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ANDROGEN INCREASES ANGIOTENSIN RECEPTOR TYPE 1A ON SMOOTH MUSCLE CELLS TO PROMOTE ANGIOTENSIN II-INDUCED ABDOMINAL AORTIC ANEURYSMS

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ABSTRACT OF DISSERTATION

Xuan Zhang

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University of Kentucky
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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate Center for Toxicology at the University of Kentucky

By
Xuan Zhang
Lexington, Kentucky

Director: Dr. Lisa Cassis, Professor of Nutritional Sciences
Lexington, Kentucky

2011

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The purpose of this study was to determine whether androgen promotes AT1aR expression on smooth muscle to confer high prevalence of AngII-induced AAAs in hyperlipidemic mice. In addition, we also investigate the role of androgen in the progression of established AngII-induced AAAs.

First, we sought to examine the role of endogenous androgen in the growth of established AngII-induced AAAs. By castrating male mice, we demonstrated that removal of endogenous androgen significantly decreased the progressive lumen dilation of established AngII-induced AAAs in male ApoE-/- mice, but had no effect on external AAA diameters. These results suggest that androgen contributes to the progression of established AAAs through distinct mechanisms that differentially influence aortic lumen and wall diameters.

We also investigate whether androgen regulates aortic AT1aR expression to promote AngII-induced AAA formation. Our data demonstrated that in male and female mice, both endogenous and exogenous androgen stimulate AT1aR level particularly in abdominal aortas. This androgen-dependent enhanced expression of abdominal aortic AT1aR was correlated with increased AngII-induced AAA formation in male and female mice. Smooth muscle AT1aR deficiency significantly reduced luminal and external diameters of abdominal aortas as well as the incidence of AngII-induced AAAs in adult female mice administered exogenous androgen. Collectively, these results indicate that in adult mice androgen stimulate smooth muscle AT1aR expression to promote AngII-induced AAA formation.

To determine the role of androgen during development on AT1aR expression on SMC and AngII-induced vascular pathologies, we exposed neonatal female mice to one single dose of testosterone. Our data demonstrated that neonatal testosterone administration dramatically increased AngII-induced AAA, atherosclerosis and ascending aortic aneurysms in adult female mice. In
addition, smooth muscle AT1aR deficiency reduced effects of neonatal testosterone to promote AAAs, but had no effect on the other two AngII-induced vascular pathologies.

In summary, our findings demonstrated that androgen, both in adult life and during development, stimulate smooth muscle AT1aR expression and promote AngII-induced AAA in female hyperlipidemic mice.

KEYWORDS: Androgen, Angiotensin receptor, Abdominal aortic aneurysm, Sexual dimorphism, Smooth muscle
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Dissertation

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To My Loving Family
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CHAPTER I. BACKGROUND

1.1 Abdominal Aortic Aneurysms in Humans

1.1.1 Overview

According to the Society for Vascular Surgery and the International Society for Cardiovascular Surgery, abdominal aortic aneurysms (AAAs) are defined as a permanent dilation of the infrarenal aorta with a ratio of ≥1.5 fold increase in normal abdominal aortic diameter (Johnston, Rutherford et al. 1991). Most investigators also define an AAA as an infrarenal aortic diameter exceeding 3.0 cm (McGregor, Pollock et al. 1975; Sakalihasan, Limet et al. 2005). The prevalence of unsuspected, asymptomatic AAA in men and women over the age of 60 years are 4% to 8.5% and 0.5% to 1.5%, respectively (Wilmink, Quick et al. 1999; Lawrence-Brown, Norman et al. 2001; Ashton, Buxton et al. 2002; Cornuz, Sidoti Pinto et al. 2004; Palombo, Lucertini et al. 2010). AAAs tend to progressively expand over time with average growth rate between 0.25 and 0.32 cm/year (Lederle, Wilson et al. 2002). The risk of aneurysmal rupture increases proportionally with AAA diameter, and becomes especially high when the diameter exceeds 5.5 cm. Unfortunately, so far there are no pharmacologic treatments with proven efficacy to prevent AAA expansion and rupture. Currently the only effective way to prevent AAA rupture is open or endovascular surgical repair. A threshold of 5.0 – 5.5 cm in AAA size is the current recommendation for open or endovascular surgical repair of an AAA (Powell and Brady 2004). Recent estimates of AAA prevalence are 1.1 million
people in the US between the ages of 50 to 84 (Kent, Zwolak et al. 2010).

Ruptured AAA and complications after surgical treatment are responsible for at least 15,000 deaths per year in the United States (Gillum 1995). This number is considered as a likely underestimation because about 5% of the 200,000 people who die of sudden death each year may have died from AAA ruptures (Schermerhorn 2009).

The histopathologic features of human AAAs are characterized by chronic excessive transmural inflammatory (B-cells, T-cells and macrophage infiltration), destructive degradation of the elastic media, apoptosis of medial smooth muscle cells (SMCs) and significant medial neovascularization (Choke, Cockerill et al. 2005). However, the detailed mechanisms involved in biochemical and cellular processes during the development of human AAAs remains largely unknown. It is now well accepted that AAA initiation, progression and rupture are multifactorial biological processes that involve distinct biochemical, cellular and proteolytic influences and biomechanical factors on the vessel wall during different developmental stage of AAA pathogenesis. It is well established that the destruction of elastin and collagen largely contributes to the decrease in aortic wall tensile strength followed by aneurysmal dilatation. In rodents, elastase infusion into infrarenal aortas induces AAA formation (Halpern, Nackman et al. 1994). Increased proteolytic activity in the vessel wall has been demonstrated to play a significant role in the pathogenesis of AAAs. Increased levels of matrix metalloproteinases (MMPs), specially MMP-2 and MMP-9, were found in aneurysmal tissues of both humans and experimental models (Freestone, Turner
et al. 1995; Goodall, Porter et al. 2002) and the genetic deficiency of MMP 2 and 9 inhibit AAA formation in an experimental AAA model (Longo, Xiong et al. 2002). As reviewed by Takagi et al, higher circulating MMP-9 concentrations are associated with AAA in human (Takagi, Manabe et al. 2009). Furthermore, plasma MMP-9 levels were elevated in patients with larger AAAs (Lindholt, Vammen et al. 2000; Petersen, Gineitis et al. 2000). Increased MMP-9 level was detected at the rupture site of AAAs (Wilson, Anderton et al. 2006). Also a 4-fold increase in pre-operative plasma MMP-9 levels was associated with increased mortality >30 days post-rupture surgery, compared to >30 days post surgery survivors (Wilson, Anderton et al. 2008). All these findings demonstrate an important role of MMPs in the pathogenesis of human AAAs.

1.1.2 Clinical risk factors

Smoking, male gender, aging, and family history are considered as primary and independent risk factors of human AAA (Pleumeekers, Hoes et al. 1995; Lederle, Johnson et al. 2000; Wanhainen, Bergqvist et al. 2005). Smoking is a prominent risk factor that is strongly associated with AAAs development. Approximately 90% of people with AAAs have smoked (Powell and Greenhalgh 2003; Isselbacher 2005). People who have smoked for more than 40 years are much more likely to develop AAAs compared to non-smokers (Powell and Greenhalgh 2003). A slow decline in risk was observed with each year after cessation of smoking (Wilmink, Quick et al. 1999; Lederle, Johnson et al. 2001; Kent, Zwolak et al. 2010). Age, hypertension, hyperlipidemia and atherosclerosis
were also found to be moderate risk factors for developing AAAs (Wanhainen, Bergqvist et al. 2005; Kent, Zwolak et al. 2010). Genetics also plays a role in the development of AAAs. For example, people with a family history of AAAs have a 30% greater risk, and their aneurysms tend to develop at a younger age compared to those with no family history of the disease (Frydman, Walker et al. 2003). Recently it has been reported that the relative risk of developing AAA for first-degree relatives to persons with diagnosed AAA is approximately doubled compared to persons with no family history (Larsson, Granath et al. 2009). Genetic variations in several chromosome loci, including 19q13, 4q31 and 9p21, have been associated with AAA formation (Shibamura, Olson et al. 2004; Kuivaniemi, Kyo et al. 2006; Helgadottir, Thorleifsson et al. 2008). Recently obesity has been demonstrated to be associated with increased risk for AAA formation in human and experimental AAA model (Golledge, Clancy et al. 2007; Police, Thatcher et al. 2009). Interestingly, a negative association have been observed between AAA and diabetes mellitus (Blanchard, Armenian et al. 2000). Black and Asian race were also found to be associated with decreased risk of AAA (Iribarren, Darbinian et al. 2007).

1.1.3 Gender

Gender is considered a major independent risk factor for AAA development (Wanhainen, Bergqvist et al. 2005; Forsdahl, Singh et al. 2009). For example, men are 10 times more likely to develop an AAA of 4 cm or greater compared to age-matched women (Lederle, Johnson et al. 2001). Overall, men
exhibit greater incidence and severity of AAA compared to women. In recent decades epidemiological studies have demonstrated increased AAA incidence and rupture in western societies, especially in male gender (Best, Price et al. 2003; Acosta, Ogren et al. 2006). The well described risk of male gender has influenced clinical care, as men aged 65 to 75 years with a history of smoking are strongly recommended with regular ultrasound screening for AAA.

However, female gender has been associated with higher rupture risk of small aneurysms (3 times more frequently and at a smaller diameter compared to men), and decreased survival rate after surgical AAA repair (Noel, Gloviczki et al. 2001; Norman and Powell 2007; Grootenboer, van Sambeek et al. 2010). Furthermore, recent studies reported a significantly increased growth rate of AAAs in women compared to men (Solberg, Singh et al. 2005; Mofidi, Goldie et al. 2007; Norman and Powell 2007).

1.2 Renin-angiotensin system

1.2.1 Current view

Ever since its first discovery in 1898, the renin-angiotensin system (RAS) has emerged as a vital system to modulate many physiological and pathological processes. Besides its classical effects on regulating blood pressure and maintaining fluid volume homeostasis, the RAS also plays a pivotal role in renal and cardiovascular pathologies. Angiotensin peptides are produced through sequential enzymatic cleavage of the only known precursor, angiotensinogen as illustrated in Figure 1.1. Angiotensinogen, a 58 kD glycoprotein consisting of 452
amino acids, is mainly synthesized and secreted by hepatic cells into the circulation (Devenyi, Dauda et al. 1968; Kageyama, Ohkubo et al. 1984). However, angiotensinogen mRNA is also expressed in various tissues, including adipose tissue (Cassis 2000).

Renin is an aspartyl protease that cleaves off 10 amino acids at the amino terminus of angiotensinogen to yield the peptide angiotensin I. Secretion of renin is activated primarily in response to decreases in blood pressure and sodium content. Circulating renin and its precursor, prorenin, is synthesized and released primarily from the juxtaglomerular cells located in the glomerular afferent arterioles (Levens, Peach et al. 1981). In addition, renin has been localized to the brain (Inagami, Celio et al. 1980); blood vessels (Mizuno, Gotoh et al. 1984), ovaries (Lightman, Jones et al. 1988), testis (Pandey, Melner et al. 1984), macrophages (Lu, Rateri et al. 2008), and adipose tissue (Cassis 2000). Prorenin is more abundant in the circulation. In human blood plasma, prorenin levels are about 10-fold higher than renin (Danser, Derkx et al. 1998). For a long time prorenin had been considered to be a mere inactive precursor of renin until the (pro)renin receptor, a 350 amino acid protein with a single transmembrane domain, was identified and cloned in 2002 (Nguyen, Delarue et al. 2002). The (pro)renin receptor has been shown to bind both renin and prorenin. In humans, the prorenin receptor binds prorenin preferentially (K_d ~6-8 nM vs. >20 nM for renin) (Nabi, Kageshima et al. 2006; Jan Danser, Batenburg et al. 2007; Danser, Batenburg et al. 2008). Binding of prorenin to the (pro)renin receptor induces a conformational change in the prorenin molecule, allowing it to display full
enzymatic activity without undergoing proteolytic cleavage to renin (Batenburg, Krop et al. 2007). Recently it has been demonstrated that renin and prorenin also acted as agonists for the (pro) renin receptor and induce angiotensin-independent effects, such as triggering activation of the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2 signaling pathway and upregulating transforming growth factor β1 (TGF-β1) gene expression (Huang, Noble et al. 2007; Feldt, Batenburg et al. 2008).

Angiotensin I (AngI) is biologically inactive and is rapidly converted to AngII through an enzymatic cleavage of the last 2 amino acids at the carboxy terminus by the angiotensin-converting enzyme (ACE). Two forms of ACE are produced from a single gene through alternate promoters (Ehlers and Riordan 1989). Somatic ACE is the 160 to 170 kDa protein formed primarily in endothelial cells of the lung (Ryan, Ryan et al. 1975). It has also been found in kidney (Wallace, Bailie et al. 1978), intestine (Bruneval, Hinglais et al. 1986), placenta (Sim and Seng 1984) and adipose tissue (Karlsson, Lindell et al. 1998) as well as vascular endothelium, epithelium and macrophages (Fuchs, Xiao et al. 2004).

Germinal ACE is a 100 to 110 kDa protein produced by germ cells in the testis (Ehlers and Riordan 1989; Bernstein, Howard et al. 1992). Mice with ACE deficiency exhibits reduced blood pressure, inability to concentrate urine, reduced aldosterone production and serum potassium concentrations and defects in renal structure and function (Kim, Krege et al. 1995; Esther, Howard et al. 1996). ACE inhibitors are widely used to treat hypertension and congestive heart failure. Besides Ang I, ACE acts on a number of natural substrates.
including bradykinin (Ryan, Ryan et al. 1975), cholecystokinin (Dubreuil, Fulcrand et al. 1989) substance P (Couture and Regoli 1981) and luteinizing hormone-releasing hormone (Skidgel and Erdos 1985).

Recently a homologue of ACE, known as angiotensin-converting enzyme 2 (ACE2), was identified. ACE2 is predominantly expressed in vascular endothelial cells of the heart and kidney (Donoghue, Hsieh et al. 2000; Tipnis, Hooper et al. 2000). ACE2 expression has also been detected in adipocytes, testes, colon and lung (Hamming, Timens et al. 2004; Gupte, Boustany-Kari et al. 2008). Unlike ACE, ACE2 is a monocarboxypeptidase, cleaving a single carboxyl-terminal residue from its substrates. Both Ang I and AngII can be catabolized by ACE2 to generate Ang-(1-9), and Ang-(1-7), respectively (Donoghue, Hsieh et al. 2000; Tipnis, Hooper et al. 2000). However, ACE2 exhibits a 400-fold higher catalytic efficiency to hydrolyze AngII as a substrate compared to AngI (Vickers et al. 2002). Interestingly, Ang-(1-7) is a vasodilator and exhibits vasoprotective effects. Ang-(1-7) mediates its effects via interaction with Mas, a G protein coupled receptor. It has been demonstrated that Ang-(1-7) induces vasodilation through endothelium-dependent nitric oxide involving bradykinin receptors (Brosnihan et al. 1996). Ang-(1-7) has also been demonstrated to have antiproliferative, antitrophic and apoptotic effects. Thus, ACE2/Ang-(1-7) has been suggested as the counter balancing arm of the RAS.

As the most potent vasoconstrictor of the RAS, AngII plays a critical role in regulating blood pressure and fluid electrolyte balance (Peach, Cline et al. 1966). AngII has been demonstrated to increase blood pressure via eliciting
vasoconstriction in vascular smooth muscle cells (Chiu, Roscoe et al. 1991) and modulating vascular structure. In the vasculature, AngII exhibit mitogenic effects and cause vascular smooth muscle cell growth (Touyz and Schiffri 1997), proliferation (Stouffer and Owens 1992) and hypertrophy (Chiu, Roscoe et al. 1991). In addition, AngII increases intravascular volume to further enhance total peripheral resistance, therefore increasing blood pressure. AngII regulates water and sodium reabsorption by stimulating the synthesis and release of aldosterone from the adrenal cortex (Laragh, Ulick et al. 1960; Carpenter, Davis et al. 1961). AngII also induces water retention and a dipsogenic response by increasing the release of vasopressin from the pituitary (Epstein, Fitzsimons et al. 1969; Phillips, Rolls et al. 1985). In the adrenal medulla, AngII stimulates catecholamine release from the chromaffin cells (Peach, Cline et al. 1966). Moreover, Ang II also increases blood pressure by facilitating peripheral noradrenergic neurotransmission. AngII has been demonstrated to enhance vascular vasopressor responses to norepinephrine (Reams et al. 1987), facilitate norepinephrine release from sympathetic nerve terminals (Hughes et al. 1971) and inhibit norepinephrine reuptake during sympathetic stimulation (Campbell, Brooks et al. 1974). AngII can also act in the area postrema of the brain to increase sympathetic drive to the periphery, and thus increase blood pressure (Meldrum, Xue et al. 1984; Papouchado, Vatta et al. 1995).

AngII exerts its physiological and pathophysiological biological actions via binding to specific receptors on the cell surface. AngII receptors belong to the seven transmembrane class of G-protein-coupled receptors (GPCR), and are
divided into two main distinct subtypes: angiotensin II type 1 (AT1) and 2 (AT2) receptors (Lin and Goodfriend 1970). These two receptors were initially defined based on their differential pharmacological and biochemical properties. The majority of the well-known AngII effects, which includes vasoconstriction (Touyz and Schiffrin 1997), cellular growth and differentiation (Huang, Richards et al. 1996), tubular reabsorption (Navar, Harrison-Bernard et al. 1999) and aldosterone synthesis and secretion (Balla, Baukal et al. 1991) are mediated via AT1R. The AT1R is ubiquitously expressed in many cells and tissues, such as vascular smooth muscle cells, kidney, heart, adrenal gland, brain, lung, and adipose tissues (Murphy, Alexander et al. 1991). The intracellular signaling pathways involved in AT1R action are illustrated by Figure 1.2. AT1R triggers the activation of classic G protein coupled intracellular signalling pathways, which includes coupling to a GTP-binding protein, activation of a phospholipase C resulting in inositol trisphosphate generation, and mobilization of intracellular Ca^{2+} stores and diacylglycerol formation leading to protein kinase C activation (Farese, Larson et al. 1984). In addition to the G protein mediated pathways, AngII also act through AT1R to cross talk with several tyrosine kinases and activate mitogen-activated protein kinases (MAPKs), including extracellular signaling regulation kinase (ERK1/2), JNK and p38MAPK (Mehta and Griendling 2007). Unlike human, rodents have 2 isoforms of AT1R that share 95% amino acid sequence similarity, AT1aR on chromosome 17 and AT1bR on chromosome 2 (Elton, Stephan et al. 1992; Sasamura, Hein et al. 1992). Studies have demonstrated that mice with AT1aR deficiency, but not AT1bR deficiency, have
decreased blood pressure and an attenuated response to AngII (Ito, Oliverio et al. 1995; Chen, Li et al. 1997; Oliverio, Best et al. 2000), suggesting the AT1aR is the major receptor subtype involved in blood pressure regulation in mice.

Recently, it has been demonstrated that AT1bR predominantly mediates contraction in the isolated abdominal aorta and femoral artery (Zhou, Chen et al. 2003; Zhou, Dirksen et al. 2005; Swafford, Harrison-Bernard et al. 2007). Moreover, AngII infusion induces a pressor response in AT1aR deficient mice, which can be blocked by AT1R antagonists. These data suggest that AT1bR receptors may play a role in blood pressure regulation in the absence of AT1aR (Oliverio, Best et al. 1997).

In contrast to the AT1 receptor, the physiological significance of the AT2R is less understood. AT2R seems to counterbalance some of the effects mediated by AT1R as illustrated in Figure 1.3. It has been demonstrated that AT2 antagonize effects of the AT1 receptor by promoting vasorelaxation, growth inhibition, and proapoptotic effects (Akishita, Yamada et al. 1999). AT2R deficient mice exhibit a 10 mmHg increase in blood pressure (Gross, Obst et al. 2004) and enhanced sensitivity to the pressor effects of AngII infusion (Hein, Barsh et al. 1995; Ichiki, Labosky et al. 1995). Unlike AT1R, whose expression level remain relatively constant in adults, AT2R is mainly present during late embryonic development and in the neonate of the mouse and rat, but levels rapidly decline shortly after birth (Nakajima, Hutchinson et al. 1995; Shanmugam, Corvol et al. 1996; Horiuchi, Yamada et al. 1997). AT2R is believed to play a role in physiological development, partially through its effects on vascular remodeling. In
adults, AT2R expression is largely restricted to kidneys, adrenals, uterus, ovary, heart and specialized nuclei in the brain. Interestingly, AT2R is upregulated in many cardiovascular pathological conditions associated with inflammation or tissue remodeling such as heart or renal failure (Ohkubo, Matsubara et al. 1997), myocardial infarction (Nio, Matsubara et al. 1995), vascular injury, and wound healing (Kimura, Sumners et al. 1992).

Angiotensin (2-8) (AngIII), another biologically active peptide of the RAS is generated from Ang II by aminopeptidase A (Vaughan et al.1974; Kugler et al.1982). AngIII has high affinity for AT1 and AT2 receptors and can induce vasoconstriction, aldosterone release and sodium retention (Zini, Fournie-Zaluski et al. 1996). In the central nervous system, AngIII mediates central control of blood pressure and vasopressin release (Harding, Wright et al. 1994; Song, Wilk et al. 1997). However, AngIII was catabolized metabolically five times faster than Ang II because it is very sensitive to degradation by the enzyme aminopeptidase N, which cleaves the N-terminal arginine, generating AngIV (Zini, Fournie-Zaluski et al. 1996). AngIV is also short lived and is rapidly converted to inactive fragments by several peptidases. AngIV mediates its action via the AT4 receptor, also known as insulin-regulated amino peptide receptor (IRAP) (Albiston, McDowall et al. 2001). AT4 receptors are expressed in brain, kidney, heart and vessels. Its signaling mechanisms are largely unknown, but it influences local blood flow and is associated with cognitive processes and sensory response (de Gasparo, Catt et al. 2000)
1.2.2 Angiotensin, inflammation and cardiovascular disease (CVD)

It has been vastly recognized that RAS overactivation contributes to various cardiovascular diseases and many clinical studies have demonstrated that inhibition of the RAS through ACE inhibition or angiotensin receptor blockers (ARB) is beneficial for patients with CVD. In 1992 it was proved that an ACE-inhibitor, enalapril, in combination with the conventional heart failure therapy, significantly improved the survival of patients with severe congestive heart failure (Kjekshus, Swedberg et al. 1992). In the Survival of Myocardial Infarction Long-Term Evaluation (SMILE) study, the risk of myocardial infarction was reduced by about 37% with ACE inhibitors treatment (Borghi and Ambrosioni 1995). Results from the Heart Outcomes Prevention Evaluation (HOPE) study revealed that in patients without heart failure ACE inhibition reduced the rates of death, myocardial infarction, and stroke by 22% without major effects on blood pressure (Yusuf, Sleight et al. 2000). Furthermore, Losartan Intervention For Endpoint reduction (LIFE) trial revealed that among patients with hypertension and left ventricular hypertrophy losartan, an AT1 receptor blocker, significantly reduced the risk of combined morbidity and mortality compared to a beta-blocker, atenolol (Dahlof, Devereux et al. 2002).

As a potent pro-inflammatory factor, AngII has been implicated in the pathogenesis of CVD. Inflammation involves: 1) activation of the endothelium of blood vessels (permeability) and 2) extravasation (adhesion/transmigration) of specific leukocyte populations to the site of injury. It has been demonstrated that AngII acts via AT1 receptors, induces the expression of endothelial adhesive
molecules including selectins, vascular cell adhesion molecules-1 (VCAM-1),
intercellular adhesion molecules-1 (ICAM-1) and integrins to increase vascular
permeability (Piqueras, Kubes et al. 2000; Pueyo, Gonzalez et al. 2000; Alvarez,
Cerda-Nicolas et al. 2004). AngII also activates COX-2, which generates reactive
oxygen species (ROS) and vasoactive prostaglandins including leukotriene C4
and prostaglandins E2 to induce endothelial dysfunction (Wu, Laplante et al.
2005; Welch 2008). In addition, vascular endothelial growth factor (VEGF), has
been reported to be upregulated by AngII in human VSMCs (Williams, Baker et
al. 1995) and rat heart endothelial cells (Chua, Hamdy et al. 1998).

AngII facilitates the recruitment of infiltrating inflammatory cells into tissues
by stimulating the production of specific cytokine/chemokines. For example,
AngII induces the production of the potent monocyte chemoattractant MCP-1 in
cultured monocytes (Dai, Xu et al. 2007). In spontaneously hypertensive rats
(SHR) elevated AngII level has been associated with increased expression of
MCP-1 and one of its receptors, the C–C chemokine receptor (CCR2) in
macrophages. And AT1 receptor blockade reduced aortic inflammation and
macrophage MCP-1/CCR2 expression in SHR rats (Dai, Xu et al. 2007).
Moreover, in cultured mesangial and vascular smooth muscle cells, Ang II acts
on AT1 receptors to stimulate the expression of the Toll-like receptor 4 (TLR-4),
which promotes cellular oxidative injury, apoptosis and inflammation (Ji, Liu et al.
2009; Lv, Jia et al. 2009). It has been reported recently that AT1 receptor
blockade exerts protective effects against myocardial ischemia and reperfusion
injury by reducing TLR-4 expression and cytokine release (Yang, Jiang et al.
In addition, AngII also stimulates cytoskeletal rearrangements in T cells and triggers the release of specific cytokines and chemokines, thus facilitating T cell recruitment to the sites of inflammation (Jurewicz, McDermott et al. 2007; Crowley, Frey et al. 2008; Kvakan, Kleinewietfeld et al. 2009).

Taken together, results from both clinical trials and experimental studies infer a critical role for the RAS in CVD pathogenesis and support the beneficial effect of AngII inhibition in CVD patients.

1.2.3 Sex Hormones and the RAS

Sex hormones exert differential effects on various components of the RAS as illustrated in Figure 1.4 (Fischer, Baessler et al. 2002). The overall effect of physiological concentrations of estrogen is to inhibit the RAS while androgen generally stimulates the RAS.

Angiotensinogen, the only known precursor of AngII, has an estrogen response element (ERE) in its gene promoter which markedly stimulates angiotensinogen synthesis (Clauser, Gaillard et al. 1989; Feldmer, Kaling et al. 1991). Women with oral administration of estrogen replacement therapy exhibit higher plasma angiotensinogen levels (De Lignieres, Basdevant et al. 1986; Schunkert, Danser et al. 1997). In rats, renal angiotensinogen mRNA expression was lower in females compared to males (Ellison, Ingelfinger et al. 1989; Yanes, Sartori-Valinotti et al. 2009). Furthermore, angiotensinogen mRNA expression was reduced by castration and increased by administration of exogenous androgen (Ellison, Ingelfinger et al. 1989). Similarly, renal and hepatic angiotensinogen
mRNA levels were higher in male than female SHR rats (Chen, Naftilan et al. 1992). Castration of male SHR rats reduced, and testosterone replacement restored renal and hepatic angiotensinogen mRNA expression (Chen, Naftilan et al. 1992). Additionally, testosterone exerts considerable stimulatory effects on angiotensinogen gene expression in a variety of other tissues, including the adipose tissue (Ming, Sikstrom et al. 1993; Serazin-Leroy, Morot et al. 2000).

In humans, plasma renin levels in postmenopausal women are lower compared to men and are further decreased by hormone replacement therapy (Schunkert, Danser et al. 1997; Danser, Derkx et al. 1998). In healthy young adult population, men exhibit higher ACE activity compared to women (Zapater, Novalbos et al. 2004). ACE is suppressed in women taking estrogen replacement therapy (Proudler, Ahmed et al. 1995; Schunkert, Danser et al. 1997). In experimental models, the circulating concentration of renin is decreased by estrogen (Oelkers 1996; Brosnihan, Weddle et al. 1997), but increased by testosterone (Wagner, Metzger et al. 1990; Chen, Naftilan et al. 1992; Kon, Endoh et al. 1995). Plasma ACE activity and ventricular ACE expression are significantly higher in male than female mice, and are reduced by castration of male mice (Freshour, Chase et al.; Lim, Retnam et al.).

In studies with estrogen replacement therapy, estrogen appears to downregulate AT1 receptor mediated effects through reductions in the receptor density and down-stream activation steps (Nickenig, Baumer et al. 1998; Owonikoko, Fabucci et al. 2004). In experimental rodents, AT1 receptor densities at heart, kidney and aortic tissues are decreased by estrogen and increased by
ovariectomy (Kisley, Sakai et al. 1999; Wu, Maric et al. 2003; Dean, Tan et al. 2005) In contrast, testosterone upregulates the AT1 receptor as castration almost abolished the AT1 receptor protein in rat epididymis (Leung, Wong et al. 1999). AT1 receptor protein expression is higher in the renal cortex of male spontaneous hypertensive rats (Sullivan, Semprun-Prieto et al. 2007). In addition, elevated contractile responses to AngII in conjunction with greater AT1 receptor mRNA and protein expression were detected in the rat aorta from male compared to female rats (Silva-Antonialli, Tostes et al. 2004). Renal AT2 receptor expression in female mice is higher than in male mice (Armando, Jezova et al. 2002; Dean, Tan et al. 2005). It has been reported that castration increases AT2 expression in male rat bladder while exogenous testosterone administration reduces its expression (Nakazawa, Tanaka et al. 2007). Most recently, higher expression of renal ACE2 in female mice has been reported and ovariectomy reduces ACE2 levels (Ji, Menini et al. 2008).

In summary, while estrogen exerts either stimulatory or inhibitory effects on different components of RAS, the overall effect of estrogen results in a down regulation of the RAS. In contrast, testosterone exerts a stimulatory effect on all major components of the RAS.

1.3. AngII-induced AAA

1.3.1 Overview

It has been well established that chronic infusion of AngII into hyperlipidemic mice (LDLR/-/- or ApoE/-/-) promotes the formation of AAAs
Based on the desired infusion rate (500-1000 ng/kg/min), osmotic mini-pumps are filled with a pre-determined concentration of AngII and implanted subcutaneously in the subscapular region of mice to achieve chronic AngII infusion. LDLR-/- or ApoE-/- mice are usually fed with a moderately high fat diet (44% kcal from fat) to augment hyperlipidemia prior to and during AngII infusion. AAAs form at a high prevalence (80%-100%) with 28 days of chronic AngII infusion (1000 ng/kg/min) in male mice. AAAs that form from AngII infusion are typically localized in suprarenal region of the aorta, which is consistent in both ApoE-/- and LDLR-/- mice. AngII-induced AAAs are characterized by medial breaks, macrophage infiltration into media, thrombus formation, extracellular matrix degradation and adventitia remodeling. In comparison, infusion of AngII into a normolipidemic background strain, C57BL/6, results in only 10-30% AAA formation in male mice (Wang 2005). It has been suggested that hyperlipidemia facilitates AngII-induced AAA formation in mice via upregulating AT1R expression. Multiple studies also have demonstrated that chronic AngII infusion increased the extent of atherosclerosis in hyperlipidemic mice (Daugherty, Rateri et al. 2004). Atherosclerotic lesions are present in the abdominal aortas of mice infused for very chronic durations with AngII (Saraff, Babamusta et al. 2003). However, atherosclerosis does not appear to be a requirement for AAA formation. For example, manipulations have been demonstrated to increase atherosclerosis, but yet decrease AngII-induced AAAs (Henriques, Huang et al. 2004). In addition, AngII infusion in nonhyperlipidemic C57BL/6 mice result in modest
AngII-induced AAA formation, but does not induce atherosclerosis, further demonstrating that atherosclerosis is not a major contributor to AngII-induced AAA formation.

As previously discussed, AngII is a potent vasoconstrictor and exerts potent effects on peripheral resistance. Mice infused with AngII (1000 ng/kg/min) typically exhibit an increased systolic blood pressure in the range of 20-30 mmHg (Daugherty, Manning et al. 2000; Manning, Cassis et al. 2002). Although hypertension has been debated as a risk factor for human AAA formation, AngII-induces AAA formation in hyperlipidemic mice appears to be independent of AngII-induced hypertension (Daugherty, Manning et al. 2000; Weiss, Kools et al. 2001). Infusion of norepinephrine to male LDLR-/- mice at the dose that resulted in a similar hypertensive response as AngII infusion, did not cause AAA formation (Cassis, Gupte et al. 2009). In addition, co-infusion of antihypertensive drug (hydralazine) with AngII abolishes the increased blood pressure response and has no impact on AngII-induced AAA formation (Cassis, Gupte et al. 2009).

AngII infusions activate endogenous RAS and promote aldosterone release by the adrenal gland. However, aldosterone does not appear to contribute to AngII-induced AAA as it has been demonstrated that male ApoE-/- mice infused with multiple doses of aldosterone failed to generate AAAs (Cassis, Helton et al. 2005). Furthermore, the aldosterone receptor antagonist spironolactone had no effect on AngII-induced AAAs. These data demonstrate that aldosterone is not involved in the development of AngII-induced AAAs.
1.3.2 Angiotensin receptors in AngII-induced AAAs

As discussed previously, AngII-induced inflammatory response and ROS production are mediated through AT1 receptor. It has been demonstrated that co-infusion of AngII and the AT1 receptor antagonist, losartan, completely prevented AngII-induced AAA formation, demonstrating that AngII infusion promotes AAA formation through its action on the AT1 receptor (Daugherty, Manning et al. 2001). AT2 receptor activation by AngII is believed to counterbalance some of the effects of AT1 receptor. Co-infusion of AngII and the AT2 receptor antagonist PD123319 enhanced AngII-induced AAA formation in male ApoE-/- mice (Daugherty, Manning et al. 2001). These results demonstrate that the AT1 receptor plays a critical role in the development of AngII-induced AAA while the AT2 receptor protects against AAA formation. Of the two AT1 receptor subtypes in mice, results have demonstrated that, despite the abundance of AT1b receptor in the aortas, deficiency of the AT1a receptor subtype completely ablates the development of AngII-induced AAAs (Cassis, Rateri et al. 2007). Furthermore, AT1aR deficient mice did not exhibit a pressor response to AngII infusion and AngII-induced atherosclerotic lesion formation was abolished by AT1aR deficiency (Cassis, Rateri et al. 2007). Collectively, these data demonstrate that the AT1a receptor is pivotal for AngII-induced AAA and atherosclerosis formation in hyperlipidemic mice.

Macrophages are abundantly evident in AngII-induced AAA tissues and atherosclerotic lesions and AT1aR are present on macrophage. To investigate whether the activation of AT1aR on infiltrating monocytes or resident vascular
cells contributes to AngII-induced AAA formation, a series of bone marrow transplantation studies were conducted to create chimeric mice lacking AT1aR on bone marrow-derived cells. Both wild-type and AT1aR-/- recipients were irradiated and repopulated with bone marrow-derived cells from mice that was either wild type or AT1aR deficient. Interestingly, only AT1aR deficient mice were dramatically protected from AngII-induced AAA and atherosclerosis, irrespective of the donor genotype. These data indicate that AT1aR in resident vascular tissue is required for the development of AngII-induced atherosclerosis and AAAs (Cassis, Rateri et al. 2007).

1.3.3 Mechanistic insights into AngII-induced AAAs

Numerous publications have demonstrated various mechanisms contributing to the development of AngII-induced AAA (Table 1.1). In agreement with human AAAs, results from AngII-induced AAAs in mice also support a role for matrix metalloproteinases (MMPs) in AAA formation. Deficiency of MMP-2 or MMP-9 completely eliminates calcium chloride induced AAA formation (Longo, Xiong et al. 2002). Administration of doxycycline, a broad spectrum inhibitor of MMPs, has been demonstrated to attenuate AngII-induced AAA in LDLR-/- mice (Manning, Cassis et al. 2003). Metacept-1 (MCT-1), a synthesized histone deacetylase inhibitor can inhibit MMP-9 mRNA expression in metastatic cancer cells in vitro (Kim, Lee et al. 1999). Administration of MCT-1 significantly reduced AAA formation in male ApoE-/- mice (Vinh, Gaspari et al. 2008). Further evidence for MMP involvement in AngII-induced AAAs comes from recent studies
demonstrating that deficiency of group X secretory phospholipase (sPLA2) in ApoE-/- mice reduced AngII-induced AAAs (Zack, Boyanovsky et al. 2010). Aortas from group X sPLA2 that exhibited reduced AAA formation had blunted expression of MMP-2, -13 and 14. Interesting recent studies demonstrated that deficiency of telomerase in bone marrow-derived cells reduced AngII-induced AAA in LDLR-/- mice, and that these effects were associated with reduced macrophage MMP-2 expression (Findeisen, Gizard et al. 2010). Collectively, results from studies using many different approaches support a role for MMP activation in pivotal cell types in AngII-induced AAAs.

In addition, the plasminogen system has been revealed to contribute to AngII-induced AAA formation, further supporting an important role of MMPs in AAA pathogenesis. Plasminogen is converted to plasmin by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). Plasmin degrades multiple extracellular matrix (ECM) proteins and activates MMPs. Deficiency of plasminogen or uPA has been reported to protect mice from AngII-induced AAA (Deng, Martin-McNulty et al. 2003; Gong, Hart et al. 2008). Plasminogen activator inhibitor-1(PAI-1) is a major inhibitor of the plasminogen system and overexpression of PAI-1 has been demonstrated to reduce AngII-induced AAA formation (Qian, Gu et al. 2008).

Inflammation and macrophage accumulation in AAA regions are consistent and profound features of AngII-induced AAAs. Monocyte chemoattractant protein-1 (MCP-1), a prominent chemokine, interacts with its receptor, CCR2, to invoke macrophage-based immune responses. Deficiency of
CCR2 markedly attenuated the development of AngII-induced AAAs (Ishibashi, Egashira et al. 2004). Furthermore it has been revealed that CCR2 expression on bone marrow-derived cells is required for AngII-induced AAAs formation (Ishibashi, Egashira et al. 2004; Daugherty, Rateri et al. 2010). Interferon-γ (IFN-γ) is a major T-helper type 1(Th1) cytokine and an important mediator of Th1 immune response. In ApoE-/-mice AngII-induced AAA formation was greatly enhanced with deficiency of interferon-γ (IFN-γ) or IFN-γ-inducible T-cell chemokine, CXCL10 (King, Lin et al. 2009). Recently, our laboratory has demonstrated that obesity significantly promotes AngII-induced AAA formation in nonhyperlipidemic mice. The enhanced AAA formation was associated with increased macrophage accumulation in periaortic adipose tissue (Police, Thatcher et al. 2009).

Cyclooxygenase (COX) products, mainly prostaglandin E2 (PGE2), are classic mediators of inflammation. COX-2 is expressed in the vessel wall and macrophages of AAA tissues (Holmes, Wester et al. 1997; Walton, Franklin et al. 1999). In addition, COX-2 expression in human AAA is positively associated with the extent of macrophage infiltration and the degree of AAA neorevascualrization (Chapple, Parry et al. 2007). With chronic AngII infusion mice exhibited increasing levels of COX-2 mRNA in abdominal aortas and deficiency of COX-2 significantly eliminated AngII-induced AAA formation (Gitlin, Trivedi et al. 2007). Administration of Celecoxib, a COX-2 inhibitor, reduced AngII-induced AAA incidence and severity in male ApoE-/- mice (King, Trivedi et al. 2006). An important source of PGE2 in the mouse is microsomal prostaglandin (PG) E2
synthase-1 (mPGES-1), which catalyzes the conversion of the COX product PGH$_2$ to PGE$_2$. It has been demonstrated that mPGES-1 deficiency reduced oxidative stress, aortic MMP-2 activity and markedly attenuated AngII-induced AAA formation in LDLR-/- mice (Wang, Lee et al. 2008). Moreover, recent studies demonstrated that deletion of the EP4 receptor on bone marrow-derived cells enhanced AngII-induced AAAs in LDLR-/- mice, supporting a protective effect of PGE2 through the EP4 receptor (Tang, Shvartz et al. 2010).

Similar to COX enzymes, lipoxygenases also produce a variety of bioactive lipids derived from arachidonic acid. For example, 5-lipoxygenase (5-LO) catalyzes the generation of Leukotriene B4, which exerts its effects through binding to its G protein coupled receptor, BLT-1. Deficiency of BLT-1 reduced AngII-induced AAAs in ApoE-/- mice (Ahluwalia, Lin et al. 2007). However, a recent study has demonstrated that both genetic deficiency and pharmacological inhibition of 5-LO failed to influence the AngII-induced AAAs in ApoE-/- mice (Cao, Adams et al. 2007). Thus, whether 5-LO signaling cascade contributes to AAA pathogenesis remains under debate.

Human AAA tissues exhibit increased superoxide anion (O$_2^-$), hydroxyl radical and reactive nitrogen species compared to normal aortic tissue (Miller, Sharp et al. 2002). As discussed previously, AngII acts via AT1 receptors to activate NADPH oxidase and produce O$_2^-$ in cultured vascular smooth muscle cells (Griendling, Minieri et al. 1994). NADPH oxidase is a primary source of ROS in the vasculature. Oxidative stress can regulate inflammation, MMP activation, and vascular smooth muscle cell apoptosis, thus influencing AAA
development. Dietary enrichment of vitamin E, an antioxidant, has been shown to attenuate AngII-induced AAA formation in ApoE-/- mice (Gavrila, Li et al. 2005). In contrast, a recent study found no effect of dietary enrichment of vitamins E and C on AngII-induced AAA rupture in aged ApoE-/- mice (Jiang, Jones et al. 2007). However, it has been demonstrated that deficiency of p47phox, a cytosolic subunit of NADPH oxidase, decreases aortic macrophage, aortic MMP-2 activity and greatly reduced AngII-induced AAA formation in male ApoE-/- mice (Thomas, Gavrila et al. 2006). In addition, deficiency of NOX1, a transmembrane subunit of NADPH oxidase, has been reported to attenuate AngII-induced AAA in C57BL/6 mice (Gavazzi, Deffert et al. 2007). Recently, deficiency of cyclophilin, an oxidative stress response mediator, was demonstrated to ablate AngII-induced AAA formation in ApoE-/- mice (Satoh, Nigro et al. 2009). Overall, these findings suggest a potential role of and oxidative stress and NADPH oxidase in the development of AngII-induced AAA.

Initial studies demonstrated that administration of neutralizing antibody to TGF-β to ApoE-/-/Cxcl10-/- mice decreased AngII-induced aortic dilation (King, Lin et al. 2009). However, recent studies demonstrated that inhibition of TGF-β has been reported to enhance inflammation and profoundly increase aortic dissection in AngII infused C57BL/6 mice (Wang, Ait-Oufella et al. 2010). The role of TGF-β in AngII-induced AAAs, as well as human AAA, is unclear.

SMC apoptosis is another evident pathological features frequently observed in AAA tissues. Administration of nonspecific caspase inhibitor was reported to reduce SMC apoptosis, medial inflammation and AngII-induced AAA
formation (Yamanouchi, Morgan et al. 2010). Several studies targeting intracellular signaling pathways have provided further mechanistic insights in AngII-induced AAAs. It has been reported that Fasudil, a Rho-kinasse inhibitor, reduces AngII-induced AAAs formation in ApoE-/- mice by inhibiting apoptosis and proteolysis (Wang, Martin-McNulty et al. 2005). Administration of a JNK inhibitor, SP600125, attenuated the aneurysm development in CaCl2 induced AAA mouse model (Yoshimura, Aoki et al. 2005). In addition, when SP600125 was chronically administered for 8 weeks after 4 weeks AngII infusion, the luminal diameters of formed AngII-induced AAAs showed a significant 18% reduction and there was no change in aortic diameter of control mice administered with vehicle (Yoshimura, Aoki et al. 2005). So far his is the only animal study that exhibits AAA regression with pharmacologic intervention. Further studies are required to confirm these findings and elucidate the mechanisms of AAA progression/regression. The investigation of factors and mechanisms influencing AAA expansion and progression may guide us to new drug targets and other nonpharmaceutical interventions for AAA treatments.

A feature of human AAAs that has been recently explored in AngII-induced AAAs is neovascularization to support continued aneurysm growth and progression. ApoE-/- mice administered human recombinant vascular endothelial growth factor (VEGF) exhibited increased incidence and size of AngII-induced AAAs (Choke, Cockerill et al. 2010). Studies in our laboratory demonstrated that weight loss in obese mice decreased adventitial neovascularization and prevented continued growth of established AAAs (Police,
Putnam et al. 2010). These studies suggest that neovasculization is an important mechanism contributing to continued progression of AngII-induced AAAs.

1.3.4 Gender in AngII-induced AAAs

Similar to the human condition, male mice are much more susceptible to AngII-induced AAA formation. The AngII-induced AAA incidence of male mice is 4-5 fold higher compared to that of females (Manning, Cassis et al. 2002; Henriques, Huang et al. 2004). Interestingly, AngII-induced atherosclerosis and hypertension are similar between males and females despite the striking gender difference in AAA formation. Multiple studies have been conducted to investigate if estrogen exerts some protective effects in female mice to limit AngII-induced AAA formation. Martin-McNulty et al. demonstrated that administration of 17-beta estradiol decreased AngII-induced AAA and atherosclerosis in male ApoE-/- mice (Martin-McNulty, Tham et al. 2003). These reductions were associated with decreased levels of pro-inflammatory and adhesion molecules. However, our laboratories have reported that elimination of endogenous estrogen by ovariectomy of female ApoE-/- does not alter AngII-induced AAAs or atherosclerosis (Henriques, Huang et al. 2004). Notably, orchiectomy of male ApoE-/- mice markedly reduced AngII-induced AAA formation, resulting in a similar AAA incidence as that of female mice (Henriques, Huang et al. 2004). Furthermore, AngII-induced AAA formation in castrated male mice was fully restored by exogenous androgen, dihydrotestosterone (DHT) (Henriques, Zhang
et al. 2008). Interestingly, even female ApoE-/- mice responded to DHT and exhibit a 3-fold increase in AngII-induced AAA formation (Henriques, Zhang et al. 2008). As summarized in Table 1.2, all these results demonstrate that androgen is a primary mediator of gender difference in AngII-induced AAA formation.

1.3.5 Gender in elastase-induced AAAs

In vasculature elastin fibers are crucial for the maintenance of structural integrity of blood vessels. Progressive destruction of structural matrix proteins, particularly elastin and collagen, is considered as one of the most important pathogenesis characteristics in human AAAs. First described in rats, transient intraluminal perfusion of abdominal aortas with porcine pancreatic elastase provides another widely used animal model of AAAs (Anidjar, Salzmann et al. 1990; Anidjar, Dobrin et al. 1992; Anidjar, Dobrin et al. 1994). Briefly, a segment of infrarenal abdominal aorta is isolated by a distal suture and a perfusion catheter is introduced at the iliac bifurcation. Porcine pancreatic elastase is instilled into the isolated segment of infrarenal abdominal aorta for 5 minutes at 100 mmHg pressure before restoration of flow. This transient intraluminal infusion of elastase induces mild aortic dilation immediately after the perfusion. Aortic diameter remains stable for up to 7 days followed by a rapid and significant dilation of the abdominal aorta. AAAs, which are defined by at least 100% increase in aortic diameter over the normal (pre-perfusion) aortas, typically form by day 14. The development of AAAs in this elastase perfusion model is
associated with transmural aortic wall infiltration by mononuclear phagocytes, increased local production of MMPs and massive destruction of the medial elastic lamellae (Thompson, Curci et al. 2006).

Recently, the application of this elastase model to mice have assisted investigators identify and study various mediators of AAA formation. For example, administration of doxycycline, a broad-spectrum inhibitor of MMPs, significantly attenuated the development of elastase-induced AAA formation (Bartoli, Parodi et al. 2006). In agreement to the findings in AngII-induced AAA model, deficiency of MMP-9 has been shown to totally ablate elastase-induced AAA formation (Pyo, Lee et al. 2000). In contrast, deficiency of MMP-12, alone or with MMP-9 deficiency, did not influence the occurrence of elastase-induced AAAs (Pyo, Lee et al. 2000). Smoking, as a primary risk factor of human AAA, also significantly enhanced the development of elastase-induced AAAs (Buckley, Wyble et al. 2004). Interestingly, inhibition of RAS by AT1 blocker administration has been demonstrated to suppress elastase-induced AAAs formation, suggesting RAS also contribute to the development of AAA in the elastase perfusion model (Fujiwara, Shiraya et al. 2008).

As summarized in Table 1.2, elastase-induced AAA model also exhibits gender difference, similar to AngII-induced AAA model. For example, male rats had larger AAAs and higher AAA incidence than female rats (Ailawadi, Eliason et al. 2004). Male rat aortas showed more significant macrophage infiltrates and increased MMP-9 production and activity (Ailawadi, Eliason et al. 2004). In addition, male rats demonstrated smaller aneurysms, less
macrophage/neutrophil infiltrate and MMP-9 when treated with estradiol or tamoxifen, a selective estrogen receptor modulator (Ailawadi, Eliason et al. 2004; Grigoryants, Hannawa et al. 2005). Ovariectomized female rats exhibited a higher aneurysm dilatation rate and significantly higher MMP-2 and MMP-9 expressions compared with sham-operated control females (Cho, Woodrum et al. 2009; Wu, Zhang et al. 2009). Exogenous estrogen administration decreased aneurysm dilatation rate and MMPs expression in ovariectomized females (Cho, Woodrum et al. 2009; Wu, Zhang et al. 2009). It has been demonstrated that male rats shows higher MMP2 in aortic smooth muscle cells and in vivo estrogen exposure greatly decreases male aortic MMP2 production to levels seen in the female aorta (Woodrum, Ford et al. 2009). These data suggest an important protective role of estrogen in the development of elastase-induced AAAs. Furthermore, orchiectomized male rats had significantly smaller AAAs compared with sham control males and exogenous androgen administration significantly increased elastase-induced AAAs dilation in castrated male rats (Cho, Woodrum et al. 2009). It suggests that, in agreement to the observations in AngII-induced AAAs, androgen contributes to the pathogenesis of AAAs in elastase perfusion model. However, exogenous androgen had no effect on the development of elastase-induced AAA in female rats (Cho, Woodrum et al. 2009). Overall, these studies implicate a definitive protective role of estrogen and suggest a likely contribution of androgen in elastase-induced AAAs.
1.4 Androgen

In males, androgens are essential for the development and function of the testes, maturation of secondary sexual characteristics, masculinization of the bone-muscle apparatus, libido, and stimulation of spermatogenesis. Testosterone and its more active metabolite, DHT, are the key androgens responsible for the development of male genitalia in utero as well as secondary sex characteristics during puberty. In adulthood, androgens remain essential for the maintenance of male reproductive function and sexual behavior. The primary circulating androgen in males is testosterone. The site of testosterone production in the testis is the Leydig cell. Both synthesis and secretion of testosterone are under regulation of pituitary luteinizing hormone (LH) and local factors (Lei et al. 2001; Sriramam et al. 2005). It has been reported that in women androgens play a role in regulating libido, energy, muscle and bone strength (Schneider 2003; Bolour and Braunstein 2005).

Like all steroid hormones, testosterone is derived from cholesterol. This transformation goes through different enzymatic steps in which the side chain of cholesterol is shortened through oxidation. The complete pathway for testosterone synthesis is depicted in Figure 1.5. The rate limiting step in the synthesis of testosterone is the cleavage of cholesterol to pregnenalone, which is catalyzed by the P450-linked side chain cleaving enzyme (P450scc) found in the mitochondria of steroid producing cells. Testosterone regulates its own synthesis through a negative feedback loop which regulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary and
gonadotropin releasing hormone (GnRH) by the hypothalamus. Low levels of circulating testosterone decrease the inhibition on GnRH synthesis, leading to elevated FSH and LH levels, resulting in increased testosterone synthesis.

1.4.1 Metabolism of androgen

Testosterone can be also converted to another two important hormones: DHT through 5α-reduction and estradiol through aromatization. While only a small amount of circulating testosterone (<5%) gets converted to active metabolites, which typically occurs at the tissue level, this process can amplify and diversify testosterone function (Liu, Death et al. 2003). Conversion of testosterone to DHT is irreversibly catalyzed by the enzyme 5α-reductase. Like testosterone, DHT binds to androgen receptors and amplifies testosterone action. Importantly, DHT binds with higher molar potency due to greater affinity and a slower dissociation rate (Wilbert, Griffin et al. 1983; Grino, Griffin et al. 1990). Furthermore, DHT activates gene expression more efficiently than testosterone (Deslypere, Young et al. 1992). Therefore, testosterone, through conversion to DHT, can exert more potent effects in tissues expressing 5α-reductase compared to tissues where the enzyme is not expressed. Two forms of the 5α-reductase enzyme have been identified: type I, is predominantly found in nongenital skin and the liver, and type II, which is primarily located in urogenitals of men and genital skin in both men and women. Both forms of the enzyme have been located in vascular tissue based on immunoreactivity (Eicheler, Tuohimaa
et al. 1994; Eicheler, Dreher et al. 1995) and enzymatic activity (Milewich, Kaimal et al. 1987; Fujimoto, Morimoto et al. 1994).

Testosterone actions are diversified by its conversion to estradiol and the subsequent binding to the ER. The conversion reaction is irreversible and catalyzed by the enzyme aromatase (CYP19). Aromatase is expressed in many tissues, particularly in the liver and adipose tissue. Additionally, aromatase gene expression, protein levels and enzymatic activity have been detected in vascular tissue, particularly in the endothelium and smooth muscle (Diano, Horvath et al. 1999; Harada, Sasano et al. 1999; Murakami, Harada et al. 2001). Approximately 85% of circulating estradiol in men is derived from testosterone conversion by aromatase, with the remainder being secreted by the leydig cells of the testes (Aiman, Griffin et al. 1979).

1.4.2 Androgen receptor

Like other steroid hormones, androgens mediate their biological action through binding to androgen receptors (AR), members of the steroid hormone receptor superfamily. AR is widely expressed in many various tissues (Wilson and McPhaul 1996). In the vasculature, ARs are expressed in various cell types, including endothelial cells, vascular smooth muscle cells, macrophages, and platelets. Interestingly, it has been demonstrated that males exhibits higher AR density in vascular tissue compared to females (Higashiura, Mathur et al. 1997). In macrophage, AR density has been reported to be 4-fold higher in males compared to females (McCrohon, Death et al. 2000; Ng, Quinn et al. 2003).
AR is produced from a single-copy gene located on the X-chromosome (Brown, Goss et al. 1989; Kokontis and Liao 1999). The genomic DNA encoding AR spans about 90 kb and codes for a 2,757-base pair open reading frame within a 10.6-kb mRNA and the coding sequence of human AR gene consists of eight exons (Roy, Tyagi et al. 2001). The AR is a ligand-inducible transcription factor which regulates the expression of target genes in response to ligand binding (Heinlein and Chang 2002). Similar to other members of the nuclear receptor superfamily, AR has four major functional regions; the N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD (Mangelsdorf, Thummel et al. 1995). The DBD of the AR exhibits a high degree of amino acid sequence homology to other steroid hormone receptors including the glucocorticoid receptor (GR), the progesterone receptor (PR), and mineralocorticoid receptor (MR). Consequently, the four receptors recognize very similar hormone response elements (HREs). The transcriptional activity of AR is mediated by a constitutively active activation function (AF-1) originating in the N-terminal TAD and a ligand-dependent activation function (AF-2) in the LBD (Gronemeyer and Laudet 1995). The hinge region was initially considered to be a flexible linker between the DBD and LBD. However, this region has been revealed to be involved in DNA binding as well as AR dimerization and to attenuate transcriptional activity of the AR (Wang, Lu et al. 2001; Haelens, Tanner et al. 2007).
1.4.3   Mechanism of action of ARs

The unbound AR is typically found in the cytoplasm of target cells, where it is sequestered as a multi-protein complex with heat shock proteins and immunophillins (DeFranco 1999). In the absence of the agonist, the ligand-binding domain (LBD) at the carboxyl terminus of the AR prevents the transactivation function of the N-terminal domain. Deletion of the LBD makes the receptor constitutively active by allowing its interaction with coactivator proteins (Roy, Tyagi et al. 2001). Upon ligand binding, a conformational change occurs which causes the dissociation of heat shock proteins, dimerization and translocation to the nucleus, thus activating androgen response elements (AREs) (Roy, Tyagi et al. 2001). Within the nucleus, the AR scans the genome for AREs in the promoters and enhancers of target genes. ARE is a 15-bp palindromic sequence which consist of two hexameric half-sites arranged as inverted repeats with a 3-bp spacer. Once bound to the hormone response element of a target gene, the receptor dimer recruits and interacts with classes of co-regulators and key transcriptional machinery to induce or inhibit transcription of a particular target gene. Both testosterone and DHT bind to the LBD of the AR, allowing the receptor complex to bind to specific responsive genes and stimulate the expression of those target genes (Brinkmann and Trapman 2000). However, if the AR is antagonist bound, this receptor dimer binds to the ARE and recruits co-repressors such as N-CoR and SMRT and, indirectly, histone deacetylases, leading to transrepression of target genes (Aarnisalo, Palvimo et al. 1998; Slagsvold, Kraus et al. 2001; Kinyamu and Archer 2004).
As described previously, one critical AR-mediated transcription through co-regulators is epigenetic chromatin modification (Heemers and Tindall 2007). It involves two primary types of coregulatory complexes (Kinyamu and Archer 2004). The first is the adenosine triphosphate (ATP)-dependant chromatin remodeling complexes, such as the switch/sucrose nonfermenting (SWI/SNF) complex. The ATP-dependent chromatin remodeling complexes primarily mediates the opening of chromatin (Cartwright, Hertzberg et al. 1983; Gross and Garrard 1988), facilitates formation of nucleosome arrays, homologous strand pairing and DNA transcription (Lusser and Kadonaga 2003). The second type of coregulatory is histone modifiers such as histone deacetylases (HDAC), histone acetyltransferases (HAT) and histone methyltransferases (HMT). Some androgen receptor coactivators, such as CBP/p300 and LSD1, mediate transcription through their histone modifying enzymatic activities (Baek, Ohgi et al. 2006; Kahl, Gullotti et al. 2006). Modification of a histone residue changes the net charge of the nucleosome, which results in loosening or tightening of the DNA-histone interactions, thus affecting transcription efficiency. Histone modifications mediate transcription through acetylation/deacetylation of lysine residues on histone H4, and through methylation of histone lysine and arginine residues (Strahl and Allis 2000; Kraus and Wong 2002).

Furthermore, androgen also exert non genomic effects of androgens which are faster than genomic effect through AR (Losel and Wehling 2003). Most nongenomic effects involve a membrane receptor, and putative binding sites are described for all major classes of steroids, including androgens (Heinlein and
Chang 2002). In general, nongenomic androgen effects involve the rapid induction of conventional second messenger signal transduction cascades, including increases in cytosolic calcium and activation of protein kinase A, protein kinase C, and MAPK, leading to diverse cellular effects. Nongenomic androgen action is insensitivity to inhibition of RNA and protein synthesis and classic AR antagonists (Liu, Death et al. 2003).

1.4.4 Androgens and cardiovascular disease (CVD)

In general, men are more than twice as likely as women to die from coronary heart disease in all populations and male gender is considered to be an independent risk factor for CVD (British Heart Foundation Statistics Database 1998). Androgens, specifically testosterone, are believed to be associated with a higher risk of CVD in men (Foreman 1986; Kalin and Zumoff 1990). Increased premature cardiovascular events have been observed in male athletes using high levels of anabolic steroids (Bagatell and Bremner 1996). Researchers in recent years have turned with increasing interest in elucidating the role of androgen on the development of CVD. Interestingly, a 10 year-follow up cohort study has revealed that testosterone levels are not different between men who develop CVD and their healthy cohorts (Arnlov, Pencina et al. 2006). In large prospective cohort studies (Smith, Ben-Shlomo et al. 2005; Arnlov, Pencina et al. 2006), baseline testosterone levels had not been associated with incidence of cardiovascular events. These data do not support the hypothesis that androgen increases CVD risk.
In addition, a retrospective data analysis on male veterans showed an association of low androgen levels with increased mortality (Shores, Matsumoto et al. 2006). In a large cohort study older men with testosterone insufficiency were found to have an increased risk of death over 20 years of follow-up (Laughlin, Barrett-Connor et al. 2008). It has been well demonstrated that men with coronary artery disease had a higher prevalence of hypoandrogenemia (Eckardstein and Wu 2003; Wu and von Eckardstein 2003; Pinthus, Trachtenberg et al. 2006). In many studies ultrasonography was used to evaluate vessel intima thickness, a marker used to investigate the progression of atherosclerosis. Using this method, plasma testosterone levels were negatively correlated with atherosclerosis progression (Demirbag, Yilmaz et al. 2005). Rosano et al examined 129 patients with CVD symptoms and found a clear inverse relationship between the degree of CAD and plasma testosterone levels (Rosano, Sheiban et al. 2007). Furthermore, a number of studies that correlated intima-media thickness to endogenous androgen levels seem to have confirmed similar findings. As reviewed by Manolakou et al, in most clinical studies, subjects with high endogenous testosterone levels corresponded with statistically significant lower atherosclerosis compared to subjects with lower testosterone (Manolakou, Angelopoulou et al. 2009). Notably, this finding persisted in both genders, including men (van den Beld, Bots et al. 2003; Muller, van den Beld et al. 2004; Makinen, Jarvisalo et al. 2005), premenopausal and postmenopausal women (Bernini, Sgro et al. 1999; Golden, Maguire et al. 2002; Debing, Peeters et al. 2007). The cardiovascular protective effect of endogenous androgen
observed in premenopausal women suggest it involves direct androgen action, independent of conversion to estrogen. All these findings demonstrate low endogenous testosterone correlates positively with higher prevalence CVD and suggest that endogenous androgen exerts protective effects from CVD development.

The effects of exogenous androgen on CVD pathogenesis are less well studied. Testosterone administration among 293 female-to-male transsexuals showed no increase in cardiovascular deaths in a 20-year follow up study (van Kesteren, Asscheman et al. 1997). In addition, testosterone supplementation in low to normal levels has mostly been found to be beneficial to cardiovascular system in hypogonadal men. Several studies have reported beneficial actions of exogenous testosterone therapy on coronary blood flow and exercise-induced myocardial ischaemia in men (Malkin, Pugh et al. 2004; Malkin, Jones et al. 2009). However, exogenous testosterone administration in supraphysiological doses, such as anabolic steroid abuse, seems to have adverse effects such as development of myocardial infarction, left ventricular hypertrophy, hypertension, arrhythmia, cardiac failure, pulmonary embolism, stroke, and sudden death (Ferrera, Putnam et al. 1997; Karila, Karjalainen et al. 2003; Maravelias, Dona et al. 2005). Androgen therapy is used as a performance enhancer in women. However, the role of testosterone role in the development of CAD in women is still not completely understood. The long-term cardiovascular effects of androgen replacement therapy in women have not been studied. Thus, further studies are needed to clarify the exact roles of androgen in the development of CVD in
different genders and the knowledge can also help us understand the mechanisms underlying cardiovascular manifestations in general.

1.4.5 Androgen during development and sex dimorphism

Male sexual differentiation has been revealed to be initiated by the testis-determining factor SRY, a transcription factor encoded on the Y chromosome (Sinclair, Berta et al. 1990). By interacting with other factors, SRY induces the development of testis, which secretes androgen responsible for male secondary sexual differentiation. The differentiated testis starts to synthesize low level of testosterone in Leydig cells from day 15 in the rat (El-Gehani, Zhang et al. 1998) and at 12-17 weeks of pregnancy in humans (Diez d'Aux and Pearson Murphy 1974). In male rats, androgen secretion from the testis leads to 2 perinatal peaks of plasma testosterone concentration: the first occurs on day 18 of gestation and the second approximately 2 hour after birth (Weisz and Ward 1980; Corbier, Edwards et al. 1992). This perinatal androgen surge has been observed in many other species, such as mice, horses and primates (Corbier, Edwards et al. 1992). Androgen during embryonic and neonatal life mediates the initial growth and differentiation of the male reproductive tract, such as the Wolffian ducts, urogenital sinus, and external genitalia primordial (Bentvelsen, Brinkmann et al. 1995).

Besides the well characterized sexual dimorphism of the reproductive system, other tissues and organs, including kidney, liver, and brain, have been reported to exhibits sex differences with regard to expression of certain genes.
A recent microarray analysis of 23,574 transcripts revealed a much greater extent of sexual dimorphism in gene expression than previously recognized. In all the active genes that were tested, sexual dimorphism ranged from 14% (in the brain) to 70% (in the liver) (Yang, Schadt et al. 2006).

A pioneering study in 1959 demonstrated that female guinea pigs exposed to prenatal androgen exhibited male-like behaviors (Phoenix, Goy et al. 1959). The experimental method used by Phoenix et al has been adapted by numerous investigators in the study of sex differences in the brain and behavior (Becker, Arnold et al. 2005). In this approach, the short-term exogenous androgen exposure is imposed upon females prenatally or neonatally, mimicking effects of perinatal androgen surge in males during development (Motelica-Heino, Castanier et al. 1988; Corbier, Edwards et al. 1992). As thoroughly reviewed, exposure of females during the prenatal or neonatal period to androgen has been used previously to induce male-like, or androgenized, behaviors (Pfaff and Zigmond 1971; Hrabovszky and Hutson 2002; Wallen 2005). Sexual dimorphism in the brain may take many forms, including difference in structure (e.g., the size or number of neurons), connectivity (e.g., axon projection patterns or synapse number), and neurochemistry (e.g., the expression of neuropeptides, neurotransmitters or receptors). Previous results indicated that exposure of females to androgen during development mediates many cellular/molecular mechanisms, such as apoptosis, cell migration and synaptogenesis, that
influence neuron numbers, dendrite morphology and neurotransmitter phenotypes in the brain (Simerly 2002; Foecking, McDevitt et al. 2008).

One of the well-studies sexual dimorphic features in mammalian brain is cell number. The sex dimorphism may exist either in the total number of cells in a specific brain region or in the number of cells of a particular phenotype. In many cases, sex differences in cell number have been attributed to the action of sex hormones during development, particularly testosterone and testosterone metabolites such as estradiol (Forger 2006). For example, the bed nucleus of the stria terminalis (BSNT) is a limbic forebrain structure involved in the control of sexual behavior, gonadotrophic release, stress and anxiety (Emery and Sachs 1976; Beltramino and Taleisnik 1980). The principal nucleus of the BNST (BNSTp) is larger in males compared to females across many species including rats, mice, guinea pigs, and humans (Hines, Davis et al. 1985; Allen and Gorski 1990; Walker, Toufexis et al. 2003). In rodents, the gender difference in BNSTp volume is attributed to an increased number of cells in males and neonatal testosterone administration in females elevated the cell number to a similar level of males (Guillamon, Segovia et al. 1988). Similarly, in spinal nucleus of the bulbocavernosus (SNB), which is a cluster of motoneurons located in the lumbar spinal cord, male rodents have more neurons than do females (Breedlove and Arnold 1980; Wee and Clemens 1987). SNB motoneurons innervate striated muscles of the perineum that control copulation, including the bulbocavernosus (BC) and levator ani (LA). Interestingly, these muscles also exhibit marked sexual dimorphism: the BC is completely absent and the LA is greatly reduced in
females (Breedlove and Arnold 1980; Wee and Clemens 1987). Neonatal testosterone was shown to increase SNB motoneuron number and BC/LA muscle volume (Breedlove and Arnold 1983). During development, over 50% of neurons initially generated in utero undergo apoptosis in a highly restricted time window that varies among different regions of the brain (Oppenheim 1991). It has been well established that cell death contributes to sex dimorphism of cell numbers in the brain (Forger 2006). For example, perinatal testosterone administration has been reported to rescue cells in SNB from death (Breedlove and Arnold 1983). Bax and Bak are pro-apoptotic factors that belong to the Bcl-2 family of apoptosis-regulating proteins. Deficiency of Bax has been reported to eliminate the gender difference in SNB motoneuron number (Jacob, Bengston et al. 2005). In addition, the BC and LA muscles were markedly increased in the female mice with both Bax and Bak deficiency (Jacob, Ray et al. 2008).

These changes in brain neurons of females androgenized during development have been reported to be permanent and are considered to be “organizational”, allowing for male-like behaviors that persist to adulthood (Arnold 2009). A recent study in mice demonstrated that the male brain shows higher extent of chromatin modifications on histone 3, which are associated with both gene activation and suppression. Moreover, in female brains prenatal testosterone exposure reversed this dimorphism (Tsai, Grant et al. 2009). These data suggest that epigenetic mechanisms have been implicated to contribute to the development of sexual dimorphism in the brain (Tsai, Grant et al. 2009). Most recently it has been reported that 2 hepatic steroid metabolizing enzymes,
Cyp2b9 and Cyp2a4, are expressed more abundantly in females compared to males and neonatal testosterone exposure diminishes these dimorphic gene expression patterns (Ramirez, Luque et al. 2010). In contrast to studies focused on reproductive organs and the brain, little is known about developmental effects of sex hormones on other tissues/organs.

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Table 1.1 Summary of findings in AngII-induced AAAs

<table>
<thead>
<tr>
<th>Mediator</th>
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<th>Mouse Strain</th>
<th>Gender</th>
<th>Effect on AAAs</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Inflammation</td>
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<td></td>
<td>CCR2 -/-: whole body</td>
<td>ApoE-/-</td>
<td>Male</td>
<td>↓</td>
<td>(Ishibashi, Egashira et al. 2004; Daugherty, Rateri et al. 2010)</td>
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<td></td>
<td>CCR2 -/-: bone marrow</td>
<td>ApoE-/-</td>
<td>Male</td>
<td>↓</td>
<td>(Ishibashi, Egashira et al. 2004)</td>
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<td>COX-2 -/-</td>
<td>Hybrid</td>
<td>NS</td>
<td>↓</td>
<td>(Gitlin, Trivedi et al. 2007)</td>
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<td></td>
<td>COX-2 inhibition: celecoxib</td>
<td>ApoE-/-</td>
<td>Male</td>
<td>↓</td>
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<td></td>
<td>COX-1 inhibition: SC-560</td>
<td>ApoE-/-</td>
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<td>↔</td>
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<td></td>
<td>5-LO -/-</td>
<td>ApoE-/-</td>
<td>Male</td>
<td>↔</td>
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<td>5-LO inhibition: MK-0591</td>
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<td>IFN-γ -/-</td>
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<td></td>
<td>CXCL10-/-</td>
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<td>TGF-β activity inhibition</td>
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<td>High fat diet induced obesity</td>
<td>C57BL/6</td>
<td>Male</td>
<td>↑</td>
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<td></td>
<td>GX sPLA2 -/-</td>
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<td>EP4 -/-: bone marrow</td>
<td>LDLR-/-</td>
<td>Male and Female</td>
<td>↑</td>
<td>(Tang, Shvartz et al. 2010)</td>
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Table 1.1 Summary of findings in AngII-induced AAAs (continued)

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Application</th>
<th>Mouse Strain</th>
<th>Mouse Gender</th>
<th>Effect on AAAs</th>
<th>Reference</th>
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<td>ApoE/-</td>
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<td>↓</td>
<td>(Thomas, Gavrila et al. 2006)</td>
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<td>NOX-1 -/-</td>
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<td>↓</td>
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<tr>
<td>mPGES-1 -/-</td>
<td>LDLR/-</td>
<td>Male</td>
<td>↓</td>
<td>(Wang, Lee et al. 2006)</td>
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<td>Cyclophilin A -/-</td>
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<td>↓</td>
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<td>Vitamins E and C</td>
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<td>↔</td>
<td>(Jiang, Jones et al. 2007)</td>
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<td><strong>Proteases</strong></td>
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<td>MMP inhibitor: doxycycline</td>
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<tr>
<td>TERT-/- : bone marrow</td>
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<td>(Findeisen, Gizard et al. 2010)</td>
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<td><strong>Intracellular signaling</strong></td>
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<td>Caspase inhibitor</td>
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Table 1.1 Summary of findings in AngII-induced AAAs (continued)

<table>
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<th>Mouse Strain</th>
<th>Gender</th>
<th>Effect on AAAs</th>
<th>Reference</th>
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<td>LDLR-/-</td>
<td>Male</td>
<td>↓</td>
<td>(Cassis, Rateri et al. 2007)</td>
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<tr>
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<td>AT1aR -/- : bone marrow</td>
<td>LDLR-/-</td>
<td>Male</td>
<td>↔</td>
<td>(Cassis, Rateri et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>MR antagonism: spironolactone</td>
<td>ApoE-/-</td>
<td>Male</td>
<td>↔</td>
<td>(Cassis, Helton et al. 2005)</td>
</tr>
<tr>
<td>Hormonal</td>
<td>Orchidectomy</td>
<td>ApoE-/-</td>
<td>Male</td>
<td>↓</td>
<td>(Henriques, Huang et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Ovariectomy</td>
<td>ApoE-/-</td>
<td>Female</td>
<td>↔</td>
<td>(Henriques, Huang et al. 2004)</td>
</tr>
</tbody>
</table>

Abbreviations: 5-LO-5-lipoxygenase; AAA-Abdominal aortic aneurysm; Apo-apolipoprotein; AT1aR-angiotensin II type 1a receptor; BLT1- G-protein coupled receptor for leukotriene B(4); CCR2-chemokine receptor 2; COX-cyclooxygenase; CXCL10-IFN-γ-inducible T-cell chemoattractant IP-10; EP4-prostaglandin E receptor 4; GX sPLA2-group X secretory phospholipase; HDAC-histone deacetylase; IFN-γ-interferon γ; JNK-c-Jun N-terminal kinase; LDLR-low-density lipoprotein receptor; MCP-1-monocyte chemoattractant protein-1; MMP-matrix metalloproteinase; mPGES-1-microsomal prostaglandin (PG) E2 synthase-1; MR-mineralocorticoid receptor; NS-not specified; TERT-telomerase reverse transcriptase.
Table 1.2 Gender difference in AngII and elastase-induced AAAs.

<table>
<thead>
<tr>
<th>AAA model</th>
<th>Application</th>
<th>gender</th>
<th>species</th>
<th>effect on AAA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AngII</td>
<td>orchietomy</td>
<td>male</td>
<td>mouse</td>
<td>↓</td>
<td>(Henriques, Huang et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>orchiectomy</td>
<td>female</td>
<td>mouse</td>
<td>↔</td>
<td>(Henriques, Huang et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>exogenous androgen</td>
<td>male</td>
<td>mouse</td>
<td>↑</td>
<td>(Henriques, Zhang et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>exogenous androgen</td>
<td>female</td>
<td>mouse</td>
<td>↑</td>
<td>(Henriques, Zhang et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>exogenous estrogen</td>
<td>male</td>
<td>mouse</td>
<td>↓</td>
<td>(Martin-McNulty, Tham et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>orchiectomy</td>
<td>male</td>
<td>rat</td>
<td>↓</td>
<td>(Cho, Woodrum et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>exogenous estrogen</td>
<td>male</td>
<td>rat</td>
<td>↓</td>
<td>(Ailawadi, Eliason et al. 2004; Cho, Woodrum et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>exogenous androgen</td>
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<td>rat</td>
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<td>female</td>
<td>rat</td>
<td>↔</td>
<td>(Cho, Woodrum et al. 2009)</td>
</tr>
</tbody>
</table>
Figure 1.1 Current RAS with biologically active peptides highlighted in blue.
Figure 1.2 Signaling of the AT1 receptor.

Abbreviations: Gq, G protein; PLC, PLD, PLA2, phospholipase C, D, and A2, respectively; IP3, inositol-1, 4, 5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; MAP, mitogen activated protein; JAK, Janus kinase; STAT, signal transducers and activators of transcription. Adapted from Dinh et al Clinical Science 100(5): 481–92 (2001).
Figure 1.3 Signaling of the AT2 receptor.

Abbreviations: cGMP, cyclic guanosine monophosphate; Gi, inhibitory G protein; PTP, protein tyrosine phosphatase; PP2A, serine/threonine phosphatase 2A; ERK, extracellular signal-regulated kinase; NO, nitric oxide. Adapted from Dinh et al Clinical Science 100(5): 481–92 (2001).
Figure 1.4 Regulation of the renin angiotensin system (RAS) by male and female sex hormones. The overall effect of estrogen is to tune down the RAS while androgen tends to stimulate the RAS. Adapted from Fischer et al Cardiovascular Research 53: 672-677.
Figure 1.5 The pathway leading to synthesis of testosterone from cholesterol and its subsequent metabolism to other hormones. Adapted from http://www.hemingways.org/GIDinfo/hrt_intro.htm.
CHAPTER IA. STATEMENT OF THE PROBLEM

Abdominal aortic aneurysms (AAA) are commonly found in 4% to 8.5% of men and 0.5% to 1.5% of women in the population over the age of 60 (Wilmink, Quick et al. 1999; Lawrence-Brown, Norman et al. 2001; Ashton, Buxton et al. 2002; Cornuz, Sidoti Pinto et al. 2004; Palombo, Lucertini et al. 2010). Recently it has been estimated that AAA prevalence in the United States are 1.1 million people between the ages of 50 to 84 (Kent, Zwolak et al. 2010). AAA is the 13\textsuperscript{th} leading cause of the death and causes at least 15,000 deaths each year in the United States (Gillum 1995). Male gender is a strong independent risk factor for developing AAAs. Men exhibit greater incidence and severity of AAA compared to women. In recent decades AAA incidences and ruptures have been escalating in western societies, especially in male gender (Best, Price et al. 2003; Acosta, Ogren et al. 2006). Unfortunately, so far there is no effective pharmacological intervention to treat AAA.

Our laboratories have established that chronic infusion of AngII into hyperlipidemic mice induces the development of AAA the effect of AT1aR (Daugherty, Manning et al. 2000; Cassis, Rateri et al. 2007). Similar to human condition, AngII-induced AAAs also exhibit profound gender differences. The AngII-induced AAA incidence of male mice is 4-5 fold higher compared to that of females (Manning, Cassis et al. 2002; Henriques, Huang et al. 2004). Our laboratory has previously demonstrate that orchidectomy of male ApoE/- mice markedly reduces AngII-induced AAA formation while ovariectomy of female
ApoE-/- does not have any influence on aneurysm formation (Henriques, Huang et al. 2004). Notably, exogenous androgen administration restored AngII-induced AAA formation in castrated male mice and significantly enhanced the development of AAA in female mice (Henriques, Zhang et al. 2008). All these results demonstrate that androgen is a primary mediator of gender difference in AngII-induced AAA formation. However, mechanisms underlying the effects of androgen to mediate enhanced susceptibility to AngII-induced AAAs have yet to be defined.

Therefore, the purpose of the present research was to determine how androgens during different periods of life span promote AngII-induced AAA formation in hyperlipidemic mice. We hypothesized that androgen stimulate AT1aR expression in aortas to confer a higher prevalence of AngII-induced AAAs in male mice. We established that androgen increases SMC AT1aR expression in the vasculature to stimulate AngII-induced AAA formation in adult male and female mice. We also investigated the effect of short-term exogenous androgen exposure during development on aortic AT1aR expression and AngII-induced AAA formation in adult female hyperlipidemic mice. Moreover, we defined how castration influences the progression of established AngII-induced AAAs in male mice.
CHAPTER IB. HYPOTHESIS

Androgen promotes angiotensin receptor type 1a expression on vascular smooth muscle cells to confer higher angiotensin II-induced AAA formation in male hyperlipidemic mice.

To test this general working hypothesis, the following goals were outlined:

**Goal 1:** To examine the role of androgen on the progression of established angiotensin II-induced AAA.

**Hypothesis 1:** Removal of endogenous androgen will decrease the progression of established angiotensin II-induced AAA.

**Specific Aim 1:** To determine the effect of castration on the progression of established angiotensin II-induced AAA in male ApoE-/- mice.

**Goal 2:** To examine the role of androgen on the expression of aortic smooth muscle angiotensin receptor type 1a.

**Hypothesis 2:** Androgen will stimulate aortic smooth muscle angiotensin receptor type 1a expression to promote higher angiotensin II-induced AAA formation.

**Specific Aim 2:** To determine the effect of androgen on aortic angiotensin receptor type 1a expression and the effect of smooth muscle angiotensin receptor type 1a deficiency on angiotensin II-
induced AAA formation in female hyperlipidemic mice administered exogenous androgen.

**Goal 3:** To examine the role of androgen during development on the expression of aortic smooth muscle angiotensin receptor type 1a and angiotensin II-induced AAA in adult mice.

**Hypothesis 3:** Administration of androgen during development in female mice will increase aortic smooth muscle angiotensin receptor type 1a and promote higher angiotensin II-induced AAA formation in adulthood.

**Specific Aim 3:** To determine the effect of androgen administration in neonatal female mice on aortic smooth muscle angiotensin receptor type 1a expression and angiotensin II-induced AAA formation in adulthood.
CHAPTER II. SPECIFIC AIM 1

Determine the effect of castration on the progression of established angiotensin II (AngII)-induced AAAs in male apolipoprotein E (ApoE) -/- mice.

2.1 Summary

Previous studies demonstrated that castration profoundly reduces AngII-induced AAA incidence in males while exogenous androgen administration restores AAA formation in castrated male mice. These results demonstrated that androgen is a primary regulator of AngII-induced AAA formation in male mice. In this study, we sought to determine the effect of castration on the progression of established AngII-induced AAAs, with the hypothesis that removal of androgen will slow AAA progression and reduce the risk of aneurysmal rupture.

Male ApoE-/- mice were infused with AngII for 4 weeks to induce AAA formation. Mice with AAAs were identified by ultrasound. Mice with AAAs of different sizes were evenly distributed into groups that were sham-operated or castrated. After surgery, mice from both groups were then chronically infused for an additional 8 weeks with AngII. During prolonged AngII infusion, mice from sham control groups exhibited marked progressive lumen dilation beyond that observed following 1 month of AngII infusion. Castration significantly decreased abdominal aortic lumen diameters of male mice chronically infused with AngII (day 56, 1.74 ± 0.05 vs 1.50 ± 0.04 mm; day 70, 1.87 ± 0.05 vs 1.62 ± 0.04 mm; day 84, 1.88 ± 0.05 mm vs 1.63 ± 0.04 mm; P<0.05; sham vs castration,
respectively). However, maximal external abdominal aortic diameters of cleaned, excised AAAs were not significantly different between sham-operated and castrated mice. AngII-induced atherosclerosis was not altered by castration.

In conclusion, these data demonstrate that removal of endogenous androgen has a significant impact on the luminal dilation of established AngII-induced AAA, but does not influence the aortic diameter of AAAs.
2.2 Introduction

The natural history of AAA progression is gradual expansion of aortic diameters obtained by ultrasound with increased risk of rupture as AAA size increases. Ruptured AAAs are a leading cause of death in western countries and result in at least 15,000 deaths per year in the United States. Based on current clinical practice guidelines, the only effective therapeutic option to prevent AAA rupture is open repair or endovascular surgery if AAA size (by ultrasound) exceeds 5-5.5 cm. Patients with small AAAs are usually excluded from AAA surgical repairs and so far no pharmaceutical drugs have been proven to markedly reduce AAA size progression and/or prevent ruptures of small AAAs. It has been generally acknowledged that AAA rupture risk increases while aneurysms are growing and expanding. The mean growth rate of small AAAs is 2.6 to 3.2 mm per year (Lederle, Wilson et al. 2002). It has been demonstrated that AAA expansion rate is positively associated with cigarette smoking and aneurysm diameter at baseline (Lindholt, Heegaard et al. 2001; Brady, Thompson et al. 2004) and negatively associated with diabetes and peripheral arterial diseases (Brady, Thompson et al. 2004). Therefore, it is highly likely that interventions that slow AAA progression will decrease the risk of AAA rupture in patients and immensely improve the outcome of AAA medical treatment.

The renin angiotensin system (RAS) has been demonstrated to be important in AAA pathogenesis in experimental models, and is gaining importance as a contributor to human AAA pathology. Chronic infusion of AngII
induces AAA formation in hyperlipidemic male mice (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000). Enhanced AngII levels and AngII producing enzymes, namely chymase and ACE, have been detected in human AAA tissues (Nishimoto, Takai et al. 2002; Tsunemi, Takai et al. 2002). Recently, gene polymorphisms of ACE and angiotensin type 1 receptors (AT1Rs) have been positively associated with increased AAA incidence at 3 different geographic areas (Fatini, Pratesi et al. 2005; Jones, Thompson et al. 2008). Moreover, administration of an ACE inhibitor has been linked to reduced AAA ruptures in human patients (Hackam, Thiruchelvam et al. 2006). Recent data demonstrated that administration of an AT1R antagonist slowed AAA growth rate in a clinical study involving a 25-years of AAA surveillance (Thompson, Cooper et al. 2010). These data suggest that the RAS contributes to AAA progression; therefore, inhibition of the RAS may slow AAA progression and potentially decrease the risk of AAA rupture.

Our previous studies have demonstrated that with chronic AngII infusion male mice exhibit a 4-5 fold higher prevalence of AAAs compared to female mice, and this effect is abolished by orchiectomy (Daugherty, Manning et al. 2000; Manning, Cassis et al. 2002; Henriques, Huang et al. 2004). Exogenous androgen administration restored AngII-induced AAA incidence in castrated male mice and promoted aneurysm formation in female mice (Henriques, Zhang et al. 2008), suggesting androgen is a primary regulator of pronounced gender differences in AngII-induced AAA formation. Relevance of these findings in mice to humans is demonstrated by a strong effect of male gender as a risk factor for
human AAA formation (ref). However, while male sex has been implicated as a risk factor for AAA formation, results demonstrated that median growth rate of AAAs was significantly greater in women than men (Mofidi et al., 2007). Thus, it is unclear whether effects of androgen to promote formation of AngII-induced AAAs extend to an increased progression of established AAAs.

To investigate if androgen also plays a role in AAA progression, we hypothesized that castration reduces the growth rate of AngII-induced AAAs. We performed orchietomy to remove endogenous androgen from the male ApoE-/- mice with established AngII-induced AAAs. Results from this study demonstrate that castration significantly slows the progressive dilation of abdominal aortic lumen in male ApoE-/- chronically infused with AngII.
2.3 Methods

2.3.1 Animals

Male ApoE-/- mice were purchased from The Jackson Laboratory (2 months of age; \(n = 60\); Bar Harbor, MA). All mice were maintained in a pathogen-free environment. Water and normal laboratory diet were available ad libitum. At 2 month of age, all mice were infused with AngII (1,000 ng/kg/min) for 4 weeks to induce AAA formation. On day 25 of AngII infusion, abdominal aortas of all mice were scanned by noninvasive high-frequency ultrasound to measure aortic lumen diameter as an index of AAA formation. An AAA was defined as a >30% increase in lumen diameter compared to baseline (day 0 of infusion). Only mice defined to have an AAA continued in the study. These mice were assigned to either sham-operated or castration groups (\(n=23/\)group). Large and small AAAs, reflected by abdominal aortic lumen diameters, were evenly assigned to both sham and castration groups to control for differences in AAA size as a mediator contributing to AAA progression. After surgery on day 28, mice in each group continued to be infused with AngII for an additional 8 weeks. Abdominal aortic lumen diameters were monitored by ultrasound on days 42, 56, 70 and 84.

2.3.2 AngII Infusion

Alzet (Durect Corp; Cupertino, CA) osmotic pumps (model 1004) were filled with AngII (infusion rate of 1000 ng/kg/min) as described previously (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000). Pumps were implanted subcutaneously on the right flank via an incision in the scapular region.
AngII infusions were continued each month by replacement of new osmotic minipumps containing fresh AngII.

2.3.3 Orchiectomy

Male ApoE−/− mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). Both testes were pushed down into the scrotal sacs by gently applying pressure to the abdomen. An 8-10 mm incision through the skin was made along the midline of the scrotal sac. Another incision was made into the midline wall between the testes sacs under the covering membranes. The testis, the vas deferens and epididymal fat pad were carefully pulled out through the incision. The blood vessels supplying the testis were clamped with a hemostat and the testis was dissected away. The vas deferens and the fat pad were cauterized and placed back into the scrotal sac. This procedure was repeated for the other testis. The incision through the skin was closed using wound clips. For sham-operated controls, mice were anesthetized, both incisions were made and sex organs were localized.

2.3.4 Ultrasound Imaging

Abdominal aortas of mice were visualized with high-frequency ultrasound (Vevo 660, VisualSonics, Toronto, Canada) at baseline, days 25, 42, 56, 70 and 84 of AngII infusion, as previously described (Barisione, Charnigo et al. 2006). Briefly, mice were anesthetized with isoflurane and restrained in a supine position for ultrasonography. Short axis scans of aortas were performed on abdominal
aortas from the level of the left renal arterial branch moving vertically to the suprarenal region. Cine loops of 300 frames were acquired throughout the renal region of abdominal aortas and maximal luminal diameters in the suprarenal region of abdominal aortas were measured on images at maximal dilation.

2.3.5 Atherosclerosis and AAA Quantification

Mice were sacrificed and fixed with formalin by gravity perfusion (~120 mmHg) to quickly preserve vessel morphology. Aortas were removed and fixed with formalin overnight at room temperature. Extraneous tissue, including fat and connective tissues were carefully removed from aortas. Atherosclerotic lesions were quantified en face on the luminal surface of aortic arches as described previously (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000). Briefly, atherosclerotic lesions were determined by measuring the area of the intimal surface covered by grossly discernible atherosclerotic plaque, using ImagePro Plus software (Media Cybernetics, Bethesda, MD). The extent of AngII-induced atherosclerosis was expressed as the percentage of total lesion area to the total aortic arch intimal area. Maximal diameters of suprarenal abdominal aortas were measured on cleaned aortas by ImagePro Plus software (Media Cybernetics, Bethesda, MD).

2.3.6 Statistical Analysis

Data are represented as mean ± SEM. To compare 2 groups, student’s \( t \) test was performed for parametric data and Mann-Whitney Rank Sum Test was
utilized for nonparametric data. Two-way repeated measures ANOVA with Holm-Sidak *post hoc* analysis were performed to compare body weight and ultrasound data. The incidences of AAA were analyzed using Fisher exact test. *P*<0.05 was considered statistically significant. All statistical analyses were performed using SigmaStat (SPSS Inc).
2.4 Results

2.4.1 Castration Reduces the Progressive Lumen Dilation of Established AngII-induced AAAs in Male ApoE-/- Mice

To define the role of androgen on the expansion of established AngII-induced AAAs, we infused adult male ApoE-/- mice with AngII for 4 weeks to induce AAA formation before they were subjected to sham-operation or orchiectomy. After surgery, we continued chronic AngII infusion in all mice for another 2 months to study progression of established AAAs. After castration, male mice exhibited significantly reduced body weights compared to sham controls (Figure 2.1A). At the study endpoint, castrated male mice exhibited marked atrophy in the weights of prostate, seminal vesicle and vas deferens (Figure 2.1B).

At the end of 1 month of AngII infusion, we obtained abdominal aortic lumen diameter measurements from AngII-infused AAAs by ultrasound. Abdominal aortic lumen diameters at baseline and day 28 were not different between sham controls and castrated mice (baseline, 1.07 ± 0.05 vs 1.05 ± 0.04 mm; day 28, 1.56 ± 0.05 vs 1.56 ± 0.04 mm; sham vs castration, respectively) (Figure 2.2), demonstrating that AAAs of different sizes were evenly assigned to sham control and castrated groups. In sham control mice, suprarenal aortic lumen diameters progressively increased with continued infusion of AngII, with significant increases in lumen diameter at days 70 and 84 of AngII infusion compared to day 25 (day 25 vs day 70, 1.56 ± 0.05 vs 1.87 ± 0.05 mm; P<0.001) (Figure 2.2). In contrast, aortic lumen diameters did not exhibit a progressive
increase with continued AngII infusion in castrated male mice (day 25 vs day 70, 1.56 ± 0.04 vs 1.62 ± 0.04 mm; day 84, 1.56 ± 0.04 vs 1.63 ± 0.04 mm; P>0.23) (Figure 2.2). As a result, aortic lumen diameters were reduced by castration on days 56, 70 and 84 compared to sham operated controls (day 56, 1.74 ± 0.05 vs 1.50 ± 0.04 mm; day 70, 1.87 ± 0.05 vs 1.62 ± 0.04 mm; day 84, 1.88 ± 0.05 mm vs 1.63 ± 0.04 mm; P<0.05; sham vs castration respectively) (Figure 2.2).

2.4.2 Castration Does not Alter Maximal External Diameters of AngII-induced AAAs or AngII-induced Atherosclerosis.

During prolonged AngII infusion after the surgery, the castrated male mice showed a tendency of decreased AAA rupture incidence compared with sham controls (sham vs castration 13% vs 4%; P=0.60). At the study endpoint, aortas were formalin fixed and cleaned for quantitative measurements of AngII-induced AAA and atherosclerosis (Figure 2.4). Maximal external diameters of cleaned, excised suprarenal aortas were reduced modestly (by 0.45 mm), but not significantly by castration (sham vs castration, 2.32 ± 0.24 vs 1.87 ± 0.17 mm; P=0.09) (Figure 2.3A). AngII-induced atherosclerosis in aortic arches was not different between sham and castrated mice (sham vs castration, 6.2 ± 0.5 vs 6.6 ± 0.7 %; P=0.27) (Figure 2.3B).
2.5 Discussion

The major finding of this study is that castration substantially reduces luminal dilation of established AngII-induced AAAs in male ApoE/-/- mice. Our data support that prolonged AngII infusion causes progressive elevations in suprarenal aortic lumen diameters of male ApoE/-/- mice, consistent with previous studies (Barisone, Charnigo et al. 2006). Interestingly, despite an ability of castration to significantly blunt progressive dilation of suprarenal aortic lumen diameters with prolonged AngII infusion, castration only modestly reduced AAA external diameters. These data suggest that inhibition of androgen suppresses AAA progression.

In contrast to an ability of castration to blunt progressive increases in aortic lumen diameters, castration of male mice had no effect on atherosclerotic lesions formed from chronic AngII infusion. These results are in agreement with previous data from our laboratory demonstrating that sex differences in AngII-induced vascular diseases are restricted to AAAs (Henriques et al., 2004). Although male gender is linked to higher risks of cardiovascular diseases including AAAs, low serum testosterone in men has also been associated with increased cardiovascular risk and mortality (Shores, Matsumoto et al. 2006; Maggio, Lauretani et al. 2007; Laughlin, Barrett-Connor et al. 2008). Recently it has been demonstrated that androgen deprivation therapy for prostate cancer increased the risk of cardiovascular events (Levine, D'Amico et al.). Moreover, women often have more adverse outcomes after a cardiovascular event compared to men of a similar age (Kostis, Wilson et al. 1994; Vaccarino, Parsons...
et al. 1999; Vaccarino, Parsons et al. 2009). It has been suggested that androgen plays an important role in cardiovascular repair and regeneration through stimulating erythropoietin production and angiogenesis via VEGF-related mechanisms (Sieveking, Chow et al.). Recent animal studies also revealed that androgen is required for the preservation of NO bioavailability and exerts protective effects against AngII-induced vascular remodeling in coronary arteries and thoracic aortas from male mice by regulating oxidative stress (Ikeda, Aihara et al. 2009). Our results do not support a protective role for testosterone in the progression of AngII-induced atherosclerosis. It is possible that augmentation of atherosclerosis by chronic infusion of AngII occurs through mechanisms that are independent of those suggested to be favorably influenced by testosterone.

In contrast to a lack of effect on atherosclerosis, castration totally ablated progressive increases in suprarenal aortic lumen diameters of AngII-infused male mice. Male gender has been identified as a prominent risk factor of human AAA formation by numerous epidemiological and clinical studies. However, whether male gender influences the growth rate of formed AAA remains largely under debate. Results from the United Kingdom Small Aneurysm Trial demonstrated no association of gender with AAA growth (Brady, Thompson et al. 2004). Interestingly, despite a higher AAA incidence in males compared to females, some studies have noted increased AAA expansion rate and aneurysmal ruptures in female AAA patients (Solberg, Singh et al. 2005; Mofidi, Goldie et al. 2007; Norman and Powell 2007). In contrast, female gender also has been negatively associated with AAA progression (Thompson, Cooper et al.).
interesting recent study genotyped 74 single-nucleotide polymorphisms (SNPs) in genes determining circulating sex hormones and their action in males with and without AAAs, and demonstrated that one SNP in CYP19A1 was strongly associated with aortic diameter (Golledge, Biros et al. 2010). However, similar studies have not been performed in females. To our knowledge, no studies have examined the association of AAA progression and male/female gender in AAA animal models.

In agreement with human AAAs, we have previously demonstrated that castration strikingly reduces AngII-induced AAA incidence in male mice while exogenous androgen administration restored AAA formation in castrated males, indicating an important role of androgen in the formation of AngII-induced AAAs (Henriques, Huang et al. 2004; Henriques, Zhang et al. 2008). In this study, we demonstrate that castration significantly reduced the progressive lumen dilation of established AngII-induced AAAs, suggesting that androgen also plays a role in luminal expansion of AAAs in male mice. While castration is not an amenable therapy to treat AAAs in humans, these results suggest that androgen receptor blockade may be of benefit in advancing AAAs, or alternatively that use of anabolic steroids may be contraindicated in males of advancing age with a family history of AAAs.

Despite the ability of castration to significantly reduce aortic lumen dilation of progressing AAAs, external AAA diameters were modestly, but not significantly decreased by castration. External diameters of cleaned AAAs encompass lumen diameters and abdominal aortic vascular wall remodeling. A reduction in lumen
diameter in the face of a modest but yet insignificant decrease in external diameter suggests that aortic wall remodeling was not appreciably reduced by castration with prolonged AngII infusion. Recent studies demonstrated that testosterone inhibits vascular calcification (Son, Akishita et al. 2010), suggesting that continued aortic remodeling in castrated male mice may have resulted from removal of the inhibitory effects of castration on vascular calcification. It is unclear whether changes in lumen diameter, as compared to aortic wall diameter, are more closely linked to rupture of AAAs. This is especially difficult to ascertain as ultrasound can be used to accurately quantify aortic lumen, but not aortic wall diameters in mice, while in humans ultrasound assesses aortic diameters (lumen plus wall). Our results demonstrate a modest reduction in aneurismal rupture in castrated male mice compared to sham controls, suggesting that castration-induced decreases in aortic lumen diameter are more closely linked to rupture of AngII-induced AAAs.

In conclusion, castration significantly decreased the progressive lumen dilation of established AngII-induced AAAs in male ApoE/-/- mice, but had no effect on external AAA diameters. These studies suggest that androgen contributes to the progression of established AAAs through distinct mechanisms that differentially influence aortic lumen and wall diameters.

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Figure 2.1 Body weights through AngII infusion (A) and male sex organ tissue weights at study endpoint (B) of both sham and castration group. Data are mean ± SEM. *, P < 0.001 compared to day 28 within each group; **, P < 0.001 compared to control group.
Figure 2.2 Lumen diameters of suprarenal abdominal aortas were obtained from sham and castration groups using ultrasound through 3 months AngII infusion. Data are mean ± SEM. *, P < 0.001 compared to day 28 measurements within each group; **, P < 0.05 compared to control group.
Figure 2.3 Castration does not significantly reduce maximal external diameters of AngII-induced AAA (A; P=0.09) or have any impact on AngII-induced atherosclerosis in aortic arch (B; NS). Each circle represents the measurement from each individual sample.
Figure 2.4 Perfusion fixed aortas from sham and castration groups.
CHAPTER III. SPECIFIC AIM 2

Determine the effect of androgen on aortic angiotensin receptor type 1a expression and the effect of smooth muscle angiotensin receptor type 1a deficiency on angiotensin II-induced AAA formation in female hyperlipidemic mice administered exogenous androgen.

3.1 Summary

Our previous studies have demonstrated that AngII infusion induce AAA formation through AT1aR. Castration of male ApoE-/- reduces AngII-induced AAA to that of female mice while androgen administration restores or stimulates AAA formation in castrated male mice or female mice. Thus, we sought to determine if androgen positively regulate aortic AT1aR expression to confer a higher AngII-induced AAA incidence and if SMC AT1aR deficiency reduce the effect of androgen on increasing AAA formation in female mice.

AT1aR mRNA abundance in the AAA-prone region of abdominal aortas was 7-fold greater compared to thoracic aortas of male but not female mice. AT1aR mRNA abundance decreased after castration in abdominal but not thoracic aortas of male mice. Exogenous androgen (dihydrotestosterone, DHT) administration to castrated male mice restored AT1aR mRNA abundance in abdominal aortas but had no effect in thoracic aortas. DHT also stimulated AT1aR mRNA abundance in abdominal aortas from female mice. When treated
with testosterone, primary cultured smooth muscle cells exhibited significant increase of AT1aR mRNA abundance.

To investigate if exogenous androgen stimulates AT1aR expression on SMC to promote AngII-induced AAA in female mice, SMC specific AT1aR deficient mice were generated using Cre-LoxP technology. Both AT1aR deficient mice and wild type littermate control female mice were administered DHT for 2 week before infused with AngII. AngII-induced AAA incidence was significantly reduced with SMC AT1aR deficiency (85% vs 50%; P<0.05).

In conclusion, these studies demonstrate that enhanced abdominal aortic AT1aR level by androgen is highly correlated with increased AngII-induced AAA formation in adult mice. Furthermore, exogenous androgen positively regulates SMC AT1aR expression to promote AngII-induced AAA susceptibility in adult female hyperlipidemic mice.
3.2 Introduction

Male gender has been consistently identified as a major risk factor for human AAAs. The incidence and severity of abdominal aortic dilations are greater in men than women (Singh, Bonaa et al. 2001; Isselbacher 2005). Experimental models of this disease also exhibit similar gender differences. In the rat model of intraluminal elastase infusion induced AAAs, male rats had larger and more frequent aneurysms than females (Ailawadi, Eliason et al. 2004). Chronic AngII infusion into hyperlipidemic mice resulted in AAA formation at a 4-5 fold higher incidence in male compared to female mice (Daugherty, Manning et al. 2000; Manning, Cassis et al. 2002; Henriques, Huang et al. 2004). It has been demonstrated that removal of androgen by castration of male ApoE-/- mice profoundly reduced AngII-induced AAA incidence to a similar level of female ApoE-/- mice while ovariectomy had no significant influence on AngII-induced AAA formation in female ApoE-/- mice (Henriques, Huang et al. 2004). Recent studies also demonstrated that exogenous androgen administration restores AngII-induced AAA formation in castrated male ApoE-/- mice and significantly augments AAA incidence in female ApoE-/- mice (Henriques, Zhang et al. 2008). These data implicate androgen as a primary mediator of gender differences in AngII-induced AAAs; however, mechanisms of androgen to promote AAA formation are unknown.

Androgen has been reported to increase the expression of each component of the renin-angiotensin system (RAS), including angiotensinogen, renin, ACE and AT1 receptors (Fischer, Baessler et al. 2002). Previous studies in
our laboratory demonstrated that both the AT1 receptor antagonist losartan administration and AT1aR deficiency abolishes AngII-induced AAAs in male mice, implicating a pivotal role of AT1aR in AngII-induced AAA formation (Daugherty, Manning et al. 2001; Cassis, Rateri et al. 2007). Moreover, using bone marrow transplantation, AT1aR deficiency of recipient mice, but not in cells used to repopulate, reduced AngII-induced AAAs (Cassis, Rateri et al. 2007). In aortic vasculature, several cell types express AT1aRs. The expression level of AT1aR is highest in SMC of vessel wall (Mehta and Griendling 2007). SMC apoptosis has been found to contribute to AngII-induced AAAs, since administration of a caspase inhibitor reduced medial apoptosis and significantly decreased AAA formation (Yamanouchi, Morgan et al. 2010). Previous studies examining temporal changes in aneurismal pathology with AngII infusion demonstrated medial smooth muscle elastin degradation occurred early in AngII-induced AAAs (Saraff, Babamusta et al. 2003). Collectively, these results demonstrate that AngII induces AAA formation through stimulation of AT1aRs, and support an essential role on vascular SMC in AAA formation.

In this study, we tested the hypothesis that androgen increases AT1aR expression on aortic SMC to promote AngII-induced AAAs in female hyperlipidemic mice. We first examined the effect of exogenous androgen on AT1aR mRNA abundance in thoracic versus abdominal aortas from castrated male and female ApoE/-/- mice. Then, we defined the role of AT1aR on aortic SMC in the effect of exogenous androgen to augment AngII-induced AAA formation in female mice.
3.3 Methods

3.3.1 Animals

For studies examining the effects of androgen on AT1aR mRNA abundance, Male and female apoE-/- mice (12 weeks old, backcrossed 10 times onto C57BL/6 background) were purchased from Taconic Farms (Germantown, PA). All mice were maintained under barrier conditions. Water and normal laboratory diet were available ad libitum. Male and female mice were castrated as described previously and 1 week later implanted in the subcutaneous space with slow release pellets (Innovative Research of America, Sarasota, FL) containing vehicle or dihydrotestosterone (DHT; 10 mg pellets/60 day sustained release; 0.16 mg/d). Castrated male mice administered vehicle or DHT were examined at 1 or 5 weeks after DHT administration (n=10 to 13 per treatment per time point). A group of ovariectomized female apoE-/- mice were included, with vehicle or DHT (0.16 mg/d) administration beginning 1 week after castration for a total duration of 5 weeks (n=8 per treatment group).

For the study determining the role of SMC AT1aR on androgen increasing AngII-induced AAA susceptibility in female mice, 3 month old female AT1aR

[fill]

and AT1aR

[fill]

KO mice were implanted AT1aR

[fill]

KO mice dihydrotestosterone (DHT; 10 mg pellets/60 day sustained release; 0.16 mg/day) 2 weeks before infused with AngII (1,000ng/kg/min, Bachem, Torrance, CA) by osmotic pumps (Alzet, model 1004, Durect Co., Cupertino, CA) for 4 weeks.
3.3.2 Orchiectomy and Ovariectomy

Ovariectomy was performed on 3-month-old female apoE/-/- mice. Mice were anesthetized with ketamine/xylazine, bilateral incisions (approximately 0.5 cm in length) were made into the abdominal cavity, and the uterus was exposed. The ovaries were carefully picked out of the incisions. Circulation to the fallopian tubes was cut off with a hemostat to minimize bleeding when dissecting the ovaries. Once the ovaries were excised, the cut end of the fallopian tubes was cauterized and all the tissues were neatly placed back into the abdominal cavity. The muscle was then sutured, and the skin was closed using wound clips. Sham controls were subject to a similar procedure without removing the ovaries. Orchiectomy surgeries were performed on 3-month-old male apoE/-/- mice as previously described.

3.3.3 Generation of $AT1aR^{SM22}$ KO mice

$AT1aR^{fl/fl}$ were generated by InGenious Targeting Laboratory (Stony Brook, NY) directly in ES cells of C57BL/6 mice and subsequently crossed to LDLR/-/- mice. SM22-Cre mice (The Jackson Laboratory, Tg(Tagln-cre)1Her/J, #004746, Bar Harbor, ME) were bred to LDLR/-/- mice and were identified by speed congenic screening (The Jackson Laboratory) to be the equivalent of N10. For experimental mice, male $AT1aR^{SM22}$ KO mice were bred to female $AT1aR^{fl/fl}$ mice to generate approximately 50:50 distribution of Cre hemizygous transgenics ($AT1aR^{SM22}$ KO) and non Cre expressing littermate controls ($AT1aR^{fl/fl}$).
3.3.4 Genotyping

AT1aR<sup>SM22</sup> KO and AT1aR<sup>fl/fl</sup> littermate controls were genotyped by PCR of genomic DNA (tail) and smooth muscle AT1aR deficiency confirmed using aortic samples at study endpoint. A small piece of infrarenal aorta was removed, cleaned of adventitia and endothelium and DNA was extracted (DNeasy; Qiagen, Alameda, CA). The primers and PCR conditions used for genotyping are listed in Table 3.1.

3.3.5 Aortic gene expression quantification by real time PCR

Total RNA was extracted from thoracic (aortic arch to diaphragm) and abdominal aortas (diaphragm to ileal bifurcation) using RNeasy fibrous tissue minikit (Qiagen, Valencia, CA). For in vitro experiments, primary mouse vascular smooth cells (passage 7-10) were treated with 10nM of testosterone propionate (Sigma Aldrich, St. Louis, MO) in 0.1% DMSO for 24 hours. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). All RNA samples were treated with Turbo DNA-free (Ambion, Austin, TX) to remove genomic DNA. RNA quality was assessed using a Bio-Rad Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). Mouse AT1aR primers were obtained from Qiagen, while AT1bR primers were from Applied Biosystems TaqMan Gene Expression Assays. Relative quantification of AT1aR mRNA abundance was performed with an iCycler (BioRad, CA) based on a standard curve method using the SYBR Green PCR core reagent (Applied Biosystems, CA). The real-time PCR conditions were: 5 min at 94º, 40 cycles for 1 min at 94º, 1 min at the annealing
temperature, 1 min at 72º, and a final elongation step for 10 min at 72º. To verify specificity of primers for AT1aR, quantification was performed on tissues from AT1aR−/− mice. 18S rRNA was used to control for DNA loading, and data are expressed as AT1aR/18S RNA ratios.

3.3.6 Measurement of aortic contractility

Aortas from intact male, castrated male, and castrated male ApoE−/− mice administered DHT (5 weeks) were removed and adventitia was carefully dissected free. Measurement of contractile activity was performed using ring segments (3 mm) from thoracic and abdominal aortas as described previously (Babamusta, Rateri et al. 2006). Aortic segments were mounted by passing two tungsten wires through the arterial lumen and bathed in wells filled with Krebs Henseleit solution. Optimal resting tension (1 gm) was maintained continuously and recorded on a Micro-Med instrument. After 30 min for equilibration, tissues were incubated with KCl (60 mM), followed by AngII (1 µM). The contractile force generated in response to AngII was normalized as a percentage of the KCl response.

3.3.7 Quantification of atherosclerosis and AAAs

Atherosclerotic lesions were quantified in the aortic arch as described previously (Daugherty, Manning et al. 2000; Daugherty and Whitman 2003). AAA incidence was quantified by grading of excised, cleaned tissue by 3 independent observers blinded to the experimental design as described previously. Abdominal
aortic lumen diameter measurements were obtained on anesthetized mice using high-resolution ultrasound (Visualsonics, Vevo 660) at baseline, day 7, day 14 and day 28 of AngII infusion. Maximal diameters of abdominal aortas were measured on cleaned, formalin-fixed aortas by ImagePro Plus software (Media Cybernetics, Bethesda, MD). AAAs were defined as > 50% dilation of lumen diameters from saline-infused mice.

3.3.8 Statistical Analyses

Data are represented as mean±SEM. Parametric data were initially analyzed using ANOVA. If differences existed between groups, post hoc analyses were performed (Tukey). The incidences of AAA were analyzed using Fisher exact test. P=0.05 was considered statistically significant. All statistical analyses were performed using SigmaStat (SPSS Inc).
3.4 Results

3.4.1 Androgen Selectively Augments Abdominal Aortic AT1aR Expression in Both Male and Female ApoE-/- mice

To determine if aortic AT1aR was regulated by androgen to promote AngII-induced AAA formation, male and female ApoE-/- mice were subjected to orchiectomy or ovariectomy operation and administered exogenous androgen, DHT for 1 or 5 weeks. Abdominal aortas, where AngII-induced AAAs form in mice, exhibit strikingly higher (7-fold) AT1aR mRNA abundance than thoracic aortas in male but not female ApoE-/- mice (Figure 3.2). AT1aR mRNA abundance progressively decreased after castration in abdominal aortas to levels observed in thoracic aortas. Administration of DHT for 1 week to castrated male apoE-/- mice resulted in complete restoration of AT1aR mRNA abundance in abdominal aortas, but had no effect in thoracic aortas (Figure 3.2A). Longer exposures to DHT (5 weeks) also increased AT1aR mRNA abundance in abdominal aortas to levels not different from intact male mice (Figure 3.2A). Interestingly, administration of DHT (total of 5 weeks) to ovariectomized female apoE-/- mice also resulted in increased AT1aR mRNA abundance in abdominal but not thoracic aortas (Figure 3.2B).

3.4.2 Androgen Fails to Regulate Aortic AT1bR Expression in ApoE-/- Mice

To determine whether androgen regulation of AT1 receptors was restricted to the AT1aR subtype, we examined AT1b mRNA abundance in thoracic and abdominal aortas from male and female mice in each group. AT1bR
mRNA abundance was greater in abdominal compared to thoracic aortas from male and female ApoE-/- mice, with similar expression levels in aortas from male and female mice (Figure 3.3). In addition, AT1bR mRNA abundance in abdominal aortas was not altered by castration or by DHT administration to castrated male or female ApoE-/- mice (Figure 3.3). Despite the presence of both AT1aR and AT1bR in aortas, aortic AT1bR has been demonstrated to be primarily responsible for AngII-mediated contractile responses of abdominal aortas (Zhou, Dirksen et al. 2005; Swafford, Harrison-Bernard et al. 2007). We defined the contractile response to Ang II in aortic rings from thoracic versus abdominal aortas of male and female ApoE-/- mice. In agreement to previous findings (Zhou, Dirksen et al. 2003), The contractile response to Ang II was greater in abdominal than thoracic aortas from male and female ApoE-/- mice and was of similar magnitude in aortas from male and female mice (Figure 3.4). Moreover, there was no difference in Ang II–induced contractile responses in castrated (5 weeks) male or female mice or in mice administered DHT (5 weeks) (Figure 3.4).

3.4.3. Androgen enhances SMC AT1aR Expression to promote AngII-induced AAAs in Female Hyperlipidemic Mice

We examined effects of testosterone on AT1aR mRNA abundance using primary cultures of mouse aortic smooth muscle cells. Incubation of mouse aortic smooth muscle cells with testosterone significantly increased AT1aR mRNA abundance, implicating smooth muscle cells as targets of androgen to
increase aortic AT1aR mRNA abundance (Figure 3.6). Previous studies demonstrated that administration of DHT to adult female ApoE−/− mice increased AngII-induced AAAs, but had no effect on atherosclerosis (Henriques, Zhang et al. 2008). Therefore, we first investigated the role of SMC AT1aR in DHT-mediated promotion of AngII-induced AAAs and atherosclerosis in adult female LDLR−/− mice.

We used Cre-LoxP technology to generate mice with deficiency of AT1aR in smooth muscle cells. Mice were engineered with loxP sites flanking exon 3 of AT1aR which includes the entire coding region for the receptor protein (Figure 3.7A). AT1aR<sup>fl/fl</sup> females on an LDLR−/− background were mated to male mice expressing Cre recombinase under the control of an SM22 promoter to generate smooth muscle AT1aR deficient mice (Kuhbandner, Brummer et al. 2000; Holtwick, Gotthardt et al. 2002) and littermate controls (AT1aR<sup>fl/fl</sup>). Depletion of AT1aR exon 3 from smooth muscle cells was verified by PCR of genomic DNA from aortas (Figure 3.7B). Female AT1aR<sup>fl/fl</sup> and AT1aR<sup>SM22</sup> KO mice were administered DHT at 2 months of age beginning 2 weeks before AngII infusion. Consistent with previous findings (Henriques, Zhang et al. 2008), administration of DHT to adult AT1aR<sup>fl/fl</sup> female mice resulted in robust AngII-induced AAAs (Figure 3.7A; AAA incidence, 87%). In contrast, AT1aR<sup>SM22</sup> KO females exhibited significantly reduced AAA formation (AAA incidence, 50%; P<0.05), abdominal aortic lumen diameters (Figure 3.8A) and maximal aortic diameters of suprarenal aortas (1.90 ± 0.22 vs. 1.35 ± 0.14 mm; P<0.05) (Figure 3.8B). In addition, aneurismal rupture in adult DHT-treated females was decreased by
$AT1aR^{SM22}$ KO (WT, 40%; SMC AT1aR KO, 13%; Figure 3.7B). In contrast, smooth muscle AT1aR deficiency had no effect on aortic arch atherosclerotic lesion sizes or ascending aortic dilation in DHT-treated females (Figure 3.9). These results indicate an important role for smooth muscle AT1aR in androgen-mediated promotion of AngII-induced AAAs in adult female mice.
3.5 Discussion

Results from this study demonstrate that male, but not female ApoE-/- mice, exhibit regional differences in AT1aR mRNA abundance in aortas, with greater receptor expression in abdominal compared to thoracic aortas. Moreover, castration of male ApoE mice resulted in a specific reduction in AT1aR mRNA abundance in abdominal aortas, which was restored by DHT administration. Interestingly, DHT also increased AT1aR mRNA abundance in abdominal aortas from female ApoE-/- mice. These effects of androgen to promote AT1aR mRNA abundance specifically in abdominal aortas were associated with increased AAA incidence and severity in both male and female castrated ApoE-/- mice.

The effect of androgen on AT1 receptor expression has been relatively unexplored. In rat epididymis, castration reduced AT1 receptor protein, that was restored when rats were treated with testosterone (Leung, Wong et al. 2002). Moreover, androgen was reported to increase AngII receptors in bovine adrenal glomerulosa cells (Carroll and Goodfriend 1984). In contrast neither castration alone or combined with androgen administration regulated AT1 receptor mRNA abundance in homogenates of renal cortex punches from male New Zealand genetically hypertensive rats (Song, Kost et al. 2006). In addition, incubation of androgen-dependent human prostate cancer cells with DHT increased AT1 receptor mRNA and protein (Uemura, Hasumi et al. 2006), supporting androgen regulation of human AT1 receptors. Our results demonstrate a specific effect of androgen to regulate AT1aR mRNA abundance in abdominal, but not thoracic...
aortas. Taken together, these results suggest that androgen exhibits tissue and/or cell-specific regulation of AT1 receptors. Interestingly, recent studies demonstrated that smooth muscle cells of the ascending and arch portions of the aorta are derived from murine neural crest, while smooth muscle cells of the abdominal aorta are derived predominately from mesenchymal cells in splanchnic mesoderm (Majesky 2007). It has been demonstrated that these mesenchymal cells during development exhibits androgen receptor (AR) activity (Cunha, Shannon et al. 1981; Majesky 2007; Wasteson, Johansson et al. 2008). Interestingly, we also demonstrate that AR mRNA abundance is greater in abdominal aortas compared to in thoracic aortas in both male and female ApoE-/- mice (Figure 3.5). This specific regional expression pattern of AR correlates with highly selective upregulation of abdominal aortic AT1aR by androgen in adult ApoE/- mice. Overall, these results suggest that the differences in smooth muscle embryonic origins along the length of the aorta may have contributed to regional differences in AT1aR regulation by androgen.

Previous investigators have demonstrated greater AngII-induced contractile responses in abdominal compared to thoracic aortic ring segments from male C57BL/6 mice (Zhou, Dirksen et al. 2003). Further studies demonstrated a prominent role for AT1b receptors in AngII-induced contractile responses (Zhou, Chen et al. 2003; Swafford, Harrison-Bernard et al. 2007). Our results extend these findings by demonstrating greater AT1aR mRNA abundance in abdominal aortas from male, but not female mice. To define whether androgen effects were restricted to the AT1aR subtype, we measured AT1bR mRNA
abundance, and the contractile response to AngII as an index of AT1b receptor function. While the contractile response to AngII exhibited a similar regional specificity to the abdominal aorta of male and female ApoE/-/- mice, AngII-induced contraction was not regulated by androgen, nor was AT1bR mRNA abundance. These results demonstrate that androgen specifically regulates aortic AT1a receptors. Given that AT1a and AT1b receptor subtypes exhibit differential cell and tissue distribution, differences in the promoters of these distinct genes may contribute to androgen-specific effects to increase AT1aR mRNA abundance.

Vascular SMC plays an essential role in AAA pathogenesis. Previous results implicated medial elastin degradation as an early event in AAA formation in adult male ApoE/-/- mice (Saraff, Babamusta et al. 2003). Apoptosis of smooth muscle has been found to contribute to AngII-induced AAAs, since administration of a caspase inhibitor reduced medial apoptosis and significantly decreased AAA formation (Yamanouchi, Morgan et al.). Moreover, genetic deficiency of cyclophilin A, a chaperone protein abundantly expressed in smooth muscle cells, abolished AngII-induced AAAs (Satoh, Nigro et al. 2009). Our results demonstrate that testosterone increases AT1aR mRNA expression in primary cultured mouse aortic smooth muscle cells. Moreover, deficiency of AT1aR in smooth muscle decreased effects of exogenous androgen to promote AngII-induced AAAs in adult female mice, but had no effect on atherosclerosis or ascending aortic dilation induced by infusion of AngII. Interestingly, in this study, AAAs that did form in female mice with smooth muscle cell AT1aR deficiency had
similar pathologic characteristics to those of wild type controls. Thus, as opposed to downstream signaling pathways, androgen may influence initiating events in the formation of AngII-induced AAAs by promoting AT1aR expression in pivotal cell types, including vascular smooth muscle cells. In conclusion, these data suggest that promotion of these AngII/AT1aR-mediated effects in smooth muscle cells contribute to enhanced AAA formation in adult female mice exposed to exogenous androgen.
Table 3.1 PCR primers and conditions used for $AT1aR^{0/0}$ and $AT1aR^{SM22}$ KO mice genotyping.

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<th>Gene screened</th>
<th>Primers</th>
<th>Product size</th>
<th>PCR anealing temperature</th>
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<tr>
<td>Cre transgene</td>
<td>5'-CTAGGCCACAGAATTGAAAGATCT 5'-AGTAGGTGGAATTCTAGCATCC</td>
<td>182 bp</td>
<td>57.5°C</td>
</tr>
<tr>
<td>IL-2</td>
<td>5'-ACCTGAAGATGTTGCGGATT 5'-CGGCACTCAACGTTTCTTTT</td>
<td>324 bp</td>
<td>57.5°C</td>
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<tr>
<td>Floxed AT1aR</td>
<td>5'-TGTTGCATCTACATCCTG 5'-TCTAAAGAAACCTCATGAAC</td>
<td>262 bp</td>
<td>53°C</td>
</tr>
<tr>
<td>Excised AT1aR</td>
<td>5'-TGTTGCATCTACATCCTG 5'-TGTTTGGGGGTTTGTGTT</td>
<td>531 bp</td>
<td>53°C</td>
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<tr>
<td>Group</td>
<td>Gender</td>
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<td>1</td>
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<td>13</td>
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<td>2</td>
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<td>9</td>
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<td>Ovariectomy</td>
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<td>7</td>
<td>Female</td>
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Figure 3.1 Schematic representations of the experimental groups and study design as described in the methods section.
Figure 3.2 DHT augments AT1aR mRNA abundance in abdominal aortas from male and female ApoE-/- mice. A, In males, AT1aR mRNA abundance was greater in abdominal than thoracic aortas. Castration decreased, whereas DHT restored AT1aR mRNA abundance in abdominal aortas. Data are mean ± SEM. * P<0.05 compared to thoracic; **P<0.01 compared to intact. B, In females, DHT increased AT1aR mRNA abundance in abdominal aortas. Data are mean ± SEM. * P<0.05 compared to thoracic; **P<0.01 compared to intact.
Figure 3.3 AT1bR mRNA abundance in abdominal and thoracic aortas from male (A) and female (B) ApoE-/- mice was not altered by castration (1 or 5 weeks), or by DHT administration (1 or 5 weeks). However, AT1bR mRNA abundance was greater in abdominal compared to thoracic aortas from male and female mice, and was similarly expressed in aortic regions from male compared to female mice. * P<0.01 compared to thoracic aortas within treatment group.
Figure 3.4 Contractile responses of aortic (thoracic and abdominal) rings to AngII (1 μM, as a % of the KCl response) in male (A) and female (B) mice was not altered by castration, or by DHT administration. However, AngII-induced contraction was greater in abdominal compared to thoracic aortas, and was similar between male and female ApoE-/- mice. * P<0.01 compared to thoracic aortas within treatment group.
Figure 3.5 Androgen receptor (AR) mRNA abundance was greater in abdominal compared to thoracic aortas both male (A) and female (B) ApoE-/- mice. * P<0.01 compared to thoracic aortas within treatment group. **P<0.05 compared to ovariectomy.
Figure 3.6 AT1aR mRNA abundance in mouse aortic smooth muscle cells was increased by testosterone (10 nM; 24 hours of incubation). All results represent 3 replicates with each treatment performed in triplicate. *, P < 0.05 compared to DMSO vehicle control.
Figure 3.7 Generation (A) and DNA genotyping (B) of smooth muscle AT1aR deficient LDLR/- mice. (A) Gene map of partial AT1aR gene locus, floxed AT1aR allele and SM22-Cre driven deletion allele. (B) PCR reactions were performed with DNA samples extracted from aortic smooth muscle cells. Left, PCR for IL-2 was used to control for DNA loading, and primers for Cre were used to identify Cre1/0 mice. Right, in mice expressing the Cre transgene (Cre 1/0), exon 3 of AT1aR was deleted as demonstrated by the presence of a 531bp DNA product (AT1aR^{SM22 KO}) and the disappearance of 262bp DNA product (AT1aR^{fl/fl}).
Figure 3.8 SMC AT1aR deficiency reduces AAAs in adult female mice administered DHT and infused with AngII. (A) AAA incidence in AT1aR^{fl/fl} or AT1aR^{SM22 KO} female mice. (B) Survival curves for AT1aR^{fl/fl} or AT1aR^{SM22 KO} female mice. *, P<0.05 compared to AT1aR^{fl/fl}.
Figure 3.9 SMC AT1aR deficiency decreases abdominal aortic lumen diameters (A) and maximal external abdominal diameters (B) in adult female mice administered DHT and infused with AngII. Triangles are individual mice with circles representing mean ± SEM. *, P<0.05 compared to AT1aR<sup>fl/fl</sup>. 
Figure 3.10 SMC AT1aR deficiency has no effect on AngII-induced atherosclerosis in aortic arch or ascending aortic dilation. (A) Atherosclerotic lesion sizes in aortic arch of mice from each genotype. (B) Aortic arch areas, dictating ascending aortic dilation, from each genotype. Triangles are individual mice with circles representing mean ± SEM.
CHAPTER IV. SPECIFIC AIM 3

Determine the effect of androgen administration in neonatal female mice on aortic smooth muscle angiotensin receptor type 1a expression and angiotensin II-induced AAA formation in adulthood.

4.1 Summary

Our previous studies have demonstrated that androgen promotes AAA pathology in adult males through upregulation of AT1aR in abdominal aortas. Mechanisms for regulation of AT1aR in abdominal aortas by testosterone are unclear, but may relate to developmental effects on vascular wall cells. Thus, we sought to determine if administration of testosterone to neonatal female apolipoprotein E (ApoE) -/- or LDL receptor (LDLR)-/- mice confers adult susceptibility to AngII-induced AAAs.

One day old female mice were administered testosterone and AngII-induced vascular diseases were defined in adult females. Neonatal exposure to testosterone increased aortic AT1aR expression and promoted a striking increase in AngII-induced atherosclerosis, ascending aortic aneurysm and AAA (20% vs 64%; P<0.05) in adult female hyperlipidemic mice. These effects were independent of a requirement for circulating testosterone. Deficiency of AT1aR in smooth muscle cells reduced effects of neonatal or adult testosterone to promote AAAs in adult females, but did not alter atherosclerosis or aortic arch aneurysms. These data define a previously unrecognized role of sex hormone
exposures during development to confer enhanced susceptibility to distinct vascular diseases.
4.2 Introduction

Pioneering studies by Barker et al. initiated the concept that exposure to environmental stimuli, including sex hormones, during critical periods of development influence disease susceptibility in adult life. It is well established that several types of cardiovascular diseases exhibit sex differences, typically with enhanced disease development in males compared to females. Sex hormone influences have been demonstrated to contribute to sex differences between adult males and females, most commonly studied in the context of loss of female sex hormones during menopause as a contributor to higher cardiovascular disease development in aging females. By comparison, influences of sex hormone exposure during development on cardiovascular disease susceptibility in later life remains poorly defined.

Previous studies in our laboratories demonstrated that chronic AngII infusion in hypercholesterolemic male mice increases atherosclerosis and induces formation of aneurysms in the ascending and suprarenal aorta (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000; Daugherty, Rateri et al. 2010). These AngII-induced vascular pathologies are mediated through angiotensin type 1a receptors (AT1aR) (Daugherty, Rateri et al. 2004; Cassis, Rateri et al. 2007). Interestingly, despite being induced by infusion of the same peptide, only AAAs exhibit marked sex differences with higher prevalence (4-fold) in male compared to female mice (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000; Manning, Cassis et al. 2002; Henriques, Huang et al. 2004). Testosterone was found to be the primary mediator of higher AAA prevalence in
adult AngII-infused male mice (Henriques, Huang et al. 2004; Henriques, Zhang et al. 2008). As a mechanistic target of testosterone to promote AngII-induced AAAs, AT1aR mRNA abundance was greater in abdominal compared to thoracic aortas of male, but not female mice, and this regional difference was abolished by orchiectomy and restored in castrated males by androgen administration (Henriques, Zhang et al. 2008).

Vascular wall cells along the aortic length derive from different embryonic origins that exhibit distinct sensitivity to testosterone during development (Cunha, Shannon et al. 1981; Majesky 2007; Wasteson, Johansson et al. 2008). For example, smooth muscle of the abdominal aorta, where AT1aR expression is pronounced in males, derives from mesenchymal cell origin, and this embryonic cell type exhibits androgen receptor (AR) activity (Cunha, Shannon et al. 1981; Majesky 2007; Wasteson, Johansson et al. 2008). It is unclear whether AR stimulation of mesenchymal cells during developmental testosterone surges contributes to regional differences in AT1aR expression in aortas of adult males and impacts AAA susceptibility. Exposure of females during the neonatal period to testosterone has been used previously to induce male-like, or androgenized, behaviors (Pfaff and Zigmond 1971; Hrabovszky and Hutson 2002; Wallen 2005). This method, which imposes upon females exposure to testosterone shortly after birth, mimics developmental effects of androgen in males during the neonatal period (Motelica-Heino, Castanier et al. 1988; Corbier, Edwards et al. 1992). In this study, we sought to define effects of exposure of neonatal females to testosterone on regional expression of aortic AT1aR and susceptibility to
AngII-induced vascular diseases as adults. Remarkably, we found that exposure of neonatal female mice to a single dose of testosterone increased AT1aR mRNA abundance specifically in abdominal aortas of adult females. In addition, females exposed to testosterone as neonates exhibited a striking increase in adult susceptibility to AngII-induced AAAs, atherosclerosis and ascending aortic aneurysm. Deficiency of AT1aR in smooth muscle cells reduced effects of neonatal testosterone to promote AngII-induced AAAs in adult females, but had no effect on atherosclerosis or ascending aortic aneurysm. Our findings indicate that during a critical period of neonatal development transient exposure to testosterone in females is capable of developmentally programming enhanced susceptibility to three different AngII-induced vascular pathologies through distinct mechanisms.
4.3 Methods

4.3.1 Animals

Female ApoE-/- and LDLR-/- mice (N10 C57BL/6 background) were bred to males in house. Within 24 hours of birth, female mice were injected once with either vehicle (corn oil) or testosterone propionate (400 µg/mouse, s.c., Sigma Aldrich, St. Louis, MO). At 3 months of age, female ApoE-/- and LDLR-/- mice were sacrificed for aortic gene expression analysis. Age-matched ApoE-/- females were infused with saline or AngII (500, 750 or 1,000ng/kg/min, Bachem, Torrance, CA) by osmotic pumps (Alzet, model 1004, Durect Co., Cupertino, CA) for 4 weeks. Female ApoE-/- and LDLR-/- mice were maintained on standard diet through the study.

AT1aR\(^{SM22}\) KO and littermate controls AT1aR\(^{fl/fl}\) mice were generated and bred as previously described. Within 24 hours of birth, female AT1aR\(^{fl/fl}\) and AT1aR\(^{SM22}\) KO mice were injected once with either vehicle (corn oil) or testosterone propionate (400 µg/mouse, s.c.). At 3 months of age, females were infused with saline or AngII (1,000ng/kg/min, Bachem, Torrance, CA) by osmotic pumps for 4 weeks as previously described. The mice were fed a high fat diet containing 21% milk fat and 0.2% cholesterol (TD88137, Harlan Teklad, Indianapolis, IN) 1 week prior to pump implantation and through study endpoint. All experiments were performed in accordance with the University of Kentucky Institutional Animal Care and Use Committee.
4.3.2 Measurement of systolic blood pressure

Systolic blood pressures were measured one week prior to osmotic mini pump implantation and during the third week of AngII infusion using a noninvasive tail-cuff system (BP-2000 Blood Pressure Analysis System, Visitech Systems).

4.3.3 Quantification of Atherosclerosis, AAA and Ascending Aortic Aneurysm

Atherosclerotic lesions were quantified in the aortic arch and root as described previously. Aortic roots from representative samples (n=5/group) were sectioned and immunostained for CD68 (5 μg/mL; rat monoclonal ab53444, Abcam, Cambridge MA). AAA incidence was quantified by 3 independent observers blinded to the experimental design as described previously (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000). Abdominal aortic lumen diameter measurements were made on anesthetized mice using high-resolution ultrasound (Visualsonics, Vevo 660) at baseline, day 7, day 14 and day 28 of AngII infusion. Maximal diameters of abdominal aortas were measured on cleaned, formalin-fixed aortas by ImagePro Plus software (Media Cybernetics). AAAs were defined as > 50% dilation of lumen diameters from saline-infused mice.

The extent of ascending aortic dilation as an index of aortic arch aneurysms was quantified by measuring aortic arch intimal areas (to 3 mm distal to the subclavian branch) and ascending aortic diameters (Daugherty, Rateri et al. 2010). Representative ascending aortic aneurysms (n=4-5/group) were sectioned
and stained with H&E, and macrophages (Accurate Chemical, catalog No. AIAD31240, dilution 1:1000). Stained sections were magnified and photographed using a Nikon Eclipse 80i microscope and digital camera. Medial thickness was measured on H&E stained sections.

4.3.4 Aneurismal Tissue Characterization

To compare AAAs between different groups, three suprarenal aortas (closest to the mean external diameter of each group) were chosen for vascular characterization. Briefly, cleaned, formalin-fixed aortas were injected with Tissue-Tek® O.C.T. (Optimal Cutting Temperature) compound (Ted Pella, Inc., Redding, CA). The suprarenal aorta segment containing AAA was embedded in O.C.T. compound and serially sectioned using a cryostat as previously described (Police, Putnam et al. 2010). Sections were fixed with absolute alcohol and stained with hematoxylin and eosin (H&E), and Movat's staining for vascular characterization.

4.3.5 Blood Analysis

Blood was obtained from the right ventricle of anesthetized mice (ketamine/xylazine, 100/10 mg/kg, ip) for measurement of monocyte and red blood cell counts (Hemavet, Drew Scientific Group, Dallas, TX). Total serum cholesterol concentrations were measured from individual mice using enzymatic assay kits (Wako Pure Chemical, Catalog No. 439-17501). Serum samples (50 ul; n = 5) were fractionated by size exclusion chromatography (Daugherty, Manning
et al. 2000), and cholesterol concentrations determined using enzymatic kits. Serum testosterone concentrations and plasma aldosterone concentrations were determined using commercial RIA DSL-4000 and RIA DSL-8600 kits, respectively (Diagnostic Systems, Inc.).

4.3.6 Aortic gene quantification by Real Time PCR

Thoracic (aortic arch to diaphragm) and abdominal aortas (diaphragm to ileal bifurcation) were harvested from age-matched adult female mice. Aorta samples were stored in RNA Later (Ambion, Austin, TX) and cleaned of all adherent tissue. Total RNA was extracted using Rneasy Fibrous Minikit (Qiagen, Alameda, CA) (Henriques, Zhang et al. 2008). For in vitro experiments, primary mouse vascular smooth cells (passage 7-10) were treated with 10nM of testosterone propionate (Sigma Aldrich, St. Louis, MO) in 0.1% DMSO for 24 hours. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). All RNA samples were treated with Turbo DNA-free (Ambion, Austin, TX) to remove genomic DNA. RNA quantity was assessed spectrophotometrically, and quality was evaluated using a Bio-Rad Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). Mouse AT1aR primers were obtained from Qiagen (Alameda, CA). Relative quantification of aortic AT1aR mRNA abundance was performed using a BioRad iCycler (BioRad, Hercules, CA) (Henriques, Zhang et al. 2008). 18S rRNA was used to normalize for loading and data are expressed as AT1aR/18S ratios.
Customized PCR arrays were obtained from SABioscience (Frederick, MD) to test 43 genes that have been recently reported to differentially expressed in the between AAA-prone and non AAA-prone aortic segments in male ApoE-/- mice (Rush, Nyara et al. 2009). After total RNA samples were extracted from thoracic and abdominal aortas, reverse transcription and subsequent PCR reactions were performed using RT² First Strand Kit and RT² qPCR Master Mixes respectively, based on manufacturer recommendation (SABioscience, Frederick, MD).

4.3.7 Statistical Analysis

Data are represented as mean ± SEM. Comparison of AAA incidence between groups was analyzed using Fisher’s exact test. Unpaired student’s t test was used to compare parametric data on two groups. Multiple groups were analyzed with 2-way ANOVA followed by Tukey’s test for more than two groups. Analyses were performed using Sigmastat. Statistical significance was defined as P<0.05.
4.4 Results

4.4.1 Neonatal testosterone exposure developmentally programs increased AT1aR expression in abdominal aortas from adult female hyperlipidemic mice

Previous results demonstrated a requirement for AT1aR in AngII-induced atherosclerosis and AAA formation in adult male mice (Daugherty, Rateri et al. 2004; Cassis, Rateri et al. 2007). Recent studies demonstrated greater AT1aR mRNA abundance in abdominal compared to thoracic aortas of male, but not female ApoE-/- mice (Henriques, Zhang et al. 2008). Moreover, androgen-mediated regulation of AT1aR mRNA abundance in abdominal aortas of male and female ApoE-/- mice paralleled susceptibility to AngII-induced AAAs (Henriques, Zhang et al. 2008). Therefore, we determined if testosterone administration to neonatal females imparted regional differences in AT1aR expression along the aortic length in adult females. We examined two hypercholesterolemic strains of female mice to determine if background genetics influenced developmental effects of testosterone. As reported previously (Henriques, Zhang et al. 2008), adult female ApoE-/- or LDLR-/- mice administered vehicle as neonates exhibited a similar AT1aR mRNA abundance in thoracic compared to abdominal aortas (Figure 4.1). In contrast, administration of testosterone to neonatal females increased AT1aR mRNA abundance in abdominal, but not thoracic aortas of adult female mice (P<0.001) (Figure 4.1). Thus, similar to observed results previously in males (Henriques, Zhang et al. 2008), exposure of neonatal female mice to testosterone imparted
increased localization of AT1aR mRNA expression specifically to abdominal aortas of adult females. However, unlike males, increased abundance of AT1aR mRNA in adult females exposed neonatally to testosterone persisted even though adult females exhibited low serum concentrations of testosterone (Table 4.1).

A recent study utilizing whole genome expression analysis has demonstrated that over 300 transcripts are differentially expressed between AAA-prone and non AAA-prone aortic segments in male ApoE-/- mice (Rush, Nyara et al. 2009). To test if any of these genes were regulated by neonatal testosterone, we selected 43 gene targets and performed PCR array analysis to examine their mRNA expression levels along the length of the aorta. Interestingly, mRNA abundance of neutrophil cytosolic factor 1 (Ncf1, a component of NADPH oxidase) in thoracic aortas, and aryl hydrocarbon receptor nuclear translocator-like (Arntl) in both thoracic and abdominal aortas were significantly increased in adult females administered testosterone as neonates (Figure 4.14).

4.4.2 Administration of testosterone to neonatal female ApoE-/- mice increases AngII-induced AAAs in adult females.

Since previous studies demonstrated that AngII-induced AAAs are promoted by a hypercholesterolemic environment in male mice (Daugherty and Cassis 1999; Daugherty, Manning et al. 2000), initial studies were performed in hypercholesterolemic female mice. Female ApoE-/- mice were administered a single dose of testosterone or vehicle within the first 24 hours after birth to mimic
neonatal testosterone surges in males (Motelica-Heino, Castanier et al. 1988; Corbier, Edwards et al. 1992), and then adult female ApoE-/- mice were infused with different doses of AngII. Neonatal administration of testosterone resulted in a modest but significant increase in body weight in adult females but had no effect on serum cholesterol concentrations, blood pressure, blood monocyte counts (Table 4.1) or serum lipoprotein cholesterol distribution (Figure 4.5). Moreover, serum testosterone concentrations were not elevated in adult female mice administered testosterone as neonates (Table 4.1), and were 7-fold less than those observed previously in adult male ApoE-/- mice (Henriques, Huang et al. 2004).

Infusion of AngII to adult females administered vehicle as neonates resulted in modest but significant increases in abdominal aortic lumen and external diameters, indicative of aneurysm formation in a small percentage of mice (Figure 4.2-4.4). AAA incidence, which included mice that died from aneurismal rupture, was increased dose-dependently by AngII in female mice administered vehicle as neonates, but did not rise above 20% at the highest dose (1,000 ng/kg/min) of AngII infusion (Figure 4.3). Notably, adult female mice that were exposed to testosterone as neonates exhibited striking increases in abdominal aortic lumen dilation (1.29 ± 0.10 vs. 1.69 ± 0.11mm; P<0.01) and external diameters (1.31 ± 0.115 vs. 1.85 ± 0.16mm; P<0.01) at the highest dose (1,000 ng/kg/min) of AngII infusion, with pronounced aneurismal pathology similar to that previously observed in adult males (Manning, Cassis et al. 2002; Henriques, Huang et al. 2004) (Figure 4.3, 4.4). AAA incidence was greater at
each dose of infused AngII in adult females administered testosterone as neonates compared to vehicle controls (Figure 4.2), with a 3-fold increase in AAA incidence at the infusion dose of 1,000 ng/kg/min of AngII (21% vs. 64%; P<0.05). An increase in mortality from aneurismal rupture contributed to higher AAA incidences in females administered testosterone as neonates (rupture: vehicle, 7%; testosterone, 20%; NS).

4.4.3 Neonatal testosterone exposure developmentally programs AngII-induced atherosclerosis and ascending aortic aneurysms in female LDLR-/- mice independent of smooth muscle AT1aR

Since smooth muscle cell AT1aRs were pivotal in androgen promotion of AngII-induced AAAs in adult females, we determined if smooth muscle AT1aR deficiency influenced effects of neonatal testosterone to promote AngII-induced vascular pathologies in adult females. In addition, we quantified ascending aortic aneurysms (Daugherty, Rateri et al. 2010) and atherosclerosis (Daugherty, Manning et al. 2000) as additional vascular pathologies induced by infusion of AngII that do not exhibit sex differences between adult males and females (21, unpublished observations). Female AT1aR<sup>fl/fl</sup> control and AT1aR<sup>SM22</sup> KO mice were administered a single dose of vehicle or testosterone at 1 day of age, and then infused with AngII at 3 months of age. Neonatal administration of testosterone, as well as smooth muscle cell AT1aR deficiency, had no effect on baseline systolic blood pressures, AngII-induced hypertension, plasma aldosterone concentrations, serum cholesterol concentrations (Table 4.2) or
serum lipoprotein cholesterol distributions (Figure 4.6). Moreover, serum testosterone concentrations did not differ across study groups (Table 4.2). In females administered vehicle as neonates, $AT1aR^{SM22}$ KO had no effect on AngII-induced atherosclerosis in the aortic arch or aortic root (Figure 4.7). Interestingly, administration of testosterone to neonatal female mice resulted in pronounced increases in AngII-induced atherosclerosis in both $AT1aR^{fl/fl}$ (11.3 ± 1.1 vs. 16.3 ± 1.6 %; P<0.01) and $AT1aR^{SM22}$ KO (10.4 ± 1.0 vs. 14.9 ± 1.5 %; P<0.05) adult female mice (Figure 4.7). Lesional macrophages, as evidenced by quantification of CD68 immunostaining in aortic root sections from adult females, were increased by neonatal administration of testosterone (Figure 4.8).

However, deficiency of AT1aR in smooth muscle cells did not influence the ability of neonatal testosterone administration to promote AngII-induced atherosclerosis (Figure 4.7, 4.8).

In addition to promoting atherosclerosis, neonatal testosterone administration to female LDLR-/- mice augmented AngII-induced increases in ascending aortic diameters (P<0.05) (Figure 4.9A) and ascending aortic intimal areas (P<0.05) (Figure 4.9B), indicative of aortic arch aneurysms. Cross sections from ascending aortas of female mice exposed to testosterone as neonates and then infused with AngII as adults exhibited medial thickening (P<0.001) (Figure 4.10) and aortic ulceration (P<0.05) (Figure 4.11). Macrophage immunostaining was prominent in media and adventitia of aortic arch sections from AngII-infused adult female mice administered testosterone as neonates (P<0.05) (Figure 4.10), similar to results previously observed in aortic
arches of adult male mice (Daugherty, Rateri et al. 2010). Deficiency of AT1aR in smooth muscle did not influence the ability of neonatal testosterone administration to promote AngII-induced aneurysms in the aortic arch (Figure 4.9-4.11).

4.4.4 Smooth muscle AT1aR deficiency attenuates neonatal testosterone programming of AngII-induced AAAs in adult female LDLR-/- mice

Similar to findings in ApoE-/- females exposed neonatally to testosterone, adult AT1aR<sup>fl/fl</sup> females administered testosterone as neonates exhibited a striking increase in AngII-induced AAAs (AAA incidence, 15 vs. 64 %, P<0.001; Maximal external abdominal aortic diameter, 0.98 ± 0.10 vs. 1.55 ± 0.09 mm, P<0.001) (Figure 4.12,). Interestingly, smooth muscle cell specific AT1aR deficiency reduced, but did not ablate effects of neonatal testosterone to promote AngII-induced AAAs (AAA incidence, 64 vs. 33 %, P<0.05; Maximal external abdominal aortic diameter, 1.55 ± 0.09 vs. 1.22 ± 0.12 mm, P<0.05) (Figure 4.12). The magnitude of effect of smooth muscle AT1aR deficiency to reduce AngII-induced AAAs was similar between neonatal and adult females exposed to androgen (Figure 4.12A vs Figure 3.8). We examined tissue characteristics of AAAs of equivalent sizes that formed in female mice of each genotype exposed to neonatal testosterone. AAAs that formed in both groups exhibited typical characteristics of medial elastin degradation and pronounced thrombus in the adventitia (Figure 4.13). These data demonstrate that exposure of neonatal
females to testosterone increases adult susceptibility of females to AngII-induced
AAAs partially through smooth muscle cell AT1aR.
4.5 Discussion

Our findings reveal that neonatal exposure of female mice to testosterone developmentally programs three distinct AngII-induced vascular pathologies, namely atherosclerosis, ascending arch aneurysms, and AAAs. As a mechanism contributing to sex differences in AngII-induced AAAs between adult males and females (Manning, Cassis et al. 2002; Henriques, Huang et al. 2004), exposure of neonatal females to testosterone developmentally programs a regional increase in abdominal aortic AT1aR mRNA abundance. To gain insights into the cell target of testosterone during development to increase aortic AT1aR mRNA abundance and enhance AngII-induced vascular pathologies, we performed studies in female mice lacking AT1aR in smooth muscle cells. Smooth muscle deficiency of AT1aR reduced neonatal effects of testosterone to promote AngII-induced AAAs, but had no effect on augmented atherosclerosis or aortic arch aneurysms. These results demonstrate that distinct mechanisms mediate effects of developmental testosterone to enhance three different AngII-induced vascular pathologies in adult females. These studies are the first to demonstrate that sex hormone exposures during development can markedly alter vascular disease susceptibility in adults.

Exposure of neonatal females to testosterone, designed to mimic a surge of testosterone in males shortly after birth (Motelica-Heino, Castanier et al. 1988; Corbier, Edwards et al. 1992), has been used by several investigators to study sexual differentiation of the brain. Permanent changes in brain neurons of females androgenized during development are considered to be “organizational”,

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allowing for male-like behaviors that persist to adulthood (Arnold 2009). In contrast to studies focused on brain, little is known about developmental effects of sex hormones on other traits. We focused on effects of sex hormones during development on regional variations in AT1aR abundance in aortas since previous studies demonstrated a critical requirement of AT1aR for all three AngII-induced vascular pathologies (Cassis, Rateri et al. 2007; Daugherty, Rateri et al. 2010). Moreover, previous results demonstrated higher expression of AT1aR in abdominal compared to thoracic aortas of male but not female mice (Henriques, Zhang et al. 2008). In this study we demonstrated that administration of testosterone to neonatal female mice initiated greater abdominal aortic AT1aR mRNA abundance and markedly enhanced AAA formation in adult females. Interestingly, unlike males (Henriques, Huang et al. 2004; Henriques, Zhang et al. 2008), exposure of neonatal females to testosterone resulted in long-lasting effects that persist into adulthood and did not require the continued presence of circulating testosterone. Thus, similar to effects of testosterone to permanently influence brain behaviors (Arnold 2009), aortic AT1aR abundance and AAA susceptibility can be programmed developmentally by testosterone and sustained in adulthood with low circulating testosterone in females.

In mice, aortic smooth muscle cells are highly heterogeneous. The diversity of smooth muscle cell embryonic origins has been suggested to contribute to region-specific aortic pathologies, including those induced by infusion of AngII (Majesky 2007; Tromp, Kuivaniemi et al. 2010). For example, thoracic and abdominal aortic smooth muscle cells respond distinctively to
transforming growth factor-β (TGF-β) (Topouzis and Majesky 1996). Inhibition of TGF-β has a beneficial effect in a mouse model of ascending aortic aneurysms while TGF-β protects against aortic dissection of AngII-induced AAAs (Habashi, Judge et al. 2006; Wang, Ait-Oufella et al. 2010). Our results demonstrate that thoracic and abdominal aortas differ in the regulation of expression of AT1aR, and that effects of testosterone to increase aortic AT1aR expression were specific to abdominal aortas. Recent results demonstrated that infusion of AngII to C57BL/6 male mice resulted in hyperplasia of smooth muscle cells in the ascending aorta, but hypertrophy of smooth muscle in other aortic regions (Owens, Subramanian et al. 2010). Interestingly, despite differences in AT1aR-mediated regulation of vascular smooth muscle growth along the aortic length, all growth-related responses of aortic smooth muscle to AngII were abolished in AT1aR deficient mice (Owens, Subramanian et al. 2010). Our results demonstrate that even though testosterone promoted a region-specific increase in AT1aR mRNA abundance to abdominal aortas, all three AngII-induced pathologies were increased in neonatal females exposed to testosterone. While smooth muscle cell specific deficiency of AT1aR reduced effects of testosterone to promote AngII-induced AAAs, aortic arch aneurysms and atherosclerosis were not influenced by AT1aR on this cell type. Moreover, our results demonstrate that neonatal testosterone exposure increase mRNA abundance of neutrophil cytosolic factor 1 (Ncf1, a component of NADPH oxidase) in thoracic aortas, and aryl hydrocarbon receptor nuclear translocator-like (Arntl) in both thoracic and abdominal aortas of adult females (Figure 4.14), indicating that multiple genes
can be modulated by developmental androgen in a regional specific manner. These results demonstrate that distinct mechanisms, regulated differentially by testosterone, contribute to AngII-induced initiation of these vascular pathologies.

In contrast to AAAs, previous findings from our laboratory do not support sex differences in AngII-induced atherosclerosis (Henriques, Zhang et al. 2008). Moreover, aortic arch aneurysms occur at similar levels between adult male and female hyperlipidemic mice infused with AngII (unpublished observations). Our results demonstrate that even though adult males and females do not exhibit robust sex differences in these two AngII-induced vascular pathologies, androgenization of females during development programmed increases in atherosclerosis and aortic arch aneurysms. These results suggest that male and female mice differ in their response to testosterone during the neonatal period of development. Unfortunately, technical difficulties preclude measurement of testosterone concentrations in neonatal female mice to determine if levels of testosterone in androgenized females are different than those normally experienced by neonatal males. It is conceivable that the dose (400 µg) of testosterone administered to neonatal females exceeded levels experienced by males, resulting in increased susceptibility to pathologies that do not typically exhibit sex differences. Alternatively, recent studies demonstrated that sex chromosomes contribute to differences in AngII-induced hypertension between male and female mice (Ji, Zheng et al. 2010). An ability of neonatal testosterone to mediate increased AngII-induced atherosclerosis and aortic arch aneurysms in adult females may have arisen from sex chromosome effects. Future studies
examining sex chromosome effects on AngII-induced vascular pathologies may reveal a role for sex chromosomes as contributors to differences in effects of neonatal testosterone in males compared to females.

In conclusion, these data demonstrate that developmental androgenization of female mice confers increased susceptibility to AngII-induced AAAs, atherosclerosis and ascending aortic aneurysms as adults. An ability of testosterone to developmentally program three distinct vascular pathologies in females indicates that short term hormonal exposures can markedly impact vascular disease susceptibility of adults. Based on these findings, targeting of specific environmental risk factors during development may provide new insights into the prevention, diagnosis and treatment of these life-threatening vascular diseases.
Table 4.1 Characteristics of female ApoE-/- mice subjected to neonatal administration with either vehicle or testosterone.

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>Liver (%BW)</th>
<th>Serum testosterone (pg/ml)</th>
<th>WBC (K/µl)</th>
<th>RBC (M/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>22.0±0.4</td>
<td>4.63±0.08</td>
<td>133±9</td>
<td>1.92±0.37</td>
<td>8.01±0.38</td>
</tr>
<tr>
<td>Testosterone</td>
<td>23.5±0.3*</td>
<td>4.95±0.08*</td>
<td>94±9</td>
<td>2.24±0.32</td>
<td>8.75±0.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Total serum cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline 500 750 1000 AngII</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>123±3 130±5 148±3** 160±6**</td>
<td>368.1±19.5</td>
</tr>
<tr>
<td>Testosterone</td>
<td>120±2 133±8 142±5** 159±8**</td>
<td>351.7±18.3</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM from n = 20-25 mice/group.

*P<0.01 compared to vehicle group.

**P<0.001 compared to baseline measurement within treatment (vehicle, testosterone).
Table 4.2 Characteristics of adult female $AT1aR^{fl/fl}$ and $AT1aR^{SM22}$ KO administered vehicle or testosterone as neonates.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>BW (g)</th>
<th>Serum testosterone (pg/ml)</th>
<th>Total serum cholesterol (mg/dl)</th>
<th>Plasma aldosterone (pg/ml)</th>
<th>Systolic blood pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>baseline, AngII</td>
</tr>
<tr>
<td>$AT1aR^{fl/fl}$ + vehicle</td>
<td>26</td>
<td>24.0 ± 0.6</td>
<td>185 ± 28</td>
<td>1412 ± 136</td>
<td>739 ± 131</td>
<td>121 ± 4, 154 ± 8**</td>
</tr>
<tr>
<td>$AT1aR^{SM22}$ KO + vehicle</td>
<td>22</td>
<td>24.4 ± 0.4</td>
<td>142 ± 16</td>
<td>1456 ± 181</td>
<td>601 ± 123</td>
<td>112 ± 5, 150 ± 8**</td>
</tr>
<tr>
<td>$AT1aR^{fl/fl}$ + testosterone</td>
<td>33</td>
<td>26.6 ± 0.6*</td>
<td>155 ± 15</td>
<td>1424 ± 119</td>
<td>548 ± 125</td>
<td>115 ± 3, 156 ± 4**</td>
</tr>
<tr>
<td>$AT1aR^{SM22}$ KO + testosterone</td>
<td>18</td>
<td>27.0 ± 0.7*</td>
<td>130 ± 20</td>
<td>1414 ± 147</td>
<td>456 ± 77</td>
<td>116 ± 6, 153 ± 8**</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM.

*P<0.001 compared to vehicle group within genotype. **P<0.001 compared to baseline blood pressure.
Figure 4.1 AT1aR mRNA abundance in adult female ApoE-/- mice (A) (n = 8-10/group) and LDLR-/- mice (B) (n = 5-6/group) administered testosterone as neonates. *, P < 0.001 compared to vehicle group within aortic region; **, P<0.001 compared to thoracic within treatment.
Figure 4.2 Neonatal testosterone administration significantly increases AngII-induced AAAs in adult female ApoE-/- mice. (A) Representative aortas from each AngII-infused group; scale bar, 2 mm. (B) AAA incidences in mice infused with 500, 750 or 1000 ng/kg/min AngII (n=14-25/group). *, P<0.05 compared to 500 ng/kg/min AngII infused group within testosterone treatment; **, P<0.05 compared to vehicle within 1000 ng/kg/min AngII-infused group.
Figure 4.3 Neonatal testosterone administration significantly increases abdominal aortic lumen diameters in adult female ApoE-/- mice infused with 500, 750 or 1000 ng/kg/min AngII (n=14-25/group). *, P<0.05 compared to 500 ng/kg/min AngII infused group within treatment; †, P<0.05 compared to 750 ng/kg/min AngII infused group within testosterone treatment; **, P<0.05 compared to vehicle within 1000 ng/kg/min AngII-infused group. Triangles represent measurements from individual mice, circles represent mean ±SEM.
Figure 4.4 Neonatal testosterone administration significantly increases maximal external diameters of the abdominal aortas in adult female ApoE-/- mice infused with 500, 750 or 1000 ng/kg/min AngII (n=14-25/group). *, P<0.05 compared to 500 ng/kg/min AngII infused group within treatment; **, P<0.01 compared to vehicle within 1000 ng/kg/min AngII-infused group. Triangles represent measurements from individual mice, circles represent mean ±SEM.
Figure 4.5 Serum lipoprotein cholesterol distributions in AngII-infused female ApoE/- mice administered with either vehicle or testosterone as neonates. Neonatal testosterone administration does not alter lipoprotein profiles in adult female mice. Data are the mean ± SEM of 5 individual mice per group.
Figure 4.6 Serum lipoprotein cholesterol distribution in AngII-infused female wild type and SMC AT1aR deficient mice administered with either vehicle or testosterone as neonates. Neither neonatal testosterone administration nor SMC AT1aR deficiency altered lipoprotein profiles in adult female mice. Data are the mean ± SEM of 5 individual mice per group.
Figure 4.7 Neonatal testosterone administration increases AngII-induced atherosclerosis in adult $AT1aR^{fl/fl}$ and $AT1aR^{SM22 KO}$ female LDLR/- mice. (A) Atherosclerotic lesion formation (% intimal surface area) in the aortic arch. *P<0.05 compared to vehicle group within genotype. (B) Quantification of atherosclerotic lesions stained with Oil Red O in aortic root sections. *, P<0.05 compared to vehicle within genotype.
Figure 4.8 Neonatal testosterone administration increases macrophage accumulation in aortic root atherosclerotic lesions from adult AT1aRfl/fl and AT1aRSM22 KO female LDLR−/− mice. (A) Representative aortic root sections stained with oil red O (top) and CD68 (bottom). (B) Quantification of CD68 positive staining as measurement of macrophages in aortic root atherosclerotic lesions. *, P<0.05 compared to vehicle within genotype.
Figure 4.9 Neonatal testosterone administration increases AngII-induced ascending aortic aneurysms in adult $AT1aR^{fl/fl}$ and $AT1aR^{SM22\text{KO}}$ female LDLR-/- mice. (A) Ascending aortic diameters as an index of ascending aortic aneurysms. *, $P<0.001$ compared to vehicle group within genotype. (B) Aortic arch areas. *, $P<0.05$ compared to vehicle within genotype. Circles and triangles (A,B) represent individual mice, diamonds are means ±SEM.
Figure 4.10 Neonatal testosterone administration increases AngII-induced ascending aortic pathologies in adult AT1aR<sub>fl/fl</sub> and AT1aR<sub>SM22</sub> KO female LDLR<sup>-/-</sup> mice. (A) Cross sections of ascending aortas were stained with H&E (top), or for macrophages (bottom). The orientation of aortic sections is described by L, lumen; M, media; A, adventitia. Magnification, X200. (B) Medial thickness of aortic arch sections (n=5-6/group). *, P<0.001 compared to vehicle within genotype.
Figure 4.11 Neonatal testosterone administration increases ascending aortic ulceration in adult AT1aR/+/fl and AT1aR SM22 KO female LDLR−/− mice. (A) Representative example of aortic ulceration in ascending aorta. Scale bar, 1 mm. (B) The incidence of ascending aortic ulceration from each group. *, P<0.05 compared to vehicle within genotype.
Figure 4.12 Smooth muscle AT1aR deficiency reduces AngII-induced AAAs in adult female mice administered testosterone as neonates. (A) AAA incidence. (B) Maximal external diameters of abdominal aortas. Circles and triangles represent individual mice, diamonds are means ±SEM. *P<0.001 compared to vehicle group within genotype; **P<0.05 compared to WT within treatment.
Figure 4.13 Cross sections of AngII-induced AAA from $AT1aR^{fl/fl}$ and $AT1aR^{SM22}$ KO mice administered testosterone as neonates. Top, H&E sections; Magnification, X40. Bottom, Movat’s stain for elastin (black), collagen (yellow) and smooth muscle (red); Magnification, X200.
Figure 4.14 Aortic genes were differentially regulated by neonatal testosterone exposure in adult female LDLR-/- mice. (A) Ncf1, encoding the cystic unit p47phox of NADPH oxidase, was significantly increased by neonatal testosterone in thoracic, not abdominal aortas. N=3/each group. * P<0.01 compared to thoracic within treatment; **, P<0.05 compared to vehicle within aortic region. (B) Arntl was upregulated in both thoracic and abdominal aortas. N=3/each group. **, P<0.01 compared to vehicle within aortic region.
CHAPTER V. GENERAL DISCUSSION

5.1 Summary

The purpose of this study is to examine the hypothesis that androgen promotes AT1aR expression on SMC to confer high prevalence of AngII-induced AAAs in hyperlipidemic mice. In addition, we also investigate the role of endogenous androgen in the progression of established AngII-induced AAAs in male mice.

First, we sought to examine the role of endogenous androgen in the growth of established AngII-induced AAAs in male mice. Since previous studies demonstrated that removal of endogenous androgen markedly reduced AAA formation in male mice (Henriques, Huang et al. 2004), we hypothesized that removal of endogenous androgen would also decrease the progressive expansion of established AAAs. Interestingly, castration significantly decreased the progressive lumen dilation of established AngII-induced AAAs in male ApoE-/- mice, but had no effect on external AAA diameters. These results suggest that androgen contributes to the progression of established AAAs through distinct mechanisms that differentially influence aortic lumen and wall diameters.

Next, we investigated if androgen regulates aortic AT1aR expression to promote AngII-induced AAA formation. We hypothesized that androgen promotes AT1aR expression on SMC to confer higher susceptibility of AngII-induced AAAs in male mice. Our data demonstrated that in male and female mice, both endogenous and exogenous androgen stimulate AT1aR level particularly in
abdominal aortas where AngII-induced AAAs are usually located. This androgen-dependent enhanced mRNA abundance of abdominal aortic AT1aR was highly associated with increased AngII-induced AAA formation in male and female mice. To further examine if AT1aR on SMC were regulated by androgen to confer high prevalence of AngII-induced AAA formation in mice, we utilized mice with SMC specific AT1aR deficiency to determine the role of SMC AT1aR in enhanced AngII-induced AAA formation in female hyperlipidemic mice administered exogenous androgen. SMC AT1aR deficiency significantly reduced luminal and external diameters of abdominal aortas as well as the incidence of AngII-induced AAAs. Collectively, these results indicate that in adult mice androgen stimulate SMC AT1aR expression to promote AngII-induced AAA formation.

Furthermore, we determined the role of androgen during development on AT1aR expression on SMC and AngII-induced AAA formation in adult female hyperlipidemic mice. We hypothesized that neonatal androgen administration in female mice stimulates aortic SMC AT1aR expression and results in increased susceptibility of AngII-induced AAAs in adulthood. Our data demonstrated that neonatal testosterone administration dramatically increased AngII-induced AAA formation in adult female mice and this was associated with long-lasting enhanced AT1aR expression specifically in abdominal aortas. In addition, deficiency of SMC AT1aR reduced effects of neonatal testosterone administration. AngII-induced atherosclerosis and ascending aortic aneurysms were also enhanced by neonatal administration of testosterone while SMC
AT1aR deficiency had no effect on the pathogenesis of these two AngII-induced vascular pathologies. In summary, these results demonstrated that androgen, both in adult life and during development, stimulates SMC AT1aR expression and promotes AngII-induced AAA formation in adult female hyperlipidemic mice.

5.2 Potential mechanisms of androgen to influence the development of AngII-induced AAAs

5.2.1 The role of androgen during adult life in AngII-induced AAA formation and progression

In humans, male gender is a strong independent risk factor of AAA. A similar gender difference has been observed in the experimental model of AngII-induced AAA. In agreement with previous findings, results from this study confirm a critical role for testosterone as a primary mediator of gender differences in the pathogenesis of AngII-induced AAA. It has been demonstrated that removal of endogenous androgen profoundly reduced AngII-induced AAA formation in male ApoE-/- mice (Henriques, Huang et al. 2004). Our laboratory also demonstrated that exogenous androgen administration can restore a high incidence of AngII-induced AAA in castrated male mice and also stimulate AAA formation in female mice (Henriques, Zhang et al. 2008). Interestingly, estrogen has been reported to exert protective effects against AAA development. For example, others demonstrated that male mice administered supraphysiological level of estrogen exhibited a significant reduction in AngII-induced AAA formation (Martin-McNulty, Tham et al. 2003) and a similar finding has been observed in male rats infused
with elastase in the abdominal aorta to induce aortic dilation (Ailawadi, Eliason et al. 2004; Cho, Woodrum et al. 2009). The removal of endogenous estrogen was shown to enhance AAA incidence of female rats in elastase infusion induced AAAs (Wu, Zhang et al. 2009), but not in others (Cho, Woodrum et al. 2009). However, most studies indicate that testosterone promotes AAA formation in different models of this disease, including AngII-induced AAAs.

To investigate mechanisms of androgen in promoting AngII-induced AAA formation, we hypothesized that androgen increases the expression of AT1aR in the abdominal aorta to confer a higher prevalence of AAA in male mice. This was primarily due to the observation that deficiency of AT1aR totally ablates AngII-induced AAA formation in male mice, suggesting AT1aR is required for AAA development (Cassis, Rateri et al. 2007). In addition, bone marrow transplantation studies revealed that AT1aR on monocytes may not be involved in AngII-induced AAAs, indicating a potential role of AT1aR on resident cells presumably vascular wall cells of the abdominal aorta as targets of testosterone and/or AngII (Cassis, Rateri et al. 2007). Indeed, in these studies we demonstrate that in both male and female ApoE-/- mice, androgen positively regulates the mRNA abundance of AT1aR in abdominal, but not thoracic aorta (Chapter III). In the presence of androgen, either endogenous or exogenous, the increased level of abdominal aortic AT1aR is highly correlated with higher prevalence of AngII-induced AAAs in male and female mice. In contrast, the region specific pattern of aortic AT1bR was not altered by endogenous or exogenous androgen. Differences in the promoters of AT1aR and AT1bR genes
may contribute to androgen-specific effects to increase AT1aR mRNA abundance. We have performed a preliminary scan of the rat AT1 receptor promoter for potential transcription factor binding sites using Genomatix database. Several putative androgen response elements (AREs) binding sites were identified at around 515, 2660 and 2826 upstream of the transcription start site. In addition, it has been reported that the actions of AR coregulators also depend on the state of chromatin at the loci of target genes. For example, one of the AR coregulators, CBP acts as a corepressor of AR when recruited to pericentric regions, but as a coactivator when recruited to euchromatin (Zhao, Takeyama et al. 2009). AT1aR and AT1bR are encoded by two distinct genes located on two different chromosomes. It is possible that the differences in the context of chromatins at AT1aR and AT1bR loci may contribute to the specific stimulatory effect of androgen on AT1aR expression. In humans, there is one gene encoding the AT1 receptor. DHT increased AT1 receptor mRNA and protein levels in androgen-dependent human prostate cancer cells (Uemura, Hasumi et al. 2006). These results suggest that testosterone positive regulation of AT1 receptor expression is not restricted to mice. However, no studies have examined whether human AT1 receptors are differentially expressed between thoracic and abdominal aorta.

It has been previously reported AR are expressed at lower levels in the vasculature of female compared to male rats (Higashiura, Mathur et al. 1997). However, our results demonstrate that female ApoE-/- mice express abundant ARs in abdominal aortas and can respond to exogenous androgen similar to
males. Interestingly, our results also demonstrate that similar to AT1aR expression, the abdominal aortas of male and female ApoE-/- mice express higher levels of AR compared to the thoracic region. Collectively, these results demonstrate marked heterogeneity of the aorta along its length in males and females, with sex hormones contributing to differential expression of some of these region-specific genes.

Furthermore, we utilized SMC AT1aR deficient mice to confirm that androgen positively regulates the expression of AT1aR on smooth muscle cells and promotes AngII-induced AAA formation. Deficiency of SMC AT1aR resulted in a 35% reduction of AAA incidence from 85% to 50% in adult female mice administered exogenous androgen. Although this significant 35% reduction suggests a pivotal role SMC AT1aR in AAA development, deficiency of SMC AT1aR did not totally ablate the effect of exogenous androgen on AngII-induced AAA formation in female mice. These results suggest that androgen may regulate AT1aR level on other cells in the vasculature, such as endothelial cells or fibroblasts in the adventitia, to promote AAA formation. Alternatively, there may be other potential mechanisms for androgen regulation of gender differences in AngII-induced AAA formation. For example, androgen has been demonstrated to stimulate MMP-2 expression in prostate cancer cells (Liao, Thrasher et al. 2003). Recently it has been demonstrated that male rats exhibit increased MMP-13 mRNA expression and proteins level in the aortic tissues as well as larger AAA compared to females in the experimental model of elastase infusion induced AAAs (Cho, Roelofs et al. 2009).
Overall, our studies demonstrate that AT1aR plays a critical role in the development of AngII-induced AAAs and is an important mechanism by which androgen mediates gender differences in experimental AAAs. Indeed, administration of losartan, an AT1 receptor inhibitor, fully protects male mice from developing AngII-induced AAAs (Daugherty, Manning et al. 2001). Recently, it has also been reported that AT1 receptor inhibition reduces macrophage infiltration, elastin degradation and prevents AAA formation in the rat model of elastase infusion induced AAA (Sweeting, Thompson et al. 2010).

In humans, men are more likely to develop AAAs. As our results from the AngII infusion model of AAAs show a clear association of androgen with high AAA prevalence in males via regulation of AT1aR in abdominal aortas, it is conceivable that androgen may also influence the growth and expansion of formed AAAs. Therefore, we hypothesized that removal of endogenous androgen will decrease the progression of established Ang II-induced AAAs. As is shown in Chapter II, in male ApoE-/- mice with established AAAs, castration significantly reduced luminal dilation of abdominal aortas compared to sham control males during an additional 8 weeks of continuous AngII infusion. A modest, but not significant decrease in maximal external diameters of abdominal aortas was also observed at the endpoint of the study. In contrast, it has been recently reported that formed AAAs in nonhyperlipidemic C57BL/6 mice exhibit no further lumen dilation when the mice are fed a high fat diet to induce obesity and then subjected to 3 month chronic AngII infusion (Police, Putnam et al. 2010).
discrepancy may be explained by the different mouse models and experimental
design used in these studies.

As discussed previously, androgen exerts stimulatory effects on most
components of the RAS, including the AT1 receptor. It is conceivable that
castration reduces the progressive luminal dilation of formed AngII-induced AAAs
through inhibiting the RAS, possibly the AT1 receptor. We acknowledge that in
the current study we have not measured components of the RAS, systemically or
at local AAA tissues. Notably, a retrospective case-control study using a large
Canadian administrative database showed that patients with an AAA taking ACE
inhibitors were less likely to present with ruptured aneurysm (Hackam,
Thiruchelvam et al. 2006). In the rat elastase model of AAA, an ACE inhibitor has
been proven to significantly reduce AAA formation (Liao, Miralles et al. 2001). In
the mouse model of fibrillin 1 deficiency mimicking Marfan syndrome, losartan
has been demonstrated to effectively prevent the development of ascending
aortic aneurysm by antagonizing TGF-β, which plays a role in the progression of
tissue changes associated with Marfan syndrome (Neptune, Frischmeyer et al.
2003; Habashi, Judge et al. 2006). In the future, comparing the levels of RAS
components may provide us with insights how castration limits the luminal
expansion of established AngII-induced AAAs.

Our laboratory has previously demonstrated that weight loss in obese
C57BL/6 mice limits adventitial expansion of established Ang II-induced AAAs,
resulting in a reduction of external aneurysm diameters (Police, Putnam et al.
2010). Limited adventitial expansion in mice undergoing weight loss was
associated with a striking decrease in neovascularization of the vessel wall (Police, Putnam et al. 2010). Importantly, it is well known that androgen acts via its receptor to regulate angiogenesis in the prostate and promote tumor neovascularization growth (Colombel, Filleur et al. 2005). Indeed, androgens have been reported to stimulate the expression of VEGF and hypoxia inducible factor-1 (HIF-1), key mediators of angiogenesis, in the prostate (Mabjeesh, Willard et al. 2003; Boddy, Fox et al. 2005). Therefore, one hypothesis to explain the ability of castration to blunt AAA progression is that castration decrease neovascularization within expanding aneurysms, limiting adventitial expansion and resulting in a reduction in the size of established AngII-induced AAAs. Unfortunately, this hypothesis is discordant with our findings that castration decreased aortic lumen diameters, but not aortic wall diameters of formed AAAs. Recent studies demonstrated that testosterone inhibits vascular calcification (Son, Akishita et al. 2010), suggesting that castration may increase vascular calcification and stimulate the remodeling of vessel wall. These findings suggest that androgen may exert differential effect on luminal dilation and aortic remodeling of established AngII-induced AAAs, via distinct mechanisms.

In conclusion, androgen stimulates AT1aR expression, particularly on SMC, to promote AngII-induced AAA formation in adult mice. Furthermore, castration significantly decreased the progressive lumen dilation of established AngII-induced AAAs in male mice, suggesting that androgen plays a role in the formation and expansion of AAAs.
5.2.2 The role of developmental androgen in AngII-induced AAA formation

As discussed previously, during development androgen plays a vital role in sexual differentiation of the brain and adult male behaviors as well as sexual dimorphism of other organs. We have identified that androgen in adult mouse mediates the prominent gender differences in AngII-induced AAAs partially by promoting AT1aR on vascular smooth muscle cells. To investigate if developmental androgen also contributes to a higher prevalence of AAAs in males, we hypothesized that administration of androgen during development to female mice will increase SMC AT1aR expression and promote Ang II-induced AAA formation in adult females. We administered a single dose of testosterone to female mice right after birth to mimic the effect of perinatal androgen surges which naturally occurs in males (Weisz and Ward 1980; Corbier, Edwards et al. 1992). The neonatal administration of testosterone significantly stimulated abdominal aortic AT1aR expression and strikingly promoted AngII-induced AAA formation in adult female ApoE-/− and LDLR-/− mice (Chapter IV). Deficiency of SMC AT1aR ablated the effect of neonatal testosterone on AngII-induced AAA formation.

Notably, these effects of neonatal testosterone administration are independent of endogenous androgen in adulthood as serum testosterone concentrations in adult female mice were not altered by the short term exposure to androgen during development. However, as is thoroughly reviewed by Foecking et al, the neurosecretory control system of neonatally androgenized females is structurally and functionally altered and therefore incapable of
supporting cyclic GnRH and gonadotropin secretion in adulthood (Foecking, McDevitt et al. 2008). Females that were androgenized by testosterone as neonates exhibit a persistent estrus syndrome including anovulation, acyclic LH secretion and attenuated pituitary responsive to GnRH (Foecking, McDevitt et al. 2008). Thus it is likely that these alterations in the endocrine system can lead to changes in the estrogen production and secretion in androgenized female mice. We acknowledge that estrogen measurements were not obtained in this study due to technical difficulties. However, since previous studies for our laboratory have demonstrated that endogenous estrogen does not regulate the formation of AngII-induced AAA (Henriques, Huang et al. 2004), changes in estrogen levels in androgenized females most likely did not mediate the striking increases in susceptibility to AngII-induced AAAs of adult female mice.

Interestingly, in female mice administered testosterone as neonates increased expression levels of abdominal aortic AT1aR were acquired following a single exposure to testosterone and maintained through adulthood without a continued requirement for circulating testosterone. In contrast, our results demonstrate that male mice continue to require circulating androgen to maintain abdominal aortic AT1aR expression in adult life. This is important as males would be naturally under the influence of perinatal androgen surges during development. AR mediated gene transcription often involves epigenetic mechanisms, in which the coregulatory factors of AR modulate chromatin structure and influence epigenetic marks. However, it has been demonstrated that the distribution and expression level of some AR coregulators vary between
males and females. For example, in rats many AR coregulatory factors, such as SCR-1, CBP/p300, NCoR and SMRT, are differentially expressed in various tissues (Misiti, Schomburg et al. 1998). Therefore, gender differences in the expression of AR coregulators may result in variable responses to androgen between male and female mice. In addition, recent studies have also revealed that sex chromosomes influence the different responses to sex hormones in the experimental model of AngII-induced hypertension (Ji, Zheng et al. 2010). It is also feasible that the presence of an additional X chromosome, or alternatively the absence of a Y chromosome in female mice may contribute to the different response to neonatal androgen between male and female mice.

In addition, many investigators have discovered that the specific impacts of developmental androgen on the adult animal vary greatly, depending on: 1) at what “critical” period during development androgen is administered; 2) the dose and duration of androgen exposure and 3) the animal species. For example, it is well established that androgen (usually a single dose) is administered perinatally, during late gestation or right after birth, to induce neuroendocrine alterations and androgenized male-like behaviors in female rodents, sheep and non-human primate (Pfaff and Zigmond 1971; Hrabovszky and Hutson 2002; Wallen 2005). Recently excess androgen exposure in utero has emerged as a promising tool to generate experimental model of polycystic ovary syndrome (PCOS) (Xita and Tsatsoulis 2006). PCOS is a common endocrine and metabolic disorder characterized by excess androgen production of ovarian and/or adrenal origin, luteinizing hormone (LH) hypersecretion, hyperinsulinemia from insulin
resistance and reduced fecundity. PCOS imposes an increased risk for type 2 diabetes and cardiovascular disease in women. It has been reported that PCOS phenotypes can be obtained from repeated injections of pregnant rhesus monkeys carrying female fetuses with testosterone for 15 to 35 days starting on either days 40–60 (early gestation) or days 100–115 (late gestation) during a total 165-day gestation (Dumesic, Abbott et al. 2007). In comparison, the administration of testosterone during early gestation produces more traits of PCOS, such as LH hypersecretion and increased type 2 diabetes. In this study, we administered exogenous androgen neonatally to distinguish from the PCOS experimental model as the metabolic syndrome phenotypes in PCOS model would complicate the interpretation of our results. However, it is not clear whether or not the effect of developmental androgen on abdominal aortic AT1aR expression and AngII-induced AAA formation would sustain if exogenous androgen was administered early in development.

Interesting, in our studies neonatal testosterone administration also significantly enhanced AngII-induced atherosclerosis and ascending aortic aneurysm. Previously we have demonstrated that gender differences were restricted to AngII-induced AAAs, as male and female mice exhibited similar levels of atherosclerosis following AngII infusion (Henriques, Zhang et al. 2008). Recently the pathological characteristics of AngII-induced ascending aortic aneurysms have been reported (Daugherty, Rateri et al. 2010). Preliminary observations suggest that gender differences do not exist in AngII-induced aortic arch aneurysms. We acknowledge that the dose of testosterone used in this
study (400 µg/mouse) is higher than what has been used by many investigators to androgenize females in the study of sexual dimorphism of brain (100 µg/mouse). This may contribute to the unexpected observation that two AngII-induced vascular pathologies that do not display gender differences were influenced by neonatal testosterone administration. In addition, SMC AT1aR deficiency reduced the effect of neonatal testosterone to promote AngII-induced AAAs, but had no effect on atherosclerosis or ascending aortic aneurysms in adult female mice infused with AngII. However, the AT1aR is required for these AngII-induced vascular pathologies. It is possible that neonatal testosterone regulates AT1aR expression on other cells in the vasculature to promote AngII-induced atherosclerosis and ascending aortic aneurysms. Furthermore, the mRNA abundance of p47phox, one of the cytosolic units of NADPH oxidase, was significantly increased in thoracic aortas of female mice administered testosterone as neonates. It is conceivable that the increased ROS production and oxidative stress in the vasculature of thoracic aortas may play a role in enhanced AngII-induced atherosclerosis and ascending aortic aneurysm in this region. Future studies should include full DNA array analysis on the aortas from androgenized and control female mice to provide potential gene targets that may be regulated by neonatal testosterone exposure and contribute to these AngII-induced vascular pathologies in female mice.

In conclusion, our findings have demonstrated that a short-term exposure to testosterone during a critical period of development increases AngII-induced
AAA formation, via regulating SMC AT1aR, as well as other AngII-induced vascular pathologies in adult female mice.

5.3 Future directions

Results from these studies demonstrate that androgen increases AT1aR expression, particularly in abdominal aortic SMCs, to promote AngII-induced AAA formation. Our studies focus on investigating the role of androgen in adult life and during development in the formation of AngII-induced AAAs. In both studies we have demonstrated that the enhanced AAA formation by androgen is highly associated with the increased expression of abdominal aortic AT1aR. However, we did not address the molecular mechanisms responsible for androgen stimulation of AT1aR expression, especially in the abdominal aortas. Therefore, an interesting study will be to examine the exact coregulatory factors that interact with ARs recruited to the AT1aR promoter to increase transcription of AT1aR in abdominal aortas.

Our results suggest that female mice exhibit long-lasting enhancement of abdominal aortic AT1aR expression once exposed to neonatal androgen while male mice require constant high circulating androgen to maintain higher AT1aR expression. Our study did not address why female mice seem to be more sensitive to the effects of neonatal testosterone administration. It is possible that the dose of testosterone we used in the study (400 µg/mouse) causes the circulating testosterone level in fetal females to exceed those normally found in fetal males. Therefore, one of the future experiments could focus on defining
effects of a lower dose testosterone, 100 µg/mouse, on AngII-induced AAA formation as well as atherosclerosis and ascending aortic aneurysms in adult female mice.

Another possibility is that sex chromosomes may influence the differential responses to neonatal testosterone between male and female mice. Recently, a powerful mouse model, name the 4 core genotypes, has been demonstrated to enable studying the sex chromosome effects independent of the influences of sex hormones (Arnold and Chen 2009). The 4 core genotypes includes alternative gonadal female mice (XY\textsuperscript{−}) generated by the deletion of Sry gene, the dominant testis-determining gene, from the Y chromosome. The XY\textsuperscript{−} mouse does not develop testes but develops ovaries and expresses a female gonadal hormone phenotype instead. Similarly, The Sry gene was also inserted onto an autosome, creating XY\textsuperscript{−}Sry and XXSry transgenic mice which are gonadal male mice regardless of the sex chromosome complement. Therefore, by comparing among XX and XY females and XX and XY males the effect of sex chromosome complement can be uncoupled from the influences of sex hormone and studied independently. One interesting study in the future should utilize the 4 core genotype and compare XX and XY females to determine if neonatal testosterone administration induces the same extent of AngII-induced vascular pathologies between XX and XY females. It will allow us to dissect out the possible effect of sex chromosomes in the different responses to neonatal androgen between the genders.
5.4 Clinical application

5.4.1 AAA prevention

In agreement to observations in human AAAs, our studies in experimental AngII-induced AAAs further confirm male gender is a potent risk factor for AAA pathogenesis. Moreover, our results suggest that male sex hormone, namely testosterone, is a primary contributor to AAA formation and progression. In men, androgen replacement therapy has been used to treat a variety of symptoms associated with aging, including loss of energy, mood depression and erectile dysfunction. Women also use exogenous androgen administration as part of hormone replacement therapy to improve performance. Our data demonstrate that androgen promotes AngII-induced AAA formation in both male and female mice via upregulation of AT1aR. A significant increased risk of developing AAA may be identified in the aging men and women who take exogenous androgen as hormone replacement therapy as well as male and female athletes/bodybuilders who abuse anabolic hormones. These populations with a potentially high AAA risk should exercise precaution in using androgen as hormone replacement therapy, look for other alternative therapy options and undergo regular ultrasound monitoring to effective prevent AAA formation.

Although in general female gender is considered as a negative risk factor for AAAs, the consequences tend to be more vicious once AAAs are formed in women. For example, The UK Small Aneurysm trial reported that in patients kept under surveillance, a 3-times higher risk of aneurysm rupture for women was found even at a smaller diameter than men (Brown and Powell 1999). Women
also tend to have a lower survival rate after the surgical repair of AAAs (Dueck, Kucey et al. 2004). In addition, multiple studies have demonstrated a faster growth rate of AAAs in women compared with men (Solberg, Singh et al. 2005; Schouten, van Laanen et al. 2006; Mofidi, Goldie et al. 2007). Therefore, it is important to identify the particular female population that exhibits high risk of developing AAAs. In addition, our studies demonstrate that a short-term androgen exposure during a critical period of development dramatically predisposes to AAA in females when they become adults. In humans, women suffering from PCOS experience high circulating androgen levels after puberty. It has been reported that plasma androgen levels are higher in women with PCOS during pregnancy (Falbo, Rocca et al. 2010). Based on 6.6% estimated prevalence of PCOS in reproductive aged women, there are at least 4 million women with this disease in the United States. The female offspring of these PCOS women may become more susceptible to AAA and possibly other vascular pathologies in adult life due to the impact of excess androgen exposure during fetal development. It will be beneficial to raise the awareness of the potential high risk of AAA in this particular female population.

5.4.2 AAA treatment

Currently there is no effective pharmacologic treatment to slow AAA progression and reduce the chance of aneysymal ruptures. Recently the drugs inhibiting the RAS, such as ACE inhibitors and AT1 receptor blockers, have been emerged as potential treatments for this disease due to the positively protective
effects against AAA formation observed in experimental models of AAA (Daugherty, Manning et al. 2001; Liao, Miralles et al. 2001; Sweeting, Thompson et al. 2010). Despite the promising findings in animal models, the effects of RAS inhibitors on human AAA remains controversial and requires more clinical studies to confirm the findings. For example, ACE inhibitor has been associated with reduced AAA ruptures in a retrospective study (Hackam, Thiruchelvam et al. 2006), while in a different study the use of ACE inhibitors was recently associated with increased growth rate of AAAs in human patients (Sweeting, Thompson et al. 2010). In addition, preoperative RAS inhibition before AAA surgical repair has been reported to increase mortality in AAA patients (Railton, Wolpin et al. 2010). However, in a recent study involving 25 years of surveillance, treatment with an angiotensin receptor antagonist was reported to be associated with slower AAA growth rates (Thompson, Cooper et al. 2010). Our data demonstrate that androgen both in adult life and during development promotes AngII-induced AAA formation through stimulating SMC AT1aR expression of the abdominal aortas in female mice. Our findings support an important role of AT1 receptors in the pathogenesis of AAAs and indicate ARB may be beneficial as a treatment of human AAA.

5.5 Concluding remarks

In conclusion, findings of this dissertation demonstrated that androgen, both during development and in adult life, mediates AngII-induced AAA formation in adult hyperlipidemic mice. These findings implicate increased expression of
AT1a receptor specifically in abdominal aortas as a potential mechanism underlying androgen as a primary mediator of high AAA prevalence in male gender. In addition, endogenous androgen modulates the lumen expansion of established AngII-induced AAAs. These findings support AT1 receptor blocker as a potential option to treat AAA and also raise the possibility of androgen deprivation as a pharmacologic therapeutic option to limit AAA progression and ruptures for patients diagnosed with AAA.


proteinases with matrix destruction and inflammatory cell response."


Henriques, T., X. Zhang, et al. (2008). "Androgen increases AT1a receptor expression in abdominal aortas to promote angiotensin II-induced AAAs in..."


predominant role of angiotensin III in the control of vasopressin release."

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