitchell et al. 2001) and that AT_1R activation facilitates the intracellular compartmentalizing and collection of Ang II (van Kats, de Lannoy et al. 1997). Additionally, increases in intrarenal RAS function have been linked to the development of hypertension-associated renal injury (Navar, Mitchell et al. 2001, Kobori, Prieto-Carrasquero et al. 2004), with a positive correlation between renal function and androgen deficiency in many clinical cases of renal failure (Thirumavalavan, Wilken et al. 2015). Serum creatinine, used to estimate GFR, is greater in TES-deficient men , and serum TES levels are used to predict the progression of renal failure and the risk for

developing cardiovascular related mortality in men with chronic kidney disease (Yildiz, Seyrek et al. 2005, Dousdampanis, Trigka et al. 2014).

The enzymes necessary for synthesizing TES have been identified throughout various structures of the kidney (Quinkler, Diederich et al. 2004, Zhao, Leppert et al. 2016), yet the direct effects of androgens and androgen deficiency on renal function have not been fully explored. In human males, TES therapy delays the onset of end-stage-renal disease, which suggests that TES has a protective role in renal function (Kurniawan, Hsu et al. 2019). In male SHR, TES deficiency impairs intrarenal RAS function, while TES therapy increases Agt and AT_1R gene expression in the kidney (Chen 1996, Reckelhoff, Zhang et al. 1999), with similar results in intact male SD rats, where TES increases Na^+/H^+ exchange proteins and up-regulates intrarenal Agt expression (Quan, Chakravarty et al. 2004, Kienitz and Quinkler 2008). Interestingly, the TES metabolite, 5α-DHT, increases AT_2R expression, which may suggest intrarenal regulation between androgens (Toot, Jenkins et al. 2008, Mishra, Hankins et al. 2016).

2.1.4 Specific aims of project 1

The clinical studies and epidemiological observations that hypertension and CAD occur more frequently in men than in premenopausal women have led to the dogmatic view that TES and other androgens exert deleterious effects on cardiovascular health and exacerbate the development of CVD in men. While there are many reports of beneficial effects of estrogen on the female cardiovascular system (Mendelsohn and Karas 1994, Farhat, Lavigne et al. 1996), TES is considered to exacerbate CVD in males (Mendoza, Zerpa et al. 1983, Chute, Baron et al. 1987). Most previous animal studies have provided support for the dogmatic view that TES exacerbates CVD (Crofton, Share et al. 1989,

Reckelhoff, Zhang et al. 1998, Reckelhoff, Zhang et al. 1999). The clinical and epidemiological studies that suggest deleterious effects of TES are controversial, at best. Interestingly, the clinical studies from the 1940s that first documented the beneficial effects of TES on angina pectoris (Hamm 1942, Walker 1942), hypertension, and peripheral vascular disease (Edwards, Hamilton et al. 1939, Lesser 1946), gained support from the more recent clinical trials, which demonstrated that acute intracoronary injections of TES improve myocardial ischemia and increase arterial dilation and blood flow in men with CAD (Webb, McNeill et al. 1999). In parallel, accumulating evidence, from more recent animal studies employing isolated blood vessels *in vitro*, reveals that TES and other androgen metabolites exert beneficial effects through rapid, nongenomic vasorelaxation mechanisms in a variety of large arteries and smaller resistance arteries at physiological and pharmacological androgen concentrations (Perusquia and Stallone 2010). Although the mechanisms underlying the acute vasodilatory effects of TES have been established *in vitro*, and to a lesser extent *in vivo*, little is known about the long-term systemic effects of TES on BP. Thus, the **central hypothesis of the present investigation is that:**

Endogenous and exogenous androgens exert beneficial antihypertensive effects on BP regulation through suppression of the renin-angiotensin system, resulting in enhanced renal salt and water excretion in male SD and AR-deficient Tfm rats.

This hypothesis will be tested by addressing the following specific aims:

Specific Aim 1: Determine the short-term and long-term effects of endogenous and exogenous TES on systemic blood pressure in normal male and Tfm rats.

Specific Aim 2: Determine the systemic and renal mechanisms underlying the effects of TES on systemic blood pressure and RAS function in normal male and Tfm rats.

2.2 Project II – Pregnancy-induced hypertension and preeclampsia

The DOCA-salt rat model of PE was used to investigate to role of androgens and the mechanisms of androgen-induced vasorelaxation in the UA of normal pregnant and PE pregnant female SD rats.

2.2.1 Adaptations to pregnancy

During pregnancy, females undergo significant physiological adaptations, mediated by a series of dynamic shifts in sex steroid hormone production. From the onset of ovulation, through conception and implantation, and into placental and fetal development, changes in the maternal endocrine, metabolic, cardiovascular, and renal systems maintain an environment conducive to fetal growth and development (Sherwood 2013). In reproductively sound females, cyclic changes in the pituitary hormones, follicle stimulating hormones (FSH) and luteinizing hormones (LH), mediate the female ovulation cycle. Fluctuations in circulating concentrations of estrogens and progesterone facilitate ovulation and prepare the female reproductive system for fertilization and pregnancy. Following ovulation, haploid oocytes fertilized in the fallopian tube form a diploid zygote that undergoes continuous cellular division as it migrates towards the uterus. The rapidly dividing ball of cells begins to differentiate and self-organize into a multicellular blastocyst as it enters the uterus. These cells consist of two distinct cell layers: embryoblast and trophectoderm. The embryoblast, also known as the inner cell mass, gives rise to all

embryonic tissue, while the trophectoderm differentiates into syncytiotrophoblast and cytotrophoblast cells which form the placenta and fetoplacental unit (FPU). Fetal cytotrophoblast cells invade the maternal vasculature and remodel maternal uterine spiral arteries into high-capacity, high-flow, low-resistance vessels to form the foundation of the FPU, which will serve as a major source of hormone and protein production throughout gestation. FPU proteins and hormones are produced from the substrates that pass between maternal circulation, the placenta, and fetal circulation, and drive many of the cardiovascular and metabolic adaptations observed during pregnancy (Clifton, Stark et al. 2012).

2.2.1.1 Endocrine adaptations

Pregnancy involves a combination of autocrine, paracrine, and endocrine signaling factors among maternal, placental, and fetal tissues. As the blastocyst implants in the uterine wall, differentiated trophoblast cells begin to secrete human chorionic gonadotropin (hCG), which stimulates the ovaries to continue progesterone production. Progesterone secretion from the corpus luteum of the ovaries is responsible for maintaining the pregnancy for the first seven to nine weeks of gestation until the placenta is established. During a luteal-placental transition phase, the placenta takes over the role of estrogen and progesterone production and becomes the primary endocrine organ of pregnancy. Throughout gestation, the placenta functions independently of the maternal endocrine system to ensure adequate fetal growth and development regardless of maternal homeostatic mechanisms (Tal R 2000).

The biosynthesis of progesterone and other steroid hormones of pregnancy, such as estrogen and TES, relies upon several precursors including cholesterol and

dehydroepiandrosterone sulfate (DHEAS). Progesterone, the main hormone of pregnancy, is produced by the corpus luteum during early pregnancy and by the placenta throughout the remainder of gestation. Progesterone production plays an important role in suppression of the maternal immunological response to fetal antigens and is vital to proper placental implantation. Further, progesterone promotes myometrial quiescence and prevents the onset of uterine contractions throughout gestation (Reshef Tal 2015). Estrogen production relies upon the pro-hormone dehydroepiandrosterone (DHEA), which is produced by the fetal adrenal glands, hydroxylated in the fetal liver, and aromatized to estrogen in the placenta. Estrogen production increases progressively with gestational age and promotes changes in the myometrium, which are necessary to prepare the uterus for parturition at term. Additionally, the pro-hormones DHEA and androstenedione (A4) further facilitate androgen production during pregnancy. The maternal adrenal glands, ovaries, and peripheral tissues, in addition to fetal and placental tissues, utilize these pro-hormones to facilitate the production of TES and DHT. While a specific fetal zone in the fetal adrenal gland converts cholesterol and pregnenolone into fetal derived DHEAS, the maternal adrenal glands produce cytochrome P450 enzymes, P450 SCC and P450 c17, which catalyze DHEAS into DHEA. The circulating androgens vary throughout pregnancy, as TES production increases in both early and late pregnancy, A4 increases late in gestation, and DHEAS decreases throughout gestation (Makieva, Saunders et al. 2014). While fetal TES levels are elevated in male offspring, estrogen and DHT remain similar between sexes (Diez d'Aux and Pearson Murphy 1974, Rodeck, Gill et al. 1985).

2.2.1.2 Metabolic adaptations

Metabolic adaptations during pregnancy involve dynamic changes in the metabolism of carbohydrates, lipids, and protein. The metabolic changes that occur in early pregnancy are proportional to increases in circulating progesterone concentrations, which induce smooth muscle relaxation, decrease gut motility, and promote increases in nutrient absorption within the first few weeks of pregnancy. Increases in pancreatic insulin secretion and sensitivity during the first trimester of pregnancy are followed by marked increases in insulin resistance during the second and third trimesters of pregnancy (Newbern and Freemark 2011) as the metabolic demands of the developing fetus increase. As pregnancy progresses, the maternal metabolism shifts from the use of glucose for fuel to a dependency on lipolysis for energy requirements. Using lipids for energy ensures an adequate preservation of circulating amino acids and glucose levels for the developing fetus (Soma-Pillay, Nelson-Piercy et al. 2016). In addition to the increased nutrient requirements of pregnancy, Ca^{2+} and phosphate requirements increase as well. Ca^{2+} is required for fertilization and implantation during early pregnancy. During the third trimester of pregnancy, 80% of the fetal mineral requirements are derived from the maternal diet as intestinal absorption increases during pregnancy. The increase is driven by calcitriol production in the maternal reproductive and renal systems and in the fetal renal system (Kovacs 2000). These metabolic changes during pregnancy promote and ensure a continuous nutrient supply to the developing fetus (Angueira, Ludvik et al. 2015).

2.2.1.3 Hemodynamic adaptations

The maternal cardiovascular system undergoes a 30-50% increase in ECF volume during pregnancy. In response to this increase in circulating blood volume, myocardial

contractility, CO, and SV increase, yet, paradoxically, systemic BP decreases by approximately 10 mmHg. The decrease in systolic BP is largely attributed to a decrease in systemic vascular sensitivity and the resulting reduction in vascular tone both systemically and in the uterine artery (Cheung and Lafayette 2013).

In the uterine artery, the luminal diameter doubles in size during pregnancy with marked reductions in vascular tone and vasoconstrictor sensitivity. Pregnancy-induced increases in sheer stress and exposure to changes in various circulating chemical messengers result in a subsequent vascular remodeling of the uterine arteries during pregnancy. Continuous exposure to estriol, the dominant estrogen of pregnancy, and vascular endothelial growth factor (VEGF) enhance endothelial NO production and reduce vascular tone (Osol and Mandala 2009). Compared to non-pregnant women, pregnant women have a decreased sensitivity to the pressor effects of Ang II infusions (Broughton Pipkin and Baker 1997). During early pregnancy, a marked decrease in AT_1R expression reduces uterine artery sensitivity to the pressor effects of Ang II and exacerbates the vasodilatory effects of NO. The pressor effects of Ang II increase markedly in pregnant women administered prostaglandin (PG) synthesis inhibitors, such as aspirin and indomethacin. This suggests that vasodilatory PG plays a role in suppressed vascular reactivity (Magness, Osei-Boaten et al. 1985). Clinical observations suggest a placental origin for the suppressed vascular response to Ang II, as the suppression was lost within 15-30 minutes after delivering the placenta. Bolus administration of progesterone during labor reduces the pressor effects of Ang II and mitigates the vasoconstrictive actions of Ang II (Abdul-Karim and Assali 1961). These findings suggest that the pressor effects of Ang II during pregnancy might not be completely controlled by PG, but rather a PG

metabolite that initiates a similar vascular response (Gant, Worley et al. 1980). The PG most likely responsible for vasodilation during pregnancy is prostacyclin (PGI2). PG is metabolized into 5α-dihydroprogesterone (5α-DHP) by the enzyme 5α-reductase, the same enzyme responsible for the conversion of TES into 5α-DHT. Interestingly, 5α-DHT is elevated in the placenta, and infusion of 5α-DHT restores vascular refractoriness to Ang II. These studies suggest that reduced sensitivity to Ang II is potentially mediated through a 5α-reductase enzyme-mediated increase in 5α-DHT to reduce vascular tone and BP (Gant, Worley et al. 1980).

2.2.1.4 Renal adaptations

Cardiovascular and endocrine adaptations during pregnancy influence the renal system. Increases in the hormones of pregnancy, and increases in plasma volume, decreases in colloid osmotic pressure, and decreases in systemic vascular resistance facilitate changes within the renal vasculature and tubular function. A direct correlation between increases in estrogen production and increases in RAS function are marked by elevated plasma Agt, renin, Ang II, and aldosterone during the third trimester of pregnancy (Cheung and Lafayette 2013). Ang II and aldosterone increase salt and water retention in the renal system. Relaxin from the placenta initiates a marked increase in renal blood flow and GFR during pregnancy (Conrad 2011). Overall, renal blood flow increases by approximately 80% during early pregnancy and GFR increases by 50% (Dunlop 1981). Hyperfiltration by the glomerulus results from decreases in oncotic pressure during normal pregnancy. Increases in GFR reduce tubular reabsorption and increase urinary excretion of small amounts of protein in normal pregnancy, yet marked increases in protein excretion indicate potential pregnancy related disorders (Cheung and Lafayette 2013).

2.2.2 Epidemiology and pathogenesis of preeclampsia

Hypertensive disorders that develop during pregnancy are classified as essential hypertension, gestational hypertension, or preeclampsia (PE). PE is one of the most dangerous pregnancy-specific forms of hypertension that is characterized by the sudden onset of high BP and multisystem dysfunction during the second trimester. PE complicates about 3-10% of all pregnancies in the United States (Jeyabalan 2013) and is a leading cause of maternal mortality worldwide. While marked improvements have been made in diagnosing and treating PE, the pathogenesis of the disease remains poorly understood.

Risk factors for PE include genetic factors, in which both a maternal and/or paternal family history of the disease can predispose women to PE (Esplin, Fausett et al. 2001). Additional risk is associated with a previous history of PE and in women with women antiphospholipid syndrome (Duckitt and Harrington 2005). Preexisting cardiovascular and metabolic risk factors such as hypertension, diabetes mellitus, insulin resistance, and obesity also increase the incidence of PE during pregnancy (Rana, Lemoine et al. 2019). Clinical diagnosis of PE is characterized by blood pressures greater than 140/90 combined with urine protein excretion greater than 300 mg per day (Sibai and Stella 2009). Several theories suggest that improper placental implantation and vascularization leads to endothelial dysfunction and the development of PE. During normal implantation, cytotrophoblasts invade the maternal uterine wall and remodel the maternal decidual arteries to generate a low-resistance high-flow vascular system capable of maintaining an adequate nutrient supply to the developing fetus. Improper or shallow vascular remodeling during placental implantation leads to the development of placental ischemia and the progression of PE as the vascular perfusion capacity is unable to accommodate the

increasing nutrient demands of the developing fetus (Zhou, Damsky et al. 1993). Doppler ultrasounds of women with PE reveal significant impairments in diastolic blood flow in the uterine arteries (Struijk, Ursem et al. 2001, Pariente, Shwarzman et al. 2013), with marked reductions in the luminal diameter and increases in the vascular resistance (Osol and Mandala 2009), which results in intrauterine growth restriction (IUGR) of the fetus and elevated BP in the maternal vascular system (Jeyabalan 2013).

Pro-angiogenic factors such as VEGF promote endothelial cell proliferation and contribute to vascular health and blood pressure regulation (Facemire, Nixon et al. 2009). VEGF infusion increases endothelial NO and prostacyclin production and induces VSM cell relaxation in coronary arteries (Ku, Zaleski et al. 1993). Placental growth factor (PIGF), a VEGF homolog, is a pro-angiogenic factor expressed by the placenta that potentiates the actions of VEGF, and together, VEGF and PIGF, are essential for the proliferation and survival of endothelial cells during placental angiogenesis. VEGF binds to Flt-1, an anti-angiogenic receptor, and kinase insert domain receptor (KDR), a pro-angiogenic receptor, on vascular endothelial cells. PIGF acts to displace VEGF and Flt-1 biding to facilitate VEGF and KDR, which promotes angiogenesis. Flt-1 expresses tyrosine kinase-1 ($sFlt_1$), a soluble VEGF signaling inhibitor, which binds VEGF and further modulates VEGF bioavailability (Park, Chen et al. 1994). The anti-angiogenic properties of Flt-1 with other factors such as TGF-β proteins, which facilitate angiogenesis at low levels and antagonize cell proliferation at high concentrations, contribute to the development of PE. Prior to the onset of PE, elevated concentrations of $sFlt_1$ and endoglin (sEng) have been identified in maternal circulation. As a soluble TGF-β co-factor expressed by the placenta, sEng dysregulates eNOS activation and subsequent NO-induced

vasodilation (Venkatesha, Toporsian et al. 2006). Interaction with $SFlt_1$ and the intrarenal RAS may contribute to the symptoms of PE, as women with pregnancy-induced hypertension and PE are profoundly more sensitive to Ang II infusion than women with normal pregnancies (van der Graaf, Toering et al. 2012). In experimental animal studies, Ang II increases placental $sFlt_1$, an effect mitigated by Losartan, an AT_1R antagonist (Delforce, Lumbers et al. 2019). Despite an increase in vascular sensitivity to Ang II, PE women have suppressed Agt, renin, and Ang II production (Gant, Daley et al. 1973).

2.2.3 Animal models of Preeclampsia

Several animal models have been developed to mimic the characteristics of PE; however, since PE is a pregnancy-induced disease specific to humans, many of these animal models fail to accurately represent the human pathology of the disease. Most of the animal models of PE focus on a specific pathogenic mechanism including uteroplacental ischemia, impaired NO production, overactive RAS function, or impaired proteins of angiogenesis, and these mechanisms are employed in several rodent and non-rodent species, which each offer its own unique set of experimental limitations.

The uterine ischemia model of PE reduces uterine blood flow during pregnancy to mimic the 50-70% reduction in uteroplacental blood flow observed in PE women (Lunell, Lewander et al. 1984). This model was first developed in pregnant dogs using an abdominal aorta clamp technique to surgically-induce uterine ischemia, which decreased uteroplacental perfusion by approximately 50% and increased systolic BP within minutes (Young and Gynaecology 1914). This technique has also been employed in several other rodent and non-rodent animal species with variable effects on BP. In rodents, aortic constriction induces hypertension, proteinuria, IUGR, and endothelial dysfunction and

significantly reduces systemic NO production (Alexander, Kassab et al. 2001). In rabbits, aortic clamping increases α-adrenergic tone and systolic BP which results in proteinuria and deleterious effects on fetal development (Abitbol, Gallo et al. 1976). Interestingly, in sheep, aorta clamping has no effect on systolic BP; however, the addition of a high salt diet results in significant hypertension (Leffler, Hessler et al. 1986). In rodents, the reduced uterine perfusion pressure (RUPP) model was developed as an alternative placental ischemia model. At time points equivalent to second trimester, the RUPP model is created with surgical clamping of the abdominal aorta and main uterine arteries to more closely mimic the ischemic effects of PE. While the RUPP model induces hypertension, proteinuria, and impaired renal function, this model is not conducive to research targeting therapeutic improvements to uterine blood flow or investigating the uteroplacental vascular response. Additionally, these models of ischemia fail to mimic the progression of PE observed in humans. The timing of reduced uteroplacental perfusion occurs after normal placental implantation has been established, which is contradictory to the impaired placental implantation in human PE (Li, LaMarca et al. 2012).

Several induced rodent models of PE utilize mechanisms such as Ang II and NO inhibition to mimic the maternal manifestations of PE at the vascular level. In normal pregnancy circulating RAS components increase, while the pressor effects of Ang II decrease. In contrast, decreased levels of circulating RAS components and increased reactivity to Ang II is observed in PE pregnancy (Baylis, Beinder et al. 1998). Thus, rodent models using increasing Ang II levels to induce hypertension may not be relevant to human disease. While the Ang II model of PE does not mimic the progression of human disease, the animal model of chronic NO inhibition mimics the molecular progression of PE on

endothelial function. In rodents, chronic NOS inhibition produces sustained hypertension in non-pregnant animals (Zatz and Baylis 1998); however, eNOS knockout mice exhibited a decrease in BP during pregnancy (Shesely, Gilbert et al. 2001), which contradicts findings from additional studies (Hefler, Tempfer et al. 2001). In rats, chronic NOS inhibition induced proteinuria and caused fetal malformation, which were later attributed to the effects of the administered NOS inhibitors (Baylis, Engels et al. 1992). Thus, the use of the chronic NOS inhibitor model remains controversial. Other induced animal models of PE target angiogenic proteins of pregnancy. In humans, PE is associated with upregulated placental sFlt-1 (Cui, Shu et al. 2018) and sEng compared to normal pregnancy. While this has generated interest in the use of anti-angiogenic animal models of PE (Venkatesha, Toporsian et al. 2006), research suggest that upregulated antiangiogenic factors are not the cause of PE, but rather a byproduct of the disorder (Alasztics, Gullai et al. 2014).

Genetically altered animal models of hypertension such as the SHR and Dahl salt-sensitive rat have been used as animal models of PE. These models develop hypertension irrespective of pregnancy status, and fail to mimic the sudden onset of pregnancy-induced hypertension associated with PE (Takeda 1964). While pregnancy in the SHR does not exacerbate hypertension, systolic BP increases during pregnancy in the Dahl-salt sensitive rat (Gillis, Williams et al. 2015), which is beneficial in experimental animal studies that target the effects of pregnancy on preexisting maternal hypertension, but not to understanding the pathogenesis of PE. There is increasing interest in the use of several transgenic models of PE; however, these models have not been fully developed.

While several animal models of PE have been identified to resemble the characteristics of PE in humans, there is no single model that fully mimics the human progression of PE. For the present study, the DOCA-salt model was chosen to investigate the vasodilatory effects of androgens on the uteroplacental vasculature in PE. The DOCA-salt animal model is a well-established hypertensive animal model that targets neurogenic, renal, and vascular response elements. An imbalance of renal Na^+ and water reabsorption results in hypervolemia, which mimics the effects of fluid volume expansion observed in early gestation. While DOCA-salt treatment increases RAS activity in the brain, which generates a neurologic effect that alters renal function and systemic vascular resistance (Ueno, Mohara et al. 1988), circulating renin and Ang II levels remain the same. Further, DOCA-salt rats are characterized by systemic endothelial dysfunction, with enhanced vascular reactivity in isolated blood vessels (Nunes, Fortes et al. 2000).

2.2.4 Androgens in pregnancy

Sex steroid hormones such as estrogens, androgens, and progesterone are known to increase during pregnancy and there is increasing interest in the role androgens play in maintaining utero-placental blood flow during pregnancy. While sex steroid hormones are maternally produced by the maternal ovaries, adrenal glands, and adipose tissue, local production of hormones at the uteroplacental interface suggests local regulation of utero-placental blood flow. Uterine blood flow is critical to maintaining an intrauterine environment conducive to fetal growth and development throughout gestation. Estrogen and progesterone increase endothelial production of PGI_2, a potent vasodilator, in the UA (Rupnow, Phernetton et al. 2002), and systemic infusion of estradiol-17β increases uterine blood flow without effecting systemic parameters (Magness and Rosenfeld 1989). Some

studies report that TES metabolites, specifically 5β-DHT, are more potent vasodilators with a greater binding affinity to AR than TES (Ishimaru, Edmiston et al. 1978, Kumar, Crozat et al. 1999). In line with this evidence, differences have been observed in the sensitivity and reactivity of isolated resistance vessels to each of the androgens. Vasorelaxation of resistance vessels increases in response to 5β-DHT compared to TES or 5α-DHT (Isidoro, Ferrer et al. 2018). Similarly, in human umbilical arteries, DHEA and 5β-DHT-induced vasorelaxation is greater compared to TES or other female sex steroid hormones (Perusquia, Navarrete et al. 2007). Systemically, the anti-hypertensive effects of 5β-DHT and DHEA appear to be significantly greater than the moderate effects of TES and 5α-DHT on arterial BP in conscious pregnant female rats (Perusquia, Hanson et al. 2018). Thus, uterine blood flow may depend upon the vascular reactivity to each of the androgens or a local increase in production of key regulating hormones at the uteroplacental interface.

2.2.5 Specific aims of project II

In recent *in vitro* and *in vivo* studies, TES and its metabolites exert acute vasodilatory effects on resistance vessels. Androgen production increases during pregnancy, and it is possible that the vasodilatory effects of placental androgens play a role in the maintenance of fetal/placental blood flow during pregnancy. The relative importance of these hormones in healthy vs. diseased maternal vasculature remains unclear. **Thus, the central hypothesis to be tested is that:**

The deleterious effects of preeclampsia on uteroplacental blood flow may result from reduced vasodilatory effects of placental androgens on uterine artery function.

This hypothesis will be tested by addressing the following specific aims:

Specific Aim 1: Validation of the DOCA-salt preeclampsia model in the rat

Specific Aim 2: Determine the vascular response of the UA to TES, 5α-DHT, and 5β-DHT in normal pregnant vs. PE pregnant rats.

Specific Aim 3: Determine the mechanisms of androgen-induced vasorelaxation of the UA from normal pregnant vs. PE pregnant rats.

3. ANTIHYPERTENSIVE EFFECTS OF ENDOGENOUS AND EXOGENOUS ANDROGENS IN THE MALE SPRAGUE-DAWLEY RAT

3.1 Introduction

The observations that hypertension and CAD occur more frequently in men than age-matched pre-menopausal women has led to the dogmatic view that TES has deleterious effects on cardiovascular function and exacerbates the development of CVD in men. This view is driven by abundant clinical trials and epidemiological data which demonstrate the deleterious effects of exogenous androgen supplementation on CAD (Death, McGrath et al. 2004) and is supported by most experimental animal studies, which suggest that castration ameliorates the development of hypertension in various rat models, such as the SHR (Crofton, Share et al. 1989, Chen and Meng 1991, Reckelhoff, Zhang et al. 1998, Yanes, Sartori-Valinotti et al. 2009). While the laboratory rat is a well-established experimental animal model used to study human cardiovascular disease (Miller, Kaplan et al. 2011), the SHR exhibits marked differences in systemic BP, renal function, calcium metabolism, and calcification of the vasculature, irrespective of androgen status (Shibata and Ghishan 1991, Meng, Zhao et al. 2018). Additionally, many of these experimental studies are relatively short-term in nature and fail to mimic the natural long-term progression of hypertension in human disease. These limitations in the rat models of hypertension and in experimental design, may explain the discrepancy with findings of the most recent clinical trials, which reveal that androgens exert beneficial effects on cardiovascular and metabolic health (Traish, Haider et al. 2017).

The most recent clinical data suggest a direct correlation between TES deficiency and the progression of many cardiovascular diseases such as hypertension and atherosclerosis (Rezanezhad, Borgquist et al. 2018). Further, intravenous infusion of TES improves myocardial ischemia, reduces BP, and increases coronary artery dilation in hypogonadal men (Traish, Haider et al. 2017). In support of these clinical findings, accumulating recent evidence from both *in vitro* and *in vivo* experimental animal studies demonstrate beneficial and even cardio-protective effects of TES. Thus, in isolated large arteries and smaller resistance vessels, TES induces rapid, non-genomic vasorelaxation (Perusquia and Stallone 2010), while acute intravenous infusions or bolus injections of androgens decrease arterial BP in conscious male SD and WKY rats *in vivo* (Perusquia, Greenway et al. 2015, Perusquia, Herrera et al. 2017). Although there is accumulating evidence that acute androgen infusions exert beneficial effects on vascular tone and systemic BP, little is known about the long-term effects of androgens on BP regulation. **Thus, in the present study, the central hypothesis to be tested is that endogenous and exogenous androgens exert beneficial anti-hypertensive effects on systolic BP in normal male SD rats.**

3.2 Methods

3.2.1 Ethical approval

All animal protocols were written in accordance with regulations of the National Institutes of Health as detailed in the "Guide for the Care and Use of Laboratory Animals" and approved by the Institutional Animal Care and Use Committee at Texas A&M University.

3.2.2 Experimental animals

12-week-old male SD rats were purchased from Envigo (Houston, TX) and housed at the Laboratory Animal Resource and Research facilities at Texas A&M University in College Station, TX. The rats were maintained in well-ventilated rooms with regulated temperatures (21-26°C), relative humidity (~50%), and photoperiod (12:12-hr light: dark cycle). Animals were pair-housed in standard plastic laboratory rat cages and fed a 16% protein rat chow that was soybean- and alfalfa-free (Teklad Global diet; Houston, TX). This diet is nominally free of dietary phytoestrogens, which have been reported to confound studies investigating sex differences in vascular function (Li and Stallone 2005). Food and water were provided *ad libitum*. Rats were randomly divided into one of three experimental groups: intact controls (InT-controls), castrated (CsX), and castrated with TES therapy (CsX-TRT).

Parallel studies using age-matched Tfm rats bred in house were used to separate the non-genomic and genomic effects of TES. Affected male Tfm rats exhibit a recessive X-linked single gene mutation, leading to a defective cytosolic AR that eliminates the classical genomic effects of TES (Shapiro, Levine et al. 1980, Yarbrough, Quarmby et al. 1990). Affected males were identified at birth as Tfm rats are genotypically male (XY), but phenotypically female with internal testes and external female genitalia, which is easily visualized in pups 1-3 days old. The affected pups were ear notched for identification after weaning. This phenotypic presentation is due to the loss of AR-mediated differentiation and development of the male reproductive tract. These Tfm rats served as a natural AR "knockout" model to investigate the genomic vs. non-genomic effects of endogenous and exogenous TES (Stallone, Salisbury et al. 2001).

3.2.3 Systolic blood pressure

Systolic BP was recorded using a non-invasive tail cuff plethysmograph system (IITC Life Science; Woodland Hills, CA) connected to a Power Lab Data Acquisition System and analyzed with Lab Chart Software (AD Instruments; Colorado Springs, CO). Prior to data collection, animals were acclimated to daily handling, and trained to rest quietly in a darkened, heated, cylindrical restraint. Week 0 systolic BP and BW were recorded as baseline for each animal prior to castration or experimental treatments and Week 1 was 7 days post-operative for the castrated rats.

3.2.4 Surgical procedure (bilateral orchiectomy)

Male SD rats in CsX and CsX-TRT groups underwent a bilateral orchiectomy, using standard surgical procedures. Briefly, animals were pretreated with atropine sulfate (0.05 $mg \cdot kg^{-1}$; SC) and anesthetized with isoflurane. Ophthalmic ointment was applied to the eyes of each animal as a preventative for corneal dryness. During the entire surgical procedure, animals were provided supplemental heat via a heating pad and continuously monitored for changes in respiratory rate, membrane color, and reflex response to ensure an adequate surgical plane of anesthesia. The surgical site was clipped, aseptically prepared, using alternating scrub cycles of iodine and 70% isopropyl alcohol, and the area was covered with a surgical drape. A single 1-2 cm incision was made in the scrotum and the muscular cremaster tunic, which encloses each testis, was bluntly dissected. Additional 1 cm incisions were made through the left and the right tunic to externalize each of the testes. The spermatic cords were ligated with surgical silk suture, transected, and the cut ends were swabbed with betadine and allowed to retract into the tunic. The tunics were closed with 2 absorbable sutures (Ethicon 4-0 Vicryl®) and the scrotum was closed with

3-5 absorbable sutures (Ethicon 4-0 Vicryl®). Flunixin Meglumine (2.5 mg·kg^{-1}; IM) was administered for analgesia and the animals were transferred to a recovery cage.

The Tfm rats in the CsX and CsX-TRT groups also underwent bilateral orchidectomy. Castration of Tfm rats involves a laparotomy to remove the abdominal testes in these animals. The testicular arteries were ligated and transected and the testes removed. The body wall musculature was closed with 4-5 absorbable sutures (Ethicon 4-0 Vicryl®) and the skin was closed with 3-4 stainless steel wound clips.

3.2.5 Experimental design

Weekly measurements of systolic BP and BW for InT-control, CsX and CsX-TRT SD and Tfm rats resumed 7 days following surgery and continued weekly for the entire 15-week experimental period. Following BP and BW measurements on wk 10, CsX-TRT rats began receiving bi-weekly subcutaneous (SC) injections of TES-enanthate dissolved in 90% olive oil/10% DMSO vehicle (1.75 mg·kg^{-1}). CsX animals not receiving androgen therapy received bi-weekly injections (SC) of the olive oil-DMSO vehicle-control at equivalent volumes to the TES-enanthate. Systemically, TES can be reduced to DHT or aromatized to estrogen. To confirm that the observed effects of TES on BP regulation did not result from conversion to estrogen, the TES metabolite, DHT, which cannot be aromatized, was administered as the androgen therapy in a separate group of castrated male SD rats (CsX-DRT). Similar to TES therapy, 5α-DHT therapy began on wk 10 following castration, with bi-weekly injections of 5α-DHT-enanthate (1.00 mg·kg^{-1}; SC) dissolved in the olive oil-DMSO vehicle.

In a separate group of SD rats, Losartan-potassium (LST), an AT_1R antagonist, was used to investigate the role of the RAS in androgen-dependent changes in BP. Male rats

were randomly assigned to one of three groups: CsX, CsX-LST, or CsX-LST-TRT. Animals were acclimated to weekly systolic BP measurements, and CsX animals underwent bilateral orchiectomy surgery as previously described. CsX-LST and CsX-LST-TRT groups received *ad libitum* drinking water with LST (250 $mg \cdot L^{-1}$). Weekly systolic BP and BW was recorded for CsX, CsX-LST and CsX-LST-TRT rats for the entire 15-week experimental period. CsX-LST-TRT animals began bi-weekly injections of TES-enanthate (1.75mg·kg-1; SC 2x/wk) following BP and BP measurements on wk10. CsX and CsX-LST animals not receiving androgen therapy received equivalent volumes of the olive oil-DMSO vehicle-control (SC 2x/wk).

3.2.6 Urinary sodium and water excretion

Metabolic studies were performed on the three main experimental groups at weeks 0, 4, 10, 12 and 15. Animals were single-housed in metabolic cages for 24-hours, where water consumption and urine excretion were determined gravimetrically. After 24-hours, urine sample aliquots (1-2 mL) were collected and stored at -80°C for later analyses. During the metabolic studies, animals were fasted to prevent sample contamination. Samples were analyzed for potassium (K^+), Na^+, and creatinine concentrations at the Texas A&M Clinical Pathology Laboratory (ClinPath; College Station, TX). Na^+ and fluid excretion were normalized by creatinine as an index of GFR.

3.2.7 Plasma panel and hormone concentrations

Animals were humanly euthanized via rapid decapitation at wk 10 or 15 for plasma and tissue collection. Rapid decapitation minimizes potential neural and hormonal artifacts that result during anesthesia and euthanasia (Boivin, Hickman et al. 2017). Trunk blood was collected in chilled 13 x 100 mm glass test tubes containing 100-200 units of heparin

sodium and immediately centrifuged at 10,000 RPM for 5 minutes to separate plasma. The plasma was frozen and stored at -80°C for later analysis. The plasma samples were analyzed by ClinPath for Na^+ and creatinine. Selective radioimmunoassays were used to assess plasma concentrations of TES and total estrogens (MP Biomedicals, LLC; Santa Ana, CA). Plasma renin was analyzed by the Core Lab Facilities at Wake Forest University (Winston-Salem, NC). Briefly, plasma renin concentrations, defined as the rate of Ang I generated by renin in the plasma sample, in the presence of excess exogenous angiotensinogen isolated from nephrectomized rat plasma were determined by incubating the plasma samples at a pH 6.5 for 90 minutes and then quantifying Ang I by radioimmunoassay (Cisbio Bioassays; Codolet, France).

3.2.8 mRNA expression of RAS components in the thoracic aorta and kidney

The kidneys and thoracic aorta were collected from InT-control, CsX and CsX-TRT rats at wk 0, 10, and 15, and analyzed for mRNA expression of RAS components by real time-polymerase chain reaction (rt-PCR). Following euthanasia, the thoracic aorta was immediately isolated, snap frozen in liquid nitrogen, and stored at -80°C. The left kidney was isolated, cleaned of connective tissue and fat, sectioned, snap frozen on dry ice, and stored at -80°C. Portions of the aorta and kidney samples (0.3-0.5 g) were used to analyze angiotensinogen (Agt), angiotensin converting enzyme (ACE), angiotensin type 1 receptor (AT_1R), and renin mRNA expression using previously established rt-PCR methods (Chang, Skiles et al. 2017). Briefly, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen), according to manufacturer's instructions. Individual RNA samples were tested for potency and total purified RNA samples (900-1500 ng) were seeded into a reverse transcription reaction using the SuperScriptII system (Invitrogen),

according to the recommended protocol for the high capacity cDNA reverse transcription kit (Invitrogen), by combining 2.0µL random hexamer oligonucleotides, 0.8µL of 100 mM dNTP, 2.0 µL RT Buffer, 1.0 µL MultiScribe™ reverse transcriptase, and 4.2 µL purified RNA plus water. Samples were brought to 25°C for 5 min, 42°C for 50 min, 45°C for 20 min, 50°C for 15 min, and 70°C for 5 min. Relative levels of candidate gene transcripts were analyzed using the Dynamo Flash SYBR Green qPCR (Thermo Scientific), according to the recommended protocol. Reactions were quantified on a CFX38 touch rt-PCR detection system (BioRad). The primers for Agt, ACE, AT_1R, renin, and Elongation factor-1 (Ef-1; reference gene) are listed in Table 1.

Table 1. Primer sequence for renin angiotensin system components.

Gene	Sequence
Angiotensinogen (Agt)	Sense: AGCACGGACAGCACCCTATT Antisense: AGAACTCATGGAGCCCAGTCA
Angiotensin Converting Enzyme (ACE)	Sense: CTGCCTCCCAACGAGTTAGAA Antisense: CGGGACGTGGCCATTATATT
Angiotensin II Type I Receptor (AT_1R)	Sense: TATCACAGTGTGCGCGTTTCA Antisense: TGGTAAGGCCCAGCCCTAT
Renin	Sense: GCTACATGGAGAATGGGACTGAA Antisense: ACCACATCTTGGCTGAGGAAAC
Reference Gene: Elongation Factor-1 (Ef-1)	Sense: GCAAGCCCATGTGTGTTGAA Antisense: TGATGACACCCACAGCAACTG

3.3 Chemical Reagents and Drugs

The following drugs and reagents were used: TES-enanthate (17β-hydroxy-4-androsten-3-one-enanthate; Steraloids, Newport, RI), 5α-DHT-enanthate (17β-hydroxy-5α-androstan-3-one-enanthate; Steraloids, Newport, RI), and Losartan-potassium (Selleck Chemical, Houston, TX). All other chemicals were of reagent grade quality, and were

purchased from Sigma Chemical (St Louis, MO). TES-enanthate and 5α-DHT-enanthate for androgen therapy experiments were dissolved in 90% olive oil: 10% DMSO at 5.0 $mg \cdot ml^{-1}$.

3.4 Data Analysis

All data are expressed as the means ± standard error of the mean (SEM) and n indicates the number of animals in each experimental group. A repeated measures analysis of the variance (ANOVA) was used to analyze differences in BP over time among different treatment groups (InT vs. CsX vs. CsX-TRT vs. CsX-DRT vs. CsX-LST vs. CsX-LST-TRT). Other data groups (e.g. mRNA expression) was first subjected to one- or two-way ANOVA to detect significant differences among means of the experimental groups; differences among means were accepted as significant if $P < 0.05$. Factors compared for one- or two-way ANOVA include: gonadal status (InT vs. CsX vs. CsX-TRT vs. CsX-DRT) or RAS inhibition (CsX vs. CsX-LST vs. CsX-LST-TRT). If main effects were identified, then pair-wise Student's t-tests were performed on group means to detect significant pair-wise differences among the means of the various experimental groups. A Bonferroni correction was employed for type I errors associated with multiple comparisons and differences between the means. Differences between any two means were accepted as significant if $P < 0.05$.

3.5 Results

3.5.1 Effect of androgens on body weight and seminal vesicle weight

The effects of endogenous and exogenous androgens on BW and seminal vesicle weight are summarized in Table 2. BW on wk 0 was slightly lower in the InT-controls and slightly higher in CsX-DRT compared to the other experimental groups. At wk 10 BW

was significantly increased in CsX-DRT and CsX-TES-Tfm ($P \leq 0.0001$), while over the entire 15-wk experimental period, BW increased proportionally in all groups. Seminal vesicle weight decreased more than 90% between CsX (0.02 ± 0.001 g·100g BW^{-1}) vs. InT-control rats (0.29 ± 0.013 g·100g BW^{-1}, $P \leq 0.0001$), while TRT fully restored seminal vesicle weight (CsX-TRT, 0.33 ± 0.018 g·100g BW^{-1}) to levels similar to those of InT-control rats ($P > 0.05$). 5α-DHT therapy partially restored seminal vesicle weight (34%), while treatment with LST had no effects on seminal vesicle weight in either CsX-LST or CsX-LST-TRT rats ($P > 0.05$).

Table 2. Body weight (BW) and seminal vesicle weight of InT-control, CsX, CsX-TRT, CsX-DRT, CsX-LST, CsX-LST-TRT and CsX-TRT-Tfm rats.

Group	wk 0 BW(g)	wk 10 BW(g)	wk 15 BW(g)	Seminal Vesicle (g·100g of BW^{-1})
InT-control	$339 \pm 8^{a+}$	$433 \pm 12^{a\#}$	$439 \pm 14^{a\#}$	0.29 ± 0.013^{a}
CsX	$383 \pm 22^{b+}$	$457 \pm 22^{a\#}$	$458 \pm 27^{a\#}$	0.02 ± 0.001^{c}
CsX-TRT	$374 \pm 7^{b+}$	$433 \pm 7^{a\#}$	$446 \pm 8^{a\#}$	0.33 ± 0.018^{a}
CsX-DRT	$519 \pm 13^{c+}$	$594 \pm 17^{c\#}$	$670 \pm 26^{c*}$	0.10 ± 0.003^{d}
CsX-LST	$385 \pm 4^{b+}$	$456 \pm 8^{a\#}$	$462 \pm 11^{a\#}$	0.03 ± 0.001^{b}
CsX-LST-TRT	$374 \pm 3^{b+}$	$432 \pm 4^{a\#}$	$453 \pm 5^{a*}$	0.34 ± 0.025^{a}
CsX-TRT-Tfm	$401 \pm 23^{b+}$	$487 \pm 16^{b\#}$	$525 \pm 20^{b\#}$	–

Sprague Dawley (SD) male rats either intact (InT), bilaterally castrated (CsX), or CsX with TES therapy (CsX-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}) or CsX with 5α-DHT therapy (CsX-DRT; 1.00 mg·kg^{-1}; SC 2x·wk^{1}). In a separate experiment, CsX males SD rats were placed on losartan drinking water (250 mg ·L^{-1}) without TES therapy (CsX-LST) or with TES therapy (CsX-LST-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}). Testicular feminized male (Tfm) rats were castrated and treated with TES therapy (CsX-TRT-Tfm). Data are means ± SEM (n=6-13 rats/group). $^{a\text{-}d}$ Mean values within each column for body weight (wk 0, 10, and 15) and seminal vesicle weight without common superscript are significantly different ($0.0001 \leq P \leq 0.02$). $^{+\,\#\,*}$ Mean values within each row over time without common superscript are significantly different ($0.0001 \leq P \leq 0.02$).

3.5.2 Effects of castration and androgen therapy on plasma sex hormone concentrations

The effects of castration and androgen therapy on plasma TES and total estrogen concentrations are summarized in Table 3. As expected, castration drastically reduced plasma TES concentrations. CsX and CsX-LST plasma TES concentrations were below the detectible limits of the radioimmunoassay (TES<0.01ng·mL^{-1}). The plasma TES concentrations were restored in CsX-TRT to levels similar to those of InT-control rats (*P*> 0.16). Plasma estrogen levels were low among all experimental groups, with no significant differences among TES therapy or LST treatment (*P*> 0.09).

Table 3. Testosterone (TES) and total estrogen concentrations in plasma of InT-control, CsX, CsX-TRT, CsX-LST, and CsX-LST-TRT male rats.

Group	TES (ng·mL^{-1})	Total Estrogen (pg·mL^{-1})
InT-control	0.72 ± 0.2[a]	30.7 ± 0.6[a]
CsX	–	30.9 ± 1.2[a]
CsX-TRT	0.96 ± 0.1[a]	31.3 ± 0.5[a]
CsX-LST	–	32.7 ± 0.2[a]
CsX-LST-TRT	1.14 ± 0.1[a]	33.2 ± 0.6[a]

Sprague Dawley (SD) male rats remained intact (InT-control) or were bilaterally castrated (CsX) without or with TES therapy (CsX-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}). In a separate experiment, CsX male SD rats were placed on losartan-potassium drinking water (250 mg ·L^{-1}) without TES therapy (CsX-LST) or with TES therapy (CsX-LST-TRT). Data are means ± SEM (n= 6-13 rats/group). There were no significant differences between groups (*P* > 0.09).

3.5.3 Effects of androgens on systolic blood pressure

Systolic BP at wk 0 was similar among all experimental groups (*P* > 0.05). A repeated measures ANOVA revealed significant changes in BP over time and among treatment groups (P < 0.001). Systolic BP in Int-control rats slightly increased over the

first 2 wks and stabilized around 114 ± 5 mmHg, which was maintained throughout the remainder of the 15-week experiment. CsX rats exhibited a progressive and significant increase in systolic BP from wk 0 (109 ± 3.0 mmHg) to a maximal pressure at wk 9 (143 ± 3.5 mmHg), which then plateaued from wk 10 to 15. Systolic BP at wk 15 was significantly higher in CsX (140 ± 1.2 mmHg) compared to InT-control (113 ± 1.8 mmHg; $P \leq 0.0001$). In a separate group of CsX, TES therapy administered from wk 10 to 15 resulted in a progressive decline in systolic BP, which completely normalized by wk 15 (CsX-TRT, 113 ± 1.3 mmHg) to levels virtually identical to those of InT-control (113 ± 1.8; $P > 0.05$). The effects of CsX on systolic BP were also determined in AR-deficient Tfm rats. Similar to the SD rats, castration of Tfm rats increased systolic BP and TES therapy fully normalized the BP measurements. CsX-Tfm systolic BP averaged 109 ± 0.7 mmHg at wk 0, plateaued by wk 10 (139 ± 0.4 mmHg), and was fully normalized by TES therapy at wk 15 (109 ± 2.5 mmHg). Compared to SD rats, Tfm rats exhibited a delay in the initial development of hypertension and a more rapid onset of hypertension from wks 5-10. TRT in Tfm rats resulted in a distinctly more rapid and greater overall decline in systolic BP compared to CsX-TRT SD rats. The weekly systolic BP measurements of InT-control, CsX, CsX-TRT and CsX-TRT-Tfm rats are shown in Figure 3.

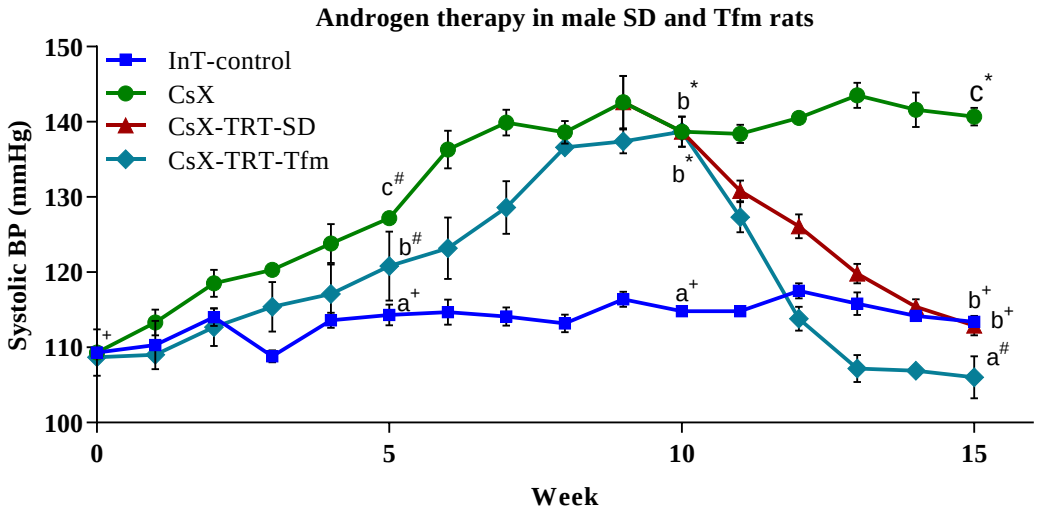

Figure 3. Effects of endogenous and exogenous testosterone on systolic BP.
Sprague-Dawley (SD) male rats that were intact (InT-control), bilaterally castrated (CsX), or CsX with TES therapy administered from weeks 10-15 (CsX-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}). A separate groups of testicular feminized male (Tfm) rats were CsX with TES therapy administered from weeks 10-15 (CsX-TRT-Tfm). Data points are means ± SEM (n= 5-10 rats/group). There were no significant differences between groups at wk 0. $^{a\text{-}c}$ Mean values at each time point (wk 5, 10, and 15) for each group (InT-control, CsX, CsX-TRT-SD, or CsX-TRT-Tfm) without common script are significantly different ($0.0001 \leq P \leq 0.01$). $^{+\,*\,\#}$ Mean values for each group over time (wk 5 vs. 10 vs. 15) without common script are significantly different ($0.0001 \leq P \leq 0.01$).

3.5.3.1 Mechanisms underlying the anti-hypertensive effects of androgens

The role of the RAS in the development of castration-induced hypertension is summarized in Figure 4. Systolic BP declined significantly in CsX SD rats treated with Losartan-potassium in the drinking water (CsX-LST; 250 mg·L^{-1}), an AT_1R antagonist. Repeated measures ANOVA revealed significant changes in BP over time and by treatment groups ($P < 0.001$). Systolic BP declined until wk 4 (CsX-LST, 100 ± 1.5 vs. CsX, 124 ± 2.6 mmHg; $P \leq 0.001$) and remained significantly lower throughout the 15-week experimental period (CsX-LST, 99 ± 0.4 vs. CsX, 141 ± 1.2 mmHg; $P \leq 0.0001$), with no statistically significant differences between CsX-LST (99 ± 0.4 mmHg) vs. CsX-LST-TRT

(101 ± 0.08 mmHg; $P > 0.05$). CsX-DRT exhibited similar effects on systolic BP compared to CsX-TRT. Systolic BP normalized identically between groups (CsX-DRT, 114 ± 1.3 vs. CsX-TRT, 113 ± 1.3 mmHg; $P > 0.05$).

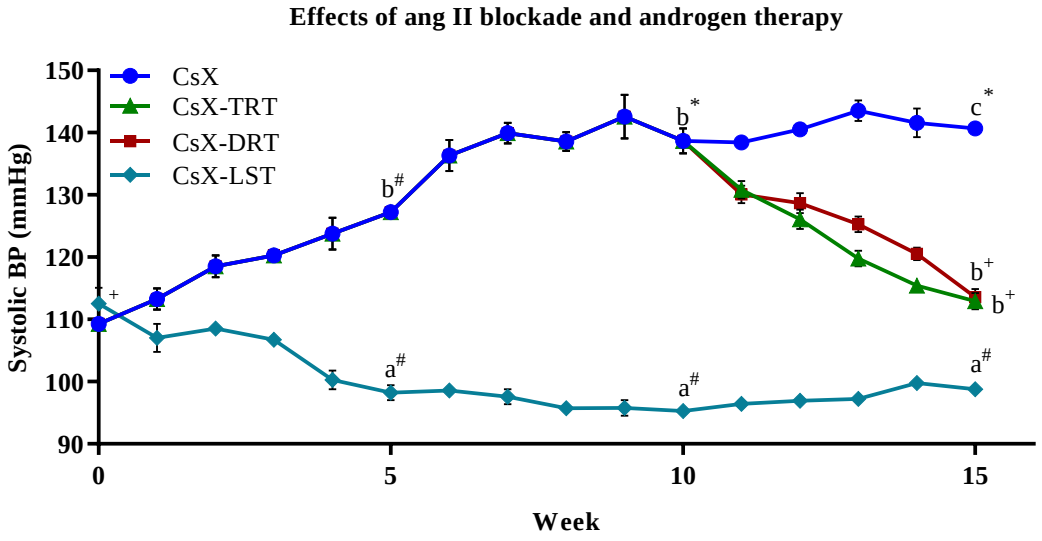

Figure 4. Effects of angiotensin II blockade and androgen therapy on systolic BP.
Castrated Sprague-Dawley (SD) male rats (CsX), CsX with TES therapy administered from weeks 10-15 (CsX-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}), or CsX with 5α-DHT therapy administered from weeks 10-15 (CsX-DRT; 1.00 mg·kg^{-1}; SC 2x·wk^{-1}). A separate group of CsX SD rats received Losartan-potassium (LST), an AT_1R antagonist in their drinking water (CsX-LST; 250 mg·L^{-1}). Data points are means ± SEM (n= 5-10 rats/group). There were no significant differences between groups at week 0. $^{a\text{-}c}$ Mean values at each time point (wk 5, 10 and 15) without common script are significantly different ($0.0001 \leq P \leq 0.02$). $^{+\,*\,\#}$ Mean values within each group over time (wk 5 vs. 10 vs.15) without common script are significantly different ($0.0001 \leq P \leq 0.01$).

3.5.4 Effect of androgens on urinary fluid and sodium excretion

The effects of castration and TRT on 24-hour urinary sodium and fluid excretion (normalized to BW) at weeks 0, 10, and 15 are summarized in Table 4. Trends in urine volume and Na^+ excretion reduced over time in the CsX rats and TES therapy increased

these variables transiently over weeks 10-15. The results are inconclusive due to significant variability among the data sets.

Table 4. Urine excretion volume, Na^+ excretion, and creatinine excretion of InT-control, CsX, CsX-TRT, CsX-LST, CsX-LST-TRT or CsX-DRT male rats.

Group	wk 0	wk 10	wk15
Urine Volume ($mL \cdot kg^{-1}$)			
InT-control	2.16 ± 0.4^a	1.45 ± 0.4^a	1.27 ± 0.2^a
CsX	1.99 ± 0.7^a	1.17 ± 0.2^a	1.32 ± 0.3^a
CsX-TRT	1.72 ± 0.4^a	0.92 ± 0.2^a	0.94 ± 0.2^a
CsX-LST	1.34 ± 0.2^a	0.76 ± 0.2^b	0.74 ± 0.1^b
CsX-LST-TRT	1.23 ± 0.1^a	0.92 ± 0.1^b	0.71 ± 0.1^c
CsX-DRT	1.85 ± 0.5^a	1.28 ± 0.1^a	0.95 ± 0.3^a
Na^+ Excretion ($mL \cdot kg^{-1}$)			
InT-control	0.07 ± 0.004^a	0.04 ± 0.006^b	0.02 ± 0.011^c
CsX	0.07 ± 0.009^a	0.05 ± 0.006^a	0.05 ± 0.011^a
CsX-TRT	0.06 ± 0.017^a	0.02 ± 0.006^b	0.01 ± 0.002^c
CsX-LST	0.04 ± 0.006^a	0.02 ± 0.006^b	0.02 ± 0.005^b
CsX-LST-TRT	0.03 ± 0.006^a	0.02 ± 0.007^a	0.01 ± 0.004^b
CsX-DRT	0.07 ± 0.004^a	0.07 ± 0.005^a	0.04 ± 0.007^b
Creatinine Excretion ($mg \cdot kg^{-1}$)			
InT-control	8.43 ± 0.5^a	14.09 ± 0.7^b	13.57 ± 0.6^b
CsX	15.59 ± 3.2^a	13.15 ± 0.9^a	13.03 ± 1.2^a
CsX-TRT	10.20 ± 1.4^a	13.99 ± 1.1^b	15.17 ± 1.0^b

Sprague Dawley (SD) male rats either intact (InT), bilaterally castrated (CsX), or CsX with TES therapy (CsX-TRT; 1.75 $mg \cdot kg^{-1}$; SC $2x \cdot wk^{-1}$) or 5α-DHT therapy (CsX-DRT; 1.00 $mg \cdot kg^{-1}$; SC $2x \cdot wk^{-1}$) from weeks 10-15. In a separate experiment, CsX males were placed on losartan drinking water (250 mg $\cdot L^{-1}$) without TES therapy (CsX-LST) or with TES therapy (CsX-LST-TRT; 1.75 $mg \cdot kg^{-1}$; SC $2x \cdot wk^{-1}$) from weeks 10-15. Data are means ± SEM (n= 4-6 rats/group). $^{a-c}$ Mean values for urine volume, Na^+ excretion, and creatinine excretion among the six experimental groups at each time point (wk 0, 10, and 15) without common superscript are significantly different ($0.0005 \leq P \leq 0.050$).

3.5.5 Effect of androgens on plasma renin concentrations

Plasma renin concentrations are summarized in Figure 5 and Table 5. CsX increased mean plasma renin concentration significantly (38%) compared to InT-control

rats ($P \leq 0.02$), while TES therapy reduced plasma renin concentration to levels similar to those of InT-control rats ($P > 0.05$).

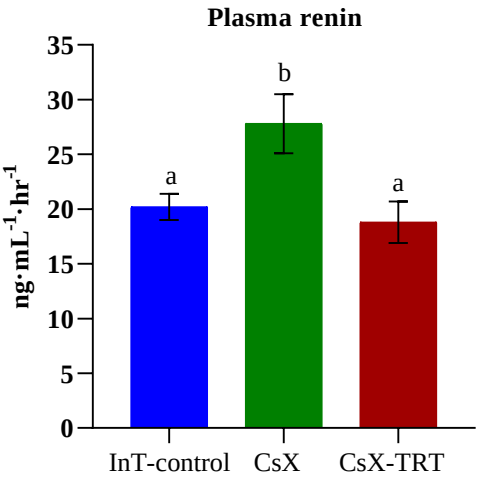

Figure 5. Effects of testosterone on plasma renin concentrations.
Male Sprague Dawley (SD) rats either intact (InT-control), bilaterally castrated (CsX), or CsX with TES therapy (CsX-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}). Bars represent means ± SEM (n= 5-6 rats/group). $^{a\text{-}b}$ Mean plasma concentrations of experimental groups (InT-control, CsX, and CsX-TRT) without common scripts are significantly different ($0.01 \leq P \leq 0.02$)

Table 5. Plasma renin concentration for InT-control, CsX, and CsX-TRT male rats.

	InT-control	CsX	CsX-TRT
Plasma renin (ng·mL^{-1}·hr^{-1})	20.2 ± 1.2^{a}	27.8 ± 2.7^{b}	18.8 ± 1.9^{a}

Plasma renin concentration for male Sprague Dawley (SD) rats either intact (InT-control), bilaterally castrated (CsX), or CsX with TES therapy (CsX-TRT; 1.75 mg·kg^{-1}; SC 2x·wk^{-1}). Data are means ± SEM (n= 5-6 rats/group). $^{a\text{-}b}$ Mean plasma concentrations without common script are significantly different ($0.01 \leq P \leq 0.02$).

3.5.6 Effect of androgens on mRNA expression of RAS components

mRNA expression of RAS components, as measured by rt-PCR, in the kidney and aorta of InT-control, CsX, and CsX-TRT male SD rats is summarized in Table 6 and illustrated in Figure 6. In the kidney, CsX significantly increased expression of mRNA for renin (92%), AT_1R (80%), and ACE (58%) compared to InT-control rats ($0.0013 \leq P \leq 0.033$), while TES therapy normalized the expression of mRNA for renin, ACE, and AT_1R to levels similar to those of InT-control rats ($P > 0.05$). In contrast, mRNA expression of renal Agt was reduced 55% in CsX rats compared to InT-control rats ($P \leq 0.0006$), while TES therapy restored 73% of mRNA expression of Agt compared to InT-control rats ($P \leq 0.01$). In the thoracic aorta, neither CsX nor CsX-TRT had any statistically significant effects on expression of mRNA for renin, ACE, AT_1R, or Ang ($P > 0.09$).

Table 6. Effects of TES on the mRNA expression of renin, angiotensin type I receptor (AT_1R), angiotensin converting enzyme (ACE), and angiotensinogen (Agt) in InT-control, CsX, and CsX-TRT male rats.

Group	**Renin (AU)**	**AT_1R (AU)**	**ACE (AU)**	**Agt (AU)**
Kidney				
InT-control	1.2 ± 0.20^a	1.0 ± 0.07^a	1.2 ± 0.26^a	1.1 ± 0.15^a
CsX	2.3 ± 0.50^b	1.8 ± 0.32^b	1.9 ± 0.34^b	0.5 ± 0.06^b
CsX-TRT	1.3 ± 0.17^a	1.1 ± 0.07^a	0.4 ± 0.03^c	0.8 ± 0.05^a
Aorta				
InT-control	1.0 ± 0.18^a	1.0 ± 0.17^a	1.0 ± 0.11^a	1.0 ± 0.22^a
CsX	1.5 ± 0.27^a	1.4 ± 0.36^a	1.2 ± 0.05^a	0.3 ± 0.52^a
CsX-TRT	0.6 ± 0.38^a	1.0 ± 0.09^a	0.9 ± 0.30^a	0.6 ± 0.03^a

rt-PCR for mRNA expression of the RAS components in the kidney and aorta of intact (InT-control), bilaterally castrated (CsX), or CsX with TES therapy (CsX-TRT; 1.75 mg·kg-1; SC 2x·wk-1) male Sprague Dawley (SD) rats. Data are means ± SEM (n= 3-9 rats/group). $^{a\text{-}c}$ Mean values of mRNA expression for renin, AT_1R, ACE, or Agt in the kidney or aorta without common superscript are significantly different ($0.0001 \leq P \leq 0.033$).

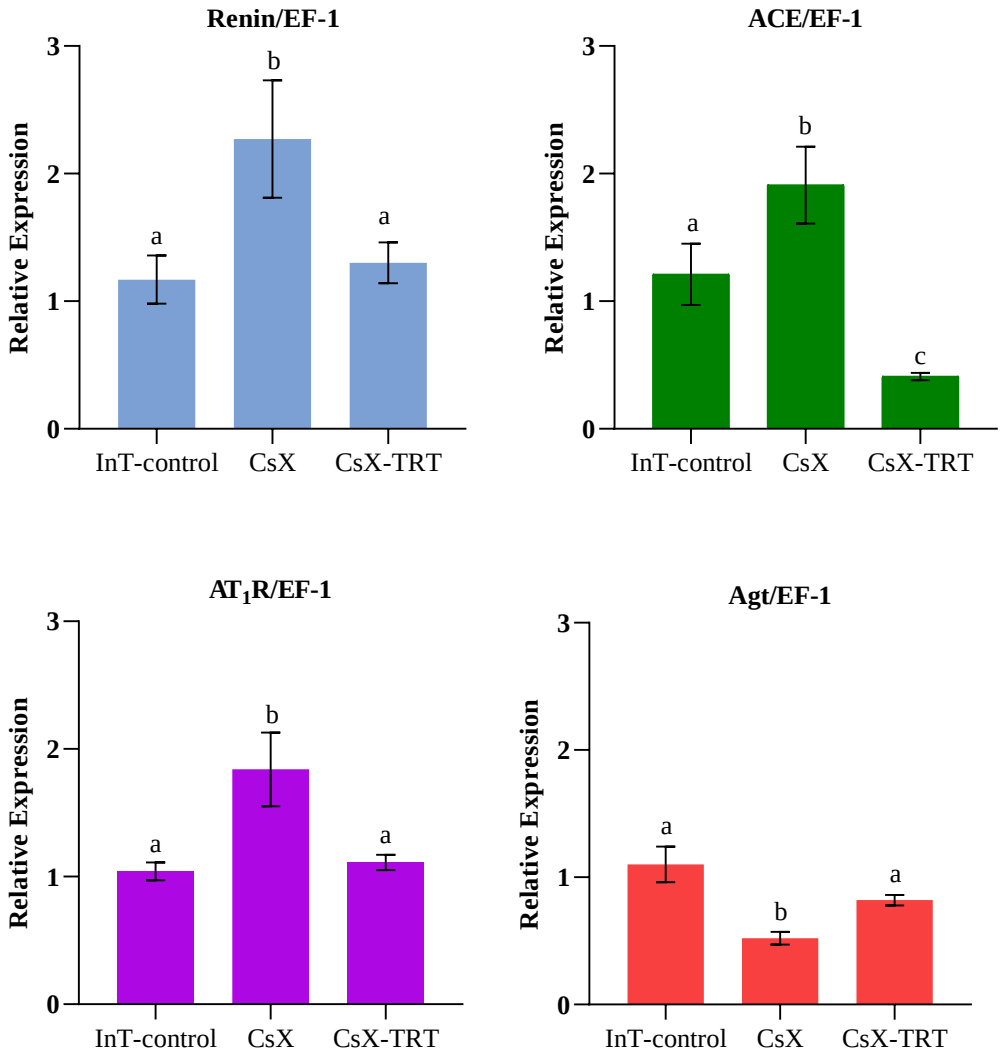

Figure 6. Effects of testosterone on the mRNA expression of renin, angiotensin type 1 receptor (AT_1R), angiotensin converting enzyme (ACE) and angiotensinogen (Agt) in the kidney.
Kidney mRNA expression of RAS components in male Sprague-Dawley (SD) rats either Intact (InT-control), bilaterally castrated (CsX), or CsX with TES therapy (CsX-TRT; 1.75 mg·kg-1; SC 2x·wk-1). Bars represent means ± SEM (n= 6-9 rats/group). [a-c] Mean values for mRNA expression of renin, AT_1R, ACE, or Agt in each of the three experimental groups (InT-control, CsX, and CsX-TRT) without common script are significantly different ($0.0013 \leq P \leq 0.033$).

3.6 Discussion

In the present investigation, the long-term effects of endogenous and exogenous TES on systemic BP were studied in male SD and Tfm rats. The results reveal that androgens (TES and 5α-DHT) exert novel long-term anti-hypertensive effects on systemic BP that appear to involve estrogen-independent, non-genomic and possibly genomic mechanisms, which reduce RAS component expression in the kidney. Thus, the long-term anti-hypertensive effects of TES may involve enhanced fluid and electrolyte excretion as well as systemic vasodilation. Castration of InT-control male SD rats resulted in a dramatic reduction in plasma TES concentration and seminal vesicle mass (an important target tissue for TES), while TES therapy restored plasma TES concentration and seminal vesicle mass to nearly identical values as InT-control male SD rats. Additionally, there were no significant differences in plasma total estrogen concentrations among InT-control, CsX, and CsX-TRT male SD rats. Thus, TES therapy provided physiological replacement of circulating TES concentrations without any measurable changes in total estrogen levels. While the dose of TES therapy employed in the present study produced physiological concentrations and biological effects on reproductive target tissues (i.e. seminal vesicle mass), the dose of 5α-DHT employed for androgen therapy only partially restored seminal vesicle mass. Since 5α-DHT is a local tissue hormone and not a circulating systemic androgen, it may not be as effective as the local conversion of TES to 5α-DHT which typically occurs within the seminal vesicle. Thus, the dose of 5α-DHT employed was either too low and/or systemic administration was not effective. It should be noted however, that the dose of 5α-DHT employed did exert virtually identical anti-hypertensive effects on systemic BP as TES over the 5-week treatment period. Thus, the anti-

hypertensive effects of TES observed in the present studies do not likely involve aromatization of TES to estrogen.

The epidemiological observations that hypertension and CAD occur more frequently in men than in premenopausal women have led to the firmly entrenched dogma that TES exerts deleterious effects on cardiovascular health. Recent clinical trials reported allegedly deleterious effects of androgen therapy (Basaria, Coviello et al. 2010, Vigen, O'Donnell et al. 2013, Finkle, Greenland et al. 2014) have been criticized by the scientific community for errors in reporting and flaws in experimental design and inclusion/exclusion criteria (Haddad, Kennedy et al. 2007, Morgentaler 2017, Gagliano-Juca and Basaria 2018, Morgentaler 2018). Additionally, this dogma is supported by many experimental animal studies that routinely employ genetic or induced animal models of hypertension, such as the SHR and the DOCA-salt rat models. The relevance of these models to human hypertension is limited because the development and progression of the hypertension are independent of androgen status and they fail to mimic the natural age-dependent pathogenesis of the disease as it occurs in human males.

There is accumulating evidence from recent clinical and experimental animal studies that challenge the dogma that androgens are deleterious to cardiovascular function (Traish, Abdou et al. 2009, Perusquia and Stallone 2010, Perusquia, Espinoza et al. 2012, Traish, Guay et al. 2014, Traish and Zitzmann 2015, Traish, Haider et al. 2017). These studies reveal a beneficial and even cardio-protective role for androgens in cardiovascular health (Saad 2012, Traish, Haider et al. 2017). There is also accumulating evidence that TES deficiency is associated with metabolic complications such as increased insulin resistance and hyperlipidemia, which together with hypertension are risk factors for

cardiovascular disease (Wang, Nieschlag et al. 2008, Bhasin, Cunningham et al. 2010, Francomano, Bruzziches et al. 2014). The current epidemiological data in hypogonadal men suggests that the progression of TES deficiency is proportional to the progression of atherosclerosis and hypertension (Slowinska-Srzednicka, Zgliczynski et al. 1989, Phillips, Pinkernell et al. 1994, Aversa, Bruzziches et al. 2010) and low TES concentrations increase risk factors for the development of diabetes (Keating, O'Malley et al. 2010) and CVD (Wu and von Eckardstein 2003, Malkin, Pugh et al. 2010, Yeap, Alfonso et al. 2014). Additionally, TES therapy improves metabolic profiles, lowers cholesterol, and reduces inflammation in hypogonadal men (Kovac, Pastuszak et al. 2014) and improves glucose and insulin dynamics of hypogonadal diabetic patients (Traish and Zitzmann 2015). In the cardiovascular system, TES reduces coronary artery plaque (Budoff, Ellenberg et al. 2017) and improves overall cardiac function and BP (Traish, Abdou et al. 2009). Acute TES injections improve arterial stiffness (Francomano, Fattorini et al. 2016) and reduce exercised-induced myocardial ischemia in hypogonadal men with CAD (Webb, Adamson et al. 1999).

It is estimated that within the next 20 years, TES deficiency will play a role in the development of an estimated 1.3 million cases of CVD, 1.1 million cases of diabetes, and 600,000 cases of osteoporosis-related fractures in the United States (Moskovic, Araujo et al. 2013). In agreement with the most recent clinical data, several experimental animal studies report similar observations regarding the adverse effects of TES deficiency on metabolic profiles and cardiovascular function, which improves with physiological levels of TES therapy. In these studies, castration impairs insulin sensitivity and metabolic profiles, which are restored with exogenous TES therapy (Holmang and Bjorntorp 1992).

In vivo studies reveal that acute TES infusions produce hypotensive effects in normotensive conscious male SD rats (Perusquia, Greenway et al. 2015) and anti-hypertensive effects in castrated male WKY rats (Perusquia, Contreras et al. 2019). Similarly, i*n vitro* studies utilizing isolated blood vessels reveal that exogenous TES produces rapid endothelium-independent vasorelaxation (Costarella, Stallone et al. 1996, Deenadayalu, White et al. 2001, Perusquia, Navarrete et al. 2007, Deenadayalu, Puttabyatappa et al. 2012). Taken together, the data on acute effects of TES provide valuable insights into the effects and underlying mechanisms of androgens on cardiovascular health; however, the present study is the first to investigate the long-term effects of endogenous and exogenous androgens on BP regulation. The main findings of this study reveal that androgens exert novel long-term anti-hypertensive effects on systemic BP through non-genomic, estrogen-independent mechanisms that involve inhibition of RAS function. In normotensive male SD and Tfm rats, castration induced a long-term progressive increase in systolic BP, which was reversed with androgen therapy. In a separate group of male SD rats, the castration-induced hypertension was abolished by LST, an AT_1R antagonist. TES-deficiency increased plasma renin concentrations and upregulated RAS component mRNA expression in the kidney. Thus, it appears that the antihypertensive effects of endogenous and exogenous androgens involve suppression of intrarenal RAS function, as well as the systemic vasodilation reported in previous studies.

3.6.1 Effect of castration and androgen therapy androgen concentrations

In the present study, castration was used to induce endogenous androgen-deficiency, while exogenous androgen therapy was used to restore physiological concentrations. The physiological replacement of TES was determined through plasma

TES and total estrogen concentrations and restoration of seminal vesicle mass. TES is known to influence nearly every system within the body, which includes the cardiovascular, endocrine, integumentary, renal, and reproductive systems. Studies have shown that castration significantly reduces circulating TES and 5α-DHT concentrations (Coyotupa, Parlow et al. 1973) and that TES therapy can restore TES concentrations to physiological levels (Ward and Abdel-Rahman 2005). While TES deficiency adversely affects metabolic function and cardiovascular health (Traish, Haider et al. 2017), excess TES can also influence metabolic and cardiovascular function (Bassil, Alkaade et al. 2009). Elevated TES levels are associated with cerebrovascular disease (Pal, Hadjadj et al. 2019) and hypertension (Achar, Rostamian et al. 2010). Additionally, excess serum TES can facilitate the aromatization of TES into estrogen, which alters the physiological TES/estrogen ratio and impacts the male reproductive and cardiovascular system (Zheng, Li et al. 2012). Clinically, men undergoing androgen therapy are routinely screened for serum androgen levels, and as an additional marker, seminal vesicle mass can be used as a direct indicator of circulating TES concentrations (Sasagawa, Nakada et al. 1989). The seminal vesicles are an androgen-responsive target organ regulated by circulating TES levels and local metabolism to 5α-DHT (Kashiwagi, Shibata et al. 2005). Studies suggest that seminal vesicle mass atrophies by 80% within 14 days of castration, however, within days after the initiation of TES therapy, protein synthesis is fully restored (Higgins, Burchell et al. 1976). In the present study, castration in the male SD rat resulted in a dramatic reduction of plasma TES concentrations and seminal vesicle mass, while exogenous TES therapy fully restored circulating TES concentrations and seminal vesicle mass to physiologically normal states. Additionally, there were no significant differences

in plasma total estrogen concentrations between the intact and the castrated rats undergoing androgen therapy. While the dose of TES therapy employed in the present study restored physiological concentrations, the targeted therapeutic 5α-DHT did not restore seminal vesicle mass. Thus, the dose of 5α-DHT employed was either too low and/or systemic administration of 5α-DHT may not be as effective as the local conversion of TES to 5α-DHT that occurs within the seminal vesicle, since 5α-DHT is a local tissue hormone and not a circulating systemic androgen. It should be noted however, that the dose of 5α-DHT employed did exert virtually identical anti-hypertensive effects on systemic BP as TES over the 5-week treatment period.

Under normal physiological conditions, the ratio of circulating TES to 5α-DHT is approximately 15 to 2 nmol/L, respectively (Starka, Duskova et al. 2008). Compared to TES, 5α-DHT is considered the most efficacious and potent androgen and normally circulates at low physiological concentrations. Most 5α-DHT is produced locally in specific target tissues such as the seminal vesicles where circulating TES is rapidly reduced to 5α-DHT (Toth and Zakar 1982, Zakar and Toth 1982). Thus, circulating 5α-DHT levels may not reflect local production or accumulation of 5α-DHT in the seminal vesicles.

3.6.2 Effects of castration and androgen therapy on systolic BP

In the present study, castration of male SD rats produced a progressive increase in systolic BP that attained a stable plateau after 10-12 weeks. Androgen therapy of CsX rats with either TES or 5α-DHT at physiological levels, beginning at 10 weeks post-CsX, completely reversed the hypertension and restored systolic BP to levels comparable to those of normotensive InT-control male SD rats. These findings are similar to a recent report in which castration produced hypertension in both Wistar and Wistar-Kyoto (WKY)

male rats, which was reduced with rapid bolus injections of TES and 5α-DHT (Perusquia, Herrera et al. 2017). Several studies suggest that androgens reduce total vascular resistance with accumulating evidence that androgens exert rapid non-genomic vasorelaxation of VSM cells. In several experimental animal studies, TES exerts acute vasorelaxation in isolated conduit arteries, such as the aorta, and in smaller resistance vessels, such as the coronary, mesenteric, and pulmonary arteries (Deenadayalu, White et al. 2001, Ding and Stallone 2001, Yildiz, Seyrek et al. 2005, Malkin, Jones et al. 2006, Perusquia, Navarrete et al. 2007, Seyrek, Yildiz et al. 2007, Cairrao, Alvarez et al. 2008). In humans, similar acute vasorelaxation has been reported in resistance vessels infused with TES (Malkin, Jones et al. 2006). The mechanisms of TES-induced vasorelaxation appear to increase endothelial NO production (Chou, Sudhir et al. 1996, Molinari, Battaglia et al. 2002), inhibit Ca^{2+} channels (Scragg, Jones et al. 2004, Hall, Jones et al. 2006, Scragg, Dallas et al. 2007), and/or activate membrane bound K^{+} channels (Deenadayalu, White et al. 2001, Han, Chae et al. 2008). The vascular effects decrease tone, reduce vascular resistance, and reduce systemic BP. These antihypertensive effects of TES coincide with the current epidemiological evidence that the progression of hypertension is proportional to TES deficiency (Traish, Haider et al. 2017). In contrast, many experimental animal studies suggest that TES has deleterious effects on the cardiovascular system. These studies suggest that castration mitigates the progression of hypertension, while TES therapy exacerbates the disease (Reckelhoff, Zhang et al. 1998, Reckelhoff, Zhang et al. 2000, Reckelhoff 2001, Reckelhoff and Samson 2015, Oloyo, Sofola et al. 2016, Loh, Giribabu et al. 2017, Reckelhoff 2018). Most of the studies that conclude that TES exerts detrimental effects employ hypertensive animal models, such as the SHR. There are many

defects in the SHR that may contribute to hypertension such as an altered signaling pathways and immune response, but it is unclear which of these defects are involved in the pathogenesis of androgen-dependent hypertension. One theory is that and increase in tyrosine hydroxylase, an enzyme involved in catecholamine biosynthesis, may contribute to hypertension in the SHR. Tyrosine hydroxylase production in the vasculature and renal medulla elevates catecholamine production, which results in increased BP. Androgen-induced elevations in tyrosine hydroxylase were not observed in normotensive rats (Kumai, Tanaka et al. 1994, Kumai, Tanaka et al. 1995). These animal models of hypertension fail to elucidate the role of androgens in the pathogenesis of hypertension and fail to mimic the age-dependent progression of hypertension in human males. This may explain why studies utilizing the SHR and other rat models are incongruous with the recent human clinical findings, which demonstrate beneficial effects of the androgens on BP regulation (Lerman, Chade et al. 2005, Lerman, Kurtz et al. 2019). In the present study, normotensive male SD rats were used to mimic the natural, age-dependent progression of hypertension in hypogonadal men.

3.6.2.1 Estrogen-independent mechanisms of androgens in BP regulation

An important experimental control of the present study was to demonstrate that the long-term, antihypertensive effects of TES are independent of its aromatization to estrogen. While TES is the major circulating androgen in men, the local conversion of TES into the active metabolites estrogen and 5α-DHT can facilitate various actions at specific sites, such as adipose tissue, bone, seminal vesicles, vascular endothelium, and several sites within the brain (Szalay, Krieg et al. 1975, Martini, Celotti et al. 1990). The local site metabolism of TES is regulated by the cellular expression of aromatase and

reductase enzymes (Simpson 2003). For example, in adipose tissue TES is aromatized to estrogen, while in the seminal vesicle TES is reduced to 5α-DHT. The beneficial effects of estrogen on the vascular system are well documented (Yang and Reckelhoff 2011, Perusquia, Espinoza et al. 2012). Acute estrogen infusion induces rapid, non-genomic vasodilation which includes increases in prostanoid and endothelial NO production. The sustained effects of estrogen include a cardio-protective role in preventing atherosclerosis and improved metabolic function by optimizing lipoprotein and triglyceride metabolism (Mendelsohn 2002). In the present study, the TES metabolite 5α-DHT was used to differentiate the cardiovascular effects of TES from estrogen. 5α--DHT is unable to be aromatized to estrogen; thus, the effects of 5α-DHT therapy are independent of estrogen metabolism. The results of this study revealed virtually identical anti-hypertensive properties of both TES and 5α-DHT. Both TES and 5β-DHT cause acute, dose-dependent vasodilation in isolated blood vessels (Ding and Stallone 2001, Perusquia and Stallone 2010, Perusquia, Espinoza et al. 2012), and both androgens produced acute systemic hypotension in conscious male rats (Ding and Stallone 2001, Perusquia, Greenway et al. 2015, Perusquia, Herrera et al. 2017). With no significant differences in plasma estrogen concentrations among treatment groups, the anti-hypertensive effects of TES appear to be estrogen independent.

3.6.2.2 Non-genomic mechanisms of androgens in blood pressure regulation

The AR-deficient Tfm rat was used in the present study to identify the non-genomic effects of androgens on BP regulation. In the Tfm rats, castration induced hypertension, and androgen therapy reversed these effects. This finding strongly suggests that the antihypertensive effects of endogenous and exogenous androgens on systemic BP involve

non-genomic mechanisms. The structurally specific non-genomic effects of the androgens are fundamentally different than their translational genomic effects in reproductive targets (Ding and Stallone 2001). Non-genomic mechanisms may include activation of kinase-signaling cascades or modulation of membrane ion channel function. These non-genomic effects are characterized by their rapid onset compared to the genomic effects. Based on the rapid vascular response to androgen infusion, many studies consider androgen-induced vasorelaxation to be a non-genomic mechanism (Perusquia, Greenway et al. 2015, Perusquia, Herrera et al. 2017). In the present study, there was a progressive increase in systolic BP for both the castrated SD and Tfm rats. There was a delay in the onset of hypertension in the Tfm rats compared to the male SD rats, yet the progression was much more dramatic. This suggests a more active role for non-genomic mechanisms in BP regulation in the Tfm rat, or that in SD rats, interactions between the non-genomic and genomic effects of TES are responsible for the antihypertensive effects of TES. Circulating levels of TES are increased in Tfm rats compared to wild type controls (Yarbrough, Quarmby et al. 1990). This difference in androgen production may alter receptor activation and influence receptor expression. Thus, differences in androgen production prior to experimental manipulation may contribute to the differences in blood pressure regulation observed in response to castration and androgen therapy in the SD vs. Tfm rats. While androgens are known efficacious and acute vasodilators (Perusquia, Contreras et al. 2019), the changes in BP observed in this study were not acute in nature and occurred over the course of five weeks. This suggest a possible role for both rapid non-genomic and long-term genomic effects of androgens on systemic BP regulation.

3.6.2.3 Androgen-induced effects on the renin-angiotensin system

LST is therapeutically used to lower BP and improve blood flow (Ripley and Hirsch 2010). In the kidney, LST reduces salt and fluid reabsorption in the proximal and distal tubules, yet LST has variable effects on GFR (Zhuo, Imig et al. 2002). Since renal autoregulation integrates intrarenal mechanisms to regulate GFR, LST will only affect GFR when there are impairments to the autoregulatory system. LST decreases mesangial cell function, increases GFR, inhibits inflammation, and mitigates renal fibrosis formation (Inscho, Imig et al. 1999). In the present study, LST treatment abolished the development of castration-induced hypertension. While the direct effects of androgen deficiency, LST treatment, and renal function have not been fully explored, studies have used LST treatment to evaluate the effects on fibrosis biomarkers in the reproductive system of castrated animals with an interesting connection between castration and fibrotic tissue development mitigated by LST treatment (Kucukdurmaz, Efe et al. 2018).

To further investigate the role of the RAS in androgen-induced BP regulation in the present study, circulating plasma renin and mRNA expression of RAS components in the aorta and kidney were measured. Castration significantly increased renal expression of renin, AT_1R, and ACE mRNA compared to InT male rats, while TES therapy completely reversed these effects of castration. Numerous other studies have investigated the effects of androgens on RAS expression; however, most of these experimental animal studies employ hypertensive animal models. In both the SHR and Dahl-salt sensitive models, castration suppresses RAS component mRNA expression in the kidney, while TES therapy reverses these effects (Chen, Naftilan et al. 1992, Yanes, Sartori-Valinotti et al. 2009). However these studies fail to elucidate the mechanisms underlying the effects of androgen

deficiency on renal RAS function, as these models are predisposed to hypertension-dependent differences in renal RAS component mRNA expression independent of androgen status when compared to normotensive models (Tikellis, Cooper et al. 2006). Further, these studies fail to reconcile the documented differences in the detrimental effects of androgens in animal models of hypertension vs. the beneficial effects of TES therapy in hypertension as observed in human disease. Additionally, many of these studies that support the deleterious effects of TES utilize inappropriate therapeutic doses of androgens. High concentrations of 5α-DHT therapy decreased serum Ang II concentrations, increased renal angiotensinogen expression, and increased proximal tubule Na^+ reabsorption in SD rats (Quan, Chakravarty et al. 2004). In this study, verified physiological doses of androgens suppressed expression of RAS component mRNAs in the kidney, effects consistent with androgen-induced reductions in BP.

3.6.3 Physiological relevance

The results of the present study provide important new and novel information on the long-term, non-genomic, estrogen-independent, anti-hypertensive effects of androgens on BP regulation. The anti-hypertensive effects of LST treatment in castrated rats, the TES-induced suppression of renal RAS component mRNA expression, and the androgen-induced reduction in plasma renin concentrations that were observed in this study suggest that the antihypertensive effects of TES involve down regulation of the intrarenal RAS function. These findings agree with the current clinical and epidemiological belief that TES deficiency has adverse effects on metabolic profiles and cardiovascular health, and that TES deficiency exacerbates cardiovascular disease. The present findings are

important for future therapeutic application of TES therapy and increased understanding of the mechanisms underlying androgen-induced BP regulation.

4 THE ROLE OF ANDROGENS IN PREGNANCY-INDUCED HYPERTENSION

4.1 Introduction

PE is a multi-system hypertensive disorder that typically occurs during the second and third trimesters of pregnancy. Characterized as newly-onset, pregnancy-specific maternal hypertension, PE complicates approximately 7-10% of all pregnancies worldwide and is a major cause of maternal and fetal morbidity and mortality (Steegers, von Dadelszen et al. 2010). While the pathogenesis of PE remains controversial, improper placental implantation is thought to contribute to inadequate cardiovascular adaptations that result in maternal hypertension, proteinuria, and fetal IUGR (Robertson, Brosens et al. 1985, Pijnenborg, Vercruysse et al. 1998, Bosio, McKenna et al. 1999). During pregnancy, females undergo significant physiological adaptations that are targeted at maintaining an environment conducive to fetal growth and development (Sherwood 2013). In healthy pregnancies, maternal BP remains relatively stable during the first trimester of pregnancy and gradually decreases into the second trimester of pregnancy, despite a 40% increase in circulating blood volume (Ueland 1976). To accommodate these significant increases in blood volume, systemic and regional hemodynamic changes reduce vascular responsiveness to vasoconstrictors (Wolff, Nisell et al. 1993) and increase vascular sensitivity to vasodilators (Nelson, Steinsland et al. 1998). Any impairments in these systemic and regional hemodynamic changes during pregnancy are attributable to the progression of PE.

Increases in sex steroid hormone production, including androgens, estrogens, and progesterone, are associated with normal pregnancy (Bammann, Coulam et al. 1980, McClamrock and Adashi 1992). TES and 5α-DHT production increases significantly during the first and last trimesters of pregnancy (Buster, Chang et al. 1979, Wilke and Utley 1987), which is attributed to decreases in metabolic clearance during the first trimester of pregnancy (Bammann, Coulam et al. 1980) and increases in TES production at the fetoplacental interface throughout gestation (Escobar, Patel et al. 2011). Interestingly, circulating concentrations of TES increase two- to three-times greater in women with PE compared to women with normal pregnancies (Acromite, Mantzoros et al. 1999, Serin, Kula et al. 2001, Sharifzadeh, Kashanian et al. 2012). Further, increases in AR expression have been observed in the placental tissue of women with PE (Hsu, Lan et al. 2009). Thus, these findings suggest that TES is associated with the pathogenesis of PE. There are several experimental animal studies that support these findings and suggest that TES has deleterious effects on pregnancy. Increases in TES levels contribute to the development of hypertension in pregnant SD rats (Chinnathambi, Balakrishnan et al. 2013, Blesson, Chinnathambi et al. 2015); however, TES was administered late in gestation after the establishment of normal placental implantation. Thus, these studies fail to mimic the natural progression of human disease and require further investigation for definitive causative effect in the pathogenesis of PE. In contrast, there is increasing evidence that TES and its metabolites are beneficial to BP and vascular function during pregnancy. For example, acute bolus infusions of androgens exert beneficial, anti-hypertensive effects on the arterial BP of PE pregnant rats (Perusquia, Hanson et al. 2018), with similar observations reported in normotensive and hypertensive male rats (Perusquia, Greenway

et al. 2015, Perusquia, Herrera et al. 2017, Perusquia, Contreras et al. 2019). Interestingly, TES and other androgens produce rapid, vasorelaxation of large arteries and smaller resistance vessels *in vitro* (Perusquia and Stallone 2010). Several of these *in vitro* studies demonstrate differences in the efficacy and/or potency of various androgen metabolites to produce vasorelaxation, which suggest regional differences among the androgens. The TES precursor DHEA and the TES metabolite 5β-DHT evoke greater vasorelaxation of human umbilical arteries compared to TES and other female sex steroid hormones (Perusquia, Navarrete et al. 2007). These regional differences in androgen efficacy and/or potency may contribute to the local regulation of blood flow at the fetoplacental interface and may play a role in the maintenance of BP during pregnancy. *In vivo,* the anti-hypertensive effects of DHEA and 5β-DHT are more potent than the moderate anti-hypertensive effects of TES and 5α-DHT in PE pregnant rats (Perusquia, Hanson et al. 2018). While there is accumulating evidence that androgens exert rapid vasorelaxation and contribute to systemic BP regulation during pregnancy, there is little evidence on the direct effects of androgens on the UA. Thus, in the present study, the vascular effects of TES and its key metabolites 5α- and 5β-DHT and the androgen-induced signaling mechanism were studied in the UA of normal pregnant and PE pregnant female SD rats. Further, the effects of PE on vascular reactivity and mRNA expression of androgen reducing enzymes, such as 5α- and 5β- reductase, were investigated near the uteroplacental interface. It was hypothesized that the deleterious effects of PE on uterine blood flow during pregnancy may result from reduced reactivity and/or production of placental androgens, specifically 5β-DHT. This study utilized a DOCA-salt pregnant rat model of PE to investigate the effects of PE on UA vascular function.

4.2 Methods

4.2.1 Ethical approval

All animal protocols were written in accordance with regulations of the National Institutes of Health as detailed in the "Guide for the Care and Use of Laboratory Animals" and approved by the Institutional Animal Care and Use Committee at Texas A&M University.

4.2.2 Experimental animals

Female SD rats 12-15 weeks of age were purchased from Envigo (Houston, TX) and housed at the Laboratory Animal Resource and Research facility at Texas A&M University in College Station, TX. The rats were housed in well-ventilated rooms with controlled temperatures (21-26°C), relative humidity (~50%), and photoperiod (12:12-hr light: dark cycle). Animals were pair housed in standard plastic laboratory rat cages and fed a 16% protein rat chow that was soybean- and alfalfa-free (Teklad Global Diet; Houston, TX). This diet is nominally free of dietary phytoestrogens, since these compounds have been reported to confound data investigating sex differences in vascular function (Li and Stallone 2005). Food and water were provided *ad libitum*. Rats were randomly divided into two experimental groups: normal pregnant (NP) or preeclampsia pregnant (PEP) rats.

Female rats were determined to be regularly cycling through microscopic evaluation of vaginal cytology. Female rats in proestrus were placed in overnight breeding cages with a male SD rat (Envigo; Houston, TX). The breeding cages were equipped with a wire bottom rack to enable the visual conformation of a vaginal plug, which was

considered the first day of pregnancy (day 1). Once successful breeding occurred, the pregnant females were returned to their original housing.

4.2.3 Surgical procedure

The DOCA-salt rat is a well-established model of hypertension that targets neurogenic, renal, and vascular response elements that results in an imbalance in renal sodium and water reabsorption with subsequent hypervolemia. DOCA-salt rats characteristically have endothelial dysfunction and enhanced vascular reactivity of isolated blood vessels (Nunes, Fortes et al. 2000). While DOCA-salt treatment increases RAS activity in the brain, which generates a neurologic (SNS) effect that alters renal function and systemic vascular resistance (Ueno, Mohara et al. 1988), circulating renin and Ang II levels remain normal. PEP rats underwent subcutaneous (SC) implantation of DOCA-salt impregnated medical grade silicone rubber on day 1 of pregnancy (DOCA; 200 $mg \cdot kg^{-1}$) using methods previously described (Stallone 1995). Briefly, the female rats were pretreated with atropine sulfate (0.05 $mg \cdot kg^{-1}$; SC) and anesthetized with isoflurane. Ophthalmic ointment was applied to the eyes of each animal as a preventative for corneal dryness. During the entire surgical procedure, animals were provided supplemental heat via heating lamp and continuously monitored for changes in respiratory rate and reflex response in order to ensure an adequate surgical plane of anesthesia. The surgical site was clipped and aseptically prepared, using alternating scrub cycles of iodine and 70% isopropyl alcohol. A 1-2 cm incision was made between the shoulder blades and hemostats were used to bluntly dissect the SC adventitia and create a pocket for the DOCA silicone implant. The implant was placed, and the incision was then closed with 2-3 wound clips. Flunixin meglumine (2.5 $mg \cdot kg^{-1}$; IM) was administered for analgesia and the animals

were transferred to a recovery cage. After DOCA implantation surgery, the PEP rats were individually housed, and their drinking water was replaced with saline water containing 1.0% sodium chloride (NaCl)/0.2% potassium chloride (KCl). The wound clips were removed 10-14 days post-operative and the animals were humanely euthanized on day 20 ± 2 of pregnancy.

4.2.4 Systolic blood pressure

Systolic BP was measured using a non-invasive tail cuff plethysmography system (IITC Life Science; Woodland Hills, CA) connected to a PowerLab Data Acquisition System and analyzed with Lab Chart Software (AD Instruments; Colorado Springs, CO). Prior to data collection, animals were conditioned to minimal restraint in a warming chamber for at least 5 days. After 5-10 min of acclimation in the warmed restraint chamber, the inflation-deflation cycle of the tail cuff BP measurement was conducted for 5-7 repetitions to determine the systolic BP. The daily BP for each animal was calculated by eliminating the highest and lowest BP values for each session and averaging the remaining measurements. The mean BP and BW of each animal was recorded prior to pregnancy and/or experimental treatment on day 0, and again on days 6, 12, and 18 of pregnancy.

4.2.5 Urinary protein excretion

On day 19, NP and PEP rats were placed in metabolic cages for 24-hr urine collection. During the procedure, rats were restricted from food to prevent sample contamination. After 24-hrs, the female rats were returned to their original cages. Total urine collection was weighed to determine volume, and samples were then thoroughly mixed, aliquoted into 1.5 mL Eppendorf tubes, and stored at -80°C for later analysis.

Individual rat urinary protein concentrations were determined using the Bradford protein assay, which uses the Coomassie Blue (BioRad) dye to bind various concentrations of basic and aromatic amino acid residues. For each animal, the total 24-hr protein excretion was calculated as the product of total 24-hr urine excretion volume ($mL \cdot 24\text{-}hr^{-1}$) x urinary protein concentration ($mg \cdot mL^{-1}$).

4.2.6 Markers of preeclampsia

At term (pregnancy day 20 ± 2), animals were humanely euthanized via rapid decapitation, which minimizes potential neural and hormonal artifacts that result during anesthesia and euthanasia (Boivin, Hickman et al. 2017). Immediately following euthanasia, the UA were isolated and transferred to chilled (0-4°), continuously gassed (95% O_2/5% CO_2) Krebs-Henseleit-bicarbonate solution (KHB) buffer with the following composition (mM): NaCl (118), $NaHCO_3$ (25), glucose (10), KCl (4.74), $CaCl_2$ (2.5), $MgSO_4$ (1.18) and KH_2PO_4 (1.18). The uterus was dissected, and the placentas and fetuses were removed from the tissue. The placentas and fetuses were weighted, and values were recorded for each individual fetus, which were then immediately euthanized via rapid decapitation. The placentas were dried for 24-hrs at 80°C. After drying, the placentas were re-weighed to obtain dry weight values.

4.2.7 Determination of androgen- induced vasorelaxation - contractile force measurement

Isolated UA were dissected free of surrounding tissues with the aid of a dissection microscope. Sections of the isolated UA from each rat were prepared in quadruplicate and sectioned into 4 rings, approximately 1.5-2.0 mm in length each. Two stainless steel wire threads, 20 µM in diameter, were threaded through the lumen of each ring with careful

consideration to maintain the integrity of the surrounding endothelium. The wire-threaded rings were transferred to 10 mL tissue chambers of the wire myograph system (Danish Myotechnology, DMT; Ann Arbor, MI) with fresh, chilled, gassed KHB. The two wires were secured to the myograph support and force transducer. The UA rings underwent a 1-hr equilibration period without tension, while the tissue chamber was gradually warmed to a physiological temperature of approximately 37°C. Within the chamber, KHB was continuously gassed and replaced every 15-20 min. The length of each UA ring was individually measured and used to calculate the optimal tension for each chamber, which utilized the LabChart force-tension curve. Briefly, the internal diameter of each UA ring was gradually increased to achieve an optimal intraluminal pressure (NP, 100 mmHg; PEP, 120 mmHg). At optimal tension, the rings were allowed to rest for an additional 30 min. To condition the contractile mechanisms of the UA rings, prostaglandin F2α (PGF2α, 1.0 μM) was used to generate two consecutive vascular contractions of the UA rings. Between contractions, the tissue chambers were thoroughly rinsed with fresh KHB and the UA rings were allowed to fully relax to optimal resting tension. During the second contraction to PGF2α, the endothelial integrity of each vessel was assessed using acetylcholine (ACh, 10 μM). The vasorelaxation response of each UA ring to ACh was used to confirm that the ring preparation had an intact endothelium. After a complete rinse and an additional 30 min rest at baseline tension, the UA rings were pre-contracted with U-46619 (1.0 μM; Cayman Chemical). U-46619 is a stable analog of the endoperoxide prostaglandin H_2, which acts as a TXA_2 receptor agonist. Once a stabilized VSM cell contraction was established for each of the 4 UA rings, a cumulative concentration-response curve was obtained for vehicle (Veh; final concentration 4% EtOH) and for each of the androgens

(TES, 5α-DHT, and 5β-DHT; 0.1-1000 nM). A stable plateau response was obtained at each concentration and relaxation was calculated as a percentage of the contractile force (i.e. the amplitude of the tonic pre-contraction before the addition of the androgens). Thus, the vasorelaxation is expressed as percent relaxation (%) for each of the UA rings in NP and PEP rats.

4.2.8 Determination of nitric oxide dependent vasorelaxation

In separate experiments, isolated UA rings from NP and PEP rats, prepared in quadruplicate, were used to determine the role of NOS in androgen-induced vasorelaxation during pregnancy. The percent of contractile force was measured in each UA ring following pretreatment for 20 min with the non-selective nNOS/eNOS inhibitor L-NG - monomethyl Arginine citrate (L-NMMA, 100 μM) or the nNOS specific inhibitor NW-propyl-L-Arginine (NPA, 200 μM). Each pretreated UA ring was precontracted with U-46619, and once the contraction stabilized, a cumulative concentration-response curve was obtained for 5β-DHT and TES (0.1-1000 nM).

4.2.9 Determination of potassium channel dependent vasorelaxation

In separate experiments, isolated UA rings from NP and PEP rats, prepared in quadruplicate were used to determine the role of K^+ channels in androgen-induced vasorelaxation during pregnancy. The percent of contractile force was measured in each UA ring pretreated for 20 min with a selective K^+ channel inhibitor: Iberiotoxin (IbTx; 100 nM), 4-aminopyridine (4-AP; 3 mM), or glybenclamide (Gly; 10 μM). IbTx is a selective inhibitor of large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), while 4-AP selectively inhibits voltage- gated K^+ channels (K_v), and Gly inhibits the ATP-dependent K^+ channels (K_{ATP}). The fourth ring of each preparation served as a control (androgen). Pretreated UA

rings were then precontracted with U-46619, and once the VSM contraction stabilized, a cumulative concentration-response curve was obtained for 5β-DHT or TES (0.1-1000 nM).

4.2.10 5α- and 5β- reductase mRNA expression in the female rat

In a separate groups of animals, the placenta and UA isolated from NP and PEP rats were immediately cleaned of surrounding connective tissue and fat, and then snap frozen and stored at -80°C in RNA/DNA-free Eppendorf tubes to evaluate mRNA expression of 5α- and 5β- reductase using established rt-PCR methods (Chang, Skiles et al. 2017). Briefly, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen), according to manufacturer's instructions. Individual RNA samples were tested for potency and total purified RNA samples (500-1000 ng) were seeded into a reverse transcription reaction using the SuperScriptII system (Invitrogen), in accordance with the recommended protocol for high capacity cDNA reverse transcription (Invitrogen), which combined 2.0µL random hexamer oligonucleotides, 0.8µL of 100 mM dNTP, 2.0 µL RT Buffer, 1.0 µL MultiScribe™ reverse transcriptase, and 4.2 µL purified RNA plus water. Sample temperature was raised to 25°C for 5 min, 42°C for 50 min, 45°C for 20 min, 50°C for 15 min, and 70°C for 5 min. The relative levels of candidate gene transcripts were analyzed using the Dynamo Flash SYBR Green qPCR (Thermo Scientific), in accordance with the recommended protocol. Reactions were quantified on a CFX38 touch rt-PCR detection system (BioRad). The primers for 5α- and 5β- reductase are listed in Table 7.

Table 7. Primer sequence for 5α- and 5β- reductase rt-PCR

Gene	Sequence
5α-reductase	Sense: GAGCCAGTTTGCGGTTTATG Antisense: GGATGTGGTCTGAGTGGATATT
5β-reductase	Sense: GCAGACCCTCAAGCTAGATTAC Antisense: GGTATATCACTCGGCCATTCTC

4.3 Chemical Reagents and Drugs

PGF2α (Lutalyse; Pharmacia &Upjohn) was purchased from the TAMU Large Animal Pharmacy in a stock solution of $5mg \cdot mL^{-1}$. The solution was diluted and prepared daily with KHB. Acetylcholine chloride (Sigma Chemical, St. Louis, MO) was prepared from 10^{-2} M stock stored at -20°C and diluted in KHB. U-46619 (Cayman Chemical, Ann Arbor, MI) was dissolved in 100% ethanol and stored at -20°C and diluted daily with KHB. The androgens (Steraloids, Newport, RI) were diluted in 100% ethanol and stored as 10^{-2} stock solutions at 4°C and prepared daily with 25% ethanol in normal saline. IbTx, 4-AP, L-NMMA, and NPA (Cayman Chemical, Ann Arbor, MI) were prepared daily with either distilled water or KHB. Gly (Sigma Chemical, St Louis, MO) was prepared daily with KHB from a 10^{-2} M stock solution (DMSO/ethanol) stored at -20°C.

4.4 Data Analysis

All data are expressed as the mean ± standard error of the mean (SEM), with n= indicating the number of animals in each experimental group. Vasorelaxation responses are expressed as percentage change from precontracted force with E_{max} identifying the maximal response. The effective concentration 50 (EC_{50}) defines the potency of the androgen and is expressed as the concentration (nM) that generates half-maximal response. EC_{50} was calculated by linear regression from the cumulative log dose-response curve

obtained for each animal. For analysis of gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the reference genes. Normalized expression levels were calculated using the comparative Ct method previously described (Schmittgen and Livak 2008) and the relative fold change values from each replicate in each group were averaged for each group. To detect statistical differences among the means, data groups were first subjected to one-way or two-way analysis of the variance (ANOVA). Factors compared for two-way ANOVA include: pregnancy (NP vs. PEP) vs. hormone treatment (TES vs. 5α-DHT vs. 5β-DHT), hormone treatment vs. NOS inhibitor (L-NMMA vs. NPA) and hormone treatment vs K^+ channel inhibitor (IbTx vs. 4-AP vs. Gly). If main effects were identified, pair-wise Student's t-tests were performed on group means to detect significant pair-wise differences among the means of the various experimental groups. A Bonferroni correction was employed for type 1 errors associated with multiple comparisons and differences between the means. Significance was accepted as $P < 0.05$.

4.5 Results

4.5.1 Validation of the DOCA-salt rat model of preeclampsia

DOCA-salt treatment in pregnant female SD rats significantly increased systolic BP and urine protein excretion and reduced fetal weight, which are hallmark characteristics of PE. Systolic BP measurements for NP and PEP are summarized in Table 8 and Figure 7. On day 0, systolic BP was similar between NP (114 ± 1.4 mmHg) and PEP (118 ± 1.6 mmHg) rats and considered normotensive (BP < 140 mmHg). By experimental day 6, PEP rats developed hypertension, with significantly higher systolic BP compared to NP control rats (PEP, 142 ± 3.7 mmHg vs. NP, 119 ± 3.8 mmHg; $P \leq 0.0001$). The PEP rats remained

hypertensive vs. the normotensive NP rats throughout the entire pregnancy, with significant differences at term (NP, 113 ± 1.1 mmHg vs. PEP, 146 ± 2.8 mmHg; $P \leq 0.0001$).

Table 8. Systolic BP in NP vs. PEP rats on day 0, 6, 12, and 18 of pregnancy.

Group	Day 0	Day 6	Day 12	Day 18
NP	$114 \pm 1.4^{a\#}$	$119 \pm 3.8^{a\#}$	$116 \pm 6.0^{a\#}$	$113 \pm 1.1^{a\#}$
PEP	$118 \pm 1.6^{a\#}$	$142 \pm 3.7^{b*}$	$149 \pm 2.4^{b*}$	$146 \pm 2.8^{b*}$

Systolic blood pressure in normal pregnant (NP) vs. preeclampsia pregnant (PEP) female Sprague-Dawley (SD) rats. Data are means ± SEM (n= 5-9 rats/group. $^{a-b}$ Mean values in each column without common script are significantly different ($0.0001 \leq P \leq 0.03$). $^{\#\ *}$ Mean values in each row (NP vs. PEP) without common script are significantly different ($P \leq 0.0001$).

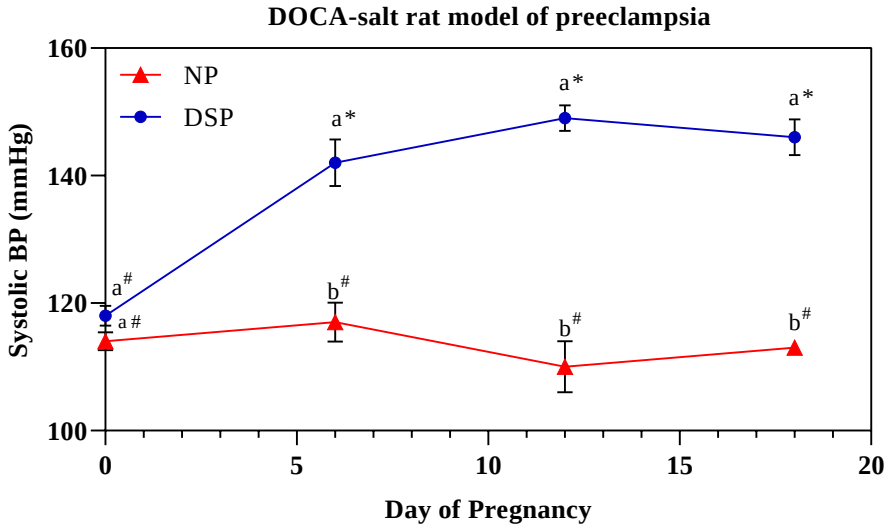

Figure 7. Validation of hypertension in the DOCA-salt rat model of preeclampsia.
Systolic blood pressure of normal pregnant vs. preeclamptic pregnant rats. Data points are means ± SEM (n=5-9 rats). $^{a-b}$ Mean value of NP vs. PEP at each day of pregnancy (day 0, 6, 12, or 18) without common script are significantly different ($0.0001 \leq P \leq 0.03$). $^{\#\ *}$ Mean values over time (day 0 vs. 6 vs. 12 vs.18) within each group (NP vs. PEP) without common script are significantly different ($P \leq 0.0001$).

NP and PEP body weight (BW), fetal number, fetal weight, placental weight (wet and dry), and urine protein excretion are summarized in Table 9. Maternal BW was significantly greater in NP vs. PEP rats (NP, 375 ± 5 vs. PEP, 355 ± 8 g; $P \leq 0.03$). While there were no significant differences in placental weight ($P > 0.05$), there was a 14% reduction in fetal numbers between NP rats (14 ± 0.5) vs. PEP (12 ± 1; $P \leq 0.01$) and a 30% reduction in fetal weight (NP, 3.24 ± 0.2 vs. PEP, 2.23 ± 0.1 g; $P \leq 0.0001$). Compared to NP control rats, urine protein excretion significantly increased 4-fold in PEP rats (NP, 9.3 ± 4.3 vs. PEP, 38.8 ± 7.6 $mg \cdot day^{-1}$; $P \leq 0.0001$).

Table 9. Maternal body weight (BW), fetal number, fetal BW, placental weight (wet and dry), and urine protein in NP vs. PEP rats at term.

Group	Maternal BW (g)	Fetal number	Fetal BW (g)	Placenta weight wet (g)	dry (g)	Urine protein ($mg \cdot 24h^{-1}$)
	n=23	n=23	n=23	n=5-9	n=5-9	n=4-5
NP	375 ± 5.0[a]	14 ± 0.5[a]	3.24 ± 0.2[a]	0.58 ± 0.03[a]	0.11 ± 0.01[a]	9.3 ± 4.3[a]
PEP	355 ± 8.0[b]	12 ± 1.0[b]	2.23 ± 0.1[b]	0.62 ± 0.05[a]	0.11 ± 0.01[a]	38.8 ± 7.6[b]

Maternal body weight (BW), fetal number, fetal BW, placental weight (wet vs. dry), and urine protein in normal pregnant (NP) vs. preeclamptic pregnant (PEP) female Sprague-Dawley (SD) rats. Data are means ± SEM. [a-b] Mean values in each column without common script are significantly different ($0.0001 \leq P \leq 0.03$).

4.5.2 Reactivity of the uterine artery to androgens

Complete concentration-response curves were obtained to compare the vascular reactivity of UA to androgens in NP vs. PEP rats. The concentration-dependent vasorelaxation of each androgen was calculated as a percent relaxation (%) relative to the pre-contractile force of each UA in response to U-46619. The vasorelaxation efficacy of

each androgen is represented as the E_{max} response, while the potency (sensitivity) to each androgen is represented as the EC_{50}. These results are summarized in Table 10. Figure 8 illustrates the complete concentration-response curve for each androgen in UA from NP and PEP rats. In both NP and PEP rats, androgens induced significant vasorelaxation of the UA. Analysis of these data by repeated measures ANOVA revealed that concentration-dependent relaxations of UA to each androgen (TES, 5α-DHT and 5β-DHT) were significant ($P \leq 0.001$), while differences in the concentration-response curves among the three androgens were not significant ($P > 0.05$). Likewise, all androgens induced significant vasorelaxation compared to the vehicle-control ($P > 0.05$). The E_{max} response to TES and other androgens was not significantly different between NP vs. PEP rats ($P > 0.05$) despite an increasing trend in androgen efficacy of PEP rats compared to NP control rats: TES (NP, 28 ± 4.2 vs. PEP, 36 ± 6.1%), 5α-DHT (NP, 29 ± 3.7 vs. PEP, 39 ± 6.6%), 5β-DHT (NP, 27 ± 3.6 vs. PEP 33 ± 5.2). The ranked potencies of androgens in NP rats was TES > 5α-DHT > 5β-DHT ($P \leq 0.04$), while the ranked potencies of androgens in the PEP rats was 5β-DHT > 5α-DHT > TES ($P \leq 0.03$). Interestingly, the sensitivity to 5β-DHT increased significantly in PEP (3.8 ± 0.7 nM) compared to NP (6.1 ± 1.6 nM) rats ($P \leq 0.0001$).

Table 10. Maximum relaxation (E_{max}) and effective concentrtaion-50 (EC_{50}) of TES, 5α-DHT, and 5β-DHT in isolated UA of NP and PEP rats.

	Androgen	NP	PEP
E_{max}	TES	$28 \pm 4.2^{a*}$	$36 \pm 6.1^{a*}$
	5α-DHT	$29 \pm 3.7^{a*}$	$39 \pm 6.6^{a*}$
	5β-DHT	$27 \pm 3.6^{a*}$	$33 \pm 5.2^{a*}$
	Veh	$5 \pm 1.0^{b*}$	$6 \pm 3.1^{b*}$
EC_{50}	TES	$4.8 \pm 1.0^{a*}$	$5.2 \pm 0.6^{a*}$
	5α-DHT	$5.5 \pm 1.1^{a*}$	$4.6 \pm 0.7^{a*}$
	5β-DHT	$6.1 \pm 1.6^{a*}$	$3.8 \pm 0.7^{a\#}$

Androgen-induced vasorelaxation of isolated uterine arteries (UA) from normal pregnant (NP) and preeclampsia pregnant (PEP) female Sprague-Dawley (SD) rats. Data are means ± SEM (n= 5-10 rats/experimental group). $^{a-b}$ Mean values for E_{max} and EC_{50} in each column without common superscript are significantly different ($P \leq 0.0001$). $^{*\#}$ Mean values in each row (NP vs. PEP) without common superscript are significantly different ($P \leq 0.034$).

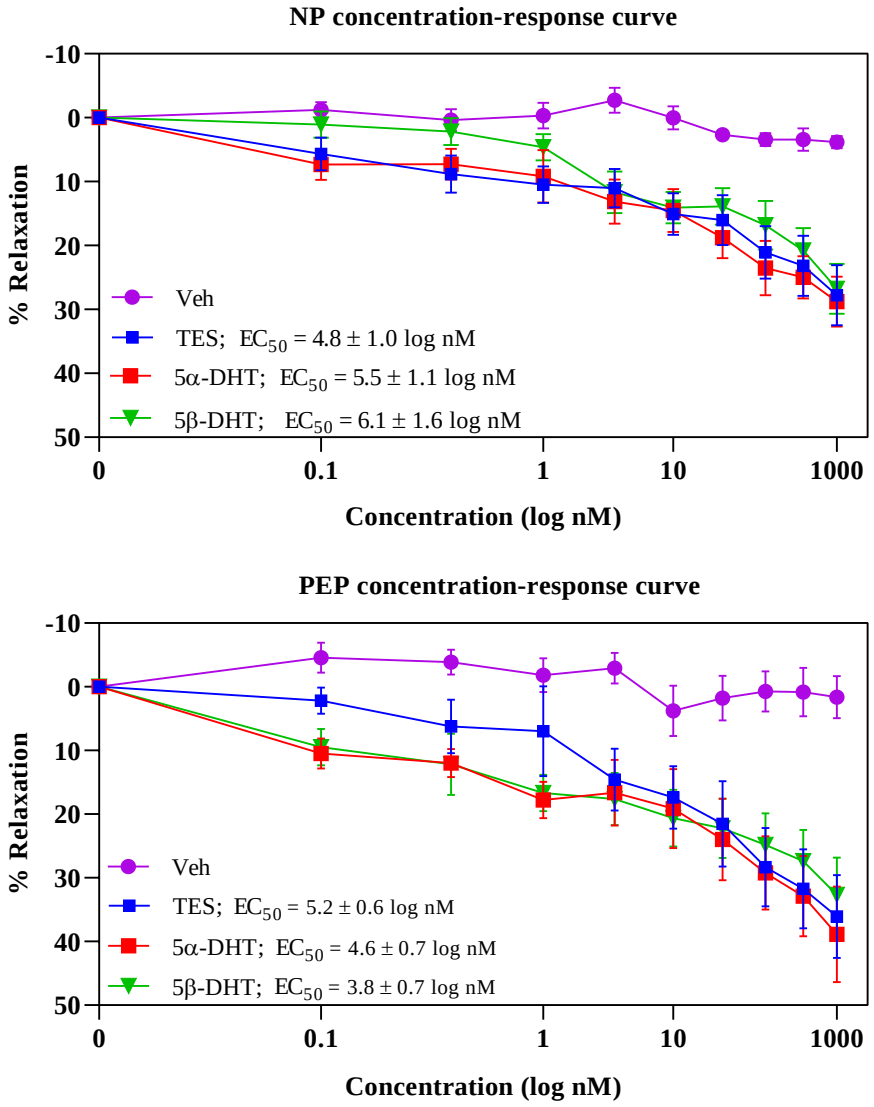

Figure 8. Vascular effects of androgens on the uterine artery.
Concentration-response curve for vehicle, testosterone, and 5α- and 5β-dihydrotestosterone in isolated uterine arteries (UA) of normal pregnant (NP) and preeclampsia pregnant (PEP) female Sprague-Dawley (SD) rats. Data points are means ± SEM (n= 5-10 rats/ group).

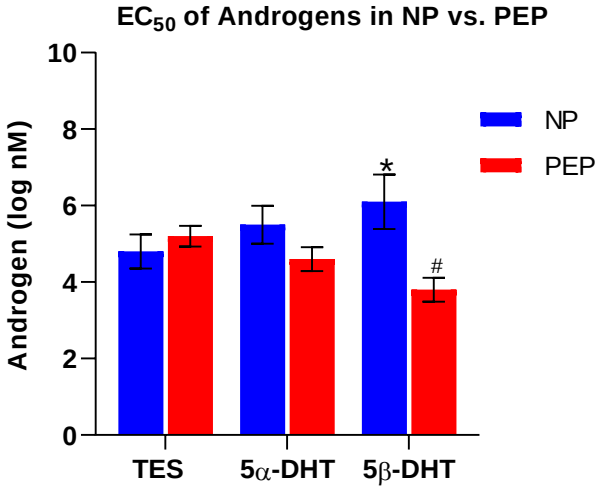

Figure 9. Uterine artery sensitivity to androgen-induced vasorelaxation in normal pregnant (NP) and preeclampsia pregnant (PEP) female Sprague-Dawley (SD) rats.
Data are means ± SEM (n= 5-10 rats/experimental group). * # Mean values for EC_{50} for 5β-DHT (NP vs. PEP) differ significantly ($P \leq 0.0001$). EC_{50} values for TES and 5α-DHT (NP vs. PEP) do not differ significantly ($P > 0.05$).

4.5.3 Effects of NOS inhibition on androgen-induced vasorelaxation

Androgen-induced vasorelaxation was markedly attenuated in UA pretreated with NOS inhibitors. The effects of NOS inhibition on androgen-induced vasorelaxation of UA in NP and PEP rats is summarized in Table 11 and Figures 9 and 10. Pretreatment with L-N^G-Arginine acetate (L-NMMA; 100 µM), a non-selective inhibitor of eNOS and nNOS, inhibited androgen-induced vasorelaxation in NP and PEP rats. Repeated measure ANOVAs revealed that UA relaxations differ significantly by both androgen concentration (TES and 5β-DHT) and NOS treatment in both NP and PEP rats ($P \leq 0.001$). The E_{max} response to TES decreased 86% in NP rats (TES; 28 ± 4.2% vs. L-NMMA; 4 ± 2.5%; $P \leq 0.0001$) and 72% in PEP rats (TES; 36 ± 6.1% vs. L-NMMA; 10 ± 5.2%; $P \leq 0.0001$). L-NMMA resulted in a similar inhibition of 5β-DHT-induced vasorelaxation. The E_{max}

response decreased 82% in NP rats (5β-DHT; 27 ± 3.6 vs. L-NMMA; 5 ± 2.6; $P \leq 0.0001$) and 70% in PEP rats (5β-DHT; 33 ± 5.2 vs. L-NMMA; 10 ± 5.7; $P \leq 0.0001$). In contrast, pretreatment with N^W-propyl-L-arginine (NPA; 200 μM), a potent and selective inhibitor of nNOS, did not significantly attenuate the E_{max} of UA from either NP or PEP rats ($P > 0.05$). With L-NMMA, the potency of TES decreased 67% in NP ($P \leq 0.0001$) and 60% in PEP rats ($P \leq 0.004$), with no significant effect on the potency of 5β-DHT. Interestingly, L-NMMA significantly increased the potency of 5β-DHT 45% in PEP vs. 15% in NP rats ($P \leq 0.0004$). NPA did not significantly affect the potency of TES or 5β-DHT in either NP or PEP rats.

Table 11. Maximum relaxation (E_{max}) and effective concentrtaion-50 (EC_{50}) of UA pretreated with NOS inhibitors L-NMMA and NPA in NP and PEP rats.

	Androgen	NP	PEP
E_{max}	TES	$28 \pm 4.2^{a*}$	$36 \pm 6.1^{a*}$
	TES + L-NMMA	$4 \pm 2.5^{b*}$	$10 \pm 5.2^{b*}$
	TES + NPA	$27 \pm 3.0^{a*}$	$34 \pm 5.5^{a*}$
	5β-DHT	$27 \pm 3.6^{a*}$	$33 \pm 5.2^{a*}$
	5β-DHT + L-NMMA	$5 \pm 2.6^{b*}$	$10 \pm 5.7^{b*}$
	5β-DHT + NPA	$23 \pm 3.6^{a*}$	$30 \pm 8.2^{a*}$
EC_{50}	TES	$4.8 \pm 1.0^{a*}$	5.2 ± 0.6a*
	TES + L-NMMA	$1.6 \pm 1.2^{b*}$	2.1 ± 0.9b*
	TES + NPA	$5.3 \pm 0.3^{a*}$	5.3 ± 0.4a*
	5β-DHT	$6.1 \pm 1.6^{a*}$	$3.8 \pm 0.7^{ab\#}$
	5β-DHT + L-NMMA	$5.2 \pm 1.5^{a*}$	$2.1 \pm 1.5^{b\#}$
	5β-DHT + NPA	$5.0 \pm 0.5^{a*}$	$4.8 \pm 0.5^{a*}$

Effects of NOS inhibitors on androgen-induced vasorelaxation in isolated uterine arteries (UA) of normal pregnant (NP**)** and preeclamptic pregnant (PEP) female Sprague-Dawley (SD) rats. Data are means ± SEM (n= 4-9 rats). $^{a-b}$ Mean values for E_{max} and EC_{50} in each column without common superscript are significantly different ($0.0001 \leq P \leq 0.02$). $^{*\#}$ Mean values in each row (NP vs. PEP) without common superscript are significantly different ($0.0001 \leq P \leq 0.045$).

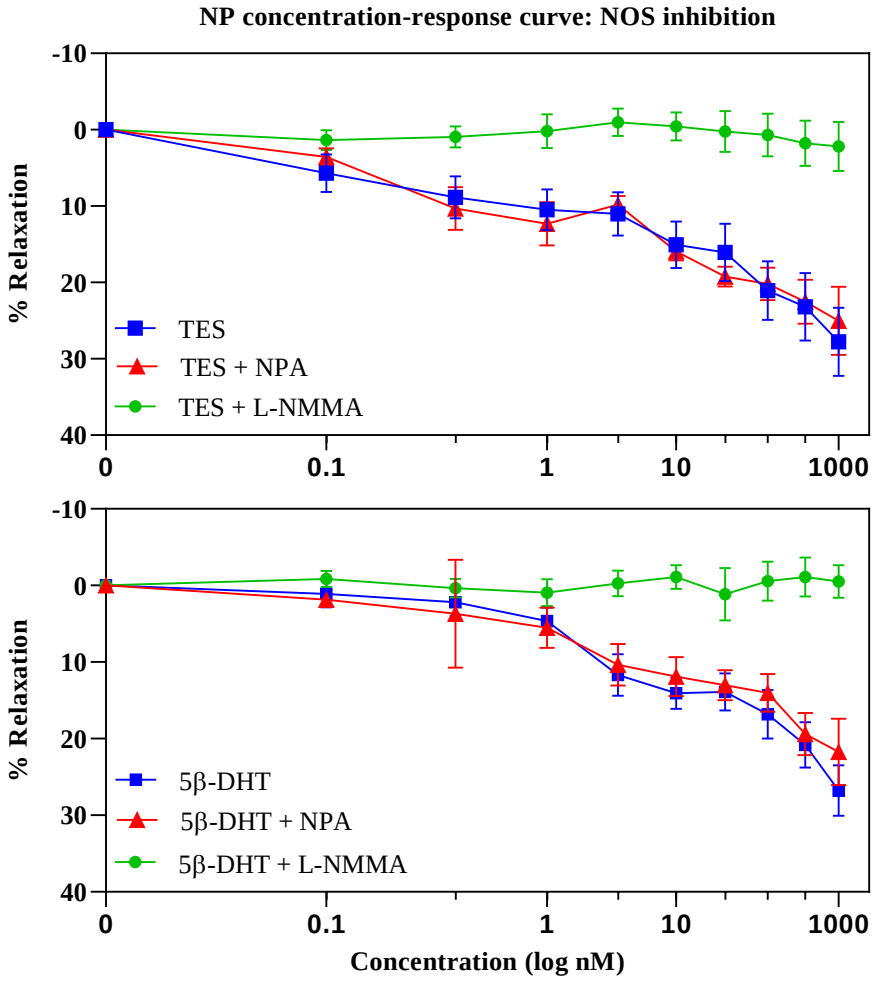

Figure 10. Effects of nitric oxide synthase inhibition on androgen-induced vasorelaxation in NP rats.

Concentration response curve of testosterone (TES) and 5β-dihydrotestosterone (5β-DHT) in isolated uterine arteries (UA) of normal pregnant (NP) female Sprague-Dawley (SD) rats. UA were pretreated with L-NG-Arginine acetate (L-NMMA; 100 µM) or NW-propyl-L-arginine (NPA; 200 µM). Data points are means ± SEM (n= 4-9 rats).

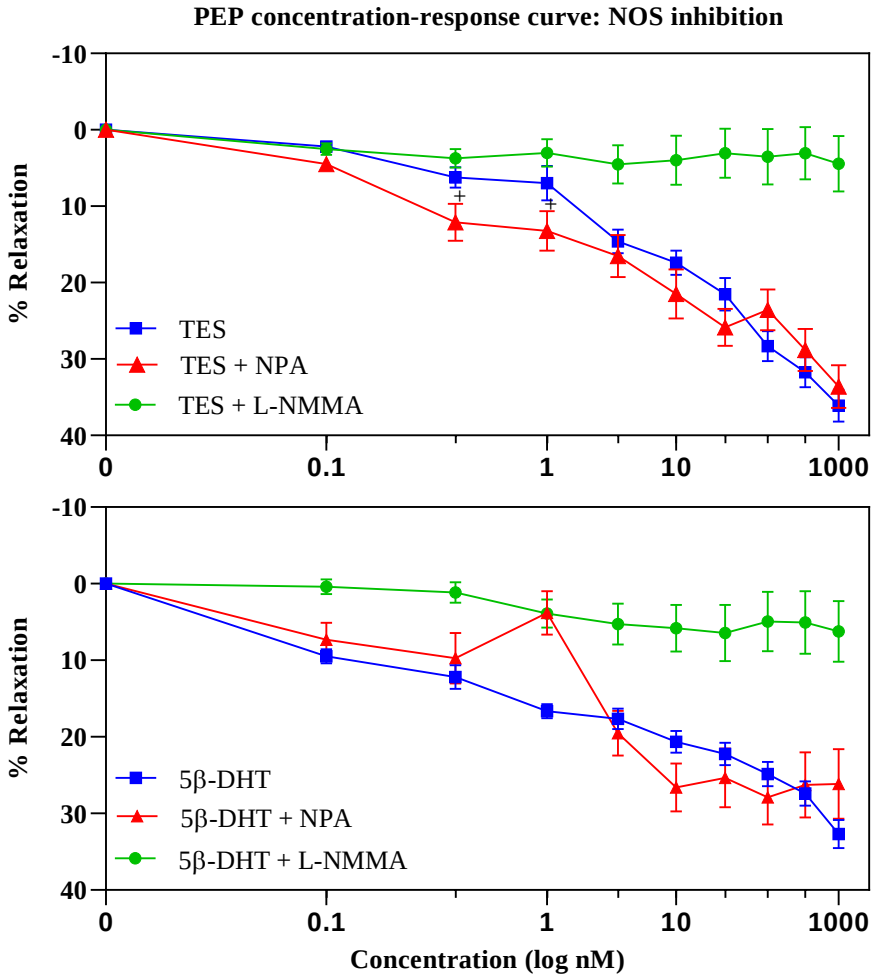

Figure 11. Effects of nitric oxide synthase inhibition on androgen-induced vasorelaxation in PEP rats.
Concentration-response curve of testosterone (TES) and 5β-dihydrotestosterone (5β-DHT) in isolated uterine arteries (UA) of preeclamptic pregnant (PEP) female Sprague-Dawley (SD) rats. UA were pretreated with L-NG-Arginine acetate (L-NMMA; 100 µM) or NW-propyl-L-arginine (NPA; 200 µM). Data points are means ± SEM (n= 4-9 rats).

4.5.4 Effects of K^+ channel inhibition on androgen-induced vasodilation

In a separate study, the relationship between K^+ channel inhibition and androgen-induced vasodilation was examined in UA of NP and PEP rats. Iberiotoxin (IbTx; 100 nM), 4-aminopyridine (4-AP; 3 mM), and glybenclamide (Gly; 10 µM) were used to

investigate the effects of Ca^{2+}-dependent (BK_{Ca}), ATP-dependent (K_{ATP}), and voltage-dependent (K_V) K^+ channel activity, respectively. The E_{max} responses and EC_{50} for the androgens are summarized in Table 12. Concentration-response curves for NP and PEP rats are illustrated in Figures 11- 13. Repeated measure ANOVAs revealed that UA relaxations differ significantly by both androgen concentration (TES and 5β-DHT) and K^+ channel inhibition in both NP and PEP rats ($P \leq 0.001$). IbTx significantly attenuated TES and 5β-DHT-induced vasorelaxation in NP rats ($0.0001 \leq P \leq 0.0040$). IbTx reduced the E_{max} response of TES 52% in NP and 50% in PEP rats, while the E_{max} response of 5β-DHT was reduced 69% in NP. Interestingly, the 5β-DHT E_{max} response in PEP rats was only reduced 30% ($P < 0.05$). 4-AP reduced the E_{max} response of TES 71% in NP rats and 73% in PEP rats, while the E_{max} response of 5β-DHT was reduced 59% in NP rats and 56% in PEP rats. In contrast to the inhibitory effects of IbTx and 4-AP, Gly increased androgen-induced vasorelaxation in both NP and PEP rats. In NP rats, Gly increased the E_{max} vasorelaxation response to TES 19% (TES; 31 ± 3.0 vs. Gly; 37 ± 4.0) and 5β-DHT 13% (5β-DHT; 32 ± 3.4 vs. Gly; 36 ± 5.3; $P > 0.05$). These effects were enhanced in PEP rats, in which Gly significantly increased androgen-induced vasorelaxation of TES 46% (TES; 26 ± 3.8 vs. Gly; 38 ± 4.8) and 56% in response to 5β-DHT (5β-DHT; 27 ± 2.6 vs. Gly; 42 ± 6.4; $P < 0.05$).

In NP rats, TES potency decreased significantly with IbTx and 4-AP, while UA sensitivity to TES-induced vasorelaxation increased with Gly. In PEP rats, 4-AP decreased UA sensitivity to TES, while IbTx and 4-AP increased the potency to TES. The potency of 5β-DHT decreased with K^+ channel inhibitors in both NP and PEP rats. Interestingly,

UA of PEP rats were less sensitive to TES and more sensitive to 5β-DHT compared to those of NP rats.

Table 12. Maximum response (E_{max}) and effective concentration-50 (EC_{50}) of UA pretreated with K^+ channel inhibitors IbTx, 4-AP, and Gly in NP and PEP rats.

	Androgen	NP	PEP
E_{max}	TES	$31 \pm 3.0^{a\#}$	$26 \pm 3.8^{a\#}$
	TES +IbTx	$15 \pm 4.0^{b\#}$	$13 \pm 2.0^{b\#}$
	TES +4-AP	$9 \pm 2.5^{b\#}$	$7 \pm 1.4^{c\#}$
	TES +Gly	$37 \pm 4.0^{a\#}$	$38 \pm 4.8^{d\#}$
	5β-DHT	$32 \pm 3.4^{a\#}$	$27 \pm 2.6^{a\#}$
	5β-DHT +IbTx	$10 \pm 1.6^{b\#}$	$19 \pm 3.7^{b*}$
	5β-DHT +4-AP	$13 \pm 2.2^{b\#}$	$12 \pm 1.6^{c\#}$
	5β-DHT +Gly	$36 \pm 5.3^{a\#}$	$42 \pm 6.4^{d\#}$
EC_{50}	TES	$9 \pm 2.1^{a\#}$	$19 \pm 6.7^{a*}$
	TES +IbTx	$97 \pm 40^{b\#}$	$12 \pm 3.5^{b*}$
	TES +4-AP	$25 \pm 7.3^{c\#}$	$63 \pm 12^{c\#}$
	TES +Gly	$5 \pm 1.3^{d\#}$	$12 \pm 5.8^{d*}$
	5β-DHT	$5 \pm 1.4^{a\#}$	$4.3 \pm 1.3^{a*}$
	5β-DHT +IbTx	$185 \pm 47^{b\#}$	$119 \pm 56^{b*}$
	5β-DHT +4-AP	$79 \pm 24^{c\#}$	$56 \pm 12^{c*}$
	5β-DHT +Gly	$14 \pm 5.0^{d\#}$	$6 \pm 1.9^{d*}$

Effects of K^+ channel inhibitors on androgen-induced vasorelaxation of isolated uterine arteries (UA) pretreated with IbTx, 4-AP, and Gly in Normal Pregnant (NP**)** and DOCA-salt Pregnant (PEP) Sprague-Dawley (SD) rats. Data are means ± SEM (n=6-9 rats/experimental group). $^{a-d}$ Mean values in each column for each androgen (TES and 5β-DHT) without common script are significantly different ($0.001 \leq P \leq 0.05$). $^{\#*}$ Mean values in each row (NP vs. PEP) without common script are significantly different ($0.001 \leq P \leq 0.05$)

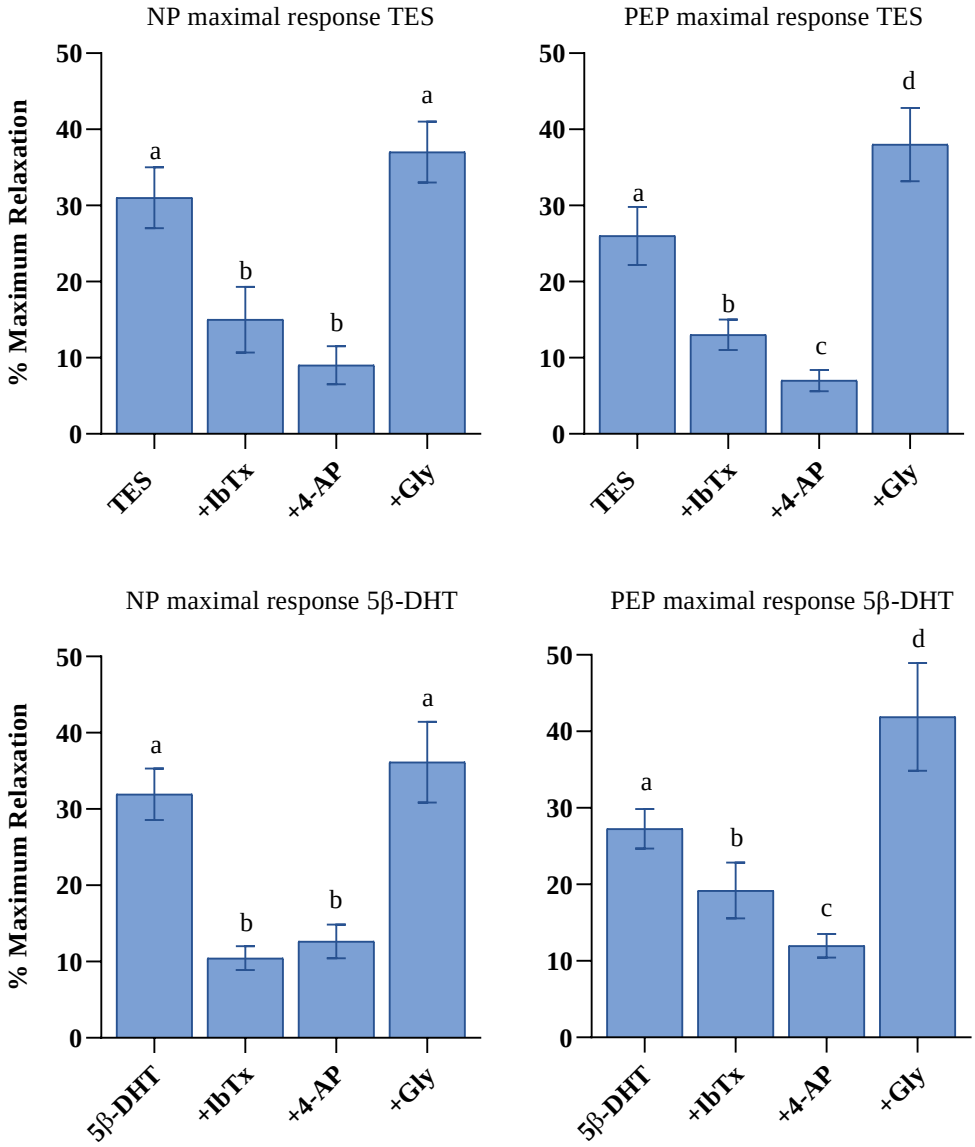

Figure 12. Maximum relaxation (E_{max}) response of testosterone and 5β-dihydrotestosterone.
E_{max} response in isolated uterine arteries (UA) of normal pregnant (NP) and PE pregnant (PEP) female Sprague-Dawley (SD) rats. The isolated UA were pretreated with K^+ channel inhibitors: iberiotoxin (IbTx; 100 nM), 4-aminopyridine (4-AP; 3 mM), or glybenclamide (Gly; 10 µM). Data are means ± SEM (n=6-9 rats/experimental group). [a-d] Mean values for androgen (TES and 5β-DHT) vs. K^+ channel inhibitor (IbTx, 4-AP, and Gly) without common script are significantly different ($0.0001 \leq P \leq 0.05$).

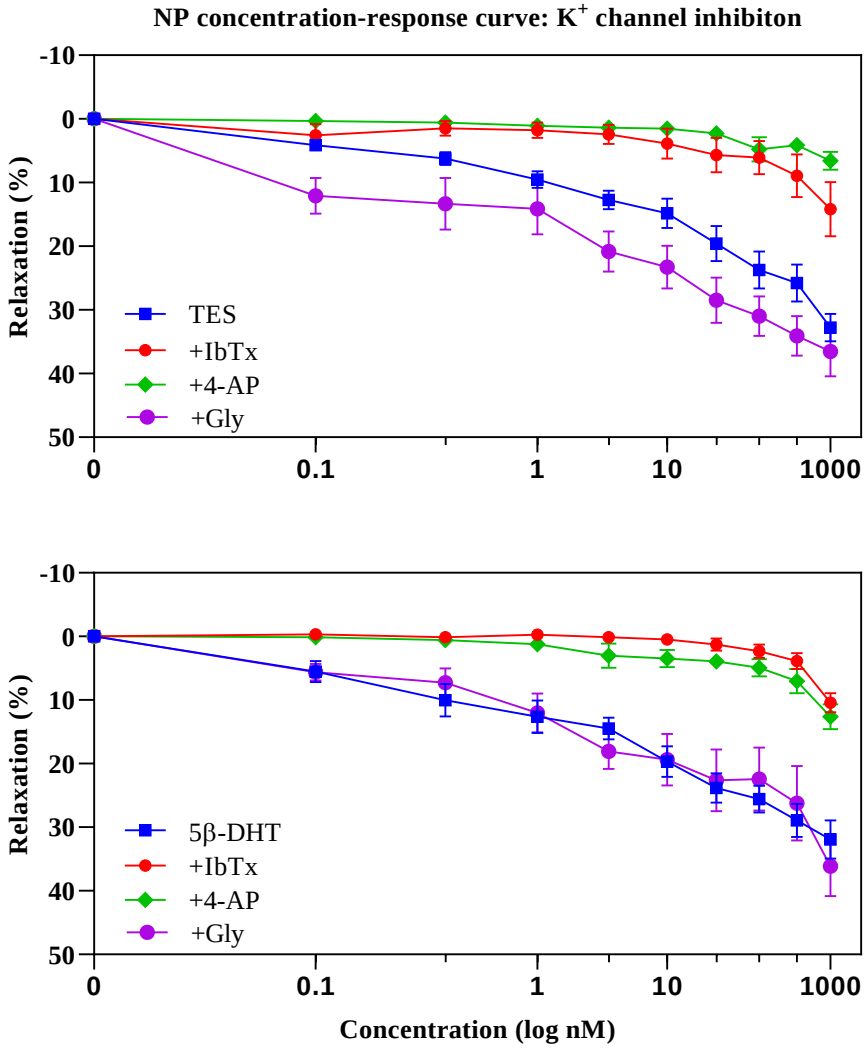

Figure 13. Effects of K^+ channel inhibition on androgen-induced vasorelaxation in normal pregnant rats.

Concentration-response curve of testosterone (TES) and 5β-dihydrotestosterone (5β-DHT) in isolated uterine arteries (UA) of PE pregnant (PEP) female Sprague-Dawley (SD) rats. The isolated UA were pretreated with K^+ channel inhibitors: iberiotoxin (IbTx; 100 nM), 4-aminopyridine (4-AP; 3 mM), or glybenclamide (Gly; 10 µM). Data points are means ± SEM (n= 6-9 rats/group).

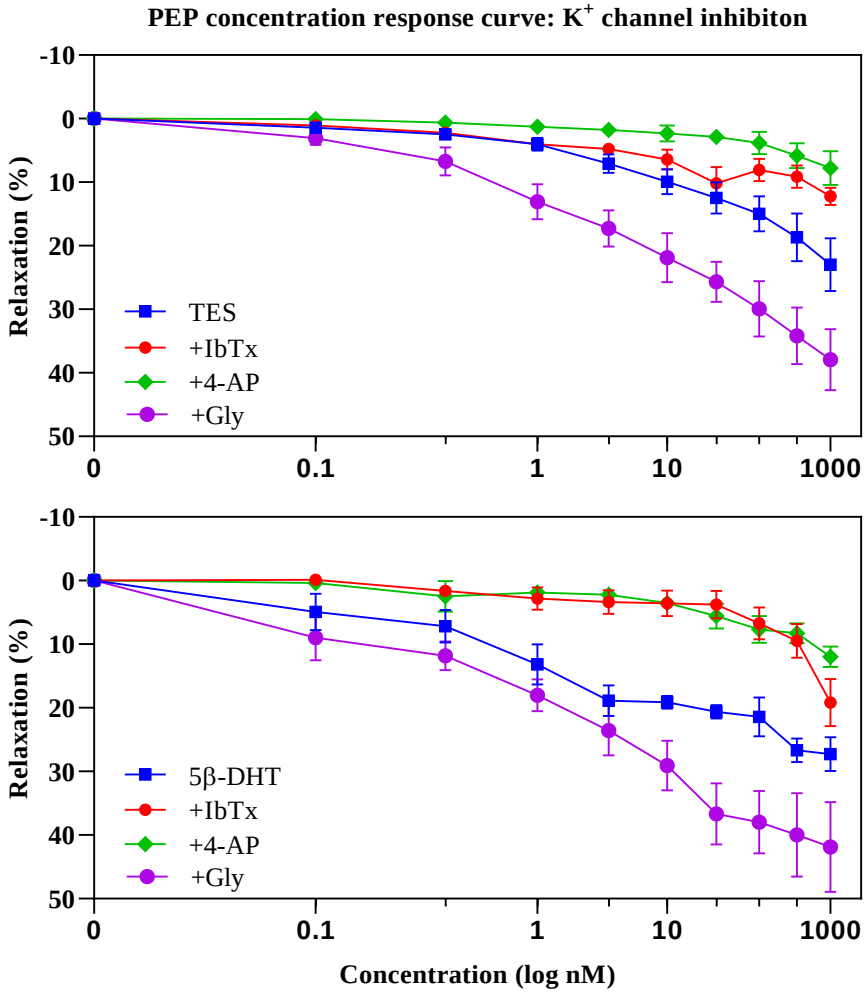

Figure 14. Effects of K^+ channel inhibition on androgen-induced vasorelaxation in PEP.
Concentration response curve of testosterone (TES) and 5β-dihydrotestosterone (5β-DHT) in isolated uterine arteries (UA) of PE pregnant (PEP) female Sprague-Dawley (SD) rats. The isolated UA were pretreated with K^+ channel inhibitors: iberiotoxin (IbTx; 100 nM), 4-aminopyridine (4-AP; 3 mM), or glybenclamide (Gly; 10 µM). Data points are means ± SEM (n= 6-9 rats/group).

4.5.5 rt-PCR mRNA expression of 5α- and 5β- reductase in the placenta and uterine arteries

The mRNA of 5β- and 5α-reductase, as measured by rt-PCR, in the placenta and uterine artery of NP and PEP rats is summarized in Figures 14. Gestational hypertension

significantly reduced mRNA expression of 5β-reductase in the placenta (36%) and uterine artery (67%) compared to NP controls ($0.0001 \leq P \leq 0.03$). There were no significant differences in 5α-reductase expression in either NP or PEP rats ($P > 0.05$).

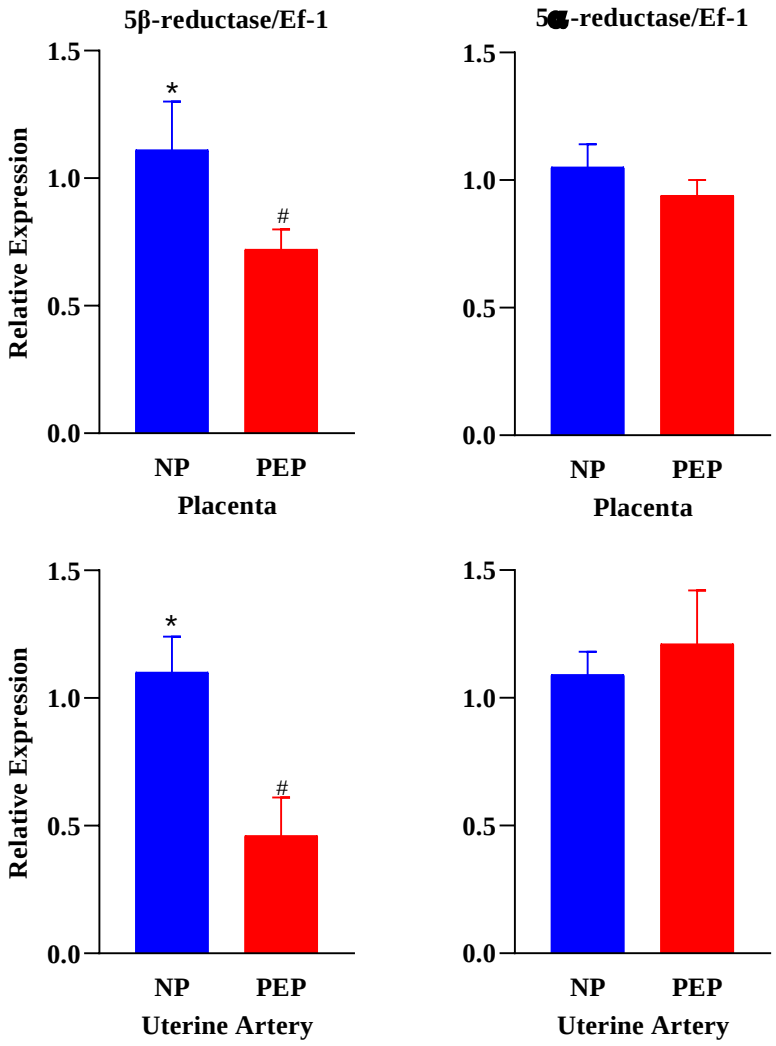

Figure 15. Effects of preeclampsia on expression of reductase enzymes in the placenta and uterine artery.
mRNA expression of 5β- and 5α- reductase in the placenta and UA of normal pregnant (NP) and PE pregnant (PEP) female Sprague-Dawley (SD) rats. Data represents means ± SEM (n= 5-7 rats/group). $^{*\#}$ Mean values for mRNA expression of 5α- and 5β- reductase in the placenta and UA between groups (NP vs. PEP) without common script are significantly different ($0.0001 \leq P \leq 0.03$).

4.6 Discussion

In the present investigation, the role of androgens (TES, 5α-DHT, and 5β-DHT) in uterine arterial function during PE were studied in isolated UA of NP and PEP female SD rats. The DOCA-salt pregnant female SD rat was validated as an acceptable animal model of PE in the present study. The results reveal that androgens elicit rapid vasorelaxation of isolated UA in both NP and PEP rats. Further, the mechanisms of androgen-induced vasorelaxation appear to be highly dependent on eNOS signaling mechanisms and the activation of K_V and BK_{Ca} channels. The DOCA-salt pregnant female SD rat was validated as an acceptable animal model of PE in the present study. In PEP rats, UA sensitivity increased significantly to the vasodilatory effects of 5β-DHT compared to the vascular response of NP control rats. Further, mRNA expression of 5β-reductase in the placenta and uterine artery was reduced in PEP vs. NP rats, with no change in the mRNA expression of 5α-reductase. Therefore, the vasodilatory effects of TES and its 5β-DHT metabolites may contribute to the regulation of uterine blood flow during normal pregnancy, while impaired production of 5β-DHT at the uteroplacental interface may reduce uterine blood flow and thereby contribute to the pathogenesis of PE. Thus, the present findings may offer a possible therapeutic role for androgens in the treatment of women with PE.

4.6.1 Validation of the DOCA-salt model of preeclampsia

The PEP rats used in the present investigation exhibited the hallmark characteristics of human PE, including maternal hypertension, proteinuria, and fetal IUGR. These results are consistent with findings from previous studies using a similar DOCA-salt rat model of PE (Puschett, Agunanne et al. 2010). PE is understood to be a human-specific pregnancy-induced hypertensive disorder attributed to improper placental implantation. In this study,

PEP rats maintained a systolic BP above 140 mmHg with a four-fold increase in urine protein excretion, a 30% reduction in fetal weight, and a 14% reduction in fetal numbers compared to NP control rats. While uterine blood flow was not directly measured in this current investigation, reduced fetal weight and decreased fetal numbers are representative of fetal IUGR, and strongly suggest reduced uterine blood flow in PEP rats. Typical laboratory rat gestation is approximately 21-23 days, with significant increases in fetal weight from gestational day 20-22 (Witlin, Li et al. 2002). While NP and PEP gestation was terminated on day 20 ± 2 of pregnancy, the relative fetal weights would still be an acceptable indicator of IUGR. Further, compared to fully-developed, age-matched fetuses of NP control rats, the fetuses of PEP rats were visually underdeveloped, with evidence of internal hemorrhaging in the amniotic fluid and underdeveloped/regressed placental tissues in many of the PEP pregnancies, which further indicates restricted uterine blood flow in PEP rats compared to NP control rats.

In humans, the characteristics of PE, specifically hypertension, are typically present during the second and third trimesters of pregnancy (Parikh, Norberg et al. 2017). PE is associated with systolic BP > 140 mmHg, which is attributed to inadequate cardiovascular adaptations in early pregnancy. Increased peripheral vasoconstriction and decreased arterial compliance associated with PE (Thadhani, Ecker et al. 2001) may contribute to multi-system dysfunction, which includes hemorrhagic stroke, hemolysis, elevated liver enzymes and low platelet count, renal failure, and pulmonary edema (Amaral, Wallace et al. 2017). In the kidneys, insufficiencies in cardiovascular adaptations damage podocyte-specific proteins in the glomerular capillaries, which contributes to increased protein filtration, resulting in subsequent increases in urine protein excretion in PE pregnancy

(Craici, Wagner et al. 2014). Further, the cardiovascular impairments of PE also result in reduced uterine blood flow during pregnancy (Ounsted, Moar et al. 1981), which contributes to increased placental ischemia and subsequent IUGR of the fetus (El-Sayed 2017). Interestingly, preterm fetal BW is approximately 12% lower in PE pregnant women compared to preterm fetal BW in normal pregnant women (Xiong, Demianczuk et al. 2002), which is consistent with the findings from the present study.

While several animal models have been developed to mimic the characteristics of PE, as a pregnancy-induced disease specific to humans, many of these animal models fail to accurately represent the human pathology of the disease. Most of the animal models of PE focus on a specific pathogenic mechanism, such as uteroplacental ischemia, impaired NO production, overactive RAS function, or inhibited angiogenic proteins. The uterine ischemia model of PE reduces uterine blood flow during pregnancy to mimic the 50-70% reduction in uteroplacental blood flow observed in PE (Lunell, Lewander et al. 1984). This technique has been employed in several rodent and non-rodent animal models, with variable effects on hypertension. Interestingly, abdominal aortic clamping has no effect on systolic BP in pregnant female sheep, yet the addition of a high salt diet induces significant increases in BP (Leffler, Hessler et al. 1986). In rodents, the RUPP model was developed as an alternative to the placental ischemia model. At time points equivalent to the second trimester of pregnancy, the RUPP model undergoes surgical constriction of the abdominal aorta and occlusion of the uterine-ovarian arteries to more closely mimic the ischemic effects of PE. While the RUPP model induces hypertension, proteinuria, and impaired renal function, this model is not conducive to research targeting therapeutic improvements to uterine blood flow or investigating the mechanisms underlying

uteroplacental vascular reactivity. Additionally, these models of ischemia fail to mimic the progression of PE observed in humans, as the timing of uteroplacental ischemia occurs after a normal placental implantation has been established, which is contradictory to the impaired placental implantation associated with human PE (Li, LaMarca et al. 2012). Several rodent models of PE utilize mechanisms such as Ang II infusion and NO inhibition to mimic the maternal manifestation of PE at the vascular level. In normal pregnancy, circulating RAS components increase, while the pressor effects of Ang II decrease. In contrast, PE pregnancy is associated with decreased levels of circulating RAS components and increased vascular reactivity to Ang II (Baylis, Beinder et al. 1998). Thus, rodent models using increasing Ang II levels to induce hypertension may not be relevant to the human disease. Use of chronic NOS inhibition produces sustained hypertension in non-pregnant animals (Lahera, Salazar et al. 1992, Zatz and Baylis 1998), while in pregnant rats, chronic NOS inhibition-induces proteinuria and causes fetal malformations, which were attributed to the pharmaceutical side effects of the selected NOS inhibitor (Baylis, Engels et al. 1992). In eNOS knockout mice BP was reduced during pregnancy (Shesely, Gilbert et al. 2001); however, similar studies report contradictory findings, with increased BP in pregnant eNOS knockout mice (Hefler, Tempfer et al. 2001). Thus, the use of the chronic NOS inhibitor models and eNOS knockout mice is problematic and controversial at best. In humans, PE has been associated with upregulated anti-angiogenic placental proteins such as sFlt-1 and sEng. These anti-angiogenic proteins inhibit the angiogenic properties of VEGF and PIGF (Cui, Shu et al. 2018) and induce characteristics of PE. Interestingly, many studies suggest that upregulated anti-angiogenic factors are not the cause of PE, but rather a byproduct of the disorder (Alasztics, Gullai et al. 2014). Thus,

these animal models fail to reconcile the underlying factors of PE. Genetically altered animal models of hypertension such as the SHR and Dahl salt-sensitive rat have also been used as animal models of PE; however, these animal models develop hypertension irrespective of pregnancy status. While these models may be beneficial to studies that target the effects of pregnancy on preexisting maternal hypertension, these studies fail to mimic the sudden onset of pregnancy-induced hypertension associated with PE (Takeda 1964). Thus, hypertensive animal models may not be an appropriate animal model of PE.

In the present study, the DOCA-salt model was chosen to investigate the vasodilatory effects of androgens on the UA. The DOCA-salt rat model is a well-established hypertensive animal model that targets neurogenic, renal, and vascular response elements. An imbalance of renal sodium and water reabsorption results in hypervolemia. Further, DOCA-salt increases RAS activity in the brain, which generates a neurologic effect that alters renal function and systemic vascular resistance (Ueno, Mohara et al. 1988). While circulating renin and Ang II levels remain normal, the neurologic effects increase vascular tone and blood volume, resulting in hypertension. In pregnancy, the DOCA-salt model would mimic the naturally occurring increase in blood volume, and the enhanced vasoconstriction that is associated with the pathogenesis of PE. Thus, the present study validates the DOCA-salt rat model of PE, with sudden onset hypertension, proteinuria, and IUGR; the hallmark characteristics of PE in humans.

4.6.2 Effects of androgens on the uterine arteries of pregnant rats

The present study is the first to clearly investigate the vasodilatory effects of androgens, specifically TES and its metabolites 5α- and 5β-DHT, on isolated UA of pregnant rats. The functional relationship between the UA and androgen-induced

vasorelaxation suggests that androgens are important regulators of uterine blood flow during pregnancy. While the efficacy of androgen-induced vasorelaxation did not differ significantly between NP vs. PEP rats, the potency of 5β-DHT-induced vasorelaxation increased significantly in PEP rats compared to NP control rats. Thus, rapid androgen-induce vasorelaxation in the UA of pregnant rats exhibited increased sensitivity to the vascular effects of 5β-DHT in rats with PE.

Circulating androgens, such as DHEA and TES, naturally increase during gestation (Bammann, Coulam et al. 1980), and are readily produced at the uteroplacental interface (Benagiano, Mancuso et al. 1968). TES and DHT production increases during the first trimester of pregnancy and significantly increases again towards term, suggesting a role in the maintenance of uterine blood flow (Buster, Chang et al. 1979, Wilke and Utley 1987, Makieva, Saunders et al. 2014). While the specific origins of each androgen remain uncertain, several fetal tissues, including the fetal liver, are known to reduce TES into metabolites, such as 5β-DHT (Benagiano, Mancuso et al. 1968). Some studies suggest that decreases in uterine blood flow and increases in maternal blood pressure are related to increases in maternal TES concentrations (Chinnathambi, Blesson et al. 2014). Interestingly, circulating TES levels are two- to three-fold greater in PE women compared to circulating concentrations of TES in women with normal pregnancy, which further suggests that increased androgens are associated with the development of PE (Salamalekis, Bakas et al. 2006). Some experimental animal studies demonstrate that administration of TES during pregnancy enhances certain characteristics of PE, such as proteinuria (Sathishkumar, Balakrishnan et al. 2011) and hypertension (Chinnathambi, Balakrishnan et al. 2013) in pregnant female rats. While these findings imply that androgens have

deleterious effects on blood flow and BP during pregnancy, many of these studies utilize high concentrations of androgens to induce the characteristics of PE late in gestation. Therefore, without further investigation, the effects of systemic TES administration on the development of PE remain unclear.

The results from the present study are fully consistent with the non-genomic androgen-induced vasorelaxation observed in the rat aorta (Perusquia, Hernandez et al. 1996), rat mesenteric arteries (Puttabyatappa, Stallone et al. 2013), and several other tissue beds from a variety of animal species (Perusquia and Stallone 2010), which include isolated human umbilical arteries (Perusquia, Navarrete et al. 2007). Interestingly, within the same species, there appear to be regional differences in vascular reactivity to androgens. For example, in the canine vasculature, coronary resistance vessels are more reactive to androgen-induced vasorelaxation than either the saphenous vein or femoral artery (Perusquia, Espinoza et al. 2012). Furthermore, some studies report regional differences in vascular reactivity to each of the androgens, with many of the studies suggesting that the TES metabolite 5β-DHT is a more potent and efficacious vasodilator than TES (Ishimaru, Edmiston et al. 1978, Kumar, Crozat et al. 1999). In the rat, 5β-DHT-induced vasorelaxation was more potent than TES or 5α-DHT-induced vasorelaxation in mesenteric arteries compared to the thoracic aorta (Isidoro, Ferrer et al. 2018). Similar differences in androgen efficacy and potency were observed in human umbilical arteries, in which DHEA and 5β-DHT-induced vasorelaxation was greater than the effects of TES or other female sex steroid hormones (Perusquia, Navarrete et al. 2007). Similar difference in androgen efficacy appear *in vivo*. The anti-hypertensive effects of 5β-DHT and DHEA were significantly greater than the moderate anti-hypertensive effects of TES and 5α-DHT

on arterial BP in PE pregnant female rats (Perusquia, Hanson et al. 2018). In the present study, TES and its DHT metabolites each induced rapid vasorelaxation of isolated UA in both NP and PEP rats, yet the sensitivity of the UA to 5β-DHT increased significantly in PEP rats. Thus, differences in uteroplacental vascular reactivity and sensitivity may contribute to differences in uterine blood flow and subsequent development of IUGR observed in PE.

4.6.3 Effects of NOS inhibition on androgen-induced vasodilation

The present study revealed that the mechanism of androgen-induced vasorelaxation involves eNOS activation in the UA of both NP and PEP rats. Pretreatment of UA with L-NMMA (non-selective NOS inhibitor) eliminated TES- and 5β-DHT-induced vasorelaxation in both NP and PEP rats, while NPA (nNOS inhibitor) resulted in no significant differences in androgen-induced vasorelaxation. Thus, the results clearly demonstrate that androgen-induced vasorelaxation involves eNOS activation in the UA of pregnant rats. These findings are consistent with previous studies investigating the role of NO in androgen-induced vasorelaxation of canine coronary arteries (Chou, Sudhir et al. 1996), rat thoracic aorta (Ding and Stallone 2001), and rat mesenteric arteries (Tep-areenan, Kendall et al. 2002). Interestingly, several studies suggest that androgen-induced vasorelaxation is NOS-independent in human radial arteries (Seyrek, Yildiz et al. 2007), rat pulmonary arteries (Jones, English et al. 2002), and rabbit coronary arteries (Yue, Chatterjee et al. 1995). The uncertainty regarding androgen-induced NOS signaling mechanisms may be attributed to the relatively small number of experimental studies that compare androgen signaling mechanisms within similar vascular beds among the same species.

Throughout gestation, NO production at the fetoplacental interface (Sladek, Magness et al. 1997) increases progressively to facilitate vasorelaxation and increase uteroplacental blood flow during pregnancy (Choi, Im et al. 2002). These findings are supported by increased concentrations of nitrates circulating in the plasma and excreted in the urine of pregnant females (Conrad 1987, Conrad and Vernier 1989, McLaughlin and Conrad 1995). Additionally, vascular sensitivity to the vasodilatory effects of NO increase during pregnancy, which is vital to maintain normotensive BP despite significant increases in circulating blood volume. Previous studies in pregnant rats have demonstrated that acute intravenous infusion of NOS inhibitors increases systemic BP (Molnar and Hertelendy 1992). The present study is the first to investigate the effects of NOS inhibition on androgen-induced vasorelaxation of the UA in the pregnant rat. Interestingly, in coronary arteries, nNOS appears to be the primary source of NO production during TES-induced vasorelaxation (Deenadayalu, Puttabyatappa et al. 2012); however, in canine coronary arteries, eNOS is the primary source of NO production (Chou, Sudhir et al. 1996). In the present study, NOS inhibition with NPA, which exhibits a 3000- and 150-fold relative selectivity for nNOS over iNOS and eNOS, respectively (Zhang, Fast et al. 1997), did not inhibit androgen-induced vasorelaxation in either the NP or PEP rats. Therefore, androgen-induced vasorelaxation of isolated UA of pregnant rats is largely dependent upon eNOS activation.

In VSM cells, NO binds to sGC with subsequent production of cGMP and mediation of smooth muscle relaxation via cGMP-dependent protein kinase pathways that reduce intracellular Ca^{2+} concentrations. While the specific mechanisms of NO-signaling are not well understood, vasorelaxation is dependent on either the inactivation of L-type

Ca^{2+} channels (Blatter and Wier 1994), with antagonistic effects on the release on intracellular Ca^{2+} concentrations from the sarcoplasmic reticulum (Furukawa, Tawada et al. 1988) and/or accelerated intracellular clearance of Ca^{2+} through membrane bound pumps (Hirata, Kohse et al. 1990), and/or involve the activation of K^+ channels (Perusquia and Stallone 2010).

4.6.4 Effects of K^+ channel inhibition on androgen-induced vasodilation

The present study investigated the activation of K^+ channels in androgen-induced vasorelaxation of isolated UA from NP and PEP rats. The results demonstrate that K_V and BK_{Ca} channels are the primary mediators of androgen-induced vasorelaxation in the UA of both NP and PEP rats. TES-induced vasorelaxation was attenuated by IbTx, a highly selective BK_{Ca} channel inhibitor, 52% in NP and 50% in PEP rats, while 5β-DHT-induced vasorelaxation was decreased 69% in NP and only 30% in PEP rats. Thus, while BK_{Ca} channel activation is a primary mediator for TES- and 5β-DHT-induced vasorelaxation in NP rats, 5β-DHT-activation of BK_{Ca} channels may be reduced in PEP rats. The K^+ channel inhibitor 4-AP, a K_V channel inhibitor, significantly attenuated TES-induced vasorelaxation in NP (71%) and PEP (73%) rats, with similar effects on 5β-DHT-induced vasorelaxation in NP (59%) and PEP (56%) rats. Thus, in addition to BK_{Ca} channels, Kv channel activation also mediates TES and 5β-DHT-induced vasorelaxation of UA in both NP and PEP rats. Interestingly, pretreatment of the UA with Gly, which is readily used as a K_{ATP} channel inhibitor, enhanced the vasodilatory effects of TES 19% in NP and 46% in PEP rats, with similar results in the vasodilatory effects of 5β-DHT in NP (13%) and PEP (56%) rats. Upregulated vasorelaxation of UA pretreated with K_{ATP} channel inhibitors suggest the possible activation of an additional intracellular signaling mechanisms. The

differences in androgen potency observed in the presence of K^+ channel inhibitors are reflective of the changes observed in androgen efficacy and reflex the inhibition of ion channel function. Thus, androgen-induced vasorelaxation of UA is not mediated by K_{ATP}, while channel inhibition appears to enhance androgen-induced vasorelaxation. Taken together, androgen-induced vasorelaxation is mediated, at least in part, by the activation of BK_{Ca} and K_V channels, with significant changes in the mechanisms of 5β-DHT-induced vasorelaxation in PEP rats that includes increased BK_{Ca} channel function.

The results of the present study extend the limited and contradictory findings regarding androgen-induced vasorelaxation signaling mechanisms. Previous studies identified the mechanism of TES-induced vasodilation as K_{ATP} channel activation in canine resistance vessels (Chou, Sudhir et al. 1996), and in canine basilar arteries TES activates K_V and BK_{Ca} channels and inhibits voltage-dependent Ca^{2+} channels (Ramirez-Rosas, Cobos-Puc et al. 2011). In the rat mesentery, TES activates BK_{Ca} channel function (Tep-areenan, Kendall et al. 2002). In the rat thoracic aorta TES appears to mediate K_V channel function (Ding and Stallone 2001); however, contradictory evidence suggests that TES-induces acute vasodilation via endothelial-derived prostanoid production, which activates BK_{Ca} channels and inhibits Ca^{2+} influx in the rat aorta (Ma, Jiang et al. 2009). Interestingly, using single-channel patch-clamp techniques, BK_{Ca} channel activation was identified as the primary target for TES signaling in porcine coronary arteries (Deenadayalu, White et al. 2001, Deenadayalu, Puttabyatappa et al. 2012). Variations in androgen-signaling mechanism may be attributed to experimentally-induced K^+ channel modifications. In the thoracic aorta of SHR and WKY-control rats, TES-induced rapid concentration-dependent vasorelaxation, which was significantly attenuated by K_{ATP}

channel inhibitors. In the SHR, K_V and BK_{Ca} channel inhibitors significantly reduced TES-induced vasorelaxation, with no effect on the WKY rats. The differences in K^+ channel activation between the SHR and WKY rats was attributed to dysfunctional K_{ATP} channels in the SHR and subsequent modification of K_V and BK_{Ca} channels to inhibit the progression of hypertension in male SHR (Honda, Unemoto et al. 1999). Further, castration of male rats decreases K_V channel expression in the thoracic aorta compared to intact control rats (Zhou, Fu et al. 2008). While the non-genomic mechanisms of androgen-induced vasodilation remain uncertain, the findings from the present study contribute to the identification of BK_{Ca} and K_V signaling mechanisms underlying androgen-induced vasorelaxation. The results from the present study were similar to findings in human umbilical arteries, (Saldanha, Cairrao et al. 2013), in which androgen-induced vasorelaxation involved both K_V and BK_{Ca} channel activation. Thus, the androgen-signaling mechanisms in the uterine arteries during pregnancy appears to be primarily dependent on K_V and BK_{Ca} channel function.

The structural differences between TES and 5β-DHT may result in differences between androgen signaling mechanisms. While 5α-DHT is widely used to verify the effects of TES independent of aromatization to estrogen (Chou, Sudhir et al. 1996, Deenadayalu, White et al. 2001, Tep-areenan, Kendall et al. 2002), many studies suggest that 5β-DHT is a more potent and efficacious vasodilator compared to TES. These effects have been verified in several blood vessels including, the rat aorta (Ding and Stallone 2001), pig coronary artery (Deenadayalu, White et al. 2001), and human umbilical artery (Perusquia, Navarrete et al. 2007). While the mechanisms of DHT are not well understood, several studies suggest that 5β-DHT-induced vasorelaxation relies on the deactivation of

Ca^{2+} channels in myometrial smooth muscle cells (Perusquia, Navarrete et al. 2005) and in human umbilical arteries (Perusquia, Navarrete et al. 2007). In the present study, the signaling mechanisms between TES vs. 5β-DHT were similar in NP rats, yet the signaling mechanisms between TES vs. 5β-DHT in PEP rats were significantly different, as BK_{Ca} channel inhibition was less efficacious at attenuating 5β-DHT-induced vasorelaxation in PEP rats compared to NP control rats. Thus, the signaling mechanism of 5β-DHT-induced vasorelaxation was less affected by BK_{Ca} channel inhibition, which suggests there is either an increase in BK_{Ca} channel expression or a decrease in BK_{Ca} signaling mechanisms in 5β-DHT-induced vasorelaxation of PEP rats vs. NP rats. This may suggest that the signaling mechanisms of 5β-DHT in PE may also involve deactivation of Ca^{2+} signaling mechanisms. Interestingly, after a 24-hr incubation of human umbilical arteries in DHT, the expression of BK_{Ca} channel β_1-subunits significantly increased, while the expression of L-type Ca^{2+} channel α-subunits decreased, and the DHT-incubated umbilical arteries became more reliant on K_V channel activation and less reliant on BK_{Ca} channel activation during TES-induced vasorelaxation compared to umbilical arteries that were not incubated in DHT (Saldanha, Cairrao et al. 2013). Taken together, androgen exposure during pregnancy may alter K^+ channel function and increase 5β-DHT sensitivity in the UA of PEP rats. Thus, the mechanisms of androgen-induced regulation of uteroplacental blood flow during pregnancy be regulated by vascular remodeling in response to androgen production during pregnancy.

4.6.5 Effects of preeclampsia on 5α- and 5β-reductase mRNA expression in the uterine artery and placenta

In the present study, the expression of 5β- reductase mRNA was reduced by 35% in the placenta and 67% in the UA of PEP rats compared to NP control rats. The placenta plays an important role in nutritional, metabolic and endocrine functions during pregnancy. As an interface between the maternal and fetal blood, the placenta transfers oxygen and essential nutrients, and relies upon adequate uterine perfusion and an extensive placental vascular network. The placenta is provided in-flowing maternal blood from the UA, which facilitates the movement of deoxygenated, nutrient-poor blood carrying fetal waste products across the placenta, into the uterine vein (UV), and back into maternal circulation. Anatomical studies suggest that the close juxtaposition of the UA and UV enable efficient counter-current transfer of biologically-active regulators including placental steroid and peptide hormones from the outgoing blood in the UV to the incoming blood in the UA, thereby enhancing their vasodilatory effects on the UA and increasing placental blood flow (Towers, Shaw et al. 1986, Zezula-Szpyra, Gawronska et al. 1997). Endocrine regulation can include systemic distribution and/or a local paracrine distribution of regulating hormones. The proximity of the UA and UV enable local regulation of uterine blood flow through the counter-current transfer of regulating hormones facilitated by the production of such hormones at the fetoplacental interface. Similar counter-current transfer has been observed in various tissues. In male rats, radioactively labeled TES was four-fold greater in the testicular arteries than in the systemic circulation. Using radioactively labeled TES, the counter-current transfer of TES was established from the outgoing spermatic vein to incoming spermatic artery in the pampiniform plexus. Similar effects occur in females, as

concentration differences of progesterone, androstenedione, and estradiol were identified between ovarian arterial blood and systemic circulation, which suggests a local transfer of follicular hormones from the ovarian vein to arteries as a means to regulate ovarian function (Hunter, Cook et al. 1983). Interestingly, the results from the present study revealed a decrease in the expression of 5β-reductase mRNA in both the placenta and UA of PEP rats compared to NP control rats. While local uterine vs. systemic androgen concentrations were not measured in the present study, the results suggest that decreased production of 5β-DHT at the uteroplacental interface may contribute to decreased UA blood flow during PE, consistent with the IUGR observed in the DOCA-salt rat model of PE studied. Thus, the findings from the present study suggest that reductions in uterine artery and placental expression of 5β-reductase enzymes result in decreases in local production of 5β-DHT during pregnancy. Thus, the decrease in outflowing concentrations of 5β-DHT from the placenta would result in a decrease of androgen concentrations in the UV and UA. Reductions in androgen production would thereby result in reduced vasodilation of the UA and reduced uterine blood flow to the placenta. This suggestion is consistent with findings observed in the PEP rats of reduced expression of 5β-reductase mRNA in the placenta and UA, increased fetal IUGR, and increased UA sensitivity to 5β-DHT *in vitro*.

4.6.6 Physiological relevance

Androgens exert rapid vasorelaxation of the vasculature at the maternal fetoplacental interface, which may maintain uterine blood flow during pregnancy. Despite accumulating evidence regarding the beneficial effects of androgens during pregnancy, the mechanisms of androgen-induced vasorelaxation are not well understood. There appear to

be differences in the reactivity and signaling mechanisms among the androgenic steroid hormones; however, the current study provides new evidence that androgens may enhance uterine blood flow during pregnancy via NOS and K^+ channel-mediated vasodilation of the UA. Furthermore, androgen production at the uteroplacental interface may contribute to local androgen production and uterine blood flow regulation during pregnancy. The juxtaposition of the UA and UV facilitate countercurrent transfer of regulatory steroid hormones produced by the placenta. The findings from this study suggest that androgen production at the uteroplacental interface may play a role in the regulation of uterine blood flow and BP during normal pregnancy, and reduced androgen productions at the uteroplacental interface may contribute to the pathogenesis of PE.

5 CONCLUSIONS

The purpose of this dissertation was to investigate the role of androgens in the regulation of BP and uterine vascular function. The first project utilized male SD and Tfm rats to evaluate the long-term effects of endogenous and exogenous androgens on systemic BP. The results reveal that androgens (TES and 5α-DHT) exert novel long-term anti-hypertensive effects on systemic BP in male rats. These anti-hypertensive mechanisms involve estrogen-independent, non-genomic, signaling pathways which reduce RAS component expression in the kidney. Thus, the antihypertensive effects of androgens appear to involve both rapid effects on systemic vasodilation and long-term effects on the kidney to promote fluid excretion. The second project utilized normal pregnant and PE pregnant (DOCA-salt model of PE) female SD rats to evaluate the vascular effects and signaling mechanisms of androgens at the uteroplacental interface during normal and PE pregnancy. The results reveal that androgens elicit rapid vasorelaxation of isolated UA from NP and PEP rats and evoke mechanisms that appear to be highly dependent on eNOS signaling and the activation of K_V and BK_{Ca} channels. In the PEP rats, UA sensitivity increased to 5β-DHT and BK_{Ca} channel inhibition was less effective at attenuating 5β-DHT-induced vasorelaxation compared to NP control rats. Further, mRNA expression of 5β- reductase is reduced in the uterine arteries and placenta of PEP rats compared to NP control rats. Thus, androgens appear to regulate uteroplacental blood flow during normal pregnancy, while reduced androgen production (5β-DHT) at the uteroplacental interface may contribute to reductions in uterine blood flow associated with the pathogenesis of PE.

Taken together, the finding from this dissertation may offer possible therapeutic roles for androgens in the treatment of hypertension in both hypogonadal men and women with PE.

5.1 Androgens in hypertension

There is a well-established sexual dimorphism in human CVD, in which men are more likely to develop hypertension and CAD compared to age-matched pre-menopausal women (Lloyd-Jones, Adams et al. 2010). Combined with earlier epidemiological data in human clinical trials, there is a firmly entrenched dogma that TES is deleterious to cardiovascular health (Mendoza, Zerpa et al. 1983, Sewdarsen, Jialal et al. 1986). This dogmatic view has driven many of the experimental animal studies which suggest that TES exacerbates hypertension (Reckelhoff, Zhang et al. 1998) and renal disease (Xu, Wells et al. 2008) in male rats, and contributes to the development and progression of PE in pregnant female rats (Kumar, Gordon et al. 2018). Interestingly, many of the experimental animal studies that suggest TES is deleterious to cardiovascular function suffer from limitations in their experimental design and/or selection of an appropriate/translatable animal model for the disease (Liu, Death et al. 2003, Wu and von Eckardstein 2003, Traish, Haider et al. 2017). These limitations may explain why findings from these studies are so incongruous with the most recent clinical and experimental animal findings that provide strong evidence that androgens are beneficial and protective against hypertension in both sexes. The findings of the present studies in this dissertation challenge the dogma that TES is deleterious to vascular health and function, and instead provide further support that TES is beneficial to BP regulation in males and uterine blood flow in pregnant females. The animal models developed in the present study may serve as useful translational models of hypogonadal hypertension in men and pregnancy-induced hypertension in PE women in

future studies to further elucidate the role of TES and its DHT metabolites in cardiovascular health and disease.

5.2 Anti-hypertensive effects of endogenous and exogenous androgens

There is increasing evidence that TES therapy significantly improves cardiovascular and metabolic functions that include reductions in diastolic BP of aging hypogonadal men (Traish, Haider et al. 2017). In the nine meta-analyses reported to date, all but one demonstrated that TES therapy is not harmful and strongly associated with significant health benefits, including improvements in diastolic BP and metabolic function, two of the most important risk factors for the development of CVD in men (Marin, Holmang et al. 1992, Traish and Zitzmann 2015). The findings from the first study in this dissertation provide important new and novel information on the long-term anti-hypertensive effects of exogenous androgens, which are entirely consistent with the much earlier human studies that demonstrate the beneficial use of acute intramuscular infusions of TES to treat angina pectoris, hypertension, and peripheral vascular disease in men with CAD (Hamm 1942, Walker 1942, Lesser 1946). Similar studies in SHR (Perusquia, Herrera et al. 2017) and WKY male rats (Perusquia, Contreras et al. 2019) reveal that castration leads to the development of hypertension, while acute intravenous infusion of TES and its metabolites provided short-term reductions in arterial BP in both male SHR and WKY rats. While the anti-hypertensive effects of androgens in pregnant rats was not studied in the present investigation, several studies in pregnant and PE pregnant female SD rats suggest that androgens exert similar anti-hypertensive effects during pregnancy. Acute bolus intravenous injections of androgens in pregnant female rats near term exert similar anti-hypertensive effects on arterial BP as seen in male rats. Interestingly, 5β-DHT and

DHEA exert strong anti-hypertensive effects on arterial BP compared moderate anti-hypertensive effects of TES and 5α-DHT in pregnant and PE pregnant rats (Perusquia, Hanson et al. 2018). Taken together with the findings from the second study in this dissertation, new information on the vascular effects of androgens at the uteroplacental interface further evidence suggest that androgen-induced vasorelaxation at the uteroplacental interface may contribute to the regulation of uterine blood flow and maternal BP during pregnancy.

5.3 Mechanisms underlying the anti-hypertensive effects of androgens

Acute infusions of TES and other androgens *in vivo* produce rapid vasodilation in human coronary arteries (Webb, McNeill et al. 1999), with similar effects in several animal species such as the dog (Chou, Sudhir et al. 1996), pig (Molinari, Battaglia et al. 2002), and rat (Perusquia, Greenway et al. 2015). I*n vitro*, TES and other androgen metabolites induce acute, non-genomic, estrogen-independent vasorelaxation of isolated blood vessels including large conduit arteries and smaller resistance vessels (Perusquia and Stallone 2010). Together, these *in vivo* and *in vitro* findings suggest short-term anti-hypertensive effects of androgens are attributed to acute vasodilation of systemic and/or regional vasculature. Interestingly, within the same species, there appear to be regional differences in vascular reactivity to each of the androgens.

In the present study, both TES and its metabolite 5α-DHT exhibit similar anti-hypertensive effects in male SD rats. Differences in the structural configuration between TES and DHT and differences in the structural configuration between 5α- and 5β-DHT may play a role in the efficacy and potency among androgens. 5α-DHT is a highly potent androgenic tissue metabolite of TES, known to mediate the effects of TES in reproductive

target tissues, while 5β-DHT is a highly potent and efficacious nongenomic vasodilator, devoid of genomic androgenic activity (Perusquia, Greenway et al. 2015). Interestingly, within the same species, there appear to be regional differences in vascular reactivity to androgens. For example, in the canine vasculature, coronary resistance vessels are more reactive to androgen-induced vasorelaxation than either the saphenous vein or femoral artery (Perusquia, Espinoza et al. 2012). Furthermore, some studies report regional differences in vascular reactivity to each of the androgens, with many of the studies suggesting that the TES metabolite 5β-DHT is a more potent and efficacious vasodilator than TES (Ishimaru, Edmiston et al. 1978, Kumar, Crozat et al. 1999). In the rat, 5β-DHT-induced vasorelaxation was more potent than TES or 5α-DHT in mesenteric arteries compared to the thoracic aorta arteries (Isidoro, Ferrer et al. 2018).

The present study investigated the long-term effects of endogenous and exogenous androgens on BP in SD and AR-deficient Tfm rats to identify the contribution of non-genomic vs. genomic anti-hypertensive mechanisms of TES and 5α-DHT. Castration of both the SD and Tfm rats induced long-term hypertension, which was reversed by androgen therapy. Thus, these findings strongly suggest that the anti-hypertensive effects of endogenous and exogenous androgens mainly involve non-genomic mechanisms. Interestingly, in the presence of a defective genomic AR in the Tfm rats, the onset of hypertension was delayed, yet the rate of progression was enhanced compared to the male SD rats. Thus, these differences between the progression of hypertension and anti-hypertensive response to TES therapy in the normal male SD vs. Tfm rats may result from interactions between non-genomic and genomic mechanisms. The non-genomic effects of androgens are fundamentally different than the genomic effects observed in reproductive

target tissues (Yue, Chatterjee et al. 1995, Ding and Stallone 2001, Perusquia and Stallone 2010), and these non-genomic mechanisms appear to involve membrane receptor activation and include kinase-signaling cascades and/or modulation of VSM ion channel function (Lucas-Herald, Alves-Lopes et al. 2017). Affected Tfm rats inherit an x-linked mutation of the AR gene from carrier females. The single gene mutation does not inhibit AR gene mRNA expression but does inhibit cytosolic AR function. This mutation involves a shift in the phosphorylation recognition site of the AR, which results in an 85-90% failure of the tissue response to androgens (Yarbrough, Quarmby et al. 1990). Because of the absence of AR-mediated negative feedback by TES, the abdominal testis of Tfm rats produce elevated levels of TES. While the rate of TES conversion to DHT is normal compared to wild type rats, aromatase activity in the affected Tfm rat is decreased (Roselli, Salisbury et al. 1987, Tejada and Rissman 2012). In this dissertation, a small parallel study of the anti-hypertensive effects of DHT therapy in castrated Tfm rats revealed that DHT therapy had similar anti-hypertensive effects compared to TES therapy. While the effects were similar, likely excluding a role for estrogen receptor activation, these findings are not conclusive regarding possible differences in estrogen receptor activation between SD and Tfm rats.

5.4 Anti-hypertensive effects of androgens on RAS function

The long time-frame for the development of hypertension in castrated male SD rats suggests that extracellular fluid volume expansion resulting from the retention of fluid and electrolytes in the absence of TES may contribute to the development of hypertension. Interestingly, long-term treatment with Losartan, a competitive antagonist of the AT_1R, completely inhibited the development of hypertension in male SD rats. Further, plasma

renin concentration and renal expression of renin, AT_1R, and ACE mRNA increased after castration and these observed effects were reversed following androgen therapy. Thus, these findings provide strong and consistent evidence that the development of long-term hypertension in male rats in the absence of TES involves up regulation of RAS function in the kidneys of these animals, and likely the retention of fluid and sodium, thereby expanding ECF volume and leading to the gradual development of hypertension. Together, the past and present studies strongly suggest that the development of hypertension involves both the loss of androgen-induced systemic vasodilation and the expansion of extracellular fluid volume resulting from upregulation of renal RAS function in the absence of endogenous androgens. The fact that hypertension develops following castration of the AR-deficient Tfm rats provides strong evidence that the anti-hypertensive effects of TES on the vasculature and kidney relies upon non-genomic mechanisms independent of the classic cytosolic AR that mediates the genomic effects of the hormone. However, the genomic and non-genomic mechanisms may not be mutually exclusive, as the genomic mechanisms of TES may modulate and influence the non-genomic effects (Wilkenfeld, Lin et al. 2018). Thus, a combination of genomic and non-genomic mechanisms may underlie the antihypertensive effects of endogenous TES observed in male SD rats.

5.5 Androgen-induced vasorelaxation in the uterine artery

There is increasing interest in the role of androgens in the pathogenesis of PE, a pregnancy-specific hypertensive disorder in human females. PE is marked by systemic vascular and hemostatic hypersensitivity to local and systemic vasoconstrictive mediators, including Ang II and TXA_2. Since androgen levels in the maternal circulation rise substantially during pregnancy and exert non-genomic vasodilatory effects on umbilical

arteries *in vitro*, it seems likely that placental androgens contribute to the maintenance of utero-placental blood flow during pregnancy. Interestingly, TES levels are three-fold higher in PE pregnancies, suggesting that TES exerts deleterious effects during pregnancy and may play a possible role in the development and progression of PE. Interestingly, several studies demonstrate beneficial effect of TES and other androgens on arterial BP in pregnant rats (Perusquia, Hanson et al. 2018), with rapid effects on vasorelaxation at the fetoplacental interface that suggest beneficial effects of TES on blood flow (Perusquia, Navarrete et al. 2007). These findings are further supported by the findings from the present studies, which reveal that TES and its metabolites 5α- and 5β-DHT induced rapid vasorelaxation of UE isolated from pregnant female SD rats. Thus, androgens appear to exert beneficial effects on BP regulation and promote uteroplacental blood flow during pregnancy. Differences in androgen reactivity were observed in human umbilical arteries, in which DHEA and 5β-DHT-induced vasorelaxation was greater compared to TES or female sex steroid hormones (Perusquia, Navarrete et al. 2007). Additionally, the anti-hypertensive effects of 5β-DHT and DHEA appear to be significantly greater than the moderate effects of TES and 5α-DHT on arterial BP in conscious pregnant female rats (Perusquia, Hanson et al. 2018). Taken together, the results of this study make for interesting speculation that 5β-DHT may be an effective treatment during pregnancy for PE.

5.6 Mechanisms of androgen-induced vasorelaxation in the uterine artery

The key mechanisms underlying androgen-induced vasorelaxation are associated with the activation of VSM ion channel function. The findings from the present study revel that the mechanism of androgen-induced vasorelaxation is dependent upon activation

of eNOS and K_V and BK_{Ca} channel function in the UA of pregnant and PE pregnant female SD rats. L-NMMA, a non-selective NOS inhibitor, decreased androgen-induced vasorelaxation of the UA more than 70% in both the NP and PEP rats, with minimal effect on vasorelaxation in UA pretreated with NPA, a selective nNOS inhibitor. Thus, androgen-induced vasorelaxation in UA is primarily dependent on eNOS activation. NO-induced vasorelaxation is associated with increased levels of cGMP in VSM cells and subsequent activation of K^+ channels (Archer, Huang et al. 1994) and/or deactivation of Ca^{2+} channels. In the present study, inhibition of K_V and BK_{Ca} ion channel function attenuated androgen-induced vasorelaxation in isolated UA, while inhibition of K_{ATP} channels exacerbated the vasodilatory effects of TES and its metabolite 5β-DHT. These findings were similar to the K_V and BK_{Ca} channel signaling mechanisms of TES-induced vasorelaxation observed in human umbilical arteries (Saldanha, Cairrao et al. 2013). Interestingly, the effects of IbTx were reduced in PEP rats compared to NP control rats. Some studies suggest androgen-induced vasorelaxation is more dependent on Ca^{2+} antagonistic actions. For example, the vasorelaxtion effects of 5β-DHT were more potent than DHEA or TES in myometrial smooth muscle cells and the mechanisms involved blocking VOCC and ROCC channels (Perusquia, Navarrete et al. 2005), with similar effects observed in human umbilical arteries (Perusquia, Navarrete et al. 2007). Changes in signaling mechanisms may be attributed to androgen exposure during pregnancy. In human umbilical arteries exposed to 5β-DHT *in vitro* have increased mRNA expression of BK_{Ca} channel β_1-subunits and decreased mRNA expression of L-type Ca^{2+} channel α-subunits (Saldanha, Cairrao et al. 2013). Thus, changes in signaling mechanism may affect the efficacy and/or potency of androgen-induced vasorelaxation.

The placenta plays a crucial role as the interface between the maternal and fetal circulation. The close anatomical juxtaposition of the UA and UV enables efficient counter-current transfer of biologically-active humoral regulators produced at the uteroplacental interface from high concentrations in the UV to the UA (Towers, Shaw et al. 1986, Zezula-Szpyra, Gawronska et al. 1997). Thus, endocrine regulation includes systemic distribution and/or local paracrine distribution of key regulating hormones from the placenta to the UA via the UV. The results from the present study revealed no significant changes in uterine artery and placental mRNA expression of 5α-reductase but a significant decrease in the mRNA expression of 5β-reductase mRNA in both the placenta and UA of PEP rats compared to NP controls. Therefore, 5β-DHT production is decreased in the uterine arteries and preeclampsia during PE. Thus, decreased production of 5β-DHT at the uteroplacental interface may contribute to decreased uterine blood flow during the development or progression of PE. This suggestion is consistent with the increased sensitivity of the UA to 5β-DHT *in vitro* and the associated IUGR observed in the PEP rats compared to the NP controls in the present study.

While several animal models of PE have been identified which share many of the characteristics of the disorder in humans, there is no single model that fully mimics the human-specific progression of PE. For the present study, the DOCA-salt model was chosen to investigate the vasodilatory effects of androgens on the uteroplacental vasculature in female rats. The DOCA-salt model of hypertension is a well-established rat model that exhibits neurogenic, renal, and vascular response elements. An imbalance in renal Na^+ and water reabsorption results in hypervolemia, which mimics the effects of fluid volume expansion observed in early gestation. While DOCA-salt treatment increases RAS

activity in the brain, which results in neural alterations in renal function and systemic vascular resistance (Ueno, Mohara et al. 1988), circulating renin and Ang II levels are unchanged. Further, DOCA-salt rats exhibit systemic endothelial dysfunction, with enhanced vascular reactivity in isolated blood vessels (Nunes, Fortes et al. 2000). Compared to the various limitations in all other animal models of PE, the DOCA-salt rat model has the least potential to confound the findings of the present study; however, the results still may not be fully applicable to human PE.

5.7 Clinical significance

The goal of this dissertation was to elucidate the roles of androgens in the regulation of systemic BP and in uterine vascular function during normal pregnancy and PE. The findings from the studies in this dissertation provide important new and novel information on the possible roles of androgens in the regulation of systemic BP in hypogonadal men and in uteroplacental blood flow during pregnancy. Further understanding of the mechanisms by which TES and other androgens exert beneficial vs. detrimental effects could lead to novel new therapeutic agents to treat systemic hypertension in men and PE in women.

TES deficiency in aging men has been well documented by several studies, which reveal that suggest free TES decreases 1.7-2.8% annually in men after 40 (Mohr, Guay et al. 2005). TES deficiency presents with symptoms including a reduction in libido, energy, physical stamina, and lean muscle mass (Bhasin, Cunningham et al. 2010), while TES therapy improves sexual function, increases stamina, improves mood, increases bone mineral density, decreases body fat mass, and increases lean body muscle mass (Bhasin, Cunningham et al. 2010, Corona, Vignozzi et al. 2013). While TES therapy appears to

improve overall wellbeing, potential adverse effects of TES therapy have been associated with increases in CVD, elevated prostate-specific antigen, and increased risk of prostate cancer. There are several recent clinical studies that suggest that TES is deleterious to cardiovascular health and function, yet all these studies suffer from significant deficiencies in experimental design and/or data analysis that limit their validity. A retrospective cohort study of men with low TES concentrations who underwent coronary angiograph in the Veterans Affairs healthcare system suggests that TES therapy increases the risk of mortality, MI, or ischemic stroke in hypogonadal men (Vigen, O'Donnell et al. 2013). This study has been criticized for errors in its statistical methodology (Traish, Guay et al. 2014). Another trial, which was a double-blind randomized control study of elderly men with limited lower limb mobility, was terminated early with reports of increased adverse cardiovascular events in the TES treated men (Basaria, Coviello et al. 2010). The results of this study have declared inconclusive by the Food and Drug Administration based on the small sample size and discrepancies in data acquisition. In contrast to these studies, the most recent clinical studies suggest that TES therapy increases coronary artery dilation and blood flow, attenuates atherosclerosis, and ameliorates metabolic syndrome (Traish 2018). Further trials are necessary to determine the differences in study outcomes. Additionally, TES therapy is associated with increased serum prostate-specific antigens in hypogonadal men and early studies suggest that castration results in a dramatic regression of prostate cancer (Huggins 1941). Interestingly, while elevated, the increased antigen levels were still within normal limits and did not definitively lead to significant increased risk of prostate cancer (Bhasin, Cunningham et al. 2010). Further, a cumulative registry study found that while mean prostate-specific antigen did rise significantly during TES

therapy, the incidence of prostate cancer in the TES treated men was lower than the incidence of prostate cancer in the general population (Traish, Haider et al. 2014). With the increased observation of hypertension and CVD in men compared to women, it is tempting to conclude that differences in sex hormones, specifically the male sex hormone TES, are responsible for the progression of these disease; however, TES may not be the single explanatory factor in the observed sex differences. Other hypotheses suggest that a lack of estrogen in men may be the root cause of these sex differences. There are several geographic and ethnic differences in risk factors associated with CVD which also suggest that other factors may play an important causative role in the progression of the disease, and further evidence that the progression of hypertension and CVD may be multifactorial (Balfour, Rodriguez et al. 2015).

The first study of this dissertation revealed that androgens appear to exert beneficial effects on BP. The anti-hypertensive effects of TES and 5α-DHT in castrated male rats provide insight into the effects of TES deficiency on systemic BP and suggest a possible therapeutic role for androgens in hypertension. Similarly, the second study of this dissertation revealed that androgens induce vasorelaxation of UA of pregnant rats, suggesting a role for androgens in the regulation of uterine blood flow during pregnancy. In PE pregnancies, increases in serum TES may result from decreases in the metabolism of TES to 5α-DHT, which is known to increase serum TES concentrations (Hong, Min et al. 2010, Enatsu, Miyake et al. 2016). The increased potency of 5β-DHT and reduced expression of 5β-reductase enzymes in the UA and placenta of PEP rats in the present study, suggests that a decrease in vasodilatory androgen production at the uteroplacental interface may reduce uterine blood flow and thereby contribute to the development and

progression of PE. Thus, it is interesting to speculate that 5β-DHT, a TES metabolite that lacks androgenic effects at the AR but is a highly efficacious and potent vasodilator, may be an effective treatment for PE by improving placental blood flow.

6 REFERENCES

Abdul-Karim, R. and N. S. Assali (1961). "Pressor response to angiotonin in pregnant and nonpregnant women." Am J Obstet Gynecol **82**(2): 246-251.

Abitbol, M. M., G. R. Gallo, C. L. Pirani and W. B. Ober (1976). "Production of experimental toxemia in the pregnant rabbit." Am J Obstet Gynecol **124**(5): 460-470.

Achar, S., A. Rostamian and S. M. Narayan (2010). "Cardiac and metabolic effects of anabolic-androgenic steroid abuse on lipids, blood pressure, left ventricular dimensions, and rhythm." Am J Cardiol **106**(6): 893-901.

Acromite, M. T., C. S. Mantzoros, R. E. Leach, J. Hurwitz and L. G. Dorey (1999). "Androgens in preeclampsia." Am J Obstet Gynecol **180**(1 Pt 1): 60-63.

Alasztics, B., N. Gullai, A. Molvarec and J. Rigo, Jr. (2014). "[The role of angiogenic factors in preeclampsia]." Orv Hetil **155**(47): 1860-1866.

Alexander, B. T., S. E. Kassab, M. T. Miller, S. R. Abram, J. F. Reckelhoff, W. A. Bennett and J. P. Granger (2001). "Reduced uterine perfusion pressure during pregnancy in the rat is associated with increases in arterial pressure and changes in renal nitric oxide." Hypertension **37**(4): 1191-1195.

Amaral, L. M., K. Wallace, M. Owens and B. LaMarca (2017). "Pathophysiology and Current Clinical Management of Preeclampsia." Curr Hypertens Rep **19**(8): 61.

Anea, C. B., B. Cheng, S. Sharma, S. Kumar, R. W. Caldwell, L. Yao, M. I. Ali, A. M. Merloiu, D. W. Stepp, S. M. Black, D. J. Fulton and R. D. Rudic (2012). "Increased superoxide and endothelial NO synthase uncoupling in blood vessels of Bmal1-knockout mice." Circ Res **111**(9): 1157-1165.

Angueira, A. R., A. E. Ludvik, T. E. Reddy, B. Wicksteed, W. L. Lowe, Jr. and B. T. Layden (2015). "New insights into gestational glucose metabolism: lessons learned from 21st century approaches." Diabetes **64**(2): 327-334.

Araujo, A. B., G. R. Esche, V. Kupelian, A. B. O'Donnell, T. G. Travison, R. E. Williams, R. V. Clark and J. B. McKinlay (2007). "Prevalence of symptomatic androgen deficiency in men." J Clin Endocrinol Metab **92**(11): 4241-4247.

Archer, S. L., J. M. Huang, V. Hampl, D. P. Nelson, P. J. Shultz and E. K. Weir (1994). "Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase." Proc Natl Acad Sci U S A **91**(16): 7583-7587.

Arnett, D. K., R. S. Blumenthal, M. A. Albert, A. B. Buroker, Z. D. Goldberger, E. J. Hahn, C. D. Himmelfarb, A. Khera, D. Lloyd-Jones, J. W. McEvoy, E. D. Michos, M. D. Miedema, D. Munoz, S. C. Smith, Jr., S. S. Virani, K. A. Williams, Sr., J. Yeboah and B. Ziaeian (2019). "2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines." J Am Coll Cardiol.

Aronow, W. S., J. L. Fleg, C. J. Pepine, N. T. Artinian, G. Bakris, A. S. Brown, K. C. Ferdinand, M. A. Forciea, W. H. Frishman, C. Jaigobin, J. B. Kostis, G. Mancia, S. Oparil, E. Ortiz, E. Reisin, M. W. Rich, D. D. Schocken, M. A. Weber, D. J. Wesley and R. A. Harrington (2011). "ACCF/AHA 2011 expert consensus document on hypertension in the elderly: a report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents." Circulation **123**(21): 2434-2506.

Aversa, A., R. Bruzziches, D. Francomano, G. Rosano, A. M. Isidori, A. Lenzi and G. Spera (2010). "Effects of testosterone undecanoate on cardiovascular risk factors and atherosclerosis in middle-aged men with late-onset hypogonadism and metabolic syndrome: results from a 24-month, randomized, double-blind, placebo-controlled study." J Sex Med **7**(10): 3495-3503.

Balfour, P. C., Jr., C. J. Rodriguez and K. C. Ferdinand (2015). "The Role of Hypertension in Race-Ethnic Disparities in Cardiovascular Disease." Curr Cardiovasc Risk Rep **9**(4).

Ballermann, B. J. and B. M. Brenner (1987). "Atrial natriuretic peptide and the kidney." Am J Kidney Dis **10**(1 Suppl 1): 7-12.

Bammann, B. L., C. B. Coulam and N. S. Jiang (1980). "Total and free testosterone during pregnancy." Am J Obstet Gynecol **137**(3): 293-298.

Barnard, R., G. Kelly, S. O. Manzetti and E. L. Harris (2001). "Neither the New Zealand genetically hypertensive strain nor Dahl salt-sensitive strain has an A1079T

transversion in the alpha1 isoform of the Na(+),K(+)-ATPase gene." Hypertension **38**(4): 786-792.

Basaria, S., A. D. Coviello, T. G. Travison, T. W. Storer, W. R. Farwell, A. M. Jette, R. Eder, S. Tennstedt, J. Ulloor, A. Zhang, K. Choong, K. M. Lakshman, N. A. Mazer, R. Miciek, J. Krasnoff, A. Elmi, P. E. Knapp, B. Brooks, E. Appleman, S. Aggarwal, G. Bhasin, L. Hede-Brierley, A. Bhatia, L. Collins, N. LeBrasseur, L. D. Fiore and S. Bhasin (2010). "Adverse events associated with testosterone administration." N Engl J Med **363**(2): 109-122.

Bassil, N., S. Alkaade and J. E. Morley (2009). "The benefits and risks of testosterone replacement therapy: a review." Ther Clin Risk Manag **5**(3): 427-448.

Baylis, C., E. Beinder, T. Suto and P. August (1998). "Recent insights into the roles of nitric oxide and renin-angiotensin in the pathophysiology of preeclamptic pregnancy." Semin Nephrol **18**(2): 208-230.

Baylis, C., K. J. C. Engels and E. H. P. B. H. i. Pregnancy (1992). "Adverse interactions between pregnancy and a new model of systemic hypertension produced by chronic blockade of endothelial derived relaxing factor (EDRF) in the rat." **11**(2-3): 117-129.

Benagiano, G., S. Mancuso, F. P. Mancuso, N. Wiqvist and E. Diczfalusy (1968). "Studies on the metabolism of C-19 steroids in the human foeto-placental unit. 3. Dehydrogenation and reduction prducts formed by previable foetuses with androstenedione and testosterone." Acta Endocrinol (Copenh) **57**(2): 187-207.

Bennett, N. C., R. A. Gardiner, J. D. Hooper, D. W. Johnson and G. C. Gobe (2010). "Molecular cell biology of androgen receptor signalling." Int J Biochem Cell Biol **42**(6): 813-827.

Benten, W. P., M. Lieberherr, C. E. Sekeris and F. Wunderlich (1997). "Testosterone induces Ca2+ influx via non-genomic surface receptors in activated T cells." FEBS Lett **407**(2): 211-214.

Benten, W. P., M. Lieberherr, O. Stamm, C. Wrehlke, Z. Guo and F. Wunderlich (1999). "Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages." Mol Biol Cell **10**(10): 3113-3123.

Berg, A. H., C. D. Rice, M. S. Rahman, J. Dong and P. Thomas (2014). "Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: I. Discovery in female atlantic croaker and evidence ZIP9 mediates testosterone-induced apoptosis of ovarian follicle cells." Endocrinology **155**(11): 4237-4249.

Berne, R. M. and M. N. Levy (2018). Berne & Levy Physiology. Philidelphia, PA, Mosby/Elsevier.

Bhasin, S., G. R. Cunningham, F. J. Hayes, A. M. Matsumoto, P. J. Snyder, R. S. Swerdloff and V. M. Montori (2010). "Testosterone therapy in men with androgen deficiency syndromes: an Endocrine Society clinical practice guideline." J Clin Endocrinol Metab **95**(6): 2536-2559.

Blatter, L. and W. J. C. c. Wier (1994). "Nitric oxide decreases [Ca2+] i in vascular smooth muscle by inhibition of the calcium current." **15**(2): 122-131.

Blesson, C. S., V. Chinnathambi, G. D. Hankins, C. Yallampalli and K. Sathishkumar (2015). "Prenatal testosterone exposure induces hypertension in adult females via androgen receptor-dependent protein kinase Cdelta-mediated mechanism." Hypertension **65**(3): 683-690.

Boivin, G. P., D. L. Hickman, M. A. Creamer-Hente, K. R. Pritchett-Corning and N. A. Bratcher (2017). "Review of CO(2) as a Euthanasia Agent for Laboratory Rats and Mice." J Am Assoc Lab Anim Sci **56**(5): 491-499.

Bosio, P. M., P. J. McKenna, R. Conroy and C. O'Herlihy (1999). "Maternal central hemodynamics in hypertensive disorders of pregnancy." Obstet Gynecol **94**(6): 978-984.

Broughton Pipkin, F. and P. N. Baker (1997). "Angiotensin II has depressor effects in pregnant and nonpregnant women." Hypertension **30**(5): 1247-1252.

Buchwalow, I. B., T. Podzuweit, W. Bocker, V. E. Samoilova, S. Thomas, M. Wellner, H. A. Baba, H. Robenek, J. Schnekenburger and M. M. Lerch (2002). "Vascular smooth muscle and nitric oxide synthase." Faseb j **16**(6): 500-508.

Budoff, M. J., S. S. Ellenberg, C. E. Lewis, E. R. Mohler, 3rd, N. K. Wenger, S. Bhasin, E. Barrett-Connor, R. S. Swerdloff, A. Stephens-Shields, J. A. Cauley, J. P. Crandall, G. R. Cunningham, K. E. Ensrud, T. M. Gill, A. M. Matsumoto, M. E. Molitch, R. Nakanishi, N. Nezarat, S. Matsumoto, X. Hou, S. Basaria, S. J. Diem, C. Wang, D. Cifelli and P. J. Snyder (2017). "Testosterone Treatment and Coronary Artery Plaque Volume in Older Men With Low Testosterone." Jama **317**(7): 708-716.

Burger, H. G. (2002). "Androgen production in women." Fertil Steril **77 Suppl 4**: S3-5.

Buster, J. E., R. J. Chang, D. L. Preston, R. M. Elashoff, L. M. Cousins, G. E. Abraham, C. J. Hobel and J. R. Marshall (1979). "Interrelationships of circulating maternal steroid concentrations in third trimester pregnancies. II. C18 and C19 steroids: estradiol, estriol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, delta 5-androstenediol, delta 4-androstenedione, testosterone, and dihydrotestosterone." J Clin Endocrinol Metab **48**(1): 139-142.

Cairrao, E., E. Alvarez, A. J. Santos-Silva and I. Verde (2008). "Potassium channels are involved in testosterone-induced vasorelaxation of human umbilical artery." Naunyn Schmiedebergs Arch Pharmacol **376**(5): 375-383.

Carson, C. C., 3rd and G. Rosano (2012). "Exogenous testosterone, cardiovascular events, and cardiovascular risk factors in elderly men: a review of trial data." J Sex Med **9**(1): 54-67.

Cato, A. C., P. Skroch, J. Weinmann, P. Butkeraitis and H. Ponta (1988). "DNA sequences outside the receptor-binding sites differently modulate the responsiveness of the mouse mammary tumour virus promoter to various steroid hormones." Embo j **7**(5): 1403-1410.

Chamorro, V., R. Wangensteen, J. Sainz, J. Duarte, F. O'Valle, A. Osuna and F. Vargas (2004). "Protective effects of the angiotensin II type 1 (AT1) receptor blockade in low-renin deoxycorticosterone acetate (DOCA)-treated spontaneously hypertensive rats." Clin Sci (Lond) **106**(3): 251-259.

Chang, R. C., W. M. Skiles, S. S. Chronister, H. Wang, G. I. Sutton, Y. S. Bedi, M. Snyder, C. R. Long and M. C. Golding (2017). "DNA methylation-independent growth restriction and altered developmental programming in a mouse model of preconception male alcohol exposure." Epigenetics **12**(10): 841-853.

Chen, Y. F. (1996). "Sexual dimorphism of hypertension." Curr Opin Nephrol Hypertens **5**(2): 181-185.

Chen, Y. F. and Q. C. Meng (1991). "Sexual dimorphism of blood pressure in spontaneously hypertensive rats is androgen dependent." Life Sci **48**(1): 85-96.

Chen, Y. F., A. J. Naftilan and S. Oparil (1992). "Androgen-dependent angiotensinogen and renin messenger RNA expression in hypertensive rats." Hypertension **19**(5): 456-463.

Cheung, K. L. and R. A. Lafayette (2013). "Renal physiology of pregnancy." Adv Chronic Kidney Dis **20**(3): 209-214.

Chinnathambi, V., M. Balakrishnan, J. Ramadoss, C. Yallampalli and K. Sathishkumar (2013). "Testosterone alters maternal vascular adaptations: role of the endothelial NO system." Hypertension **61**(3): 647-654.

Chinnathambi, V., C. S. Blesson, K. L. Vincent, G. R. Saade, G. D. Hankins, C. Yallampalli and K. Sathishkumar (2014). "Elevated testosterone levels during rat pregnancy cause hypersensitivity to angiotensin II and attenuation of endothelium-dependent vasodilation in uterine arteries." Hypertension **64**(2): 405-414.

Choi, J. W., M. W. Im and S. H. Pai (2002). "Nitric oxide production increases during normal pregnancy and decreases in preeclampsia." Ann Clin Lab Sci **32**(3): 257-263.

Chou, T. M., K. Sudhir, S. J. Hutchison, E. Ko, T. M. Amidon, P. Collins and K. Chatterjee (1996). "Testosterone induces dilation of canine coronary conductance and resistance arteries in vivo." Circulation **94**(10): 2614-2619.

Chute, C. G., J. A. Baron, S. R. Plymate, D. P. Kiel, A. T. Pavia, E. C. Lozner, T. O'Keefe and G. J. MacDonald (1987). "Sex hormones and coronary artery disease." Am J Med **83**(5): 853-859.

Clifton, V. L., M. J. Stark, A. Osei-Kumah and N. A. Hodyl (2012). "Review: The feto-placental unit, pregnancy pathology and impact on long term maternal health." Placenta **33 Suppl**: S37-41.

Conrad, K. P. (1987). "Possible mechanisms for changes in renal hemodynamics during pregnancy: studies from animal models." Am J Kidney Dis **9**(4): 253-259.

Conrad, K. P. (2011). "Maternal vasodilation in pregnancy: the emerging role of relaxin." Am J Physiol Regul Integr Comp Physiol **301**(2): R267-275.

Conrad, K. P. and K. A. Vernier (1989). "Plasma level, urinary excretion, and metabolic production of cGMP during gestation in rats." Am J Physiol **257**(4 Pt 2): R847-853.

Corona, G., L. Vignozzi, A. Sforza and M. Maggi (2013). "Risks and benefits of late onset hypogonadism treatment: an expert opinion." World J Mens Health **31**(2): 103-125.

Costa, E. D., B. A. Rezende, S. F. Cortes and V. S. Lemos (2016). "Neuronal Nitric Oxide Synthase in Vascular Physiology and Diseases." Front Physiol **7**: 206.

Costarella, C. E., J. N. Stallone, G. W. Rutecki and F. C. Whittier (1996). "Testosterone causes direct relaxation of rat thoracic aorta." J Pharmacol Exp Ther **277**(1): 34-39.

Coyotupa, J., A. F. Parlow and N. Kovacic (1973). "Serum Testosterone and Dihydrotestosterone Levels Following Orchiectomy in the Adult Rat." Endocrinology **92**(6): 1579-1581.

Craici, I. M., S. J. Wagner, T. L. Weissgerber, J. P. Grande and V. D. Garovic (2014). "Advances in the pathophysiology of pre-eclampsia and related podocyte injury." Kidney Int **86**(2): 275-285.

Crews, J. K. and R. A. Khalil (1999). "Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca2+ entry mechanisms of coronary vasoconstriction." Arterioscler Thromb Vasc Biol **19**(4): 1034-1040.

Crofton, J. T., L. Share and D. P. Brooks (1989). "Gonadectomy abolishes the sexual dimorphism in DOC-salt hypertension in the rat." Clin Exp Hypertens A **11**(7): 1249-1261.

Cui, L., C. Shu, Z. Liu, W. Tong, M. Cui, C. Wei, J. J. Tang, X. Liu, J. Hu, J. Jiang, J. He, D. Y. Zhang, F. Ye and Y. Li (2018). "The expression of serum sEGFR, sFlt-1, sEndoglin and PLGF in preeclampsia." Pregnancy Hypertens **13**: 127-132.

Curry, F. R. (2005). "Atrial natriuretic peptide: an essential physiological regulator of transvascular fluid, protein transport, and plasma volume." J Clin Invest **115**(6): 1458-1461.

Curtis, J. J., R. G. Luke, H. P. Dustan, M. Kashgarian, J. D. Whelchel, P. Jones and A. G. Diethelm (2000). "Remission of essential hypertension after renal transplantation. 1983." J Am Soc Nephrol **11**(12): 2404-2412.

Dahl, L. K., M. Heine and L. Tassinari (1962). "Role of genetic factors in susceptibility to experimental hypertension due to chronic excess salt ingestion." Nature **194**: 480-482.

Davey, R. A. and M. Grossmann (2016). "Androgen Receptor Structure, Function and Biology: From Bench to Bedside." Clin Biochem Rev **37**(1): 3-15.

Death, A. K., K. C. McGrath, M. A. Sader, S. Nakhla, W. Jessup, D. J. Handelsman and D. S. Celermajer (2004). "Dihydrotestosterone promotes vascular cell adhesion molecule-1 expression in male human endothelial cells via a nuclear factor-kappaB-dependent pathway." Endocrinology **145**(4): 1889-1897.

Deenadayalu, V., Y. Puttabyatappa, A. T. Liu, J. N. Stallone and R. E. White (2012). "Testosterone-induced relaxation of coronary arteries: activation of BKCa channels via the cGMP-dependent protein kinase." Am J Physiol Heart Circ Physiol **302**(1): H115-123.

Deenadayalu, V. P., R. E. White, J. N. Stallone, X. Gao and A. J. Garcia (2001). "Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel." Am J Physiol Heart Circ Physiol **281**(4): H1720-1727.

Delforce, S. J., E. R. Lumbers, S. K. Morosin, Y. Wang and K. G. Pringle (2019). "The Angiotensin II type 1 receptor mediates the effects of low oxygen on early placental angiogenesis." Placenta **75**: 54-61.

Diez d'Aux, R. C. and B. E. Pearson Murphy (1974). "Androgens in the human fetus." J Steroid Biochem **5**(3): 207-210.

Ding, A. Q. and J. N. Stallone (2001). "Testosterone-induced relaxation of rat aorta is androgen structure specific and involves K+ channel activation." J Appl Physiol (1985) **91**(6): 2742-2750.

Dousdampanis, P., K. Trigka, C. Fourtounas and J. M. Bargman (2014). "Role of testosterone in the pathogenesis, progression, prognosis and comorbidity of men with chronic kidney disease." Ther Apher Dial **18**(3): 220-230.

Duckitt, K. and D. Harrington (2005). "Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies." Bmj **330**(7491): 565.

Dunlop, W. (1981). "Serial changes in renal haemodynamics during normal human pregnancy." Br J Obstet Gynaecol **88**(1): 1-9.

Edwards, E., J. Hamilton and S. Duntley (1939). "Testosterone propionate as a therapeutic agent in patients with organic disease of peripheral vessels." New Engl. J. Med. **220**: 865-869.

El-Sayed, A. A. F. (2017). "Preeclampsia: A review of the pathogenesis and possible management strategies based on its pathophysiological derangements." Taiwan J Obstet Gynecol **56**(5): 593-598.

Enatsu, N., H. Miyake, T. Haraguchi, K. Chiba and M. Fujisawa (2016). "Effects of dutasteride on serum free-testosterone and clinical significance of testosterone changes." Andrologia **48**(10): 1195-1201.

English, K. M., R. D. Jones, T. H. Jones, A. H. Morice and K. S. Channer (2000). "Aging reduces the responsiveness of coronary arteries from male Wistar rats to the vasodilatory action of testosterone." Clin Sci (Lond) **99**(1): 77-82.

English, K. M., R. D. Jones, T. H. Jones, A. H. Morice and K. S. Channer (2002). "Testosterone acts as a coronary vasodilator by a calcium antagonistic action." J Endocrinol Invest **25**(5): 455-458.

Escobar, J. C., S. S. Patel, V. E. Beshay, T. Suzuki and B. R. Carr (2011). "The human placenta expresses CYP17 and generates androgens de novo." J Clin Endocrinol Metab **96**(5): 1385-1392.

Esplin, M. S., M. B. Fausett, A. Fraser, R. Kerber, G. Mineau, J. Carrillo and M. W. Varner (2001). "Paternal and maternal components of the predisposition to preeclampsia." N Engl J Med **344**(12): 867-872.

Estrada, M., A. Varshney and B. E. Ehrlich (2006). "Elevated testosterone induces apoptosis in neuronal cells." J Biol Chem **281**(35): 25492-25501.

Facemire, C. S., A. B. Nixon, R. Griffiths, H. Hurwitz and T. M. Coffman (2009). "Vascular endothelial growth factor receptor 2 controls blood pressure by regulating nitric oxide synthase expression." Hypertension **54**(3): 652-658.

Falkenstein, E., H. C. Tillmann, M. Christ, M. Feuring and M. Wehling (2000). "Multiple actions of steroid hormones--a focus on rapid, nongenomic effects." Pharmacol Rev **52**(4): 513-556.

Farhat, M. Y., M. C. Lavigne and P. W. Ramwell (1996). "The vascular protective effects of estrogen." Faseb j **10**(5): 615-624.

Feletou, M. (2011). Integrated Systems Physiology: from Molecule to Function to Disease. The Endothelium: Part 1: Multiple Functions of the Endothelial Cells-Focus on Endothelium-Derived Vasoactive Mediators. San Rafael (CA), Morgan & Claypool Life Sciences

Finkle, W. D., S. Greenland, G. K. Ridgeway, J. L. Adams, M. A. Frasco, M. B. Cook, J. F. Fraumeni, Jr. and R. N. Hoover (2014). "Increased risk of non-fatal myocardial infarction following testosterone therapy prescription in men." PLoS One **9**(1): e85805.

Forstermann, U. (2010). "Nitric oxide and oxidative stress in vascular disease." Pflugers Arch **459**(6): 923-939.

Forstermann, U., E. I. Closs, J. S. Pollock, M. Nakane, P. Schwarz, I. Gath and H. Kleinert (1994). "Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions." Hypertension **23**(6 Pt 2): 1121-1131.

Francomano, D., R. Bruzziches, G. Barbaro, A. Lenzi and A. Aversa (2014). "Effects of testosterone undecanoate replacement and withdrawal on cardio-metabolic, hormonal and body composition outcomes in severely obese hypogonadal men: a pilot study." J Endocrinol Invest **37**(4): 401-411.

Francomano, D., G. Fattorini, D. Gianfrilli, D. Paoli, P. Sgro, A. Radicioni, F. Romanelli, L. Di Luigi, L. Gandini, A. Lenzi and A. Aversa (2016). "Acute endothelial response to testosterone gel administration in men with severe hypogonadism and its relationship to androgen receptor polymorphism: a pilot study." J Endocrinol Invest **39**(3): 265-271.

Fryar, C. D., Y. Ostchega, C. M. Hales, G. Zhang and D. Kruszon-Moran (2017). "Hypertension Prevalence and Control Among Adults: United States, 2015-2016." NCHS Data Brief(289): 1-8.

Furukawa, K., Y. Tawada and M. J. J. o. B. C. Shigekawa (1988). "Regulation of the plasma membrane Ca2+ pump by cyclic nucleotides in cultured vascular smooth muscle cells." **263**(17): 8058-8065.

Gagliano-Juca, T. and S. Basaria (2018). "Trials of testosterone replacement reporting cardiovascular adverse events." Asian J Androl **20**(2): 131-137.

Gant, N. F., G. L. Daley, S. Chand, P. J. Whalley and P. C. MacDonald (1973). "A study of angiotensin II pressor response throughout primigravid pregnancy." J Clin Invest **52**(11): 2682-2689.

Gant, N. F., R. J. Worley, R. B. Everett and P. C. MacDonald (1980). "Control of vascular responsiveness during human pregnancy." Kidney Int **18**(2): 253-258.

Ghayee, H. K. and R. J. Auchus (2007). "Basic concepts and recent developments in human steroid hormone biosynthesis." Rev Endocr Metab Disord **8**(4): 289-300.

Gillis, E. E., J. M. Williams, M. R. Garrett, J. N. Mooney and J. M. Sasser (2015). "The Dahl salt-sensitive rat is a spontaneous model of superimposed preeclampsia." Am J Physiol Regul Integr Comp Physiol **309**(1): R62-70.

Goglia, L., V. Tosi, A. M. Sanchez, M. I. Flamini, X. D. Fu, S. Zullino, A. R. Genazzani and T. Simoncini (2010). "Endothelial regulation of eNOS, PAI-1 and t-PA by testosterone and dihydrotestosterone in vitro and in vivo." Mol Hum Reprod **16**(10): 761-769.

Goldblatt, H., J. Lynch, R. F. Hanzal and W. W. Summerville (1934). "STUDIES ON EXPERIMENTAL HYPERTENSION : I. THE PRODUCTION OF PERSISTENT ELEVATION OF SYSTOLIC BLOOD PRESSURE BY MEANS OF RENAL ISCHEMIA." J Exp Med **59**(3): 347-379.

Gonzalez-Montelongo, M. C., R. Marin, J. A. Perez, T. Gomez and M. Diaz (2013). "Polyamines transduce the nongenomic, androgen-induced calcium sensitization in intestinal smooth muscle." Mol Endocrinol **27**(10): 1603-1616.

Gonzalez, C., H. Rosas-Hernandez, B. Jurado-Manzano, M. A. Ramirez-Lee, S. Salazar-Garcia, P. P. Martinez-Cuevas, A. J. Velarde-Salcedo, H. Morales-Loredo, R. Espinosa-Tanguma, S. F. Ali and R. Rubio (2015). "The prolactin family hormones regulate vascular tone through NO and prostacyclin production in isolated rat aortic rings." Acta Pharmacol Sin **36**(5): 572-586.

Gorczynska, E. and D. J. Handelsman (1995). "Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells." Endocrinology **136**(5): 2052-2059.

Haddad, R. M., C. C. Kennedy, S. M. Caples, M. J. Tracz, E. R. Bolona, K. Sideras, M. V. Uraga, P. J. Erwin and V. M. Montori (2007). "Testosterone and cardiovascular risk in men: a systematic review and meta-analysis of randomized placebo-controlled trials." Mayo Clin Proc **82**(1): 29-39.

Hall, J., R. D. Jones, T. H. Jones, K. S. Channer and C. Peers (2006). "Selective inhibition of L-type Ca2+ channels in A7r5 cells by physiological levels of testosterone." Endocrinology **147**(6): 2675-2680.

Hamm, L. (1942). "Testosterone propionate in the treatment of angina pectoris." J Clin Endocrinol **2**: 325-328.

Han, D. H., M. R. Chae, J. H. Jung, I. So, J. K. Park and S. W. Lee (2008). "Effect of testosterone on potassium channel opening in human corporal smooth muscle cells." J Sex Med **5**(4): 822-832.

Hansson, N., F. Moll, D. Schultheiss and M. Krischel (2016). "Remembering Charles B. Huggins' Nobel Prize for Hormonal Treatment of Prostatic Cancer at its 50th Anniversary." Eur Urol **69**(6): 971-972.

Harman, S. M., E. J. Metter, J. D. Tobin, J. Pearson and M. R. Blackman (2001). "Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging." J Clin Endocrinol Metab **86**(2): 724-731.

Hefler, L. A., C. B. Tempfer, R. M. Moreno, W. E. O'Brien and A. R. Gregg (2001). "Endothelial-derived nitric oxide and angiotensinogen: blood pressure and metabolism during mouse pregnancy." Am J Physiol Regul Integr Comp Physiol **280**(1): R174-182.

Heinlein, C. A. and C. Chang (2002). "The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions." Mol Endocrinol **16**(10): 2181-2187.

Higgins, S. J., J. M. Burchell and W. I. Mainwaring (1976). "Testosterone control of nucleic acid content and proliferation of epithelium and stroma in rat seminal vesicles." Biochem J **160**(1): 43-48.

Hill-Eubanks, D. C., M. E. Werner, T. J. Heppner and M. T. Nelson (2011). "Calcium signaling in smooth muscle." Cold Spring Harb Perspect Biol **3**(9): a004549.

Hirata, M., K. P. Kohse, C. Chang, T. Ikebe and F. J. J. o. B. C. Murad (1990). "Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells." **265**(3): 1268-1273.

Holmang, A. and P. Bjorntorp (1992). "The effects of testosterone on insulin sensitivity in male rats." Acta Physiol Scand **146**(4): 505-510.

Honda, H., T. Unemoto and H. Kogo (1999). "Different mechanisms for testosterone-induced relaxation of aorta between normotensive and spontaneously hypertensive rats." Hypertension **34**(6): 1232-1236.

Hong, S. K., G. E. Min, S. B. Ha, S. H. Doo, M. Y. Kang, H. J. Park, C. Y. Yoon, S. J. Jeong, S. S. Byun and S. E. Lee (2010). "Effect of the dual 5alpha-reductase inhibitor, dutasteride, on serum testosterone and body mass index in men with benign prostatic hyperplasia." BJU Int **105**(7): 970-974.

Hotta, Y., T. Kataoka and K. Kimura (2019). "Testosterone Deficiency and Endothelial Dysfunction: Nitric Oxide, Asymmetric Dimethylarginine, and Endothelial Progenitor Cells." Sex Med Rev **7**(4): 661-668.

Hsu, T. Y., K. C. Lan, C. C. Tsai, C. Y. Ou, B. H. Cheng, M. Y. Tsai, H. Y. Kang, Y. H. Tung, Y. H. Wong and K. E. Huang (2009). "Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies." Taiwan J Obstet Gynecol **48**(3): 262-267.

Huang, A., D. Sun, E. G. Shesely, E. M. Levee, A. Koller and G. Kaley (2002). "Neuronal NOS-dependent dilation to flow in coronary arteries of male eNOS-KO mice." Am J Physiol Heart Circ Physiol **282**(2): H429-436.

Huggins, C., Hodges, CV (1941). "Studies on prostatic cancer, I: the effect of castration, of estrogen and of androgen injection on serum phosphateses in metastatic carcinoma of the prostate." Cancer Res **1**: 293-297.

Hunter, R. H., B. Cook and N. L. Poyser (1983). "Regulation of oviduct function in pigs by local transfer of ovarian steroids and prostaglandins: a mechanism to influence sperm transport." Eur J Obstet Gynecol Reprod Biol **14**(4): 225-232.

Ichihara, A., E. W. Inscho, J. D. Imig and L. G. Navar (1998). "Neuronal nitric oxide synthase modulates rat renal microvascular function." Am J Physiol **274**(3): F516-524.

Inscho, E. W., J. D. Imig, P. C. Deichmann and A. K. Cook (1999). "Candesartan cilexetil protects against loss of autoregulatory efficiency in angiotensin II-infused rats." J Am Soc Nephrol **10 Suppl 11**: S178-183.

Ishimaru, T., W. A. Edmiston, L. Pages and R. Horton (1978). "Splanchnic extraction and conversion of testosterone and dihydrotestosterone in man." J Clin Endocrinol Metab **46**(4): 528-533.

Isidoro, L., M. Ferrer and M. Perusquia (2018). "Vasoactive androgens: Vasorelaxing effects and their potential regulation of blood pressure." Endocr Res **43**(3): 166-175.

Jeyabalan, A. (2013). "Epidemiology of preeclampsia: impact of obesity." Nutr Rev **71 Suppl 1**(0 1): S18-25.

Jones, R. D., K. M. English, P. J. Pugh, A. H. Morice, T. H. Jones and K. S. Channer (2002). "Pulmonary vasodilatory action of testosterone: evidence of a calcium antagonistic action." J Cardiovasc Pharmacol **39**(6): 814-823.

Jones, R. D., P. J. Pugh, T. H. Jones and K. S. Channer (2003). "The vasodilatory action of testosterone: a potassium-channel opening or a calcium antagonistic action?" Br J Pharmacol **138**(5): 733-744.

Joyner, M. J. and J. K. Limberg (2014). "Blood pressure regulation: every adaptation is an integration?" Eur J Appl Physiol **114**(3): 445-450.

Kashiwagi, B., Y. Shibata, Y. Ono, K. Suzuki, S. Honma and H. Yamanaka (2005). "Effect of androgen concentration on seminal vesicle blood flow in rats-establishment of new highly sensitive simultaneous androgen measurement method." Urology **66**(1): 218-223.

Keating, N. L., A. J. O'Malley, S. J. Freedland and M. R. Smith (2010). "Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer." J Natl Cancer Inst **102**(1): 39-46.

Kelly, D. M. and T. H. Jones (2013). "Testosterone: a metabolic hormone in health and disease." J Endocrinol **217**(3): R25-45.

Kelly, D. M. and T. H. Jones (2013). "Testosterone: a vascular hormone in health and disease." J Endocrinol **217**(3): R47-71.

Kienitz, T. and M. Quinkler (2008). "Testosterone and blood pressure regulation." Kidney Blood Press Res **31**(2): 71-79.

Kobayashi, K., T. Murata, M. Hori and H. Ozaki (2011). "Prostaglandin E2-prostanoid EP3 signal induces vascular contraction via nPKC and ROCK activation in rat mesenteric artery." Eur J Pharmacol **660**(2-3): 375-380.

Kobori, H., A. Nishiyama, Y. Abe and L. G. Navar (2003). "Enhancement of intrarenal angiotensinogen in Dahl salt-sensitive rats on high salt diet." Hypertension **41**(3): 592-597.

Kobori, H., M. C. Prieto-Carrasquero, Y. Ozawa and L. G. Navar (2004). "AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension." Hypertension **43**(5): 1126-1132.

Korthuis, R. (2011). Regulation of Vascular Tone in Skeletal Muscle. S. M. C. S. R. (CA). Ca, Morgan & Claypool Life Sciences.

Kovac, J. R., A. W. Pastuszak, D. J. Lamb and L. I. Lipshultz (2014). "Testosterone supplementation therapy in the treatment of patients with metabolic syndrome." Postgrad Med **126**(7): 149-156.

Kovacs, C. S. (2000). Calcium and Phosphate Metabolism and Related Disorders During Pregnancy and Lactation. Endotext. K. R. Feingold, B. Anawalt, A. Boyce et al. South Dartmouth (MA), MDText.com, Inc.

Ku, D. D., J. K. Zaleski, S. Liu and T. A. Brock (1993). "Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries." Am J Physiol **265**(2 Pt 2): H586-592.

Kucukdurmaz, F., E. Efe, E. B. Kurutas, C. Olmez, M. Temizer and S. Resim (2018). "Losartan treatment significantly attenuates the fibrotic changes in the corpus cavernosum of castrated rats." Rev Int Androl.

Kumai, T., M. Tanaka, M. Watanabe, C. Matsumoto and S. Kobayashi (1994). "Possible involvement of androgen in increased norepinephrine synthesis in blood vessels of spontaneously hypertensive rats." Jpn J Pharmacol **66**(4): 439-444.

Kumai, T., M. Tanaka, M. Watanabe, H. Nakura and S. Kobayashi (1995). "Influence of androgen on tyrosine hydroxylase mRNA in adrenal medulla of spontaneously hypertensive rats." Hypertension **26**(1): 208-212.

Kumar, N., A. Crozat, F. Li, J. F. Catterall, C. W. Bardin and K. Sundaram (1999). "7alpha-methyl-19-nortestosterone, a synthetic androgen with high potency: structure-activity comparisons with other androgens." J Steroid Biochem Mol Biol **71**(5-6): 213-222.

Kumar, S., G. H. Gordon, D. H. Abbott and J. S. Mishra (2018). "Androgens in maternal vascular and placental function: implications for preeclampsia pathogenesis." Reproduction **156**(5): R155-r167.

Kurniawan, A. L., C. Y. Hsu, H. H. Rau, L. Y. Lin and J. C. Chao (2019). "Dietary patterns in relation to testosterone levels and severity of impaired kidney function among middle-aged and elderly men in Taiwan: a cross-sectional study." Nutr J **18**(1): 42.

Lahera, V., J. Salazar, M. G. Salom and J. C. J. J. o. h. S. o. j. o. t. I. S. o. H. Romero (1992). "Deficient production of nitric oxide induces volume-dependent hypertension." **10**(7): S173-177.

Landmesser, U., B. Hornig and H. Drexler (2004). "Endothelial function: a critical determinant in atherosclerosis?" Circulation **109**(21 Suppl 1): Ii27-33.

Leffler, C. W., J. R. Hessler, R. S. Green and A. M. Fletcher (1986). "Effects of sodium chloride on pregnant sheep with reduced uteroplacental perfusion pressure." Hypertension **8**(1): 62-65.

Leong, X. F., C. Y. Ng and K. Jaarin (2015). "Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis." Biomed Res Int **2015**: 528757.

Lerman, L. O., A. R. Chade, V. Sica and C. Napoli (2005). "Animal models of hypertension: an overview." J Lab Clin Med **146**(3): 160-173.

Lerman, L. O., T. W. Kurtz, R. M. Touyz, D. H. Ellison, A. R. Chade, S. D. Crowley, D. L. Mattson, J. J. Mullins, J. Osborn, A. Eirin, J. F. Reckelhoff, C. Iadecola and T. M. Coffman (2019). "Animal Models of Hypertension: A Scientific Statement From the American Heart Association." Hypertension **73**(6): e87-e120.

Lesser, M. A. (1946). "Testosterone propionate therapy in one hundred cases of angina pectoris." J Clin Endocrinol **6**: 549-557.

Li, J., B. LaMarca and J. F. Reckelhoff (2012). "A model of preeclampsia in rats: the reduced uterine perfusion pressure (RUPP) model." Am J Physiol Heart Circ Physiol **303**(1): H1-8.

Li, M. and J. N. Stallone (2005). "Estrogen potentiates vasopressin-induced contraction of female rat aorta by enhancing cyclooxygenase-2 and thromboxane function." Am J Physiol Heart Circ Physiol **289**(4): H1542-1550.

Lieberherr, M. and B. Grosse (1994). "Androgens increase intracellular calcium concentration and inositol 1,4,5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein." J Biol Chem **269**(10): 7217-7223.

Liu, P. Y., A. K. Death and D. J. Handelsman (2003). "Androgens and cardiovascular disease." Endocr Rev **24**(3): 313-340.

Lloyd-Jones, D., R. J. Adams, T. M. Brown, M. Carnethon, S. Dai, G. De Simone, T. B. Ferguson, E. Ford, K. Furie, C. Gillespie, A. Go, K. Greenlund, N. Haase, S. Hailpern, P. M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lackland, L. Lisabeth, A. Marelli, M. M. McDermott, J. Meigs, D. Mozaffarian, M. Mussolino, G. Nichol, V. L. Roger, W. Rosamond, R. Sacco, P. Sorlie, V. L. Roger, T. Thom, S. Wasserthiel-Smoller, N. D. Wong and J. Wylie-Rosett (2010). "Heart disease and stroke statistics--2010 update: a report from the American Heart Association." Circulation **121**(7): e46-e215.

Loh, S. Y., N. Giribabu, K. Gholami and N. Salleh (2017). "Effects of testosterone on mean arterial pressure and aquaporin (AQP)-1, 2, 3, 4, 6 and 7 expressions in the kidney of orchidectomized, adult male Sprague-Dawley rats." Arch Biochem Biophys **614**: 41-49.

Lu, D. and G. S. Kassab (2011). "Role of shear stress and stretch in vascular mechanobiology." J R Soc Interface **8**(63): 1379-1385.

Lucas-Herald, A. K., R. Alves-Lopes, A. C. Montezano, S. F. Ahmed and R. M. Touyz (2017). "Genomic and non-genomic effects of androgens in the cardiovascular system: clinical implications." Clin Sci (Lond) **131**(13): 1405-1418.

Lunell, N. O., R. Lewander, I. Mamoun, L. Nylund, S. Sarby and S. Thornstrom (1984). "Uteroplacental blood flow in pregnancy induced hypertension." Scand J Clin Lab Invest Suppl **169**: 28-35.

Ma, R., S. S. Jiang, X. M. Cheng, J. B. Gong, Q. G. Zhang and Q. S. Lin (2009). "[Testosterone at physiological level inhibits PGF2alpha-induced increase in intracellular Ca2+ in cultured vascular smooth muscle cells]." Zhonghua Nan Ke Xue **15**(4): 326-330.

Magness, R. R., K. Osei-Boaten, M. D. Mitchell and C. R. Rosenfeld (1985). "In vitro prostacyclin production by ovine uterine and systemic arteries. Effects of angiotensin II." J Clin Invest **76**(6): 2206-2212.

Magness, R. R. and C. R. Rosenfeld (1989). "Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation." Am J Physiol **256**(4 Pt 1): E536-542.

Makieva, S., P. T. Saunders and J. E. Norman (2014). "Androgens in pregnancy: roles in parturition." Hum Reprod Update **20**(4): 542-559.

Malkin, C. J., R. D. Jones, T. H. Jones and K. S. Channer (2006). "Effect of testosterone on ex vivo vascular reactivity in man." Clin Sci (Lond) **111**(4): 265-274.

Malkin, C. J., P. J. Pugh, P. D. Morris, S. Asif, T. H. Jones and K. S. Channer (2010). "Low serum testosterone and increased mortality in men with coronary heart disease." Heart **96**(22): 1821-1825.

Marin, P., S. Holmang, L. Jonsson, L. Sjostrom, H. Kvist, G. Holm, G. Lindstedt and P. Bjorntorp (1992). "The effects of testosterone treatment on body composition and metabolism in middle-aged obese men." Int J Obes Relat Metab Disord **16**(12): 991-997.

Marrachelli, V. G., F. J. Miranda, J. M. Centeno, M. C. Burguete, M. Castello-Ruiz, T. Jover-Mengual, A. M. Perez, J. B. Salom, G. Torregrosa and E. Alborch (2010). "Mechanisms involved in the relaxant action of testosterone in the renal artery from male normoglycemic and diabetic rabbits." Pharmacol Res **61**(2): 149-156.

Martini, L., F. Celotti, M. J. Lechuga, R. C. Melcangi, M. Motta, P. Negri-Cesi, A. Poletti and S. Zoppi (1990). "Androgen metabolism in different target tissues." Ann N Y Acad Sci **595**: 184-198.

McClamrock, H. D. and E. Y. Adashi (1992). "Gestational hyperandrogenism." Fertil Steril **57**(2): 257-274.

McGrath, K. C., L. S. McRobb and A. K. Heather (2008). "Androgen therapy and atherosclerotic cardiovascular disease." Vasc Health Risk Manag **4**(1): 11-21.

McLaughlin, M. K. and K. P. Conrad (1995). "Nitric oxide biosynthesis during pregnancy: implications for circulatory changes." Clin Exp Pharmacol Physiol **22**(2): 164-171.

Mei, C. C., J. Zhang and H. X. Jing (2018). "Fluid mechanics of Windkessel effect." Med Biol Eng Comput **56**(8): 1357-1366.

Meininger, G. A. and M. J. Davis (1992). "Cellular mechanisms involved in the vascular myogenic response." Am J Physiol **263**(3 Pt 2): H647-659.

Mendelsohn, M. E. (2002). "Genomic and nongenomic effects of estrogen in the vasculature." Am J Cardiol **90**(1a): 3f-6f.

Mendelsohn, M. E. and R. H. Karas (1994). "Estrogen and the blood vessel wall." Curr Opin Cardiol **9**(5): 619-626.

Mendoza, S. G., A. Zerpa, H. Carrasco, O. Colmenares, A. Rangel, P. S. Gartside and M. L. Kashyap (1983). "Estradiol, testosterone, apolipoproteins, lipoprotein cholesterol, and lipolytic enzymes in men with premature myocardial infarction and angiographically assessed coronary occlusion." Artery **12**(1): 1-23.

Meng, F., Y. Zhao, B. Wang, B. Li, Y. Sheng, M. Liu, H. Li and R. Xiu (2018). "Endothelial Cells Promote Calcification in Aortic Smooth Muscle Cells from Spontaneously Hypertensive Rats." Cell Physiol Biochem **49**(6): 2371-2381.

Merai, R., C. Siegel, M. Rakotz, P. Basch, J. Wright, B. Wong and P. Thorpe (2016). "CDC Grand Rounds: A Public Health Approach to Detect and Control Hypertension." MMWR Morb Mortal Wkly Rep **65**(45): 1261-1264.

Millar-Craig, M. W., C. N. Bishop and E. B. Raftery (1978). "Circadian variation of blood-pressure." Lancet **1**(8068): 795-797.

Miller, V. M., J. R. Kaplan, N. J. Schork, P. Ouyang, S. L. Berga, N. K. Wenger, L. J. Shaw, R. C. Webb, M. Mallampalli, M. Steiner, D. A. Taylor, C. N. Merz and J. F.

Reckelhoff (2011). "Strategies and methods to study sex differences in cardiovascular structure and function: a guide for basic scientists." Biol Sex Differ **2**: 14.

Mishra, J. S., G. D. Hankins and S. Kumar (2016). "Testosterone downregulates angiotensin II type-2 receptor via androgen receptor-mediated ERK1/2 MAP kinase pathway in rat aorta." J Renin Angiotensin Aldosterone Syst **17**(4).

Mohr, B. A., A. T. Guay, A. B. O'Donnell and J. B. McKinlay (2005). "Normal, bound and nonbound testosterone levels in normally ageing men: results from the Massachusetts Male Ageing Study." Clin Endocrinol (Oxf) **62**(1): 64-73.

Molinari, C., A. Battaglia, E. Grossini, D. A. Mary, C. Vassanelli and G. Vacca (2002). "The effect of testosterone on regional blood flow in prepubertal anaesthetized pigs." J Physiol **543**(Pt 1): 365-372.

Molnar, M. and F. Hertelendy (1992). "N omega-nitro-L-arginine, an inhibitor of nitric oxide synthesis, increases blood pressure in rats and reverses the pregnancy-induced refractoriness to vasopressor agents." Am J Obstet Gynecol **166**(5): 1560-1567.

Moncada, S. and E. A. Higgs (1995). "Molecular mechanisms and therapeutic strategies related to nitric oxide." Faseb j **9**(13): 1319-1330.

Montano, L. M., J. Espinoza, E. Flores-Soto, J. Chavez and M. Perusquia (2014). "Androgens are bronchoactive drugs that act by relaxing airway smooth muscle and preventing bronchospasm." J Endocrinol **222**(1): 1-13.

Morgentaler, A. (2017). "Testosterone Therapy in a Man with Intermediate-risk Prostate Cancer: Pro." Eur Urol Focus **3**(4-5): 316-318.

Morgentaler, A. (2018). "Testosterone therapy and medical hysteria." Nature Reviews Urology **15**(11): 659-660.

Morgentaler, A., M. M. Miner, M. Caliber, A. T. Guay, M. Khera and A. M. Traish (2015). "Testosterone therapy and cardiovascular risk: advances and controversies." Mayo Clin Proc **90**(2): 224-251.

Morgentaler, A. and A. Traish (2018). "The History of Testosterone and the Evolution of its Therapeutic Potential." Sex Med Rev.

Moskovic, D. J., A. B. Araujo, L. I. Lipshultz and M. Khera (2013). "The 20-year public health impact and direct cost of testosterone deficiency in U.S. men." J Sex Med **10**(2): 562-569.

Mount, P. F., B. E. Kemp and D. A. Power (2007). "Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation." J Mol Cell Cardiol **42**(2): 271-279.

Murphy, J. G. and R. A. Khalil (1999). "Decreased [Ca(2+)](i) during inhibition of coronary smooth muscle contraction by 17beta-estradiol, progesterone, and testosterone." J Pharmacol Exp Ther **291**(1): 44-52.

Navar, L. G., K. D. Mitchell, L. M. Harrison-Bernard, H. Kobori and A. Nishiyama (2001). "Review: Intrarenal angiotensin II levels in normal and hypertensive states." J Renin Angiotensin Aldosterone Syst **2**(1_suppl): S176-s184.

Navarro, G., C. Allard, W. Xu and F. Mauvais-Jarvis (2015). "The role of androgens in metabolism, obesity, and diabetes in males and females." Obesity (Silver Spring) **23**(4): 713-719.

Nelson, S. H., O. S. Steinsland, M. S. Suresh and N. M. Lee (1998). "Pregnancy augments nitric oxide-dependent dilator response to acetylcholine in the human uterine artery." Hum Reprod **13**(5): 1361-1367.

Newbern, D. and M. Freemark (2011). "Placental hormones and the control of maternal metabolism and fetal growth." Curr Opin Endocrinol Diabetes Obes **18**(6): 409-416.

Nieschlag, E. and S. Nieschlag (2019). "ENDOCRINE HISTORY: The history of discovery, synthesis and development of testosterone for clinical use." Eur J Endocrinol **180**(6): R201-r212.

Norman, A. W., M. T. Mizwicki and D. P. Norman (2004). "Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model." Nat Rev Drug Discov **3**(1): 27-41.

Nosarev, A. V., L. V. Smagliy, Y. Anfinogenova, S. V. Popov and L. V. Kapilevich (2014). "Exercise and NO production: relevance and implications in the cardiopulmonary system." Front Cell Dev Biol **2**: 73.

Nunes, V. W., Z. B. Fortes, D. Nigro, M. H. Carvalho, T. M. Zorn and R. Scivoletto (2000). "Influence of enalapril on the endothelial function of DOCA-salt hypertensive rats." Gen Pharmacol **34**(2): 117-125.

Oloyo, A. K., O. A. Sofola and M. A. Yakubu (2016). "Orchidectomy attenuates high-salt diet-induced increases in blood pressure, renovascular resistance, and hind limb vascular dysfunction: role of testosterone." Clin Exp Pharmacol Physiol **43**(9): 825-833.

Ong, P. J., G. Patrizi, W. C. Chong, C. M. Webb, C. S. Hayward and P. Collins (2000). "Testosterone enhances flow-mediated brachial artery reactivity in men with coronary artery disease." Am J Cardiol **85**(2): 269-272.

Osol, G. and M. Mandala (2009). "Maternal uterine vascular remodeling during pregnancy." Physiology (Bethesda) **24**: 58-71.

Ounsted, M., V. Moar and W. A. Scott (1981). "Perinatal morbidity and mortality in small-for-dates babies: the relative importance of some maternal factors." Early Hum Dev **5**(4): 367-375.

Pal, E., L. Hadjadj, Z. Fontanyi, A. Monori-Kiss, N. Lippai, E. M. Horvath, A. Magyar, E. Horvath, E. Monos, G. L. Nadasy, Z. Benyo and S. Varbiro (2019). "Gender, hyperandrogenism and vitamin D deficiency related functional and morphological alterations of rat cerebral arteries." PLoS One **14**(5): e0216951.

Pariente, G., P. Shwarzman, B. Aricha-Tamir, A. Y. Weintraub and R. Hershkovitz (2013). "Association between first trimester vaginal bleeding and uterine artery Doppler measured at second and third trimesters of pregnancy." J Matern Fetal Neonatal Med **26**(17): 1724-1727.

Parikh, N. I., M. Norberg, E. Ingelsson, S. Cnattingius, R. S. Vasan, M. Domellof, J. H. Jansson and A. K. Edstedt Bonamy (2017). "Association of Pregnancy Complications and Characteristics With Future Risk of Elevated Blood Pressure: The Vasterbotten Intervention Program." Hypertension **69**(3): 475-483.

Park, J. E., H. H. Chen, J. Winer, K. A. Houck and N. Ferrara (1994). "Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR." J Biol Chem **269**(41): 25646-25654.

Perusquia, M., D. Contreras and N. Herrera (2019). "Hypotestosteronemia is an important factor for the development of hypertension: elevated blood pressure in orchidectomized conscious rats is reversed by different androgens." Endocrine **65**(2): 416-425.

Perusquia, M., J. Espinoza, L. M. Montano and J. N. Stallone (2012). "Regional differences in the vasorelaxing effects of testosterone and its 5-reduced metabolites in the canine vasculature." Vascul Pharmacol **56**(3-4): 176-182.

Perusquia, M., C. D. Greenway, L. M. Perkins and J. N. Stallone (2015). "Systemic hypotensive effects of testosterone are androgen structure-specific and neuronal nitric oxide synthase-dependent." Am J Physiol Regul Integr Comp Physiol **309**(2): R189-195.

Perusquia, M., A. E. Hanson, C. M. Meza, C. Kubli, N. Herrera and J. N. Stallone (2018). "Antihypertensive responses of vasoactive androgens in an in vivo experimental model of preeclampsia." J Steroid Biochem Mol Biol **178**: 65-72.

Perusquia, M., R. Hernandez, M. A. Morales, M. G. Campos and C. M. Villalon (1996). "Role of endothelium in the vasodilating effect of progestins and androgens on the rat thoracic aorta." Gen Pharmacol **27**(1): 181-185.

Perusquia, M., N. Herrera, M. Ferrer and J. N. Stallone (2017). "Antihypertensive effects of androgens in conscious, spontaneously hypertensive rats." J Steroid Biochem Mol Biol **167**: 106-114.

Perusquia, M., E. Navarrete, L. Gonzalez and C. M. Villalon (2007). "The modulatory role of androgens and progestins in the induction of vasorelaxation in human umbilical artery." Life Sci **81**(12): 993-1002.

Perusquia, M., E. Navarrete, J. Jasso-Kamel and L. M. Montano (2005). "Androgens induce relaxation of contractile activity in pregnant human myometrium at term: a nongenomic action on L-type calcium channels." Biol Reprod **73**(2): 214-221.

Perusquia, M. and J. N. Stallone (2010). "Do androgens play a beneficial role in the regulation of vascular tone? Nongenomic vascular effects of testosterone metabolites." Am J Physiol Heart Circ Physiol **298**(5): H1301-1307.

Phillips, G. B., B. H. Pinkernell, T.-Y. J. A. Jing and t. a. j. o. v. biology (1994). "The association of hypotestosteronemia with coronary artery disease in men." **14**(5): 701-706.

Pijnenborg, R., L. Vercruysse, L. Verbist and F. A. Van Assche (1998). "Interaction of interstitial trophoblast with placental bed capillaries and venules of normotensive and pre-eclamptic pregnancies." Placenta **19**(8): 569-575.

Pinto, Y. M., M. Paul and D. Ganten (1998). "Lessons from rat models of hypertension: from Goldblatt to genetic engineering." Cardiovasc Res **39**(1): 77-88.

Puschett, J. B., E. Agunanne and M. N. Uddin (2010). "Marinobufagenin, resibufogenin and preeclampsia." Biochim Biophys Acta **1802**(12): 1246-1253.

Putra, M., M. M. Balasooriya, A. L. Boscia, E. Dalkiran and R. J. Sokol (2019). "The Impact of the New Hypertension Guidelines to Low-Dose Aspirin Prophylaxis Eligibility for the Prevention of Preeclampsia: A Cost-Benefit Analysis." Am J Perinatol.

Puttabyatappa, Y., J. N. Stallone, A. Ergul, A. B. El-Remessy, S. Kumar, S. Black, M. Johnson, M. P. Owen and R. E. White (2013). "Peroxynitrite mediates testosterone-induced vasodilation of microvascular resistance vessels." J Pharmacol Exp Ther **345**(1): 7-14.

Quan, A., S. Chakravarty, J. K. Chen, J. C. Chen, S. Loleh, N. Saini, R. C. Harris, J. Capdevila and R. Quigley (2004). "Androgens augment proximal tubule transport." Am J Physiol Renal Physiol **287**(3): F452-459.

Quinkler, M., S. Diederich, V. Bahr and W. Oelkers (2004). "The role of progesterone metabolism and androgen synthesis in renal blood pressure regulation." Horm Metab Res **36**(6): 381-386.

Ramirez-Rosas, M. B., L. E. Cobos-Puc, E. Munoz-Islas, A. Gonzalez-Hernandez, A. Sanchez-Lopez, C. M. Villalon, A. Maassenvandenbrink and D. Centurion (2011).

"Pharmacological evidence that Ca(2)+ channels and, to a lesser extent, K+ channels mediate the relaxation of testosterone in the canine basilar artery." Steroids **76**(4): 409-415.

Rana, S., E. Lemoine, J. Granger and S. A. Karumanchi (2019). "Preeclampsia." Circ Res **124**(7): 1094-1112.

Reckelhoff, J. F. (2001). "Gender differences in the regulation of blood pressure." Hypertension **37**(5): 1199-1208.

Reckelhoff, J. F. (2018). "Gender differences in hypertension." Curr Opin Nephrol Hypertens **27**(3): 176-181.

Reckelhoff, J. F. and W. K. Samson (2015). "Sex and gender differences in cardiovascular, renal and metabolic diseases." Am J Physiol Regul Integr Comp Physiol **309**(9): R1057-1059.

Reckelhoff, J. F., H. Zhang and J. P. Granger (1998). "Testosterone exacerbates hypertension and reduces pressure-natriuresis in male spontaneously hypertensive rats." Hypertension **31**(1 Pt 2): 435-439.

Reckelhoff, J. F., H. Zhang and K. Srivastava (2000). "Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system." Hypertension **35**(1 Pt 2): 480-483.

Reckelhoff, J. F., H. Zhang, K. Srivastava and J. P. Granger (1999). "Gender differences in hypertension in spontaneously hypertensive rats: role of androgens and androgen receptor." Hypertension **34**(4 Pt 2): 920-923.

Reshef Tal, M. D. P. D., Hugh S Taylor, M.D., Richard O Burney, M.D., M.Sc., Stephen B Mooney, M.D., and Linda C Giudice, M.D., Ph.D. (2015). "Endocrinology of Pregnancy." Endotext.

Rettig, R., C. Folberth, H. Stauss, D. Kopf, R. Waldherr and T. Unger (1990). "Role of the kidney in primary hypertension: a renal transplantation study in rats." Am J Physiol **258**(3 Pt 2): F606-611.

Rettig, R., C. G. Folberth, H. Stauss, D. Kopf, R. Waldherr, G. Baldauf and T. Unger (1990). "Hypertension in rats induced by renal grafts from renovascular hypertensive donors." Hypertension **15**(4): 429-435.

Rezanezhad, B., R. Borgquist, R. Willenheimer and S. Elzanaty (2018). "Association between serum levels of testosterone and biomarkers of subclinical atherosclerosis." Aging Male **21**(3): 182-186.

Ripley, E. and A. Hirsch (2010). "Fifteen years of losartan: what have we learned about losartan that can benefit chronic kidney disease patients?" Int J Nephrol Renovasc Dis **3**: 93-98.

Robertson, W. B., I. Brosens and W. N. Landells (1985). "Abnormal placentation." Obstet Gynecol Annu **14**: 411-426.

Rodeck, C. H., D. Gill, D. A. Rosenberg and W. P. Collins (1985). "Testosterone levels in midtrimester maternal and fetal plasma and amniotic fluid." Prenat Diagn **5**(3): 175-181.

Roselli, C. E., R. L. Salisbury and J. A. Resko (1987). "Genetic evidence for androgen-dependent and independent control of aromatase activity in the rat brain." Endocrinology **121**(6): 2205-2210.

Rowell, K. O., J. Hall, P. J. Pugh, T. H. Jones, K. S. Channer and R. D. Jones (2009). "Testosterone acts as an efficacious vasodilator in isolated human pulmonary arteries and veins: evidence for a biphasic effect at physiological and supra-physiological concentrations." J Endocrinol Invest **32**(9): 718-723.

Rupnow, H. L., T. M. Phernetton, M. L. Modrick, M. C. Wiltbank, I. M. Bird and R. R. Magness (2002). "Endothelial vasodilator production by uterine and systemic arteries. VIII. Estrogen and progesterone effects on cPLA2, COX-1, and PGIS protein expression." Biol Reprod **66**(2): 468-474.

Saad, F. (2012). "Androgen therapy in men with testosterone deficiency: can testosterone reduce the risk of cardiovascular disease?" Diabetes Metab Res Rev **28 Suppl 2**: 52-59.

Salamalekis, E., P. Bakas, N. Vitoratos, M. Eleptheriadis and G. Creatsas (2006). "Androgen levels in the third trimester of pregnancy in patients with preeclampsia." Eur J Obstet Gynecol Reprod Biol **126**(1): 16-19.

Saldanha, P. A., E. Cairrao, C. J. Maia and I. Verde (2013). "Long- and short-term effects of androgens in human umbilical artery smooth muscle." Clin Exp Pharmacol Physiol **40**(3): 181-189.

Sasagawa, I., T. Nakada, T. Kazama, T. Terada and T. Katayama (1989). "Testosterone replacement therapy and prostate/seminal vesicle volume in Klinefelter's syndrome." Arch Androl **22**(3): 245-249.

Sathishkumar, K., M. Balakrishnan, H. Gao and C. Yallampalli (2011). Elevated testosterone levels during pregnancy induce preeclampsia-like syndrome in pregnant rats. Reproductive Sciences, SAGE PUBLICATIONS INC 2455 TELLER RD, THOUSAND OAKS, CA 91320 USA.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.

Schror, K., T. A. Morinelli, A. Masuda, K. Matsuda, R. S. Mathur and P. V. Halushka (1994). "Testosterone treatment enhances thromboxane A2 mimetic induced coronary artery vasoconstriction in guinea pigs." Eur J Clin Invest **24 Suppl 1**: 50-52.

Scragg, J. L., M. L. Dallas and C. Peers (2007). "Molecular requirements for L-type Ca2+ channel blockade by testosterone." Cell Calcium **42**(1): 11-15.

Scragg, J. L., R. D. Jones, K. S. Channer, T. H. Jones and C. Peers (2004). "Testosterone is a potent inhibitor of L-type Ca(2+) channels." Biochem Biophys Res Commun **318**(2): 503-506.

Seddon, M. D., P. J. Chowienczyk, S. E. Brett, B. Casadei and A. M. Shah (2008). "Neuronal nitric oxide synthase regulates basal microvascular tone in humans in vivo." Circulation **117**(15): 1991-1996.

Selye, H., C. E. Hall and E. M. Rowley (1943). "Malignant Hypertension Produced by Treatment with Desoxycorticosterone Acetate and Sodium Chloride." Can Med Assoc J **49**(2): 88-92.

Serin, I. S., M. Kula, M. Basbug, K. Unluhizarci, S. Gucer and M. Tayyar (2001). "Androgen levels of preeclamptic patients in the third trimester of pregnancy and six weeks after delivery." Acta Obstet Gynecol Scand **80**(11): 1009-1013.

Sewdarsen, M., I. Jialal, S. Vythilingum and R. Desai (1986). "Sex hormone levels in young Indian patients with myocardial infarction." Arteriosclerosis **6**(4): 418-421.

Seyrek, M., O. Yildiz, H. B. Ulusoy and V. Yildirim (2007). "Testosterone relaxes isolated human radial artery by potassium channel opening action." J Pharmacol Sci **103**(3): 309-316.

Shabeeh, K., Jiang, Brett, Melikian, Casadei, Chowienczyk, Shah (2017). "Blood pressure in healthy humans is regulated by neuronal NO synthase." Hypertension **69**: 970-976.

Shapiro, B. H., D. C. Levine and N. T. Adler (1980). "The testicular feminized rat: a naturally occurring model of androgen independent brain masculinization." Science **209**(4454): 418-420.

Sharifzadeh, F., M. Kashanian and F. Fatemi (2012). "A comparison of serum androgens in pre-eclamptic and normotensive pregnant women during the third trimester of pregnancy." Gynecol Endocrinol **28**(10): 834-836.

Sharma, D., H. Coridon, E. Aubry, A. Houeijeh, V. Houfflin-Debarge, R. Besson, P. Deruelle and L. Storme (2018). "Vasodilator effects of dehydroepiandrosterone (DHEA) on fetal pulmonary circulation: An experimental study in pregnant sheep." PLoS One **13**(6): e0198778.

Sharma, R., O. A. Oni, K. Gupta, G. Chen, M. Sharma, B. Dawn, R. Sharma, D. Parashara, V. J. Savin, J. A. Ambrose and R. S. Barua (2015). "Normalization of testosterone level is associated with reduced incidence of myocardial infarction and mortality in men." Eur Heart J **36**(40): 2706-2715.

Sherwood, L. (2013). Human Physiology: From Cells to Systems. CA, Brooks/Cole.

Shesely, E. G., C. Gilbert, G. Granderson, C. D. Carretero, O. A. Carretero and W. H. Beierwaltes (2001). "Nitric oxide synthase gene knockout mice do not become hypertensive during pregnancy." Am J Obstet Gynecol **185**(5): 1198-1203.

Shibata, H. and F. K. Ghishan (1991). "Intestinal brush border calcium uptake in spontaneously hypertensive rats and their genetically matched WKY rats." Proc Soc Exp Biol Med **196**(1): 54-60.

Shores, M. M., K. L. Sloan, A. M. Matsumoto, V. M. Moceri, B. Felker and D. R. Kivlahan (2004). "Increased incidence of diagnosed depressive illness in hypogonadal older men." Arch Gen Psychiatry **61**(2): 162-167.

Shoskes, D. A., B. Tucky and A. S. Polackwich (2016). "Improvement of endothelial function following initiation of testosterone replacement therapy." Transl Androl Urol **5**(6): 819-823.

Sibai, B. M. and C. L. Stella (2009). "Diagnosis and management of atypical preeclampsia-eclampsia." Am J Obstet Gynecol **200**(5): 481.e481-487.

Simpson, E. R. (2003). "Sources of estrogen and their importance." J Steroid Biochem Mol Biol **86**(3-5): 225-230.

Sinha-Hikim, I., W. E. Taylor, N. F. Gonzalez-Cadavid, W. Zheng and S. Bhasin (2004). "Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment." J Clin Endocrinol Metab **89**(10): 5245-5255.

Sladek, S. M., R. R. Magness and K. P. Conrad (1997). "Nitric oxide and pregnancy." Am J Physiol **272**(2 Pt 2): R441-463.

Slowinska-Srzednicka, J., S. Zgliczynski, M. Ciswicka-Sznajderman, M. Srzednicki, P. Soszynski, M. Biernacka, M. Woroszylska, W. Ruzyllo and Z. Sadowski (1989). "Decreased plasma dehydroepiandrosterone sulfate and dihydrotestosterone concentrations in young men after myocardial infarction." Atherosclerosis **79**(2-3): 197-203.

Soma-Pillay, P., C. Nelson-Piercy, H. Tolppanen and A. Mebazaa (2016). "Physiological changes in pregnancy." Cardiovasc J Afr **27**(2): 89-94.

Stallone, J. N. (1995). "Mesenteric vascular responses to vasopressin during development of DOCA-salt hypertension in male and female rats." Am J Physiol **268**(1 Pt 2): R40-49.

Stallone, J. N., R. L. Salisbury and C. T. Fulton (2001). "Androgen-receptor defect abolishes sex differences in nitric oxide and reactivity to vasopressin in rat aorta." J Appl Physiol (1985) **91**(6): 2602-2610.

Starka, L., M. Duskova and M. J. N. L. Hill (2008). "Dihydrotestosterone and testosterone throughout the life span of Czech men." **29**(2): 201-204.

Steegers, E. A., P. von Dadelszen, J. J. Duvekot and R. Pijnenborg (2010). "Pre-eclampsia." Lancet **376**(9741): 631-644.

Struijk, P. C., N. T. Ursem, J. Mathews, E. B. Clark, B. B. Keller and J. W. Wladimiroff (2001). "Power spectrum analysis of heart rate and blood flow velocity variability measured in the umbilical and uterine arteries in early pregnancy: a comparative study." Ultrasound Obstet Gynecol **17**(4): 316-321.

Szalay, R., M. Krieg, H. Schmidt and K. D. Voigt (1975). "Metabolism and mode of action of androgens in target tissues of male rats. V. Uptake and metabolism of cyproterone acetate and its influence on the uptake and metabolism of testosterone and 5alpha-dihydrotestosterone in target organs and peripheral tissues." Acta Endocrinol (Copenh) **80**(3): 592-602.

Takeda, T. (1964). "EXPERIMENTAL STUDY ON THE BLOOD PRESSURE OF PREGNANT HYPERTENSIVE RATS. I. EFFECT OF PREGNANCY ON THE COURSE OF EXPERIMENTALLY AND SPONTANEOUSLY HYPERTENSIVE RATS." Jpn Circ J **28**: 49-54.

Tal R, T. H., Burney RO, et al. (2000). Endocrinology of Pregnancy. A. B. Feingold KR, Boyce A, et al. South Dartmouth (MA), MDText.com.

Tejada, L. D. and E. F. Rissman (2012). "Sex differences in social investigation: effects of androgen receptors, hormones and test partner." J Neuroendocrinol **24**(8): 1144-1153.

Tenover, J. S., A. M. Matsumoto, S. R. Plymate and W. J. Bremner (1987). "The effects of aging in normal men on bioavailable testosterone and luteinizing hormone secretion: response to clomiphene citrate." J Clin Endocrinol Metab **65**(6): 1118-1126.

Tep-areenan, P., D. A. Kendall and M. D. Randall (2002). "Testosterone-induced vasorelaxation in the rat mesenteric arterial bed is mediated predominantly via potassium channels." Br J Pharmacol **135**(3): 735-740.

Thadhani, R., J. L. Ecker, E. Kettyle, L. Sandler, F. D. J. O. Frigoletto Jr and Gynecology (2001). "Pulse pressure and risk of preeclampsia:: A prospective study." **97**(4): 515-520.

Thirumavalavan, N., N. A. Wilken and R. Ramasamy (2015). "Hypogonadism and renal failure: An update." Indian J Urol **31**(2): 89-93.

Thomas, R. M., E. Ruel, P. C. Shantavasinkul and L. Corsino (2015). "Endocrine hypertension: An overview on the current etiopathogenesis and management options." World J Hypertens **5**(2): 14-27.

Thorneloe, K. S. and M. T. Nelson (2005). "Ion channels in smooth muscle: regulators of intracellular calcium and contractility." Can J Physiol Pharmacol **83**(3): 215-242.

Tikellis, C., M. E. Cooper, K. Bialkowski, C. I. Johnston, W. C. Burns, R. A. Lew, A. I. Smith and M. C. Thomas (2006). "Developmental expression of ACE2 in the SHR kidney: a role in hypertension?" Kidney Int **70**(1): 34-41.

Toda, N., S. Nakanishi and S. Tanabe (2013). "Aldosterone affects blood flow and vascular tone regulated by endothelium-derived NO: therapeutic implications." Br J Pharmacol **168**(3): 519-533.

Toot, J., C. Jenkins, G. Dunphy, S. Boehme, M. Hart, A. Milsted, M. Turner and D. Ely (2008). "Testosterone influences renal electrolyte excretion in SHR/y and WKY males." BMC Physiol **8**: 5.

Toth, M. and T. Zakar (1982). "Different binding of testosterone, 19-nortestosterone and their 5 alpha-reduced derivatives to the androgen receptor of the rat seminal vesicle: a step toward the understanding of the anabolic action of nortesterone." Endokrinologie **80**(2): 163-172.

Towers, P. A., G. Shaw and M. B. Renfree (1986). "Urogenital vasculature and local steroid concentrations in the uterine branch of the ovarian vein of the female tammar wallaby (Macropus eugenii)." J Reprod Fertil **78**(1): 37-47.

Traish, A. M. (2018). "Benefits and Health Implications of Testosterone Therapy in Men With Testosterone Deficiency." Sex Med Rev **6**(1): 86-105.

Traish, A. M., R. Abdou and K. E. Kypreos (2009). "Androgen deficiency and atherosclerosis: The lipid link." Vascul Pharmacol **51**(5-6): 303-313.

Traish, A. M., A. T. Guay and A. Morgentaler (2014). "Death by testosterone? We think not!" J Sex Med **11**(3): 624-629.

Traish, A. M., A. Haider, G. Doros and F. Saad (2014). "Long-term testosterone therapy in hypogonadal men ameliorates elements of the metabolic syndrome: an observational, long-term registry study." Int J Clin Pract **68**(3): 314-329.

Traish, A. M., A. Haider, K. S. Haider, G. Doros and F. Saad (2017). "Long-Term Testosterone Therapy Improves Cardiometabolic Function and Reduces Risk of Cardiovascular Disease in Men with Hypogonadism: A Real-Life Observational Registry Study Setting Comparing Treated and Untreated (Control) Groups." J Cardiovasc Pharmacol Ther **22**(5): 414-433.

Traish, A. M. and M. Zitzmann (2015). "The complex and multifactorial relationship between testosterone deficiency (TD), obesity and vascular disease." Rev Endocr Metab Disord **16**(3): 249-268.

Trippodo, N. C. and E. D. Frohlich (1981). "Similarities of genetic (spontaneous) hypertension. Man and rat." Circ Res **48**(3): 309-319.

Tsang, S., S. Wu, J. Liu and T. M. Wong (2008). "Testosterone protects rat hearts against ischaemic insults by enhancing the effects of alpha(1)-adrenoceptor stimulation." Br J Pharmacol **153**(4): 693-709.

Ueland, K. (1976). "Maternal cardiovascular dynamics. VII. Intrapartum blood volume changes." Am J Obstet Gynecol **126**(6): 671-677.

Ueno, Y., O. Mohara, K. B. Brosnihan and C. M. Ferrario (1988). "Characteristics of hormonal and neurogenic mechanisms of deoxycorticosterone-induced hypertension." Hypertension **11**(2 Pt 2): I172-177.

Ullian, M. E. (1999). "The role of corticosteroids in the regulation of vascular tone." Cardiovascular Research **41**(1): 55-64.

van der Graaf, A. M., T. J. Toering, M. M. Faas and A. Titia Lely (2012). "From preeclampsia to renal disease: a role of angiogenic factors and the renin–angiotensin aldosterone system?" Nephrology Dialysis Transplantation **27**(suppl_3): iii51-iii57.

van Kats, J. P., L. M. de Lannoy, A. H. Jan Danser, J. R. van Meegen, P. D. Verdouw and M. A. Schalekamp (1997). "Angiotensin II type 1 (AT1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo." Hypertension **30**(1 Pt 1): 42-49.

Velasco, A. and W. Vongpatanasin (2014). "The evaluation and treatment of endocrine forms of hypertension." Curr Cardiol Rep **16**(9): 528.

Venkatesha, S., M. Toporsian, C. Lam, J. Hanai, T. Mammoto, Y. M. Kim, Y. Bdolah, K. H. Lim, H. T. Yuan, T. A. Libermann, I. E. Stillman, D. Roberts, P. A. D'Amore, F. H. Epstein, F. W. Sellke, R. Romero, V. P. Sukhatme, M. Letarte and S. A. Karumanchi (2006). "Soluble endoglin contributes to the pathogenesis of preeclampsia." Nat Med **12**(6): 642-649.

Vigen, R., C. I. O'Donnell, A. E. Baron, G. K. Grunwald, T. M. Maddox, S. M. Bradley, A. Barqawi, G. Woning, M. E. Wierman, M. E. Plomondon, J. S. Rumsfeld and P. M. Ho (2013). "Association of testosterone therapy with mortality, myocardial infarction, and stroke in men with low testosterone levels." Jama **310**(17): 1829-1836.

Walker, T. (1942). "Use of testosterone propionate and estrogenic substances in treatment of essential hypertension, angina pectoris, and peripheral vascular disease." J Clin Endocrinol **2**: 560-568.

Wallis, C. J., H. Brotherhood and P. J. Pommerville (2014). "Testosterone deficiency syndrome and cardiovascular health: An assessment of beliefs, knowledge and practice patterns of general practitioners and cardiologists in Victoria, BC." Can Urol Assoc J **8**(1-2): 30-33.

Wallis, C. J., K. Lo, Y. Lee, Y. Krakowsky, A. Garbens, R. Satkunasivam, S. Herschorn, R. T. Kodama, P. Cheung, S. A. Narod and R. K. Nam (2016). "Survival and cardiovascular events in men treated with testosterone replacement therapy: an

intention-to-treat observational cohort study." Lancet Diabetes Endocrinol **4**(6): 498-506.

Wang, C., E. Nieschlag, R. Swerdloff, H. M. Behre, W. J. Hellstrom, L. J. Gooren, J. M. Kaufman, J. J. Legros, B. Lunenfeld, A. Morales, J. E. Morley, C. Schulman, I. M. Thompson, W. Weidner and F. C. Wu (2008). "Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, EAU, EAA and ASA recommendations." Eur J Endocrinol **159**(5): 507-514.

Ward, G. R. and A. A. Abdel-Rahman (2005). "Effect of testosterone replacement or duration of castration on baroreflex bradycardia in conscious rats." BMC Pharmacol **5**: 9.

Webb, C. M., D. L. Adamson, D. de Zeigler and P. Collins (1999). "Effect of acute testosterone on myocardial ischemia in men with coronary artery disease." Am J Cardiol **83**(3): 437-439, a439.

Webb, C. M., J. G. McNeill, C. S. Hayward, D. de Zeigler and P. Collins (1999). "Effects of testosterone on coronary vasomotor regulation in men with coronary heart disease." Circulation **100**(16): 1690-1696.

Weiner, C. P., I. Lizasoain, S. A. Baylis, R. G. Knowles, I. G. Charles and S. Moncada (1994). "Induction of calcium-dependent nitric oxide synthases by sex hormones." Proc Natl Acad Sci U S A **91**(11): 5212-5216.

Weissman, B. A., C. L. Jones, Q. Liu and S. S. Gross (2002). "Activation and inactivation of neuronal nitric oxide synthase: characterization of Ca(2+)-dependent [125I]Calmodulin binding." Eur J Pharmacol **435**(1): 9-18.

White, R. E., M. P. Owen and J. N. Stallone (2007). Testosterone-induced vasorelaxation of rat mesenteric microvasculature is K+ channel-and nitric oxide-dependent but estrogen-independent, Federation of American Societies for Experimental Biology.

Wilcox, J. N., R. R. Subramanian, C. L. Sundell, W. R. Tracey, J. S. Pollock, D. G. Harrison and P. A. Marsden (1997). "Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels." Arterioscler Thromb Vasc Biol **17**(11): 2479-2488.

Wilke, T. J. and D. J. Utley (1987). "Total testosterone, free-androgen index, calculated free testosterone, and free testosterone by analog RIA compared in hirsute women and in otherwise-normal women with altered binding of sex-hormone-binding globulin." Clin Chem **33**(8): 1372-1375.

Wilkenfeld, S. R., C. Lin and D. E. Frigo (2018). "Communication between genomic and non-genomic signaling events coordinate steroid hormone actions." Steroids **133**: 2-7.

Witlin, A. G., Z. Y. Li, S. J. Wimalawansa, J. J. Grady, M. R. Grafe and C. Yallampalli (2002). "Placental and fetal growth and development in late rat gestation is dependent on adrenomedullin." Biol Reprod **67**(3): 1025-1031.

Wolff, K., H. Nisell, A. Modin, J. M. Lundberg, N. O. Lunell and B. Lindblom (1993). "Contractile effects of endothelin 1 and endothelin 3 on myometrium and small intramyometrial arteries of pregnant women at term." Gynecol Obstet Invest **36**(3): 166-171.

Won, D., S. N. Zhu, M. Chen, A. M. Teichert, J. E. Fish, C. C. Matouk, M. Bonert, M. Ojha, P. A. Marsden and M. I. Cybulsky (2007). "Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow." Am J Pathol **171**(5): 1691-1704.

Wong, K. A., Y. Ma, W. T. Cheng and T. M. Wong (2007). "Cardioprotection by the female sex hormone -- interaction with the beta(1)-adrenoceptor and its signaling pathways." Sheng Li Xue Bao **59**(5): 571-577.

Wu, F. C., A. Tajar, S. R. Pye, A. J. Silman, J. D. Finn, T. W. O'Neill, G. Bartfai, F. Casanueva, G. Forti, A. Giwercman, I. T. Huhtaniemi, K. Kula, M. Punab, S. Boonen and D. Vanderschueren (2008). "Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study." J Clin Endocrinol Metab **93**(7): 2737-2745.

Wu, F. C. and A. von Eckardstein (2003). "Androgens and coronary artery disease." Endocr Rev **24**(2): 183-217.

Wunderlich, F., W. P. Benten, M. Lieberherr, Z. Guo, O. Stamm, C. Wrehlke, C. E. Sekeris and H. Mossmann (2002). "Testosterone signaling in T cells and macrophages." Steroids **67**(6): 535-538.

Xiong, X., N. N. Demianczuk, L. D. Saunders, F. L. Wang and W. D. Fraser (2002). "Impact of preeclampsia and gestational hypertension on birth weight by gestational age." Am J Epidemiol **155**(3): 203-209.

Xu, Q., C. C. Wells, J. H. Garman, L. Asico, C. S. Escano and C. Maric (2008). "Imbalance in sex hormone levels exacerbates diabetic renal disease." Hypertension **51**(4): 1218-1224.

Yanes, L. L., J. C. Sartori-Valinotti, R. Iliescu, D. G. Romero, L. C. Racusen, H. Zhang and J. F. Reckelhoff (2009). "Testosterone-dependent hypertension and upregulation of intrarenal angiotensinogen in Dahl salt-sensitive rats." Am J Physiol Renal Physiol **296**(4): F771-779.

Yang, T. and C. Xu (2017). "Physiology and Pathophysiology of the Intrarenal Renin-Angiotensin System: An Update." J Am Soc Nephrol **28**(4): 1040-1049.

Yang, X. P. and J. F. Reckelhoff (2011). "Estrogen, hormonal replacement therapy and cardiovascular disease." Curr Opin Nephrol Hypertens **20**(2): 133-138.

Yarbrough, W. G., V. E. Quarmby, J. A. Simental, D. R. Joseph, M. Sar, D. B. Lubahn, K. L. Olsen, F. S. French and E. M. Wilson (1990). "A single base mutation in the androgen receptor gene causes androgen insensitivity in the testicular feminized rat." J Biol Chem **265**(15): 8893-8900.

Yassin, A. and G. Doros (2013). "Testosterone therapy in hypogonadal men results in sustained and clinically meaningful weight loss." Clin Obes **3**(3-4): 73-83.

Yeap, B. B., H. Alfonso, S. A. Chubb, G. J. Hankey, D. J. Handelsman, J. Golledge, O. P. Almeida, L. Flicker and P. E. Norman (2014). "In older men, higher plasma testosterone or dihydrotestosterone is an independent predictor for reduced incidence of stroke but not myocardial infarction." J Clin Endocrinol Metab **99**(12): 4565-4573.

Yildirim, E. and K. Erol (2011). "The effects of testosterone on isolated sheep coronary artery." Anadolu Kardiyol Derg **11**(4): 343-350.

Yildiz, O., M. Seyrek, H. Gul, I. Un, V. Yildirim, E. Ozal, M. Uzun and E. Bolu (2005). "Testosterone relaxes human internal mammary artery in vitro." J Cardiovasc Pharmacol **45**(6): 580-585.

Yim, H. E. and K. H. Yoo (2008). "Renin-Angiotensin system - considerations for hypertension and kidney." Electrolyte Blood Press **6**(1): 42-50.

Yoon, S. S., Q. Gu, T. Nwankwo, J. D. Wright, Y. Hong and V. Burt (2015). "Trends in blood pressure among adults with hypertension: United States, 2003 to 2012." Hypertension **65**(1): 54-61.

Young, C. N. and R. L. Davisson (2015). "Angiotensin-II, the Brain, and Hypertension: An Update." Hypertension **66**(5): 920-926.

Young, D. (2010). Control of Cardiac Output. San Rafael (CA), Morgan & Claypool Life Sciences.

Young, J. J. B. A. I. J. o. O. and Gynaecology (1914). "The Ætiology of Eclampsia and Albuminuria and their relation to Accidental Hæmorrhage. (An Anatomical and Experimental Investigation.) 1." **26**(1): 1-28.

Yu, J., M. Akishita, M. Eto, H. Koizumi, R. Hashimoto, S. Ogawa, K. Tanaka, Y. Ouchi and T. Okabe (2012). "Src kinase-mediates androgen receptor-dependent non-genomic activation of signaling cascade leading to endothelial nitric oxide synthase." Biochem Biophys Res Commun **424**(3): 538-543.

Yu, J., M. Akishita, M. Eto, S. Ogawa, B. K. Son, S. Kato, Y. Ouchi and T. Okabe (2010). "Androgen receptor-dependent activation of endothelial nitric oxide synthase in vascular endothelial cells: role of phosphatidylinositol 3-kinase/akt pathway." Endocrinology **151**(4): 1822-1828.

Yue, P., K. Chatterjee, C. Beale, P. A. Poole-Wilson and P. Collins (1995). "Testosterone relaxes rabbit coronary arteries and aorta." Circulation **91**(4): 1154-1160.

Zakar, T. and M. Toth (1982). "Steroid-binding properties of the androgen receptor of rat seminal vesicle." Endokrinologie **80**(3): 257-265.

Zatz, R. and C. Baylis (1998). "Chronic nitric oxide inhibition model six years on." Hypertension **32**(6): 958-964.

Zezula-Szpyra, A., B. Gawronska and J. Skipor (1997). "Vasa vasorum of blood and lymph vessels in the broad ligament of the sheep uterus analyzed by scanning electron microscopy." Rocz Akad Med Bialymst **42 Suppl 2**: 134-146.

Zhang, H. Q., W. Fast, M. A. Marletta, P. Martasek and R. B. Silverman (1997). "Potent and selective inhibition of neuronal nitric oxide synthase by N omega-propyl-L-arginine." J Med Chem **40**(24): 3869-3870.

Zhao, H., J. T. Leppert and D. M. Peehl (2016). "A Protective Role for Androgen Receptor in Clear Cell Renal Cell Carcinoma Based on Mining TCGA Data." PLoS One **11**(1): e0146505.

Zheng, H. Y., Y. Li, W. Dai, C. D. Wei, K. S. Sun and Y. Q. Tong (2012). "Imbalance of testosterone/estradiol promotes male CHD development." Biomed Mater Eng **22**(1-3): 179-185.

Zhou, P., L. Fu, Z. Pan, D. Ma, Y. Zhang, F. Qu, L. Guo, J. Cao, Q. Gao and Y. Han (2008). "Testosterone deprivation by castration impairs expression of voltage-dependent potassium channels in rat aorta." Eur J Pharmacol **593**(1-3): 87-91.

Zhou, Y., C. H. Damsky, K. Chiu, J. M. Roberts and S. J. Fisher (1993). "Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts." J Clin Invest **91**(3): 950-960.

Zhuo, J. L., J. D. Imig, T. G. Hammond, S. Orengo, E. Benes and L. G. Navar (2002). "Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: role of AT(1) receptor." Hypertension **39**(1): 116-121.

Zou, L. X., J. D. Imig, A. M. von Thun, A. Hymel, H. Ono and L. G. Navar (1996). "Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats." Hypertension **28**(4): 669-677.