DISSERTATION

DIHYDROTESTOSTERONE ATTENUATES ENDOTOXIN, CYTOKINE, AND HYPOXIA-INDUCED VASCULAR INFLAMMATION

Submitted by

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ABSTRACT

DIHYDROTESTOSTERONE ATTENUATES ENDOTOXIN, CYTOKINE, AND HYPOXIA-INDUCED VASCULAR INFLAMMATION

Vascular inflammation plays a key role in the etiology of cardiovascular disease, particularly stoke. Vascular inflammation is under the control of several transcription factors, including nuclear factor kappa B and hypoxia inducible factor-1 alpha (HIF-1α). Activation of these transcription factors can lead to the production of inflammatory mediators such as cyclooxygenase-2 (COX-2). COX-2 plays a role in vascular inflammation, cerebral ischemia-induced injury, and has been implicated as a source of reactive oxygen species (ROS). Inflammatory mediators, such as endotoxin or cellular breakdown products released following injury, are known to signal through the Toll-like receptor 4 (TLR4). TLR4 activation leads to NFκB activation and subsequent production of COX-2. Like COX-2, TLR4 has also been implicated in injury-induced oxidative stress and cerebral ischemia damage.

Previous studies have demonstrated that gonadal steroid hormones can also modulate vascular inflammation. Both protective and detrimental effects of androgens on the cardiovascular system have been reported. Since the potent androgen receptor (AR) agonist dihydrotestosterone (DHT) can be converted to 3β-diol, an estrogen receptor (ER) β-selective agonist, I hypothesized that ERβ may

mediate some of the protective effects of androgens, while the AR may mediate some of the detrimental effects.

The overall goal of this dissertation was to determine the mechanisms by which androgens can influence the vascular inflammatory response under both physiological and pathophysiological conditions. The hypothesis to be tested was that DHT influences vascular inflammation under both physiological and pathophysiological conditions.

In my first set of experiments, using Western blot, I found that DHT increases expression of the vascular inflammatory mediator COX-2 under physiological conditions in human coronary artery vascular smooth muscle (VSM) cells and human brain VSM cells. This effect of DHT was attenuated in the presence of the AR antagonist bicalutamide. This data indicates that the pro-inflammatory effect of DHT under normal physiological conditions is AR mediated.

In my second set of experiments, I examined the effects of DHT on vascular inflammation under a variety of pathophysiological conditions. Surprisingly, I found that DHT decreased cytokine-induced COX-2 expression and oxidative stress, endotoxin-induced COX-2 and TLR4 expression in human VSM cells. Furthermore, DHT also decreased hypoxia induced HIF-1α and COX-2 expression in human brain VSM cells and rat pial arteries. Finally, I found that DHT decreased hypoxia with glucose deprivation (HGD)-induced HIF-1α, COX-2 and TLR4 expression in human brain VSM cells. DHT‘s anti-inflammatory effects during cytokine or HGD-induced inflammation in human brain VSM cells were not blocked by the AR antagonist

bicalutamide, indicating that they were not AR mediated. These results led me to my second hypothesis, that DHT's anti-inflammatory effects are ERβ-mediated.

In my third set of experiments, I found that the DHT metabolite/ERβ selective agonist 3β-diol also decreased cytokine-induced COX-2 expression in human brain VSM cells. Furthermore, DHT's ability to reduce cytokine-induced COX-2 expression in human brain VSM cells was inhibited by the non-selective estrogen receptor antagonist ICI 182,780 and the selective ERβ antagonist PHTPP. The mRNAs for steroid metabolizing enzymes in the pathway necessary to convert DHT to 3β-diol were detected in human brain VSM cells, as were AR and ERβ mRNAs. Therefore, DHT appears to be protective against cerebrovascular inflammation via conversion to 3β-diol and subsequent activation of ERβ in human brain VSM cells.

The results of these studies indicate that: 1) DHT increases COX-2 expression under unstimulated/physiological conditions via an AR-dependent mechanism. 2) DHT decreases cytokine-, endotoxin,-hypoxia, and HGD-induced COX-2 expression via an AR-independent mechanism. 3) DHT decreases cytokine-induced reactive oxygen species. 4) DHT decreases hypoxia-induced HIF-1α expression. 5) DHT decreases HIF-1α and TLR4 expression during HGD via an AR-independent mechanism. 6) DHT's effect to attenuate cytokine-induced COX-2 expression is ERβ-mediated.

LAY RESEARCH SUMMARY

Sex differences in heart disease and stroke remain a topic of discussion both clinically and at the bench. A major contributor for the formation and progression of heart disease and stroke is inflammation of the blood vessel wall. Many studies have shown that sex hormones such as androgens and estrogens can have a significant impact on inflammatory pathways in the blood vessel wall. However, understanding “how” these hormones regulate inflammatory signaling molecules my assist in development of new prevention and treatment strategies for heart disease and stroke in both men and women. Thus, the goal of my studies was to investigate the influence of a specific androgen, dihydrotestosterone (DHT), on blood vessel wall inflammation using a human cell culture model. I found that during inflammation, DHT decreases expression of a pro-inflammatory enzyme known as the cyclooxygenase 2 (COX-2) and decreased expression of a key receptor in the innate immune response known as toll-like receptor 4 (TLR4). DHT also reduced oxidative stress and activation of a pro-inflammatory transcription factor (a protein that turns on other genes), which gets activated during ischemic conditions like stroke, known as hypoxia-inducible factor 1 alpha. Surprisingly, these anti-inflammatory effects of DHT (an androgenic compound) were found to be acting through a novel signaling pathway involving an estrogen receptor subtype known as estrogen receptor beta (ERβ). Molecular studies have demonstrated that blood

vessels from both women and men express ERβ beta and that activation of this receptor may affect blood vessel function in both the heart and brain. I believe that due to DHT's anti-inflammatory and anti-oxidant effects that I have observed in the vasculature, that low-dose androgen therapy or ERβ agonists may be beneficial in aging, androgen deficient men with atherosclerosis to prevent rupture of plaques by reducing oxidative stress and inflammation. In addition, these protective effects of DHT/ERβ agonist treatment may also be beneficial in reducing damage following stroke. This preventative hormone therapy could potentially reduce the frequency and severity of ischemic events in aging men.

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To Damian

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CHAPTER 1: INTRODUCTION

Cardiovascular diseases (coronary heart disease, heart failure, stroke, and hypertension) are the leading cause of death in humans, with approximately 2,200 Americans dying each day. Of these, stroke specifically is the third leading cause of mortality, which accounts for approximately 795,000 deaths a year in the U.S. alone (V. L. Roger et al., 2011). Ischemic strokes account for approximately 85% of all strokes, with hemorrhagic strokes accounting for the other 15% (J. Bamford et al., 1990). Epidemiological data show that cardiovascular diseases, particularly stroke, are more prevalent in men than in pre-menopausal women, yet the severity of outcome is greater in women (R. E. Petrea et al., 2009; M. Writing Group et al., 2009). Experimental studies provide evidence that these sex differences may be attributed to endogenous gonadal steroids. In experimental models of stroke, estradiol has been consistently shown to decrease infarct size, while androgens have been shown to decrease infarct size at low doses, but increase damage at high doses (T. Hawk et al., 1998; M. Uchida et al., 2009). It is possible that sex differences in stroke incidence and ischemic outcome are due to gonadal steroid regulation of vascular inflammation. The goal of this dissertation is to understand the role androgens have on regulating vascular inflammation associated with vascular events, particularly ischemic stroke since vascular inflammation plays such a large role in secondary brain injury in this type of stroke. Investigating how androgens

modulate vascular inflammation and oxidative stress may assist in the development of therapeutic strategies to circumvent pathological events associated with myocardial or cerebral ischemia.

The cerebral vasculature plays a central role in the pathogenesis of cardiovascular diseases, such as stroke (G. J. del Zoppo and T. Mabuchi, 2003), and in the initiation of inflammation after cerebral ischemia which is a key determinant in stroke outcome (H. C. A. Emsley and P. J. Tyrrell, 2002; G. J. del Zoppo and T. Mabuchi, 2003). Vascular inflammation reduces the stability of atherosclerotic plaques (S. J. Warner et al., 1989; P. Saren et al., 1996; S. J. George, 1998), thus increasing the probability of a thrombotic event such as myocardial infarction or stroke. Vascular inflammation is particularly important in the pathology of heart attack and stroke since ischemia evokes a strong inflammatory response characterized by activation and release of cytokines, chemokines, adhesion molecules, and proteolytic enzymes that exacerbate tissue damage (G. J. del Zoppo and T. Mabuchi, 2003; M. Nedergaard et al., 2003) (Figure 1.1). Following ischemia, inflammation is initiated by cytokine-induced activation of transcription factors such as nuclear factor kappa B (NFκB) and hypoxia-inducible factor 1- alpha (HIF-1α), leading to increases production of pro-inflammatory mediators (Q. Wang et al., 2007; A. Brooks et al., 2010). HIF-1α plays a particularly important role in cerebral ischemia because it is activated both by cytokines as a result of inflammation, and by low oxygen levels as a result of reduced blood flow (T. Hellwig-Burgel et al., 2005).

The transcription factor NFκB, which plays a key role in regulation of inflammation during ischemic and non-ischemic events, can be activated through the endotoxin signaling receptor, Toll-like receptor (TLR) or interleukin-1 receptor (IL-1R). The presence of one TLR member, TLR4, in smooth muscle (J. Oyama et al., 2004; L. L. Stoll et al., 2004) and endothelium (J. Pugin et al., 1993; E. Faure et al., 2000; S. M. Dauphinee and A. Karsan, 2006) suggests a role for TLR4 in vascular disease. TLR4 stimulation induces expression of a variety of pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules and pro-inflammatory enzymes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), via activation of NFκB (S. M. Dauphinee and A. Karsan, 2006). These increases in COX-2 and iNOS lead to increased oxidative stress due to the peroxidase activity of COX-2 and peroxinitrite formation during NO production. Oxidative stress is a major contributor to the progression of atherosclerosis (G. Zalba et al., 2007). TLR4 signaling can also cause detrimental effects in the absence of endotoxin. For example, TLR4 mRNA and protein significantly increase after intracerebral hemorrhage or cerebral ischemia in rodents (L. W. Weiyu Teng, Weishuang Xue, and Chao Guan, 2009; K. Hyakkoku et al., 2010), and these increases in TLR4 correlate with increased damage. Taken together, these studies suggest that TLR4 may be a critical regulator of inflammation and oxidative stress both during endotoxin-induced inflammation and during ischemic injury.

Because chronic vascular inflammation contributes significantly in the pathogenesis of cardiovascular diseases (S. J. Warner et al., 1989; P. Saren et al., 1996; S. J. George, 1998), it is important to investigate the role of androgens under

both normal and pathophysiological conditions. Both androgens and estrogen are generally credited with anti-inflammatory effects, although some pro-inflammatory effects have been reported for androgens (J. A. McCrohon et al., 1999; A. K. Death et al., 2004; R. J. Gonzales et al., 2009). Protective effects described for androgens include both anti-inflammatory and anti-oxidant actions. Anti-inflammatory effects of androgens have been demonstrated in both *in vivo* and *in vitro*. In androgen deficient men, testosterone replacement decreases cytokine levels (C. J. Malkin et al., 2004; S. Y. Kalinchenko et al., 2010a). In tumor necrosis factor alpha-stimulated human umbilical vein endothelial cells, dihydrotestosterone (DHT), a potent endogenous androgen receptor agonist, has been shown to decrease a variety of inflammatory proteins including COX-2 and TLR4. In addition, the adrenal androgen, dehydroepiandrosterone (DHEA), which is a weak androgen but also acts as a precursor to other androgens, has been shown to decrease HIF-1α accumulation during hypoxia in human pulmonary smooth muscle cells (A. Dessouroux et al., 2008). Androgens, and DHT in particular, are reported to be potent anti-oxidants both *in vitro* and *in vivo*. In mouse embryonic stem cells, hydrogen peroxide-induced reactive oxygen species (ROS) generation, lipid peroxide formation, and DNA fragmentation were inhibited by pretreatment with DHT (M. N. Lee et al., 2008). Castration has also been shown to induce oxidative stress in rat prostate epithelium, while androgen replacement partially reversed this oxidative stress in castrates (N. N. Tam et al., 2003). These studies suggest that androgens can be protective by reducing inflammation and oxidative stress. Although much of the work regarding androgens and vascular inflammation has

been conducted in endothelial cells (J. A. McCrohon et al., 1999; H. Hatakeyama et al., 2002; T. K. Mukherjee et al., 2002; A. K. Death et al., 2004; G. D. Norata et al., 2006), there are few reports on the effects of androgens on inflammation in vascular smooth muscle (VSM) (D. Somjen et al., 2009).

Investigating the effects of androgens on vascular inflammation is complicated because there are several potential receptor pathways that androgens can utilize to alter gene transcription. Using the classically described mechanism, testosterone can activate the androgen receptor (AR) directly or following its conversion by 5 alpha-reductase to the more potent androgen, DHT (R. J. Handa et al., 1987; E. D. Lephart et al., 2001). Alternatively, testosterone can be metabolized to estradiol by the aromatase enzyme (C. E. Roselli et al., 1985) and subsequently activate estrogen receptor alpha (ERα) or beta (ERβ) (G. G. Kuiper et al., 1997; G. G. Kuiper et al., 1998). A third and less well explored pathway for androgen action is through the conversion of DHT to 5α-androstane-3β,17β-diol (3β-diol), an ERβ agonist (G. G. Kuiper et al., 1996; G. G. Kuiper et al., 1997; Z. Weihua et al., 2002), by the enzymes 3beta-hydroxysteroid dehydrogenase (3β-HSD), 3alpha-hydroxysteroid dehydrogenase (3α-HSD) and 17beta-hydroxysteroid dehydrogenase (17β-HSD)(Y. Jin and T. M. Penning, 2001; Z. Weihua et al., 2002; A. Gangloff et al., 2003; S. Torn et al., 2003; S. Steckelbroeck et al., 2004). Blood vessels contain AR, ERα, ERβ, 5α-R, aromatase, and 3β-HSD (T. C. Register and M. R. Adams, 1998; G. D. Snyder et al., 2002; Y. Nakamura et al., 2005; R. J. Gonzales et al., 2007; H.-C. Shih et al., 2008) and the presence of these enzymes and receptors allows for

potential androgenic and estrogenic effects which can influence vascular inflammation.

Due to the lack of information regarding androgen's effects on inflammation of the vasculature, particularly in vascular smooth muscle cells, the goal of these studies was to determine if the potent androgen DHT could modulate vascular inflammation in human VSM cells. Due to the importance of vascular inflammation during ischemic events, such as heart attack and stroke, I chose human coronary artery and brain VSM cells as my model systems. In some cases, responses in these human cells were compared to inflammatory responses in rat arteries or human brain endothelial cells. For the majority of these studies, I chose COX-2 as a marker of interest for vascular inflammation because of the significant contribution COX-2 has on the inflammatory response in vascular tissue (C. Tsatsanis et al., 2006) and because COX-2 has been implicated in vascular diseases such as cerebral ischemia (K. Sugimoto and C. Iadecola, 2003).

Chapter 1: In the current chapter, I introduce the importance of vascular inflammation in cardiovascular disease and the role androgens may play in the etiology and outcome of ischemia.

Chapter 2: In this chapter, I review evidence for the role of gonadal steroids in vascular reactivity, cardiovascular disease, vascular inflammation, and oxidative stress.

Chapter 3: In this chapter I explore the role of the potent androgen, DHT, in regulating COX-2 expression during both normal physiological conditions and during cytokine or endotoxin-induced inflammation. A comparison is made between

responses to DHT in primary human coronary artery VSM cells and brain endothelial cells.

Chapter 4: Using two model systems, primary human brain VSM cells and isolated rat pial vessels, I explore the role of DHT on the vascular inflammatory response during hypoxia or hypoxia with glucose deprivation. In these studies, hypoxia with glucose deprivation is used as a model for some of the cellular insults that occur during ischemia (reduced oxygen and nutrient availability).

Chapter 5: TLR4 is implicated in oxidative stress associated with pathogenic endotoxin-induced inflammation and, more recently, implicated in injury-induced inflammation in the absence of endotoxin (T. V. Arumugam et al., 2009). In light of this evidence, I explore the role of DHT treatment in regulating TLR4 expression and oxidative stress following endotoxin or hypoxia with glucose deprivation in primary human coronary and brain VSM cells.

Chapter 6: Since I demonstrate an AR-independent effect of DHT on inflammatory markers, and because 3β-diol has recently been shown to reduce expression of these markers in human endothelial cells (G. D. Norata et al., 2010), I test the hypothesis that during cytokine-induced inflammation, DHT decreases the pro-inflammatory mediator COX-2 via metabolism to 3β-diol and subsequent activation of ERβ in human brain VSM cells.

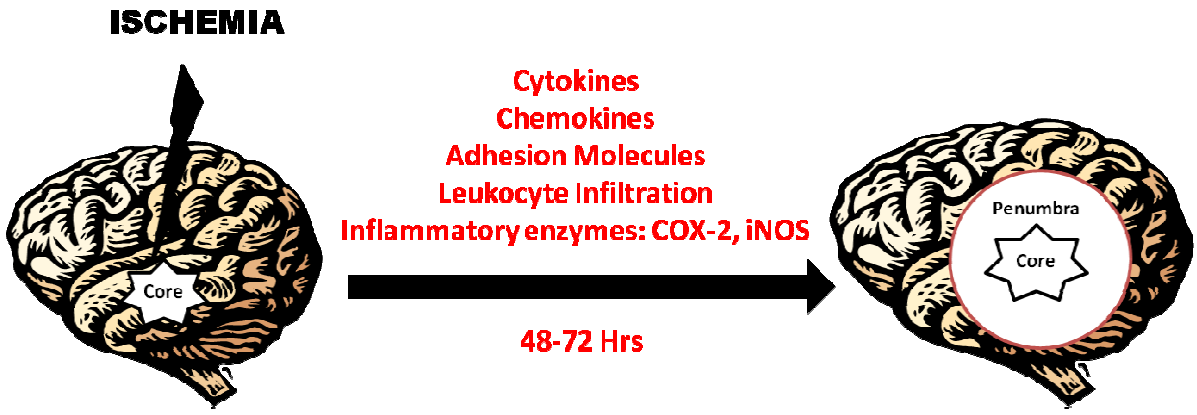


Figure 1.1. ***Inflammation contributes to secondary brain injury following cerebral ischemia.*** Core, ischemic core of dead tissue immediately following cerebral ischemia. Penumbra, expanded area of dead tissue, due to inflammation, that develops over several days.

CHAPTER 2: LITERATURE REVIEW

This review will focus on the effects of androgens in the vasculature, with an emphasis on vascular inflammation, especially as it pertains to stroke and other cardiovascular diseases. Since testosterone can be converted to estradiol and dihydrotestosterone can be converted to an estrogen receptor beta agonist, the role of estrogens and estrogen receptor beta in particular in the vasculature will also be discussed.

GONADAL STEROID HORMONES

History

In 1889, the neuroendocrinologist Charles Edward Brown-Sequard found that testicular extracts increased muscle strength, vigor and an overall sense of well-being (C. E. Brown-Sequard, 1889). In 1935, Leopold Ruzicka and Butenandt became the first to both identify the structure of testosterone and to partially synthesize testosterone from cholesterol (A. Butenandt and G. Hanisch, 1935b, a; L. Ruzicka and A. Wttstein, 1935). As a result, both Butenandt and Ruzicka received the 1939 Nobel Prize in chemistry for the synthesis of testosterone. In the 1950's and 1960's the gonadal steroid receptors were identified. Elwood Jensen discovered "estrogen binding components" later termed estrogen receptors in rat uterus (E. V. Jensen, 1962). Gerald Mueller showed that estrogen treatment

induced both RNA and protein synthesis and that uterine responses to estradiol could be blocked by inhibiting protein synthesis or RNA, showing that estradiol's effects were mediated by changes in transcription and translation (S. A. Fannon et al., 2001). In the 1970's, androgen receptor, estrogen receptor alpha, and progesterone receptor were purified (W. I. Mainwaring and R. Irving, 1970; S. A. Fannon et al., 2001); however, estrogen receptor beta was not discovered until 1996 (S. Mosselman et al., 1996).

Synthesis & Metabolism

Gonadal steroids are primarily produced by the testis, ovaries, and adrenal gland, but they are also synthesized locally in a variety of cells, including vascular smooth muscle (VSM) and endothelial cells (for reviews of steroidogenesis please see (W. L. Miller, 1988; H. J. Hsu et al., 2006)). All steroids are derived from cholesterol (Figure 2.1) and all contain the same cyclopentanophenanthrene ring as cholesterol. Gonadal steroids are divided into three main classes: androgens, estrogens, and progestins. Androgens include dehydroepiandrosterone (DHEA), androstenedione, testosterone (T), and dihydrotestosterone (DHT). Estrogens include estradiol (E), estrone, and estriol. Progestins include prenenolone, from which all other steroid hormones are derived, and progesterone.

Using the classically described mechanism, testosterone can activate the androgen receptor (AR) directly or following its conversion by 5 alpha-reductase to the more potent androgen, dihydrotestosterone (DHT) (R. J. Handa et al., 1987; E. D. Lephart et al., 2001). Alternatively, testosterone can be metabolized to estradiol

by the aromatase enzyme (C. E. Roselli et al., 1985) and subsequently activate estrogen receptor alpha (ERα) or beta (ERβ) (G. G. Kuiper et al., 1997; G. G. Kuiper et al., 1998). A final and less well explored pathway for androgen action is through the conversion of DHT to 5α-androstane-3β,17β-diol (3β-diol), an ERβ agonist (G. G. Kuiper et al., 1996; G. G. Kuiper et al., 1997; Z. Weihua et al., 2002), by the enzymes 3beta-hydroxysteroid dehydrogenase (3β-HSD), 3alpha- hydroxysteroid dehydrogenase (3α-HSD) and 17beta-hydroxysteroid dehydrogenase (17β-HSD) (Y. Jin and T. M. Penning, 2001; Z. Weihua et al., 2002; A. Gangloff et al., 2003; S. Torn et al., 2003; S. Steckelbroeck et al., 2004). The enzyme CYP7B1 can convert 3β-diol to its inactive metabolites 3alpha-triols and 7alpha-triols (M. Sundin et al., 1987; Z. Weihua et al., 2002).

Regulation of Synthesis

Gonadal steroid hormone production is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (for overview see (S. M. Breedlove et al., 2007)). In the hypothalamus, gonadotropin releasing hormone (GnRH) neurons project to the median eminence and secrete GnRH into the pypophyseal portal system. GnRH stimulates the anterior pituitary to release both luteinizing hormone (LH) and follicle stimulating hormone (FSH) into circulation. LH and FSH then stimulate the testis and ovaries to produce estradiol and testosterone. Testosterone is produced by leydig cells of the testis and to a lesser degree in both the ovary and adrenal cortex. Estradiol is produced by granulose cells of the ovary, sertoli cells of the testis, and the adrenal gland. In addition to gonadal/adrenal production, local *de novo*

synthesis of gonadal steroids occurs in many tissues, as previously mentioned. Testosterone and estradiol provide negative feedback to the anterior pituitary and hypothalamus so that when androgen/estrogen levels are high production is reduced. Gonadal hormone levels are generally low in childhood, but increase drastically after puberty. In men, T levels peak in early adulthood, then gradually decline with age (H. A. Feldman et al., 2002). In women, E levels are low in childhood, but after puberty levels increase, although they fluctuate during the reproductive cycle. E levels are highest during proestrus when the follicle is maturing. E levels fall drastically after menopause, since the ovaries no longer produce estradiol, unlike the gradual decline in T in men (E. Radwanska, 1993).

Localization of Gonadal Steroid Receptors and Steroid Metabolizing Enzymes in the Vasculature

Gonadal steroid receptors and some of the steroid metabolizing enzymes are expressed in the vasculature. ERα, ERβ, and GPR30 mRNA and protein are expressed in both male and female rat aortic VSM cells (Y. Ma et al., 2010). ERα, AR, and 5α-reductase protein is also expressed in both the smooth muscle and endothelial layer of rat cerebral arteries, and aromatase is also found in the endothelial cell layer of rat cerebral arteries (R. J. Gonzales et al., 2007). In addition to ERα, another study also found that ERβ mRNA and protein were expressed in male rat cerebral arteries (H.-C. Shih et al., 2008). ERα and ERβ mRNA are also expressed in male and female cynomolgus monkey coronary arteries and cultured aortic smooth muscle cells (T. C. Register and M. R. Adams, 1998). Expression of

ERα and ERβ has also been confirmed in human aorta. ERα and ERβ mRNA is found in human female aorta, furthermore, immunoreactivity for both receptors is found in the smooth muscle layer (Y. Nakamura et al., 2004). Expression of the enzyme 3β-HSD mRNA is also found in human aorta, with immunoreactivity showing localization of protein in VSM cells (Y. Nakamura et al., 2004). Aromatase activity has been reported in rat aortic VSM cells (G. D. Snyder et al., 2002). The presence of these enzymes and receptors allows for potential androgenic and estrogenic effects which can influence vascular inflammation.

ANDROGEN RECEPTORS

Structure and Genomic Signaling

Androgen receptors are ligand activated transcription factors (for reviews please see (D. J. Mangelsdorf et al., 1995; N. C. Bennett et al., 2010)). Unbound androgen receptors are located mostly in the cytoplasm of the cell and then translocate to the nucleus after activation (R. K. Tyagi et al., 2000). All of the gonadal steroid receptors have similar protein structures that consist of an N-terminal regulatory domain called the trans-activation domain, a ligand binding domain, a DNA binding domain, and a hinge region that contains the nuclear localization signal. The binding of a sex steroid to its receptor results in a conformational change in the receptor, dissociation of heat shock proteins, exposure of the nuclear localization signal, transport from the cytosol into the cell nucleus, and dimerization. The sex steroid receptor dimer then binds to a specific sequence of DNA known as the hormone response element and alters gene transcription (Figure

2.2). For androgens, this hormone response element is known as the androgen response element (ARE).

Two androgen receptor isoforms have been identified in humans. AR isoform 1, known as AR, is the full-length androgen receptor with a molecular weight of approximately 110 kDa. AR isoform 2, known as AR45, is a truncated form that results in a protein that is approximately 45 kDa in size that has a functional ligand binding domain, hinge region, and DNA binding domain but lacks most of the N-terminal regulatory domain which impairs its ability to recruit the necessary co-regulator molecules for transcriptional activity. AR45 inhibits transcriptional activity of AR by interacting with the N-terminal domain of AR (I. Ahrens-Fath et al., 2005). Additionally, at least 7 splice variants of AR have been identified that lack the ligand binding domain, and thus activate transcription of target genes in the absence of ligand (P. A. Watson et al., 2010).

Non-genomic Signaling

A variety of non-genomic actions of androgens have also been reported including gap junction communication, vascular relaxation, and neuronal plasticity (C. A. Heinlein and C. Chang, 2002; C. D. Foradori et al., 2008; N. C. Bennett et al., 2010). Some of the reported non-genomic mechanisms of androgen's actions are depicted in Figure 2.3. For example, DHT can activate the sex hormone binding globulin receptor to increase cyclic AMP and protein kinase A (N. Fortunati et al., 1996; A. M. Nakhla and W. Rosner, 1996). DHT can also increase intracellular calcium via an unidentified membrane-bound receptor (W. P. Benten et al., 1999b;

W. P. Benten et al., 1999a). Furthermore, several authors have also hypothesized that a membrane bound AR may exist (J. W. Gatson et al., 2006) that is not blocked by classical AR antagonists (E. F. Konoplya and E. H. Popoff, 1992; W. P. Benten et al., 1999b; W. P. Benten et al., 1999a; C. A. Heinlein and C. Chang, 2002). For example, membrane-bound T receptors, not blocked by flutamide, were found in VSM cells from aorta of rats (R. Ma et al., 2010). Below in the "Androgens & Vascular Reactivity" section I will review the evidence for androgen mediated, non-genomic vascular relaxation.

ESTROGEN RECEPTORS

Structure and Genomic Signaling

Estrogen receptors, like androgen receptors, are ligand activated transcription factors that consist of an N-terminal regulatory trans-activation domain, a ligand binding domain, a DNA binding domain, and a hinge region that contains the nuclear localization signal (D. J. Mangelsdorf et al., 1995). Following ligand binding, translocation to the nucleus and dimerization, estrogen receptors bind to estrogen response elements (EREs) on target genes to alter transcription (Figure 2.2). Two isoforms of nuclear estrogen receptors have been described, ERα and ERβ. Additionally, at least five splice variants have also been described for ERβ (M. J. Weiser et al., 2008). Although the ligand binding domains for ERα and ERβ are similar, their affinities for ligands differ. For example, propylpyrazoletriol (PTT) is 410-fold more selective for ERα than ERβ and thus is a commonly used selective

ERα agonist. Diarylpropionitrile (DPN) is an ERβ agonist with 72-fold greater affinity for ERβ than ERα (S. R. Stauffer et al., 2000; M. J. Meyers et al., 2001).

Non-genomic Signaling

A membrane-bound G protein coupled estrogen receptor, GPR30, has recently been discovered (Y. Mizukami, 2010). This receptor is thought to mediate some of the non-genomic actions of estrogens, although ERα and ERβ also mediate many of the non-genomic actions of estrogens. Some of the non-genomic actions reported for estrogens include vasodilation, decreases in intracellular calcium, increases in cAMP, activation of the mitogen activated protein kinase pathway, increases in neuronal excitability via gamma amino butyric acid (GABA) inhibition, and rapid activation of the neuroprotective phosphatidylinositol-3 kinase/Akt pathway(B. M. Schmidt et al., 2000; N. Vasudevan and D. W. Pfaff, 2008). Below in the "Estrogens and Vascular Reactivity" section I will review the evidence for estrogen receptor mediated, non-genomic vascular relaxation.

VASCULAR STRUCTURE AND REACTIVITY

Vascular Structure

The circulatory system is made up of a network of blood vessels that carry oxygen and nutrients to all tissues within the body. There are five major types of blood vessels: arteries, arterioles, capillaries, venules, and veins. Arteries and arterioles carry oxygen and nutrient rich blood from the heart to the rest of the body, capillaries are the site of nutrient and oxygen exchange, and veins and venules

return the oxygen and nutrient poor blood back to the heart. Arteries and veins consist of three layers: the intimal layer that contains endothelial cells, the medial layer that contains VSM cells and the outer advential layer that consists of connective tissue and nerves (Figure 2.4). The capillaries are much smaller and consist of endothelial cells with some surrounding connective tissue. Endothelial cells line the inner lumen of all blood vessels in a monolayer, while VSM cells make up the contractile layer of the non-cappillary blood vessels that is responsible for changes in luminal diameter. Endothelial cells respond to changes in blood flow and send signals to smooth muscle cells to contract or relax. Endothelial cells release at least three types of vasodilators onto smooth muscle cells: nitric oxide (NO), cyclooxygenase-2 (COX-2) derived prostanoids, and endothelium derived hyperpolarizing factor (EDHF). Endothelial cells can also release the potent vasoconstrictor endothelin-1. The endothelial cells in the brain also function as the blood brain barrier. For a review on vascular physiology see (M. Tennant and J. K. McGeachie, 1990; M. K. Pugsley and R. Tabrizchi, 2000).

Vascular Reactivity

Vasoconstriction, defined as the narrowing of the vessel lumen, is caused by an increase in intracellular calcium concentration within VSM cells (Figure 2.5). Calcium binds to calmodulin, which then activates myosin light chain kinase (MLCK). MLCK then phosphorylates the light chain of myosin and initiates cross bridge cycling, resulting in smooth muscle contraction and narrowing of the lumen. Phenylephrine (PE) is commonly used to elicit smooth muscle cell contraction. PE

activates alpha-1 adrenergic receptors on VSM cell membranes. Alpha-1 adrenergic receptors are G protein coupled receptors (GPCR), that when activated cause the G protein G_q to activate phospholipase C (PLC). PLC then cleaves phosphatidylinositol 4,5 bisphosphate (PIP_2) producing inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol. IP_3 then binds to the IP_3 receptor on the sarcoplasmic reticulum causing calcium release from internal stores. This calcium release into the cytoplasm causes the intracellular calcium concentration to rise enough to stimulate contraction. Acetylcholine (ACh) is commonly used to dilate blood vessels through activation of the M3 muscarinic GPCR receptor on endothelial cells. Activation of M3 causes activation of G_q leading to an increase in intracellular calcium within the endothelial cell. This increase in calcium activates endothelial nitric oxide synthase (eNOS), stimulating NO production, leading to VSM cell relaxation via soluble guanylate cyclase activation (Figure 2.5). However, if the endothelial cells are not present or damaged due to pathology, then ACh will activate M3 receptors on the smooth muscle cells and cause intracellular calcium release and smooth muscle contraction. For this reason, ACh is commonly used to test for the presence of functional endothelial cells in vessel reactivity studies.

Vasodilation, defined as widening of the vessel lumen, is caused by either a decrease in intracellular calcium concentration or dephosphorylation of myosin light chain. This occurs mainly through three mechanisms: hyperpolarization, increases in cyclic guanosine monophosphate (cGMP), or increases in cyclic adenosine monophosphate (cAMP). As previously mentioned, endothelial cells can cause vasodilation by releasing NO, prostacyclin, or EDHF. NO causes cGMP mediated-

vasodilation by stimulating soluble guanylate cyclase to produce cGMP. cGMP activates protein kinase G, which phosphorylates myosin light chain phosphatase, causing inactivation of MLCK, dephosphorylation of myosin light chain, and smooth muscle relaxation (Figure 2.5). Prostaglandins (prostacyclin, prostaglandin E_2, prostaglandin D_2) cause cAMP-mediated vasodilation by activation of their GPCRs, which stimulates adenylate cyclase to produce cAMP, which stimulates protein kinase A. Protein kinase A, like protein kinase G, inhibits MLCK leading to smooth muscle relaxation. EDHF causes vasodilation by hyperpolarization, although the exact identity and receptor for EDHF is unknown, the mechanism of action involves opening of potassium channels, which causes closing of voltage-gated calcium channels (VGCC). Closing of VGCC causes intracellular calcium levels to decrease, leading to smooth muscle relaxation. For reviews on vascular reactivity please see (C. M. Rembold, 1992; A. Horowitz et al., 1996; R. D. Jones et al., 2004; J. M. Orshal and R. A. Khalil, 2004; D. N. Krause et al., 2006).

Androgens & Vascular Reactivity

Acute androgen treatment has been shown to cause vascular relaxation in a variety of vascular beds. Whether these actions are endothelium-dependent or endothelium-independent is debated, but most studies agree that this effect is AR-independent (P. Yue et al., 1995; C. E. Costarella et al., 1996; P. Tep-areenan et al., 2002; R. D. Jones et al., 2003; J. Navarro-Dorado et al., 2008). Endothelium-dependent relaxation in response to androgens has been reported in rabbit carotid arteries (V. G. Marrachelli et al., 2010) and rat mesenteric arteries (P. Tep-areenan

et al., 2002), while endothelium-independent relaxation in response to androgens had been reported in porcine coronary and prostatic arteries (V. P. Deenadayalu et al., 2001; J. Navarro-Dorado et al., 2008). It is thought that androgens are acting via large conductance calcium activated potassium channels (BK_{Ca}), because BK_{Ca} channel inhibition or non-selective potassium channel inhibition with barium chloride greatly reduces the ability of androgens to relax coronary or mesenteric arteries (P. Yue et al., 1995; V. P. Deenadayalu et al., 2001; P. Tep-areenan et al., 2002).

In contrast to the vasodilatory effects of acute androgen treatment, several studies have shown that chronic *in vivo* androgen treatment can cause vasoconstriction. Chronic *in vivo* T treatment increased myogenic tone in middle cerebral arteries of gonadectomized male rats via an endothelium-dependent, BK_{Ca}-dependent, NO-independent mechanism (G. G. Geary et al., 2000b; R. J. Gonzales et al., 2004). Chronic *in vivo* T treatment has also been shown to increase expression of thromboxane synthase expression (thromboxane is a potent vasoconstrictor) in rat middle cerebral arteries via an endothelium-dependent mechanism. Furthermore, in the T treated group, dilation in response to thromboxane synthase inhibition or thromboxane receptor inhibition was increased (R. J. Gonzales et al., 2005). Another study found that chronic *in vitro* (24hr) T treatment increased L type calcium channels and single channel activity in cardiomyocytes and was blocked by AR antagonist, while acute T decreased L type calcium channel current and decreased calcium sparks and was AR-independent (F. Er et al., 2007). Thus the effects of androgens may differ with duration of treatment and occur through two separate pathways, one AR-mediated and the other AR-

independent. For reviews on gonadal steroids and vascular reactivity please see (R. D. Jones et al., 2004; J. M. Orshal and R. A. Khalil, 2004; D. N. Krause et al., 2006).

Estrogens & Vascular Reactivity

Estradiol treatment, *in vivo* and *in vitro*, causes vasodilation. Middle cerebral arteries from intact or ovariectomized (OVX) and E replaced rats are more dilated than arteries from OVX rats. Endothelium removal or a combination of NOS and cyclooxygenase inhibition abolished these differences. (G. G. Geary et al., 2000a). E exerts its vasodilatory effects by increasing production of the potent vasodilator nitric oxide (NO). In rodent cerebral arteries, E increases NO production, increases endothelium-dependent relaxation, and increases endothelial contraction in response to NOS inhibition (H. Momoi et al., 2003; C. G. Sobey et al., 2004). In contrast, OVX significantly decreased plasma concentrations of NO metabolites and increased thrombotic tendency, while E replacement reversed the increased thrombotic tendency caused by OVX (H. Ono et al., 2002). E increases NO production by increasing eNOS. For example, *in vivo* E treatment increases eNOS mRNA, protein expression, and activity in cerebral blood vessels causing increased NO production (A. M. McNeill et al., 1999; A. M. McNeill et al., 2002; C. Stirone et al., 2003; C. Stirone et al., 2005a), while OVX decreases eNOS protein expression (D. A. Pelligrino et al., 2000). *In vitro* estradiol treatment for 18 hours also resulted in a concentration-dependent increase in eNOS protein that was ER-dependent (A. M. McNeill et al., 2002).

Several studies have sought to determine the mechanism by which E increases eNOS activation. In intact cerebral blood vessels, it was found that E rapidly activates eNOS via a phosphoinositide-3 kinase- and ER-dependent pathway, causing increased NO production (C. Stirone et al., 2005a). In bovine microvascular and human umbilical endothelial cells, E increased phosphorylation of Akt within 1 min and this was followed by phosphorylation of eNOS, which was inhibited by a non-selective ER antagonist. E also increased NOS activity, nitrite production, vasodilatory response of aortic rings to ACh, and dilated cerebral microvascular vessels.(M. Florian et al., 2004b). E may act via changes in the cyclooxygenase pathways. For example, in middle cerebral arteries from female rat, *in vivo* E decreases cerebrovascular tone by shifting the primary end product of the endothelial cyclooxygenase-1 pathway from the constrictor prostaglandin H_2 to the vasodilator prostacyclin (J. A. Ospina et al., 2003). Calcium is also thought to play a role in E-mediated vasodilation. In rabbit isolated basilar arteries, E induces endothelium-independent relaxation by inhibiting extracellular calcium influx to VSM. This effect was not mediated by ERs, protein synthesis, or potassium channels (J. B. Salom et al., 2001). For a review on estrogen and vascular reactivity please see (S. P. Duckles and D. N. Krause, 2007).

Estrogen Receptor Beta & Vascular Reactivity

Activation of ERβ and ERα has also been shown to cause vascular relaxation/vasodilation via a non-genomic mechanism. For this review, I will focus on ERβ. In mouse and rat mesenteric artery and aorta, the ERβ agonist,

diarylpropionitrile (DPN), produced endothelium-independent relaxation. Furthermore, in aorta DPN was more effective at producing relaxation than the ERα agonist 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PTT) (K. Al Zubair et al., 2005). DPN-mediated relaxation is rapid (< 5 min) in rat mesenteric arteries (S. Montgomery et al., 2003), suggesting the mechanism is non-genomic. In porcine coronary rings, DPN induced endothelium-dependent, nitric oxide-independent relaxation and increased nitric oxide and nitrite formation in vascular endothelial but not smooth muscle cells and attenuated VSM cell superoxide formation (T. Traupe et al., 2007). DPN has also been shown to cause concentration-dependent relaxation in female rat inferior vena cava (J. D. Raffetto et al., 2010). Chronic *in vivo* treatment of OVX spontaneously hypertensive rats with the ERβ agonist 8beta-VE2 lowered elevated systolic blood pressure as well as peripheral vascular resistance. 8beta-VE2 also enhanced aortic ERβ expression, improved NO-dependent relaxation, augmented phosphorylation of the vasodilator-stimulated phosphoprotein in isolated aortic rings, increased cardiac output, and attenuated cardiac hypertrophy (V. Jazbutyte et al., 2008).

Several studies have examined the role of ERβ in E-mediated vasodilation by comparing responses in ERβ deficient mice and wild type mice. For example, *in vivo* E administration caused a rapid increase in the outer wall diameter of both elastic and muscular arteries in wild type but not in ERα or ERβ knock-out mice, implicating both receptor subtypes as mediating this E action (X. Guo et al., 2005). In small femoral arteries, ACh-induced relaxation of arteries was greater in wild type females versus males and was attributable to a greater EDHF component of

relaxation. However, this sex difference was absent in ERβ deficient mice, suggesting a role for ERβ in EDHF relaxation in females (L. Luksha et al., 2006). In femoral arteries, NOS inhibition reduced E mediated relaxation in arteries from male and female ERβ-deficient mice but not wild type mice. Furthermore, responses to DPN in arteries from wild type female and male mice did not differ after NOS inhibition (M. N. Cruz et al., 2006). These data suggest that ERβ mediated vasodilation is NO-independent, while ERα mediated vasodilation is NO-dependent.

ERβ has also been shown to attenuate vasoconstrictor responses in a variety of cell types. In rat aortic VSM cells, DPN reduced contraction to PE or potassium chloride (Y. Ma et al., 2010). Similarly, estriol, an endogenous ERβ agonist, treatment *in vivo* significantly attenuated the vasoconstrictor response to intracoronary serotonin and thromboxane receptor agonist challenge. Chronic *in vitro* estriol or 3β-diol treatment of rhesus coronary VSM cells also attenuated late calcium signals; effects of both ERβ agonists could be blocked with a selective ERβ antagonist (R. G. Mishra et al., 2006). DPN also rapidly decreased hypoxic pulmonary vasoconstriction via a rapid, non-genomic, nitric oxide-mediated mechanism (T. Lahm et al., 2008). Taken together, these studies provide evidence that ERβ activation causes vasodilation in a variety of settings.

VASCULAR INFLAMMATION

Vascular Inflammation Signaling Pathways

The transcription factor NFκB plays a key role in regulation of inflammation during ischemic and non-ischemic events. It can be activated through the endotoxin

signaling receptor, Toll-like receptor (TLR) or cytokine signaling receptor interleukin-1 receptor (IL-1R) (Figure 2.6). Both receptors share a homologous cytoplasmic signaling Toll/IL-1R (TIR) domain that when activated initiates a signaling cascade culminating in activation of NFκB and transcription of pro-inflammatory genes (S. M. Dauphinee and A. Karsan, 2006). NFκB is in the cytoplasm as a homo or heterodimer. In the cytoplasm NFκB is inactive because it is bound to the inhibitor protein of NFκB (IκB) which masks the nuclear localization signal. Endotoxin, cytokines, and free radicals all activate NFκB. NFκB activation is caused by proteolytic degradation of IκB. IκB kinases phosphorylate IκB and cause it to be ubiquinated and targeted to proteosome for degradation. The nuclear localization signal is exposed on NFκB and it translocates to the nucleus, binds to the target genes and initiates transcription (F. Mercurio and A. M. Manning, 1999). Activation of NFκB, via TLR4 or IL-1R activation, results in increased inflammation, oxidative stress, and ischemic damage due to increased transcription of a variety of pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules and pro-inflammatory enzymes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (S. M. Dauphinee and A. Karsan, 2006). These increases in COX-2 and iNOS lead to increased oxidative stress due to the peroxidase activity of COX-2 and peroxinitrite formation during NO production. Oxidative stress is a major contributor to the progression of atherosclerosis (G. Zalba et al., 2007).

Interleukin-1 Receptor

IL-1R is expressed in cerebrovascular cells, neurons, microglia, astrocytes, and oligodendrocytes (B. Fogal and S. J. Hewett, 2008). Both IL-1β and the IL-1R increase after ischemia (B. Fogal and S. J. Hewett, 2008). In both rodent and humans, ischemic damage is reduced by IL-1R inhibition (B. Fogal and S. J. Hewett, 2008). In humans, IL-1β is increased in cerebral spinal fluid after stroke (E. Tarkowski et al., 1999). In a clinical trial, the IL-1R antagonist IL-1ra has been shown to improve outcome after stroke (H. C. Emsley et al., 2005). In rodents, administration of an IL-1β neutralizing antibody or IL-1ra reduces damage from cerebral ischemia (J. K. Relton and N. J. Rothwell, 1992; D. Martin et al., 1994; A. L. Betz et al., 1995; Y. Yamasaki et al., 1995; S. A. Loddick and N. J. Rothwell, 1996; G. Y. Yang et al., 1997; G. Y. Yang et al., 1998; G. Y. Yang et al., 1999; N. J. Mulcahy et al., 2003). Furthermore, administration of IL-1β increases injury after cerebral ischemia (Y. Yamasaki et al., 1995). IL-1R deficient mice, are protected against cerebral ischemia induced injury (B. Fogal et al., 2007). These studies suggest that IL-1β and its receptor contribute to damage after cerebral ischemia.

Toll-like Receptor 4

TLRs have mainly been shown to be expressed in immune cells, including monocytes, macrophages, dendritic cells and B cells. However, expression of the TLR family member, TLR4, has also been reported in non-immune cells such as VSM (J. Oyama et al., 2004; L. L. Stoll et al., 2004; X. Yang et al., 2005) and endothelial cells (J. Pugin et al., 1993; E. Faure et al., 2000; S. M. Dauphinee and A.

Karsan, 2006; W. Wang et al., 2011). The endotoxin lipopolysaccharide (LPS), a potent TLR4 agonist, has been shown to increase TLR4 expression in human aortic VSM cells leading to increased production of inflammatory mediators such as cytokines, adhesion molecules, and pro-inflammatory enzymes (F. Y. Lin et al., 2006; H. Li et al., 2007; Y. H.-J. Heo S-K, Noh E-K, Park W-H, Park S-D, 2008). Furthermore, this increase in TLR4 was dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (an enzyme responsible for superoxide/reactive oxygen species production), providing evidence that TLR4 activation is associated with increased oxidative stress (F. Y. Lin et al., 2006). The presence of TLR4 in smooth muscle and endothelium suggest a role for TLR4 in vascular disease.

TLR4 signaling can cause detrimental effects in the absence of endotoxin. TLR4 expression is enhanced in endothelial cells from symptomatic carotid atherosclerotic plaques in humans (A. Katsargyris et al., 2010). TLR4 expression is also enhanced in VSM cells from atherosclerotic coronary arteries compared to non-atherosclerotic arteries (K. Otsui et al., 2007). In macrophages, hypoxia (low oxygen environment) has been shown to increase TLR4 expression via activation of the transcription factor hypoxia inducible factor-1 alpha (HIF-1α) (K. So Young et al., 2010). In whole brain TLR4 mRNA and protein have also been shown to significantly increase after intracerebral hemorrhage or cerebral ischemia induced by middle cerebral artery occlusion (MCAO, *in vivo* stroke model) in rodents (C. X. Ma et al., 2009; L. W. Weiyu Teng, Weishuang Xue, and Chao Guan, 2009; K. Hyakkoku et

al., 2010; X. K. Tu et al., 2010), and these increases in TLR4 correlated with increased damage.

Studies in TLR4-deficient mice have demonstrated some of the detrimental effects of TLR4 activation *in vivo*. For example, TLR4-deficient mice have smaller infarct volumes, better neurological and behavioral test outcomes, less brain inflammation, and decreased NFκB activation after cerebral ischemia than controls (J. R. Caso et al., 2007; J. R. Caso et al., 2008; F. Hua et al., 2009; K. Hyakkoku et al., 2010). In addition, TLR4 deficient mice also have lower levels of malondialdehyde (cytotoxic product of membrane lipid peroxidation caused by reactive oxygen species) after cerebral ischemia (J. R. Caso et al., 2007; J. R. Caso et al., 2008). These data further support the idea that TLR4 activation increases production of reactive oxygen species. It has also been shown that oxidative stress upregulates TLR4 expression in rat and human cerebral endothelial cells (P. Nagyoszi et al., 2010). TLR4-deficient mice sustain smaller infarctions and exhibit less inflammation after myocardial ischemia-reperfusion injury (J. Oyama et al., 2004). TLR4-deficient mice are resistant to chronic hypoxia-induced pulmonary hypertension (K. C. Young et al., 2010). Following hemorrhagic shock, mesenteric arteries from TLR4-deficient mice are protected against endothelial injury (Y. Benhamou et al., 2009). TLR4-deficient mice are also protected against LPS-induced depression of vascular contractility (S. Ehrentraut et al., 2007). Taken together, these studies all suggest that TLR4 may be a critical regulator of inflammation and oxidative stress both during endotoxin-induced inflammation and during ischemic injury.

Hypoxia Inducible Factor -1 Alpha

HIF-1α plays a particularly important role in cerebral ischemia because it is activated both by cytokines as a result of inflammation, and by low oxygen levels as a result of reduced blood flow (T. Hellwig-Burgel et al., 2005) (Figure 2.7). The HIF DNA-binding complex is comprised of a constitutively expressed HIF-1β subunit, and two hypoxia inducible subunits HIF-1α and HIF-2α. Under normoxic conditions, HIF-1α and HIF-2α are continuously degraded due to oxygen-and iron-dependent prolyl hydroxylases that target HIF α subunits for degradation by the proteosome. During hypoxia or iron deprivation, the hydroxylases are inhibited and HIFα subunits can accumulate and translocate to the nucleus where they bind HIF-1β and then bind hypoxic-response elements on the promoter regions of target genes to cause increased transcription of those genes (V. Nizet and R. S. Johnson, 2009). HIF-1α increases the transcription of several genes for proteins that promote blood flow and inflammation, including TLR4, vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), heme oxygenase-1, eNOS, iNOS, and COX-2 (H. Matsui et al., 1999; T. Hellwig-Burgel et al., 2005; Y.-N. Liu et al., 2009; K. So Young et al., 2010).

Hypoxia may contribute to the progression of atherosclerosis to an ischemic event. Hypoxia occurs in inflammatory lesions due to increased tissue oxidative metabolism and decreased vascular perfusion (T. T. Cormac and P. C. Eoin, 2009). Atherosclerotic lesions, particularly in advanced plaques, contain regions of severe hypoxia. Lesion hypoxia may be a key factor in the progression of advanced lesions by promoting lipid accumulation, inflammation, ATP depletion, and angiogenesis (L. M. Hulten and M. Levin, 2009).

Cross-Talk Between HIF-1α & NFκB

There is considerable cross-talk between HIF-1α and NFκB (Figure 2.7). NFκB activation can increase HIF-1α levels under normoxia and following tumor necrosis factor alpha (TNFα) exposure, furthermore, inhibition of NFκB impairs the hypoxia-induced HIF-1α response (P. vanÂ uden et al., 2008). Hypoxia enhances NFκB activity (K. M. Oliver et al., 2009), while prostaglandin E_2, a product of COX-2 activity, increases HIF-1α expression under normoxia (R. Ji et al., 2010). In equine endothelial cells, LPS and hypoxia have both been shown to increase HIF-1α stabilization, while the effects of combined LPS and hypoxia on HIF-1α stabilization and neutrophil adhesion were more than additive(A. Brooks et al., 2010). Furthermore, lungs of rats subjected to hemorrhagic shock show both increased HIF-1α activation and COX-2 expression (C. Hierholzer et al., 2001). IL-1β has also been shown to upregulate HIF-1α under normoxia, an effect which could be attenuated with COX-2 inhibition (Y.-J. Jung et al., 2003; O. B. D. Stasinopoulos I, Bhujwalla ZM, 2009). In human pulmonary artery smooth muscle cells, reactive oxygen species increase HIF-1α induction via an NFκB-dependent mechanism (S. Bonello et al., 2007). In addition, hypoxia increased NFκB activity and NFκB inhibition reduced HIF-1α. In addition, mutation of the NFκB binding site in the HIF-1α promoter prevented hypoxia-induced HIF-1α transcription (R. S. BelAiba et al., 2007). Hypoxia and inflammation often occur simultaneously due to prevention of adequate gas exchange.

Cyclooxygenase-2

As previously mentioned, COX-2 is a pro-inflammatory enzyme that gets upregulated during vascular inflammation. Two major isoforms of cyclooxygenase have been described, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is involved in normal physiological responses. COX-2, is the inducible isoform that is minimally expressed in vascular tissue under normal conditions, but upon induction can play an important role in mediating inflammation (J. L. Masferrer et al., 1995). COX-1 and COX-2 are responsible for converting arachidonic acid into prostaglandin H_2, which can then be converted to an array of prostanoids including prostaglandin I_2 (also known as prostacyclin), prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin D_2, and thromboxane. Production of these prostanoids can lead to many biological effects including vasodilation, vasoconstriction, platelet aggregation, anti-platelet aggregation, and apoptosis (C. Iadecola and P. B. Gorelick, 2005). COX inhibitors, also known as non-steroidal anti-inflammatory drugs, are commonly used to reduce inflammation and prevent cardiovascular events (C. Patrono and C. Baigent, 2009). Following IL-1β or LPS treatment, both human and ewe arteries have been shown to express COX-2 (K. E. Vagnoni et al., 1999; N. Foudi et al., 2009). The role of COX-2 and COX-2 derived prostanoids in vascular inflammation associated with ischemia will be discussed below.

ISCHEMIA

Cerebral ischemia, loss of blood flow to the brain, results in neuronal death due to lack of oxygen and nutrients and an increase in reactive oxygen species that

triggers cytokine release and the initiation of an inflammatory response (Q. Wang et al., 2007). Cytokines, such as IL-1β, help initiate the inflammatory response via activation of NFκB (J. Rius et al., 2008). Cerebrovascular inflammation, in particular, contributes to secondary brain injury by promoting leukocyte infiltration into the brain, blood-brain-barrier injury, hyperemia, edema, and increased intracranial pressure. Vascular-derived COX-2 prostanoids may play an important role in this response because prostanoids can promote expression of cell adhesion molecules (D. Stanimirovic et al., 1997), hyperemia (O. Miyamoto et al., 2003), blood-brain-barrier leakiness (P. Ting, 1990), and cytotoxicity (K. Seibert et al., 1995). In humans, studies have shown that plasma levels of pro-inflammatory cytokines positively correlate to infarct size and neurological deterioration following stroke (N. Vila et al., 2000). Therefore, inflammation is a modulator of stroke outcome.

Ischemic Models

Oxygen-glucose deprivation (OGD) or hypoxia with glucose deprivation (HGD) are *in vitro* models for some of the cellular insults that occur during ischemia, since during ischemia perfusion is reduced or absent, causing diminished delivery of oxygen and nutrients to affected cells/tissues. Middle cerebral artery occlusion (MCAO) is a commonly used *in vivo* model for ischemia in rodents. In this model, damage to the brain is assessed via measurement of infarct size. Infarct size is measured in thick brain sections incubated in a stain that labels live mitochondria, the areas that do not take up the dye are labeled as the infarcted/dead tissue. This serves as a relevant model for stroke because the middle cerebral artery is the most

common site of occlusion in human strokes (A. Delcker et al., 1993; W. Hacke et al., 1996), and damage to this area causes behavioral and neurological deficits in rats that can be compared between treatment groups.

Cerebral Ischemia Causes Inflammation

Cerebral ischemia, both *in vitro* and *in vivo,* has been shown to induce inflammation. OGD has been shown to induce expression of iNOS mRNA and protein and cause cell death in bovine cerebral endothelial cells (J. Xu et al., 2000). OGD has also been shown to induce human brain endothelial cell death and release of matrix metallopeptidase-9 (S. Guo et al., 2010). In astrocytes, OGD has been shown to promote cell death and increase COX-2 expression via the NFκB pathway (Y.-S. Lee et al., 2005). OGD also affects vascular reactivity. For example, in bovine middle cerebral arteries OGD inhibited contraction to serotonin to a greater extent than hypoxia alone (P. E. Vinall and F. A. Simeone, 1986). Since cerebral ischemia causes hypoxia, it is not surprising that HIF-1α expression also increases following MCAO (X. Zhang et al., 2010). TNFα, IL-1, IL-6, and iNOS increase in cerebral VSM and associated intracerebral microvessels after MCAO (A. Maddahi and L. Edvinsson, 2010). TLR4 mRNA, TLR4 protein, and NFκB activity also significantly increase in the brain after intracerebral hemorrhage in rats (L. W. Weiyu Teng, Weishuang Xue, and Chao Guan, 2009). These data indicate that ischemia causes inflammation.

Inflammation Worsens Cerebral Ischemia Outcome

Inflammation, both *in vitro* and *in vivo*, has been shown to worsen outcome following cerebral ischemia. OGD- induced cell death in bovine cerebral endothelial cells appears to be due to iNOS-generated reactive oxygen species, as evidenced by increased peroxynitrite formation, since this cell death could be attenuated by selective iNOS inhibition or non-selective NOS inhibition (J. Xu et al., 2000). Pro-inflammatory cytokine expression positively correlates to infarct size and neurological deterioration following stroke in humans (N. Vila et al., 2000) and following MCAO in rodents (X. K. Tu et al., 2010). In addition, it has also been shown that expression of TLR4, COX-2 and NFκB correlate with brain injury in rodents (X. K. Tu et al., 2010). Inhibition of iNOS reduces infarct size and NFκB activation following MCAO (R. Greco et al., 2011). HIF-1α inhibition prior to MCAO or OGD decreases brain injury, brain edema, and apoptosis in neurons (T. Higashida et al., 2011; S. H. Yeh et al., 2011). As previously mentioned, TLR4 deficient mice have lower infarct volumes, better neurological and behavioral test outcomes, decreased NFκB activation, less brain inflammation, and decreased oxidative stress after MCAO than controls (J. R. Caso et al., 2007; J. R. Caso et al., 2008; F. Hua et al., 2009; K. Hyakkoku et al., 2010).

COX-2 derived prostanoids, particularly prostaglandin E_2, are thought to contribute to injury following stroke. Evidence for the role of COX-2/prostanoids in ischemic outcome can be found in a variety of rodent studies. For example, COX-2 mRNA induction in brain after MCAO is associated with increased severity of tissue damage (Y. Collaco-Moraes et al., 1996). Furthermore, COX-2 inhibition has been

shown to reduce infarct size, inflammation, and neurobehavioral deficits following MCAO (K. Sugimoto and C. Iadecola, 2003; M. Ahmad et al., 2009; N. Fathali et al., 2010) and to reduced cell death and cytokine expression following OGD in neuronal cultures (A. Bernardi et al., 2010). The enzyme responsible for arachidonic acid, the substrate for COX-2, production from cell membrane lipids increases immediately following MCAO, followed by an increase in COX-2 derived prostaglandin E_2. Mice deficient in this enzyme have decreased neuronal prostaglandin E_2, edema, and reactive oxygen species (K. Kishimoto et al., 2010). The COX-2 endproduct, prostaglandin E_2, binds four receptor subtypes: EP1, EP2, EP3, and EP4. Activation of a majority of these receptors has been shown to increase infarct size and worsen neurological outcome following MCAO (T. Kawano et al., 2006; M. Ahmad et al., 2007; S. Saleem et al., 2007; T. Abe et al., 2008; A. S. Ahmad et al., 2008), although EP2 receptor activation has been shown to be protective during MCAO (L. McCullough et al., 2004).

Oxidative stress has also been shown to increase after MCAO (Y. S. Song et al., 2010). In addition, mice deficient in nicotinamide adenine dinucleotide phosphate oxidase (NOX), a major source of oxidants in the post-ischemic brain, had significantly smaller infarcts and less brain inflammation after MCAO than wild type mice. Furthermore, injection of IL-1β doubled infarct size in wild type, but not in the NOX deficient mice (H. Chen et al., 2011). These studies provide evidence that inflammatory proteins such as COX-2 and prostaglandins contribute to oxidative stress and ischemic damage.

Sex Differences in Cell Death Following Ischemia

Numerous studies have shown that males and females utilize different cell death pathways following cerebral ischemia (for reviews please see (J. T. Lang and L. D. McCullough, 2008; K. Vagnerova et al., 2010)) (Figure 2.8). In females, cell death following ischemia has been shown to be caspase-dependent. In females, during MCAO cell death is initiated by cytochrome c release from the mitochondria, formation of the apoptosome, caspase activation, and caspase-mediated cleavage of DNA leading to cell death (J. T. Lang and L. D. McCullough, 2008). Consequently, in female mice caspase activation is increased after MCAO, and caspase inhibition is protective. However, in males caspase inhibition has no effect (F. Liu et al., 2009). Conversely, in males, cell death following cerebral ischemia has been shown to occur via the caspase-independent pathway. In males, MCAO increases production of NO and peroxynitrite leading to DNA damage and activation of the DNA repair enzyme poly (ADP ribose) polymerase-1 (PARP-1). PARP then triggers translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus and leads to caspase-independent apoptosis (F. Liu et al., 2009). Consequently, inhibition of PARP or NOS is beneficial in males, but detrimental in females (L. D. McCullough et al., 2005). Deletion of PARP-1 also reduces infarct in males but exacerbates injury in females. Interestingly, the detrimental effects of PARP loss in females can be reversed with caspase inhibition (F. Liu et al., 2011).

There is some debate over whether these differences are due to hormonal status or to genetic sex (XX or XY chromosomes). In isolated neurons with gonadal steroids removed from the media, cytotoxic challenge causes cell death in XY

neurons via the AIF-dependent pathway, while XX neurons die via cytochrome c dependent pathway. In addition, female neurons had increased mitochondrial release of cytochrome c and caspase-3 activation, while male neurons had increased AIF levels (L. Du et al., 2004). In both intact and OVX female mice, caspase inhibition was found to be neuroprotective after MCAO, but had no effect in males, suggesting that XX chromosome status, rather than estrogen is responsible for the increased caspase-mediated cell death in females (F. Liu et al., 2009). Conversely, intact male PARP-1 knockout mice also have smaller infarcts after MCAO, but protection is lost with castration and restored with androgen replacement, suggesting androgens may mediate some of the PARP-1 selective cell death in males (K. Vagnerova et al., 2010). While it is still unclear whether genetic sex or hormone status are responsible for the sex differences in apoptosis, it is clear that males and females have sexually dimorphic mechanisms for cell death following cerebral ischemia.

EFFECTS OF ANDROGENS ON CARDIOVASCUALR DISEASE, INFLAMMATION, AND OXIDATIVE STRESS

Cardiovascular Effects of Androgens

Cardiovascular disease, particularly stroke, is more prevalent in men than in pre-menopausal women, yet the severity of outcome is greater in women (R. E. Petrea et al., 2009; M. Writing Group et al., 2009). Based on these statistics it was originally thought that androgens had detrimental effects on the cardiovascular system; surprisingly androgens have actually been shown to have a variety of

cardioprotective effects. In mice, T has been shown to reduce aortic fatty streak formation and improve HDL cholesterol (J. E. Nettleship et al., 2007). In rodents, low doses of both T and DHT have been shown to decreased infarct size after cerebral ischemia (Y. Pan et al., 2005; J. Cheng et al., 2008; Z. K. Li et al., 2008; M. Uchida et al., 2009; J. Cheng et al., 2010). The androgen DHEA has been shown to inhibit vascular remodeling and reduce neointima formation after vascular injury (M. Ii et al., 2009). DHEA has also been shown to inhibit and even reverse hypoxic pulmonary hypertension in rats (M. Oka et al., 2007).

Circulating T levels in men peak in early adulthood and then steadily decline with age at a rate of 1.6% per year for total testosterone and 2-3% per year for free (bioavailable) testosterone (H. A. Feldman et al., 2002; B. B. Yeap, 2008). In men, low androgen levels predict increased incidence of stroke (B. B. Yeap et al., 2009), aortic aneurysm (B. B. Yeap et al., 2010), hypercholesterolemia, diabetes, coronary heart disease (K. M. English et al., 2000b; A. Ponholzer et al., 2010) and hypertension (K. M. English et al., 1997). Furthermore, low androgen levels are also associated with poorer outcome after stroke (L. L. Jeppesen et al., 1996), increased vascular mortality (C. J. Malkin et al., 2010), increased arterial stiffness (M. Yaron et al., 2009), increased inflammation (V. Kupelian et al., 2010), adverse lipid profile, high levels of fibrinogen, insulin, and pro-colagulable factors (K. M. English et al., 1997). T replacement has also been used in clinical settings to relieve symptoms of angina since it causes vasodilation and thus increases bloodflow (K. M. English et al., 2000a). Furthermore, T has been shown to enhance proliferation of human VSM cells (M. R. Williams et al., 2002), potentially helping to maintain the fibrous cap of

atherosclerotic plaques (C. J. Malkin et al., 2003). Accordingly, castration of male rabbits has been shown to increase aortic atheroma by 100%, an effect which is inhibited by T replacement (P. Alexandersen et al., 1999). These studies suggest that androgen therapy may be beneficial in aging men at risk for cardiovascular disease.

Effects of Androgens During Cerebral Ischemia

Since lower testosterone levels predict increased incidence of stroke or transient ischemic attack (B. B. Yeap et al., 2009) and poorer outcome (L. L. Jeppesen et al., 1996), androgens may play a protective role during stroke. However, in rodent models of stroke the effects of androgens are less clear. Some studies have reported detrimental effects of androgens on lesions size following cerebral ischemia (T. Hawk et al., 1998; S.-H. Yang et al., 2002; J. Cheng et al., 2007), while others have reported protective effects (Y. Pan et al., 2005; J. Cheng et al., 2008; Z. K. Li et al., 2008; M. Uchida et al., 2009; J. Cheng et al., 2010). This disparity seems to be due to the fact that the effects of androgens on outcome after cerebral ischemia are both age and dose-dependent. DHT was found to be protective at a low physiological dose or in aged male rodents, but detrimental if administered at a high dose or in young male rodents (J. Cheng et al., 2008; M. Uchida et al., 2009). Nevertheless, it appears that the majority of studies report protective effects for androgens, at least at low doses. For example, T treatment of neonatal rats with hypoxic-ischemic brain damage promoted axon regeneration and morphological recovery of neurons and decreased neural apoptosis (Z. K. Li et al.,

2008). T treatment for 7 days post-MCAO accelerated functional recovery and decreased reactive hypertrophy surrounding the infarct area (Y. Pan et al., 2005). A protective dose of DHT prevented ischemia-induced histone deacetylation and increased salt-induced kinase-1, a protective gene that decreases cell death, after MCAO (J. Cheng et al., 2010). Taken together, these data show that androgens may be protective against ischemia at low doses.

Influence of Androgens on Inflammation & Oxidative Stress

Androgens have been shown to have anti-inflammatory effects *in vivo* and *in vitro*. In men, T replacement has also been shown to reduce expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, and C reactive protein), while increasing expression of the anti-inflammatory cytokine interleukin-10 (C. J. Malkin et al., 2004; S. Y. Kalinchenko et al., 2010b). In men, androgen treatment has also been shown to reduce inflammation associated with rheumatic conditions (A. Bizzarro et al., 1987; M. Cutolo et al., 1991). *In vivo* androgen replacement has been shown to reduce inflammation in rabbits fed a high cholesterol diet (Y. Qiu et al., 2010).

Androgens have been shown to reduce inflammation in a variety of cells/tissues including human monocytes (Z. G. Li et al., 1993), human macrophages (M. P. Corcoran et al., 2010), mouse macrophage (D. A. Padgett and R. M. Loria, 1998; J. A. Rettew et al., 2008; Y. Qiu et al., 2010), human prostate cancer cell line (E. T. Keller et al., 1996), human endothelial cells (H. Hatakeyama et al., 2002; T. K. Mukherjee et al., 2002; G. D. Norata et al., 2006; H. Jin et al., 2009), and human VSM cells (A. Dessouroux et al., 2008; K. L. Osterlund et al., 2010). For example, in

human umbilical vein endothelial cells DHT reduced LPS- or TNFα-induced expression of VCAM-1, inter-cellular adhesion molecule (ICAM), IL-6, monocyte chemoattractant protein, TLR4, and COX2. Furthermore, DHT decreased NFκB activation and COX-2 promoter activity (G. D. Norata et al., 2006). Another study also showed that T decreased VCAM-1 expression in human umbilical vein endothelial cells (T. K. Mukherjee et al., 2002). T has also been shown to decrease TNFα-induced VCAM-1 and NFκB in human aortic endothelium (H. Hatakeyama et al., 2002). T also was shown to exert anti-thrombotic effects on tissue factor pathway inhibitor expression during TNFα-induced inflammation by decreasing NFκB DNA-binding in human umbilical vein endothelial cells (H. Jin et al., 2009). In human pulmonary artery smooth muscle cells, the adrenal androgen, DHEA, has been shown to reduce accumulation of the pro-inflammatory transcription factor HIF-1α during hypoxia via a post-translational mechanism (A. Dessouroux et al., 2008). These data support an anti-inflammatory role for androgens in a variety of cell types.

While the majority of studies have shown anti-inflammatory effects of androgens, a few studies have reported pro-inflammatory effects of androgens in rat cerebral arteries (A. Razmara et al., 2005; R. J. Gonzales et al., 2009) and human endothelial cells (J. A. McCrohon et al., 1999; X. Zhang et al., 2002; A. K. Death et al., 2004). These apparent contradictory findings may be explained by a differential effect of androgens in the absence and presence of induced-inflammation, whereby androgens increase vascular inflammation via an AR-dependent mechanism under physiological conditions, but decrease vascular inflammation via an AR-independent mechanism during pathophysiological conditions. For example, DHT treatment *in*

vivo and *ex vivo*, increases NFκB-DNA binding activity, and increases COX-2 and iNOS protein levels in the absence of induced inflammation via an AR-dependent mechanism (R. J. Gonzales et al., 2009). This AR-mediated pro-inflammatory effect has also been reported in human coronary VSM cells in the absence of induced inflammation, however, in the presence of cytokine- or endotoxin- induced inflammation, DHT decreases COX-2 expression via an AR-independent mechanism (K. L. Osterlund et al., 2010). Additional differences may be explained by dose of androgens used, whereby androgens are protective at low doses, but detrimental at high doses as has been shown with damage following cerebral ischemia (M. Uchida et al., 2009). For example, in human umbilical vein endothelial cells, 0.1 nM DHT reduced TNFα-induced vascular inflammation (G. D. Norata et al., 2006), but in two other studies 400 nM DHT increased TNFα-induced inflammation (J. A. McCrohon et al., 1999; A. K. Death et al., 2004). These studies suggest that the effects of androgens on inflammation may be both dose- and condition-dependent.

Androgens have also been shown to have anti-oxidant effects in mouse embryonic stem cells (M. N. Lee et al., 2008), rat ventral prostate (N. N. Tam et al., 2003), rat synovium (K. Ganesan et al., 2008), and human endothelial cells (Z. R. Xu et al., 2010). In human umbilical vein endothelial cells, DHT decreased hydrogen peroxide, a stimulator of ROS, induced apoptosis (Z. R. Xu et al., 2010). These data, suggest that during pathophysiological conditions androgens play a mostly protective role against cardiovascular disease, inflammation, and oxidative stress.

EFFECTS OF ESTROGENS ON CARDIOVASCULAR DISEASE, INFLAMMATION AND OXIDATIVE STRESS

Cardiovascular Effects of Estrogen

The cardiovascular effects of estrogen are controversial. As previously mentioned, cardiovascular disease, particularly stroke, is less prevalent in pre-menopausal women compared to men, yet the severity of outcome is greater in women than men (R. E. Petrea et al., 2009; M. Writing Group et al., 2009). These statistics and many rodent studies, suggest that estrogen may play a protective role in cardiovascular disease. For example, in OVX monkeys, E treatment decreased diet-induced coronary artery atherosclerosis (M. R. Adams et al., 1990; M. R. Adams et al., 1997; T. B. Clarkson et al., 2001). Observational studies in humans also supported a protective role for E. For example, a prospective observational study found that coronary heart disease incidence was significantly decreased in women (30-55 yrs) who had previously or were currently using hormone replacement therapy (M. J. Stampfer et al., 1985). The Nurses' Health Study, a large prospective observational study, found that women who were on E replacement therapy had reduced risk of major coronary events; but stroke risk was increased with high dose E replacement (F. Grodstein et al., 2000).

However, clinical studies have actually shown adverse cardiovascular effects with estrogen treatment. The Women's Health Initiative, a large randomized clinical trial, showed that in postmenopausal women (50-79 yrs old) estrogen plus progestin treatment increase incidence of coronary heart disease and stroke (J. E. Rossouw et al., 2002). The Heart and Estrogen/Progestin Treatment Study (HERS), another

large randomized clinical trial, showed that in postmenopausal women (<80yrs) E plus progestin treatment increased the incidence of venous thromboembolic events during the first year of treatment (S. Hulley et al., 1998).

The disparities between animal studies, observational studies, and clinical studies appears to be a matter of timing, with estrogens being protective if given soon after menopause/OVX, and detrimental if given longer after menopause/OVX (T. B. Clarkson, 2007). This finding was also supported by a re-examination of the Nurses' Health Study which showed that women beginning hormone treatment near menopause had a significantly reduced risk of coronary heart disease, but protection was lost in those that initiated therapy at least 10 years after menopause (F. Grodstein et al., 2006). Therefore, it appears that E is only protective if given before menopause or soon after the onset of menopause.

Effects of Estrogen During Cerebral Ischemia

Female rats are protected against cerebral ischemia compared to males, which suggests a protective role for estrogen in cerebral ischemia. Female rodents maintain cerebral blood flow and have smaller infarcts than males after MCAO (N. J. Alkayed et al., 1998; W. Zhang et al., 2009; B. Zhang et al., 2010). The increases in cerebral blood flow in females may be due to downregulation of soluble epoxide hydrolase (sEH), the enzyme that inactivates vasodilatory epoxyeicosatrienoic acids (EETs) (I. P. Koerner et al., 2008). ETTs are fatty acid signaling molecules that are potent vasodilators with anti-inflammatory and anti-thrombotic effects in the cerebral vasculature that have been shown to be protective during cerebral ischemia (J. J. Iliff

and N. J. Alkayed, 2009). In support of this theory, sEH expression was found to be lower in female mice than males, consequently sEH knockout reduced infarct size and improved cerebral blood flow in OVX but not OVX and E replaced females, since the E replacement had already lowered the sEH levels (W. Zhang et al., 2009). It has also been shown that intravenous E injection 30 min prior to ischemia provided the same protection as chronic E treatment (T. J. K. Toung et al., 1998). In one study, E treatment of female rats was found to be beneficial only in the females that were still reproductively cycling, and was actual detrimental to the reproductively senescent females (A. Selvamani and F. Sohrabji, 2010). However, another study found that E reduced infarct in reproductively senescent female rats (N. J. Alkayed et al., 2000). Another study reported detrimental effects of estrogen in female rats after permanent (no reperfusion) MCAO (K. B. Gordon et al., 2005). Therefore, it is not clear whether E is protective or detrimental in aged females, and in different ischemic models (permanent vs. transient MCAO with reperfusion).

Estradiol also plays a protective role in males. In male rats, acute E treatment during the first 5 min of reperfusion after MCAO has been shown to reduce infarct and rapidly (10 min) improve cerebral blood flow (L. D. McCullough et al., 2001). Using a model of global forebrain ischemia (occlusion of 4 vessels for 15 min) with reperfusion, it was found that E was protective in both male and female rats, by preserving endothelial and smooth muscle-mediated vasodilation. Ischemia blunted vessel constriction to serotonin regardless of treatment (Y. Watanabe et al., 2001). These data provided evidence that E is protective in males and females during cerebral ischemia, likely via improve blood flow to the infracted area.

Influence of Estrogens on Inflammation & Oxidative Stress

Estrogen has been shown to have anti-inflammatory effects in humans and rodents. In post-menopausal women, E treatment was inversely associated with ICAM-1 and homocysteine levels (R. Karim et al., 2010). In cultured brain endothelial cells, E inhibited the basal and IL-1β mediated expression of the ICAM-1 and NFκB activation (E. Galea et al., 2002) In OVX female rats, LPS induced cerebrovascular iNOS and COX-2; however, this effect was significantly decreased with chronic *in vivo* E treatment (L. Sunday et al., 2006). In rat pial venules, *in vivo* leukocyte adhesion was greater in OVX versus intact or OVX and E replaced. Following cerebral ischemia, in OVX rats leukocyte adhesion remained elevated after 4 and 6 hours of reperfusion compared to intact or OVX and E replaced rats (R. A. Santizo et al., 2000). E is cardioprotective due to inhibition of LPS-induced TNFα expression and cardiomyocyte apoptosis (C. J. Liu et al., 2009). Anti-inflammatory effects of E may be age dependent. Following LPS treatment, aged rats had increased NFκB DNA binding activity, COX-2, and iNOS compared to young rats. E inhibited NFκB DNA binding in both groups but only inhibited COX-2 and iNOS production in the young rats (L. Sunday et al., 2007). These data demonstrate an anti-inflammatory role for estrogens in both humans and rodents.

Estrogen has also been shown to have anti-oxidant effects. *In vivo* E treatment has been shown to decrease superoxide production by NADPH oxidase in rat aorta (M. Florian et al., 2004a). In mitochondria isolated from cerebral blood vessels of female rats, in vivo E treatment decreased production of hydrogen peroxide, an indicator of oxidative stress (C. Stirone et al., 2005b). In VSM cells from

OVX mice, E decreased angiotensin II-induced free radical production and upregulated superoxide dismutase expression and activity, while OVX without E replacement increased free radical production and downregulated superoxide dismutase expression (K. Strehlow et al., 2003). Thus, E has been shown to act as an anti-oxidant and reduced oxidative stress.

Estrogen Receptor Beta Mediated Cardioprotective Effects

Selective ERβ activation, like androgen treatment, has a variety of cardioprotective and anti-inflammatory effects. Cardioprotective effects include protection against vascular injury in mice (M. D. Iafrati et al., 1997), prevention of L-glutamate-induced increases in blood pressure in male rats (S. Gingerich and T. L. Krukoff, 2006), prevention of aldosterone/high salt intake-induced increases in blood pressure, cardiac mass, and cardiac myocyte hypertrophy in rats (P. A. Arias-Loza et al., 2007), prevention of neointima formation in mice (Y. D. Krom et al., 2007), protection against damage in a rat carotid injury model (S. Makela et al., 1999), protection against cerebral ischemia in rodents(H. V. Carswell et al., 2004; N. R. Miller et al., 2005; A. Donzelli et al., 2010), protection against myocardial ischemia in mice (T. Pelzer et al., 2005; I. Nikolic et al., 2007; M. Wang et al., 2008; N. D. Vornehm et al., 2009; M. Wang et al., 2009), and protection against *in vitro* ischemia in mouse brain endothelial cells (J. Guo et al., 2010).

In humans, ERβ gene polymorphisms have been associated with higher systolic blood pressure (S. Ogawa et al., 2000; J. A. Ellis et al., 2004) and increased risk of myocardial infarction (K. M. Rexrode et al., 2007; S. Domingues-Montanari et

al., 2008). A similar phenotype in observed in ERβ knockout mice, which develop both systolic and diastolic hypertension as they age (Y. Zhu et al., 2002).

ERβ activation, like androgens, has been shown to have anti-inflammatory effects in a variety of cells/tissues such as rodent microglial (A. E. Baker et al., 2004; J. A. Smith et al., 2011), mouse brain (C. M. Brown et al., 2010), motorneurons (A. Das et al., 2011), astrocytes (D. K. Lewis et al., 2008), rat VSM cells (D. Xing et al., 2007), primate VSM (R. G. Mishra et al., 2006), and human endothelial cells (G. D. Norata et al., 2010). *In vivo* treatment with estriol, a partial ERβ agonist, attenuated aortic thromboxane receptor expression. Furthermore, *in vitro* treatment with estriol or 3β-diol downregulated thromboxane receptor expression in VSM, which was blocked for both agonists by pretreatment with an ERβ antagonist (R. G. Mishra et al., 2006). In rat VSM cells, DPN decreases TNFα-induced neutrophil chemoattractant and neutrophil chemotactic activity (D. Xing et al., 2007). In human umbilical vein endothelial cells, 3β-diol decreased TNFα-induced ICAM-1, VCAM-1, endothelial cell leukocyte adhesion molecule-1 (ELAM-1), monocyte chemoattractant protein-1, and IL-6; this effect was blocked by an ERβ antagonist. 3β-diol also decreased LPS-induced IL-6, ELAM and platelet endothelial cell adhesion molecule-1 in male mouse aorta (G. D. Norata et al., 2010). Taken together, these studies show that ERβ activation has similar protective effects to androgens.

SUMMARY

In summary, sex steroids can be synthesized and metabolized locally in vascular cells and influence vascular reactivity, inflammation, and oxidative stress. Both androgens and estrogens appear to play mostly protective roles in cardiovascular diseases via their anti-inflammatory, anti-oxidant, and vasodilatory actions. These actions occur via genomic and non-genomic mechanisms. For androgens, some the protective effects appear to be AR-independent, and thus may be mediated via the androgen metabolite 3β-diol.

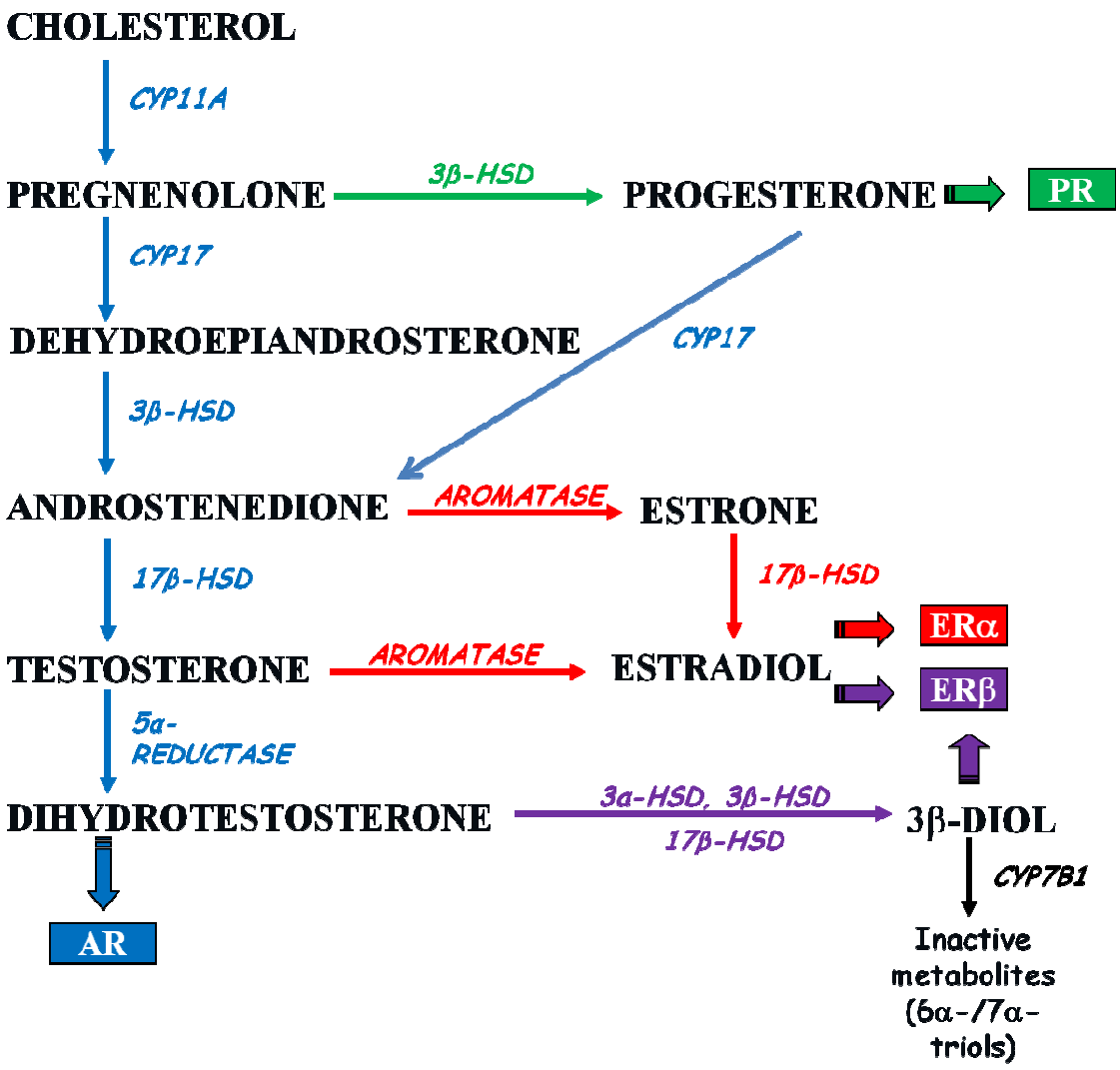


Figure 2.1. ***Gonadal Steroid Synthesis and Receptor Activation.*** Steroid common name is provided. Arrows indicate direction of enzyme reaction. 3α-HSD, 3alpha-hydroxysteroid dehydrogenase; 3β-HSD, 3beta-hydroxysteroid dehydrogenase; 3β-diol, 5α-androstane-3β,17β-diol; 17β-HSD, 17beta-hydroxysteroid dehydrogenase; CYP7B1, 25-hydroxycholesterol 7-alpha-hydroxylase; CYP11A, cholesterol side-chain cleavage enzyme; CYP17, 17alpha-hydroxylase.

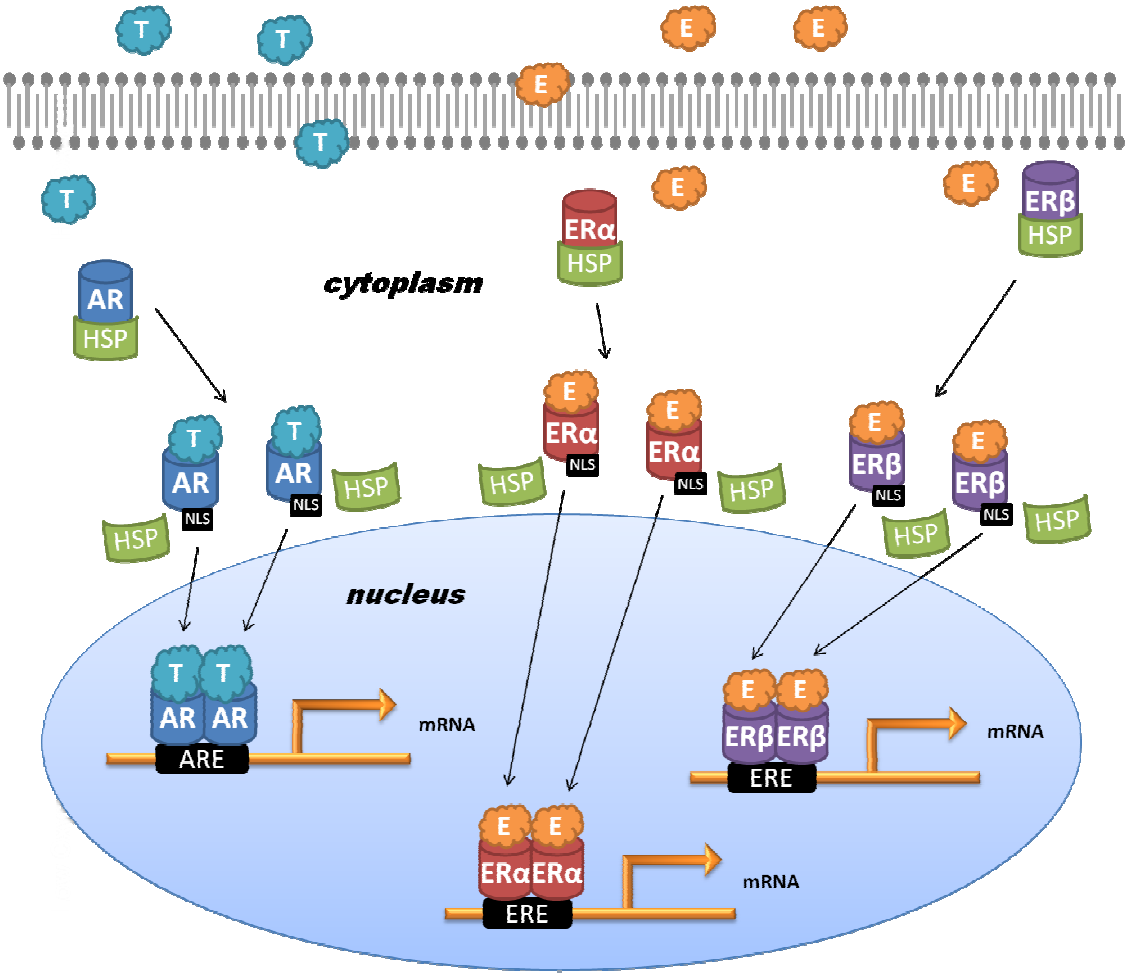


Figure 2.2. ***Gonadal Steroid Genomic Signaling.*** Schematic diagram of genomic gonadal steroid signaling. Although not shown, other androgens and estrogens, such as dihydrotestosterone and 3beta-diol, utilize the same pathways as T and E respectively. T, testosterone; E estradiol, AR, androgen receptor; ARE, androgen response element; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERE, estrogen response element; HSP, heat shock protein; mRNA, messenger RNA; NLS, nuclear localization signal.

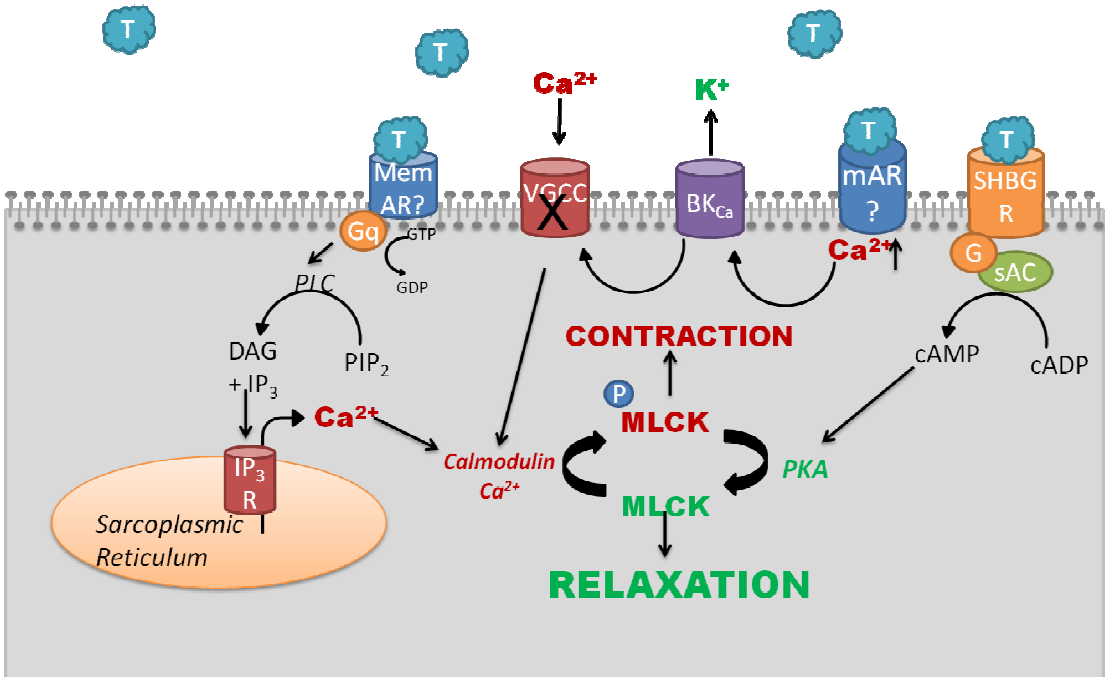


Figure 2.3. ***Non-genomic Actions of Androgens***. Mechanisms for nongenomic actions of androgens proposed by several authors are depicted. Although not shown, dihydrotestosterone is thought to work through the same pathways. BK_{Ca}, large conductance calcium activated potassium channel; Ca2+, calcium; DAG, diacylglycerol; G, G protein; GDP, guanosine diphosphate; Gq, inhibitory subunit of G protein coupled receptor; GTP, guanosine triphosphate; IP_3, inositol 1,4,5-triphosphate; IP3R, IP3 receptor; Mem AR?, unidentified membrane bound androgen receptor; MLCK, myosin light chain kinase; P, phosphorylated; PIP_2, phosphatidylinositol 4,5 bisphosphate; PKG, protein kinase G; PLC, phospholipase C; sGC, soluble guanylate cyclase; SHBG R, sex hormone binding globulin receptor; T, testosterone; VGCC, voltage gated calcium channel. X, blockage of receptor.

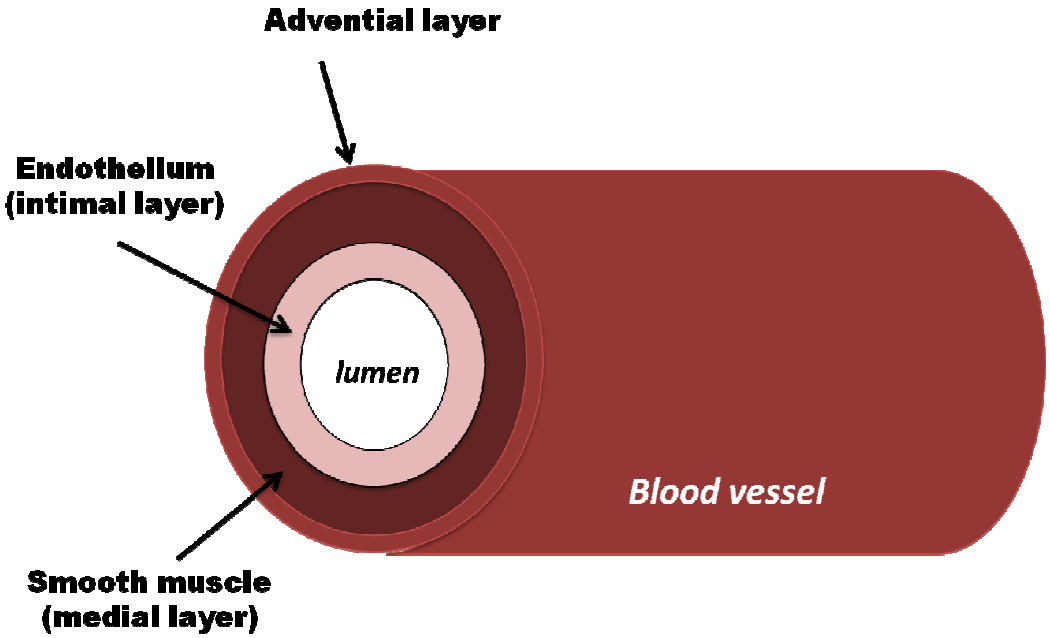


Figure 2.4. ***Vascular Structure.*** Schematic diagram of the three layers of a blood vessel.

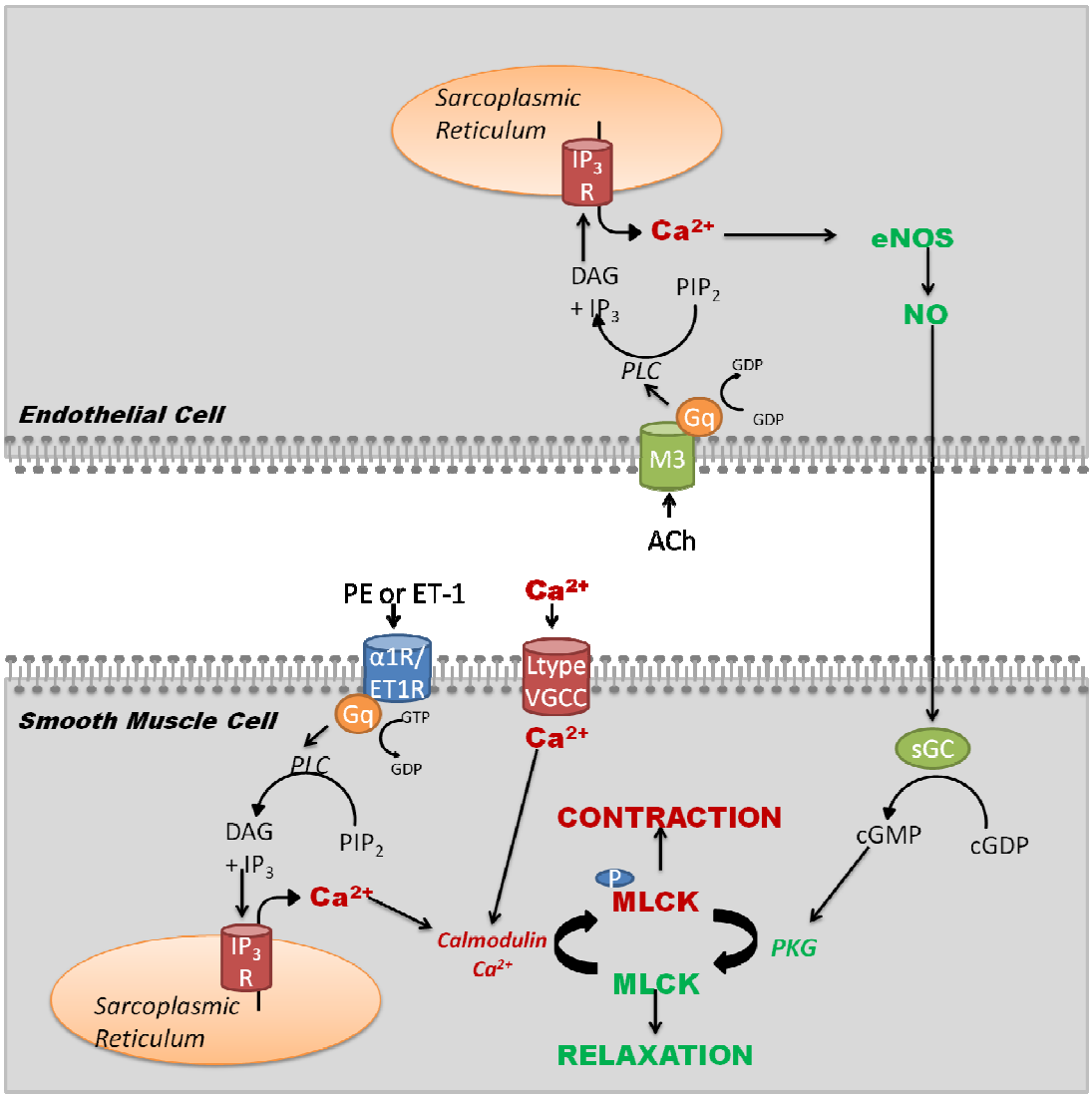


Figure 2.5. ***Vascular Reactivity.*** Pathways for PE, ET-1 and ACh are depicted. α1, alpha 1 adrenergic receptor; ACh, acetylcholine, Ca2+, calcium; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ET-1, endothelin 1; ET1R, endothelin 1 receptor, GDP, guanosine diphosphate; Gq, inhibitory subunit of G protein coupled receptor; GTP, guanosine triphosphate; IP_3, inositol 1,4,5-triphosphate; IP3R, IP3 receptor; L type VGCC, long acting voltage gated calcium channel; M3, muscarinic acetylcholine receptor 3; MLCK, myosin light chain kinase; NO, nitric oxide; P, phosphorylated; PIP_2, phosphatidylinositol 4,5 bisphosphate, PKG, protein kinase G; PLC, phospholipase C; sGC, soluble guanylate cyclase.

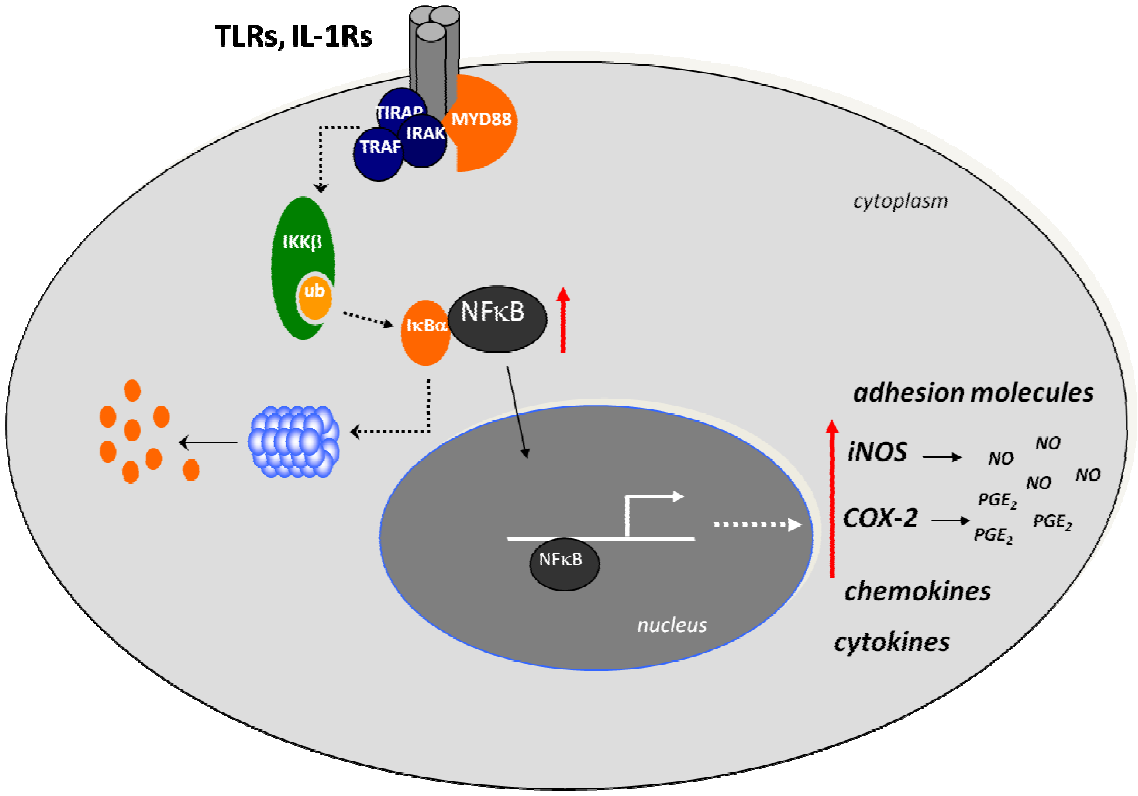


Figure 2.6. ***NFκB Signaling.*** During ischemia activation of the innate immune system through receptors, such as toll-like receptor 4 (TLR4) and the interleukin-1 receptor, leads to MyD88-dependent signaling leading to phosphorylation and degradation of IκB by the proteosome. This allows NFκB to enter the nucleus and induce transcription of pro-inflammatory genes. COX-2, cyclooxygenase-2; IκBα, inhibitor of NFκB alpha; IKKβ, inhibitor of NFκB kinase; IL-1R, interleukin-1 receptor; iNOS, inducible nitric oxide synthase; IRAK, IL-1 receptor associated kinase; NFκB, nuclear factor kappa B; NO, nitric oxide; TLR, toll-like receptor; tram, TRAF, tumor necrosis factor associated receptor; TIRAP; TIR domain containing adaptor molecule; ub, ubiquitin.

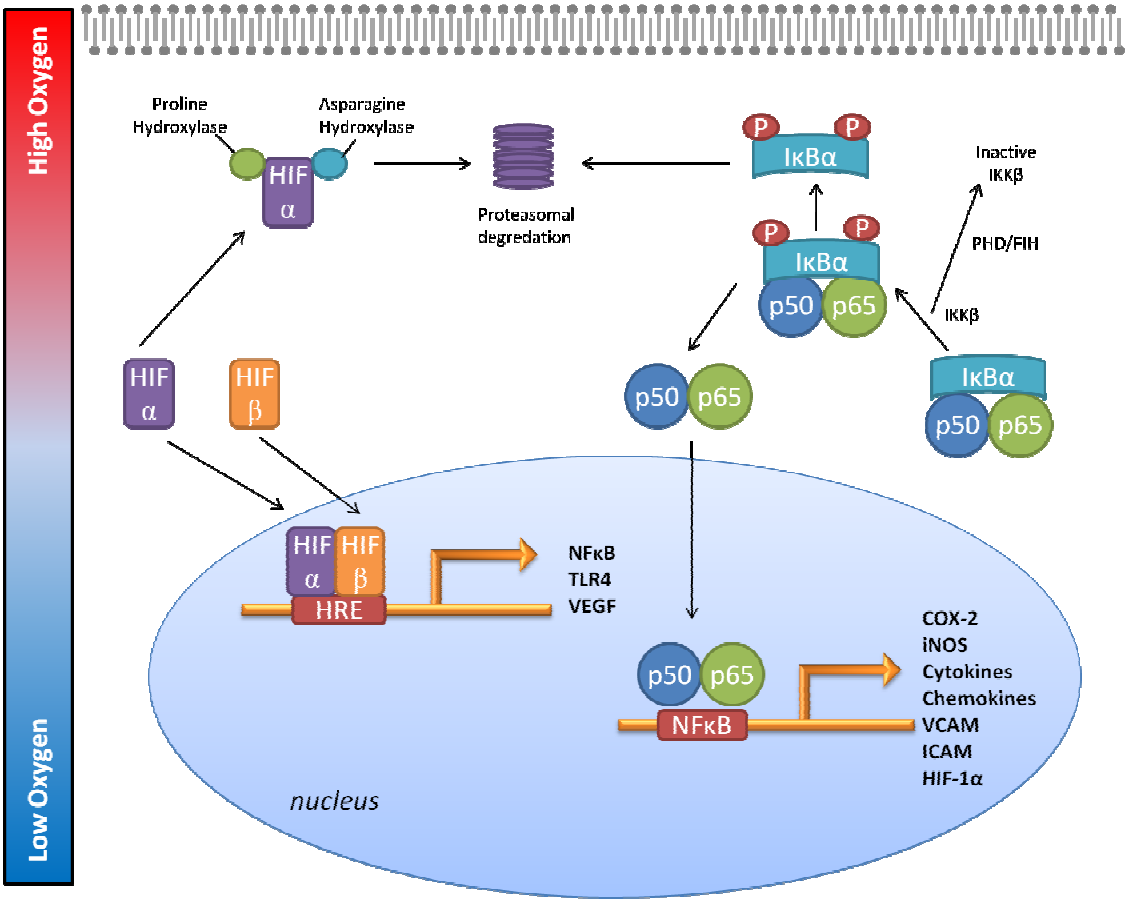


Figure 2.7. Ischemia-Induced Inflammation via NFκB and HIF-1α. During ischemia inflammation is induced by activation of the transcription factors HIF-1α and NFκB. COX-2, cyclooxygenase-2; HIFα, hypoxia inducible factor alpha subunit; HIFβ, hypoxia inducible factor beta subunit, HIF-1α, hypoxia inducible factor 1 alpha; HRE, hypoxia response element; FIH, asparaginyl hydroxylases inhibiting HIF-1α, ICAM, intercellular adhesion molecule; IκBα, inhibitor of NFκB alpha; IKKβ, inhibitor of NFκB kinase; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor kappa B; p50/p65, subunits of NFκB; PHD, prolyl hydroxylase; TLR4, toll-like receptor 4; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor. Adapted from (H. K. Eltzschig and P. Carmeliet, 2011).

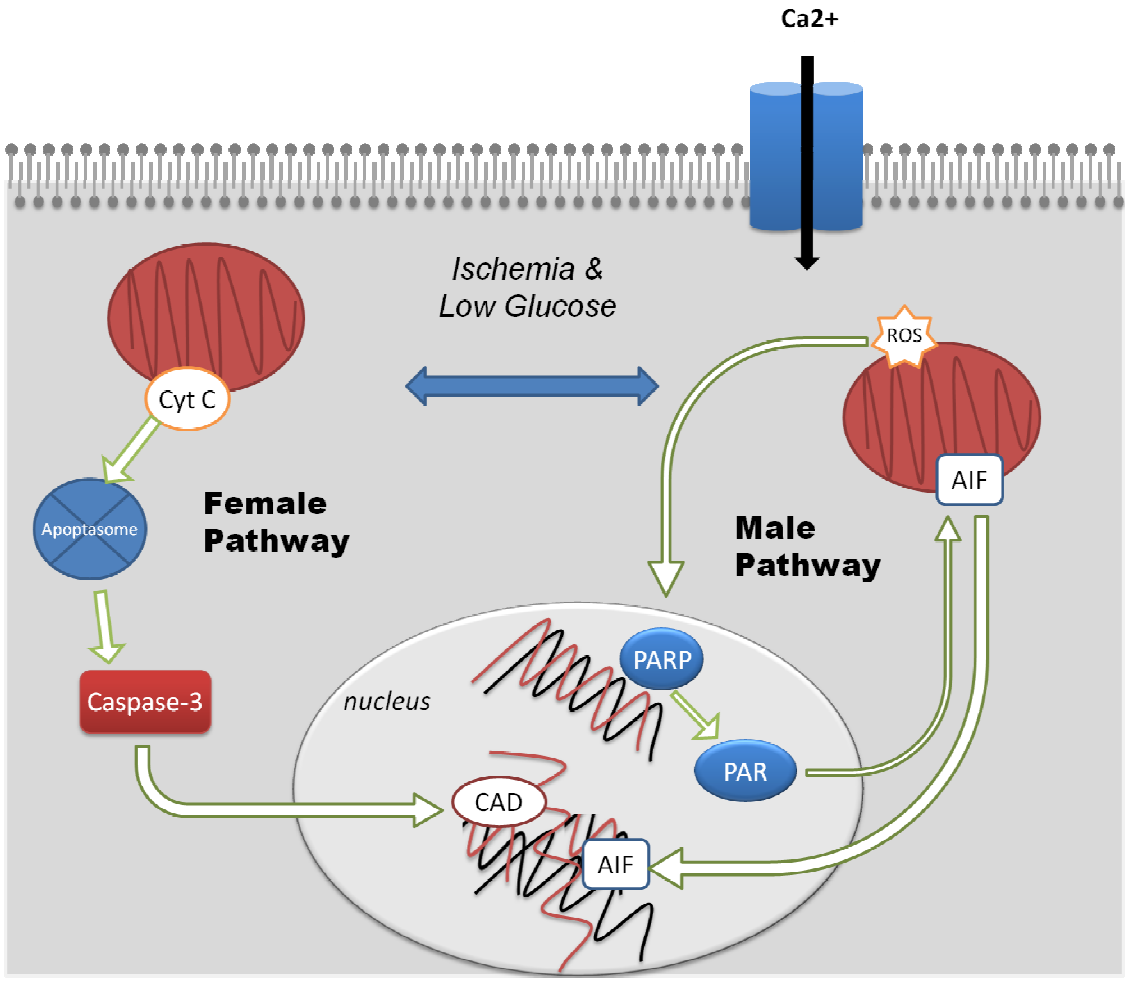


Figure 2.8. Sex Differences in Ischemic Cell Death. Schematic diagram of cell death pathways preferentially utilized by male (right) and female (left) cells. AIF, apoptosis inducing factor; Ca2+, calcium; CAD, caspase activated DNAse; Cyt C, cytochrome C; PAR, poly (ADP-ribose); PARP, PAR polymerase; ROS, reactive oxygen species. Adapted from (J. T. Lang and L. D. McCullough, 2008)

CHAPTER 3: DIHYDROTESTOSTERONE ALTERS CYCLOOXYGENASE-2 LEVELS IN HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS

Abstract

Both protective and non-protective effects of androgens on the cardiovascular system have been reported. Previous studies show that the potent androgen receptor (AR) agonist dihydrotestosterone (DHT) increases levels of the vascular inflammatory mediator cyclooxygenase-2 (COX-2) in rodent cerebral arteries independent of any inflammatory stimulus. Little is known about the effects of androgens on inflammation in human vascular tissues. Therefore, I tested the hypothesis that DHT alters COX-2 levels in both the absence and presence of induced inflammation in primary human coronary artery smooth muscle cells (HCASMC). Furthermore, I tested the ancillary hypothesis that DHT's effects on COX-2 levels are AR-dependent. Cells were treated with DHT (10 nM) or vehicle for 6 hours in the presence or absence of LPS or IL-1β. Similar to previous observations in rodent arteries, in HCASMC, DHT alone increased COX-2 levels compared to vehicle. This effect of DHT was attenuated in the presence of the AR antagonist, bicalutamide. Conversely, in the presence of LPS or IL-1β, increases in COX-2 were attenuated by co-treatment with DHT. Bicalutamide did not affect this response, suggesting that decreases in COX-2 levels by DHT occurs independently

of AR stimulation. Thus, I conclude that DHT differentially influences COX-2 levels under physiological and pathophysiological conditions in HCASMC. This effect of DHT on COX-2 involves both AR-dependent and -independent mechanisms depending on the physiological state of the cell.

Key Words

Vascular smooth muscle, interleukin-1 beta, lipopolysaccharide, inflammation, androgen

Introduction

Preclinical and clinical research suggest that vascular inflammation is a critical regulator in the etiology and progression of cardiovascular disease that, if not treated or managed, can eventually lead to fatal clinical endpoints such as stroke and myocardial infarction. Clinical data consistently show that cardiovascular disease is more prevalent in men than in pre-menopausal women (M. Writing Group et al., 2009). Numerous studies have focused on the role of estrogens in contributing to the gender-related disparities in cardiovascular disease, but the cardiovascular actions of androgens are not well understood. With the few studies documented, there is disagreement as to whether androgens exacerbate or beneficially contribute to the development and progression of cardiovascular disease. Because chronic vascular inflammation contributes significantly in the pathogenesis of cardiovascular diseases (S. J. Warner et al., 1989; P. Saren et al., 1996; S. J. George, 1998), it is important to investigate the role of androgens under both normal and pathophysiological conditions.

During vascular inflammation, monocytes and macrophages penetrate the vessel wall and produce inflammatory cytokines, (P. G. Tipping and W. W. Hancock, 1993) such as interleukin-1β (IL1β), leading to activation of nuclear factor kappa B (NFκB), a transcription factor for inflammatory mediators such as cytokines, vascular adhesion molecule-1 (VCAM-1), cyclooxygenase-2 (COX-2) and, inducible nitric oxide synthase (iNOS) (J. Rius et al., 2008). Vascular inflammation reduces the stability of atherosclerotic plaques (S. J. Warner et al., 1989; P. Saren et al., 1996; S. J. George, 1998), thus increasing the probability of a thrombotic event such as

myocardial infarction or stroke. In this study, I use COX-2 as a marker for vascular inflammation. Two major isoforms of cyclooxygenase have been described, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is involved in normal physiological responses. COX-2, is the inducible isoform that is minimally expressed in vascular tissue under normal conditions, but upon induction can play an important role in mediating inflammation (J. L. Masferrer et al., 1995). COX-1 and COX-2 are responsible for converting arachidonic acid into prostaglandin H_2, which can then be converted to an array of prostanoids. Production of these prostanoids can lead to many biological effects including vasodilation, vasoconstriction, platelet aggregation, anti-platelet aggregation, and apoptosis (C. Iadecola and P. B. Gorelick, 2005).

Previous studies have demonstrated that DHT increases COX-2 levels in rodent cerebral arteries in the absence of induced inflammation (R. J. Gonzales et al., 2009). Although much of the work regarding androgens and vascular inflammation has been conducted in endothelial cells (J. A. McCrohon et al., 1999; H. Hatakeyama et al., 2002; T. K. Mukherjee et al., 2002; A. K. Death et al., 2004; G. D. Norata et al., 2006), there are few reports on the effects of androgens on inflammation in vascular smooth muscle (D. Somjen et al., 2009).

I have hypothesized that androgens can increase COX-2 expression in both the absence and presence of induced inflammation in human coronary artery smooth muscle cells (HCASMC) grown *in vitro*. Furthermore, I hypothesized that DHT's effects on COX-2 protein levels would be androgen receptor (AR) - dependent. This study focuses on the effects of dihydrotestosterone (DHT) on the

inflammatory mediator cyclooxygenase-2 (COX-2), because of the significant contribution COX-2 has on the inflammatory response in vascular tissue (C. Tsatsanis et al., 2006) and because COX-2 has been implicated in vascular diseases (K. Sugimoto and C. Iadecola, 2003).

Materials and Methods

Cell Culture and Hormone/Drug Treatment:

Primary HCASMC (Cascade Biologics; Portland, OR) were grown in 5% CO_2, 95% room air atmosphere at 37˚C, in RPM 231 medium (Cascade Biologics) supplemented with smooth muscle growth supplement (Cascade Biologics) containing 5% FBS. Primary human brain microvascular endothelial cells (HBMEC) (Applied Cell Biology Research Institute; Kirkland, WA) were used in some of the studies for comparison. HBMEC were cultured in Cell Systems Complete (CSC) Medium containing 10% FBS (Cell Systems Corporation, Kirkland, WA).

Hormone/drug treatments were performed on cells at 70 to 80% confluency and at passage 5 or 6. Cell treatments were carried out in hormone-free media supplemented with Charcoal Stripped FBS (Cocalico Biologicals Inc., Reamstown, PA). A cytokine or endotoxin exposure time course was determined in HCASMC by treatment with either IL-1β (5 ng/ml), LPS (100 µg/ml) or vehicle (100 µl/ml PBS) for 3, 6, 9, 12 h. Since both LPS and IL-1β resulted in a significant increase in COX-2 levels at the 3, 6, and 9 h time points and a 4 h DHT treatment time has been shown to induce significant changes at COX-2 mRNA level in human endothelial cells (G. D. Norata et al., 2006), a 6 h time point was selected for subsequent studies to

investigate the effects of DHT treatment on COX-2 protein expression in the absence or presence of IL-1β- or LPS-induced inflammation. Furthermore, a 6 h time point would also allow adequate time for DHT to exert its effects while still remaining within the peak 3-9 h window for cytokine or endotoxin induced COX-2 expression. Depending on the experiment, cells were treated with DHT (1, 5, or 10 nM) or vehicle (0.001% ethanol + 100 µl/ml PBS) for 6 h in the absence or presence of IL-1β (5 ng/ml) or LPS (100 µg/ml). In a separate set of experiments, a dose-response experiment was conducted to determine the optimal dose of the AR-antagonist bicalutamide (100nM, 1µM, 10µM) for inhibiting DHT's effect on COX-2 and AR expression. Both the 1µ and 10µM bicalutamide doses were equally effective at inhibiting increases in COX-2 and AR expression in response to 10nM DHT treatment, while the 100nM bicalutamide was ineffective (data not shown). Therefore, the lowest effective dose of bicalutamide of 1µM was selected for subsequent studies. As for the protocol, HCASMC were pre-treated for 1 h with the AR-antagonist bicalutamide (1µM; dissolved in DMSO) followed by 6 h of co-treatment with bicalutamide including either vehicle (0.001% ethanol + 0.01% DMSO), DHT, or DHT and IL-1B.

Immunocytochemical Labeling for Androgen Receptor:

Immunocytochemistry was used to verify the expression of AR in HCASMC and HBMEC. Cells were plated on glass cover slips pre-coated with poly-L-lysine. Culture medium was replaced with medium containing 5% (HCASMC) or 10% (HBMEC) charcoal stripped FBS containing either DHT (10 nM) or vehicle (0.001%

ethanol). Cells were grown for an additional 4 h and then fixed (4% formaldehyde), washed in PBS (pH 7.4), and permeabilized in 1% triton X-100. Cells were first incubated in 1% BSA in PBS to block non-specific binding, incubated with anti-AR N20 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500) in PBS containing 1% BSA for 1 h, washed in PBS (5x5 min), incubated with Cy3 goat-anti mouse secondary antibody (Invitrogen Corp., Carlsbad, CA; 1:5000) for 1 h in PBS containing 1% BSA and washed in PBS (5x5 min). Coverslips were mounted on glass slides using mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to label nuclei. Cells were visualized using a Zeiss 710 confocal microscope (Carl Zeiss International, Germany). Omission of the primary antibody removed fluorescence signal and was used to determine specificity of binding (data not shown).

Western Blot for COX-1 and COX-2:

Levels of COX-1 and COX-2 protein were examined using standard immunoblotting methods. Cells were rinsed twice with ice-cold PBS containing 100 μM sodium orthovanadate, scraped from flasks over ice, and centrifuged (Sorval Legend RT+ Centrifuge; Thermo Fisher Scientific, Waltham, MA) at 800 g for 10 min. The pellet was resuspended in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1 mM EGTA, 1 mM EDTA, 1mM DTT) containing protease inhibitors (20 μM Pepstatin, 20 μM Leupeptin, 0.1 U/ml Aprotinin, 0.1 mM PMSF), homogenated, sonicated, centrifuged (Accuspin Micro 17R; Thermo Fisher Scientific) at 4500 g for 10 min at 4˚C, and lysate collected. Total protein content of

whole cell lysate was determined using a BCA Protein Assay Kit (Thermo Fischer Scientific) and measured on a MultiSkan Spectrum using SkanIt RE software (Thermo Fischer Scientific). Next, samples were diluted in Tris-Glycine SDS sample buffer (Invitrogen) and boiled for 5 min. Two color fluorescent standard (LI-CORE Biosciences, Lincoln, NE) and diluted samples were loaded into 7.5% Smart gels (LI-CORE). Proteins were separated via SDS-polyacrylamide gel electrophoresis in Smart Gel Running Buffer (LI-CORE) at 145 V using a Mini PROTEAN Tetra Electrophoresis System (Bio-Rad Laboratories, Hercules, CA). Separated proteins were transferred to nitrocellulose membranes and non-specific binding was blocked by incubation at room temperature for 30 minutes in PBS containing 1% Tween (TPBS) and 3% dried milk. Membranes were incubated in COX-2 (1:1000) or COX-1 (1:400) monoclonal antibodies (Cayman Chemical Company, Ann Arbor, MI) overnight at 4˚C in TPBS with 3% milk. Following TPBS washes (5x5 min), the membranes were incubated in Goat Anti-Mouse IR 800 Dye secondary antibody (LI-CORE) for 1 h at room temperature. COX-2 antibody specificity was verified with LPS stimulated Raw-264.7 (mouse macrophage) cell lysate (Santa Cruz), which is a positive control for COX-2 protein (data not shown). Following additional PBS washes (5x5 min), proteins were visualized using an Odyssey Infrared Imager and data was analyzed using Odyssey V3.0 software (LI-CORE). After imaging, all blots were stained with Coomassie Brilliant Blue (Bio-Rad) to verify equal loading of protein.

PGE_2 Measurements:

Prostaglandin E_2 (PGE_2), a downstream metabolite of COX-2, was measured in HCASMC incubation medium following hormone/drug treatment using a PGE_2 Monoclonal EIA Kit (Cayman Chemical). The EIA was performed according to the manufacturer's instructions and measured on a MultiSkan Spectrum using SkanIt RE software (Thermo Fischer Scientific).

Reagents:

All reagents were purchased from Sigma Aldrich Corporation (St. Louis, Missouri) unless otherwise noted.

Statistical Analysis:

Samples from each treatment were run on the same Western blot or 96-well plate (PGE_2 assay) for direct comparison and treatments were repeated for statistical analysis (n=4-24). Data from Western blots are expressed as an optical density ratio relative to vehicle. All values are reported as means ± SEM. Data were compared using analysis of variance (ANOVA), and differences were compared using post hoc tests (Student-Newman-Keuls; Prism Software, Irvine, CA). A level of $p<0.05$ was considered significant.

Results

Androgen Receptors are Present in HCASMC and HBMEC.

Using immunohistochemistry, I detected androgen receptor (AR) immunoreactivity in both the cytoplasmic and nuclear compartments in both HCASMC and HBMEC. A representative confocal image of HCASMC is shown in Figure 3.1A. Since AR activation has previously been shown to increase the expression of the AR (N. F. González-Cadavid et al., 1993), AR protein levels were measured via Western blot following treatment with vehicle, or increasing concentrations of DHT (1, 5, 10 nM). DHT treatment increased AR protein expression in HCASMC and HBMEC at all concentrations tested (Figure 3.1B).

DHT Increases COX-2 Levels in an AR-Dependent fashion.

To determine the effect of DHT treatment on COX-2 protein expression in the absence of induced inflammation, HCASMC were treated with various doses of DHT for 6 h. DHT (10 nM) increased COX-2 levels compared to vehicle ($p<0.001$); lower doses of DHT (1 or 5 nM) had no effect on COX-2 levels (Figure 3.2A). The AR-antagonist bicalutamide was used to determine if increases in COX-2 expression observed with 10 nM DHT treatment were AR-dependent. Co-treatment of cells with vehicle and bicalutamide (1 μM) had no effect on COX-2 levels compared to vehicle. However, co-treatment with DHT (10 nM) and bicalutamide (1 μM) inhibited the DHT-induced increases in COX-2 protein ($p<0.001$ vs. DHT; Figure 3.2B).

Time Course of COX-2 Increases Following Endotoxin or Cytokine Treatment in HCASMC.

A time course for COX-2 induction by either LPS or IL-1β administration was conducted. Following LPS treatment COX-2 levels were increased at the 3 h ($p<0.001$), 6 h ($p<0.001$), and 9 h ($p<0.01$) time points compared to vehicle, but COX-2 levels were not significantly different after 12 h LPS treatment (Figure 3.3A). IL-1β treatment increased COX-2 levels at all time points: 3, 6, 9, and 12 h compared to vehicle ($p<0.001$; Figure 3.3B). Based on these results, the 6 h time point for endotoxin or cytokine exposure was selected for further studies on the effects of DHT on COX-2 following an induced inflammatory stimulus.

Cytokine-Induced COX-2 Levels were more Robust in HCASMC Compared to HBMEC

To compare the inflammatory response in smooth muscle and endothelial cells, I measured COX-2 protein following IL-1β or LPS stimulation in HCASMC and HBMEC. A representative blot is illustrated in Figure 3.4A. COX-2 expression in vehicle treated HCASMC and HBMEC were not significantly different from each other (Figure 3.4). Following LPS or IL-1β stimulation, COX-2 expression was significantly elevated in both HCASMC ($p<0.001$) and HBMEC ($p<0.001$) compared to vehicle. Both IL-1β and LPS increased COX-2 levels to a greater extent in HCASMC compared to HBMEC ($p<0.001$). Because I detected a significant and robust response in the HCASMC compared to the HBMEC, I pursued this finding in HCASMC in subsequent studies.

DHT Attenuated COX-2 Levels in the Presence of LPS- or IL-1β- Induced Inflammation in HCASMC.

To determine the effect of DHT on COX-2 expression following induced inflammation, I treated HCASMC cells with either IL-1β or LPS in the absence or presence of DHT. IL-1β caused significant increases in COX-2 compared to vehicle ($p<0.001$), whereas the 10 nM dose of DHT attenuated these increases ($p<0.01$ vs. IL1β; Figure 3.5A). Lower doses of DHT (1 or 5 nM) had no effect on COX-2 induction by IL-1β (data not shown). LPS treatment of HCASMC also resulted in significant increases in COX-2 compared to vehicle treated cells ($p<0.001$). This effect was prevented by 10 nM DHT treatment ($p<0.001$ vs. LPS; Figure 3.5B).

Attenuation of IL-1B-Induced Increases in COX-2 by DHT is AR-Independent.

To determine if DHT (10 nM) attenuates IL-1B-induced increases in COX-2 via AR activation, I co-treated HCASMC with the AR antagonist bicalutamide and either vehicle or DHT and IL-1β. Consistent with my previous findings, the AR antagonist had no effect on COX-2 levels when co-treated with vehicle. DHT's attenuation of COX-2 levels in presence of IL-1B was not blocked by co-treatment with bicalutamide (Figure 3.6).

DHT Did Not Alter PGE_2 Levels in Absence or Presence of IL-1β-Induced Inflammation.

PGE_2, an end-product of the COX-2 enzymatic pathway, was measured in HCASMC culture medium by ELISA to serve as an indirect measurement of COX-2

enzymatic activity. IL-1β treatment increased PGE_2 levels compared to vehicle ($p<0.001$; Figure 3.7). However, DHT treatment (10 nM) did not alter PGE_2 levels in the absence or presence of IL-1β-induced inflammation (Figure 3.7).

Neither DHT or IL-1β Increased COX-1 Levels in HCASMC.

In order to rule out any compensatory PGE_2 production due to changes in COX-1 levels, I measured COX-1 levels in HCASMC following DHT and/or IL-1β treatment. COX-1 was detected in all treatment groups, and levels remained unchanged compared to vehicle following DHT (10 nM) treatment. In the presence of IL-1β treatment or concomitant DHT and IL-1β treatment, COX-2 levels were decreased compared to vehicle ($p<0.01$; Figure 8).

Discussion

The goal of this study was to determine the effects of androgens on inflammation in HCASMC. Using COX-2 protein levels as a marker for vascular inflammation, I examined the effects of the non-aromatizable androgen DHT on COX-2 levels in HCASMC in the absence or presence of endotoxin- or cytokine-induced inflammation. My results demonstrate that DHT treatment increased COX-2 protein levels in the absence of induced inflammation in HCASMC, an effect that was AR-dependent. In contrast, following endotoxin or cytokine-induced inflammation, DHT treatment caused an unexpected attenuation of both LPS- and IL-1β-induced increases in COX-2 levels. The inability of bicalutamide to block this response suggested that this effect was AR-independent. Thus, it appears that DHT

may modulate the inflammatory mediator COX-2 differentially under physiological conditions and pathophysiological conditions in HCASMC.

To my knowledge this is the first report of DHT's effects on COX-2 levels in the absence of induced inflammation in human vascular cell model. I found that at a 10 nM dose of DHT, COX-2 levels were significantly increased compared to vehicle treated cells however, at lower doses of DHT (1 or 5 nM) COX-2 levels were not altered. Furthermore, the DHT-dependent increases in COX-2 could be blocked with the classical AR antagonist bicalutamide. The DHT dose (10 nM) used in this study is well within the range of the 1 nM-400 nM doses of DHT that have been used in previous studies of the effects of DHT on inflammation in human endothelial cells (J. A. McCrohon et al., 1999; A. K. Death et al., 2004; G. D. Norata et al., 2006) and the 3 nM-300 nM doses that have been used to examine the effects of DHT on human vascular smooth muscle cell proliferation (D. Somjen et al., 2009). While a lower dose of DHT (0.1 nM) has been shown to reduce vascular inflammation, as measured by VCAM and COX-2 levels, in human vein endothelial cells (G. D. Norata et al., 2006), data on vascular smooth muscle cells had previously been lacking. In the current study I show that HCASMC had a greater increase in COX-2 following IL-1β or LPS stimulation compared to HBMEC. Although my two vascular cells types were isolated from different vascular beds (i.e. cerebral circulation versus heart circulation), it is possible that endothelium and smooth muscle have different thresholds for modulation of inflammation due to differences in the number of Toll-like 4 and IL-1 receptors, receptor affinity, or cytokine production. For example a comparison of the intracellular calcium response to 1 ng/ml IL-1 in human aortic

endothelial cells and human aortic smooth muscle cells revealed that only the smooth muscle cells responded with a significant increase in calcium, suggesting that that the IL-1 receptor level or receptor affinity for IL-1 may be higher in smooth muscle cells than in endothelial cells (G. Bkaily et al., 1997).

Since COX-2 levels were increased following DHT treatment in the absence of inflammation, I predicted that DHT would also increase COX-2 levels in the presence of induced inflammation. Surprisingly, DHT actually attenuated COX-2 levels in the presence of either cytokine or endotoxin stimulation. The AR antagonist bicalutamide was unable to block this effect of DHT. Thus, it appears that in the absence of an inflammatory stimulus DHT acts via the classical AR to increase COX-2 levels, but in the presence of cytokine induced inflammation DHT acts via an AR-independent mechanism to reduce COX-2 levels.

Other reports have demonstrated AR-independent actions of androgens in the vasculature and in non-vascular tissues. Aside from the AR, DHT can activate the sex hormone binding globulin receptor to increase cyclic AMP and protein kinase A (N. Fortunati et al., 1996; A. M. Nakhla and W. Rosner, 1996). DHT can also increase intracellular calcium via an unidentified membrane-bound receptor (W. P. Benten et al., 1999b; W. P. Benten et al., 1999a). Furthermore, several authors have also hypothesized that a membrane bound AR may exist that is not blocked by classical AR antagonists (E. F. Konoplya and E. H. Popoff, 1992; W. P. Benten et al., 1999b; W. P. Benten et al., 1999a; C. A. Heinlein and C. Chang, 2002).

Since DHT altered COX-2 levels in both the absence and presence of induced inflammation, I predicted that the production of COX-2 derived prostaglandins would be similarly altered. I chose to measure PGE_2 production since others have shown that this particular COX-2 product is associated with vascular inflammation (F. Cipollone et al., 2004). Surprisingly, DHT did not affect PGE_2 levels in the absence or presence of IL-1β-induced inflammation. This result could have been due to increases in COX-1 activity that arise in compensation for the decreased COX-2 protein levels; however, my data show that COX-1 protein levels were decreased by DHT in the presence of IL-1β, making compensatory COX-1 activity seem unlikely. Since PGE_2 is not the only COX-2 derived end-product, further studies will be investigated to determine which end-product (prostacyclin, $PGF_{2\alpha}$ or PGD_2) may be altered by DHT treatment.

In this study, I investigated the responses of DHT on the COX-2 pathway using primary human cultured cells. Although more clinically relevant compared to rodent studies, the use of cultured cells does provide certain limitations. First, each cell type originated from a single donor. Thus, one could argue that the differences observed in the magnitude of the COX-2 response to IL-1β or LPS between the smooth muscle and endothelial cells could have been due to inter-donor variability. However, recent preliminary studies in primary human brain vascular smooth muscle cells originating from a different donor also respond to IL-1β stimulation with a greater increase in COX-2 expression compared to that observed in the human brain endothelial cells (Osterlund and Gonzales, unpublished). Furthermore, I believe that the effects I observed with DHT in the HCAVSM cells are not due to donor

variability. In separate studies using human brain vascular smooth muscle cells similar to that reported in HCAVSM, DHT blunted increases in COX-2 levels following IL-1β stimulation (Osterlund and Gonzales, unpublished). Because similar findings were observed in rodent cerebral arteries (R. J. Gonzales et al., 2009) it is unlikely that the effect of DHT on COX-2 levels is species specific. The second major limitation of using cultured cells is that their properties change over time. For example, smooth muscle cells quickly lose their contractile properties. It is possible that receptor levels for androgens, cytokines, and endotoxins may also change in culture. To minimize the risk of changes in culture, all experiments were performed after a small number of passages (5 or 6). Furthermore, at the time of experiments the smooth muscle cells still expressed the smooth muscle cell markers smoothelin and alpha actin, while the endothelial cells still expressed the endothelial cell marker Von Willebrand Factor (data not shown).

Androgens may be both cardioprotective and anti-inflammatory. For example, low levels of testosterone (T) are associated with coronary artery disease (K. M. English et al., 2000b), hypertension, adverse lipid profile, and pro-coagulable factors (K. M. English et al., 1997). Furthermore, androgens have been shown to suppress the activity of pro-inflammatory cytokines, while enhancing the activity of anti-inflammatory factors in an induced inflammatory environment (C. J. Malkin et al., 2003). For example, androgens have been shown to decrease IL-1 production in human monocytes (Z. G. Li et al., 1993), decrease LPS-induced TNFα, IL-1, and IL-6 levels in mouse macrophages (D. A. Padgett and R. M. Loria, 1998), decrease TNFα-induced VCAM-1 and NFκB expression in human aortic endothelium (H.

Hatakeyama et al., 2002), and decrease LPS and TNFα-induced VCAM-1, IL-6, MCP-1, TLR4, and COX-2 mRNA expression in human umbilical vein endothelial cells (G. D. Norata et al., 2006). Furthermore, T has been shown to enhance proliferation of human vascular smooth muscle cells (M. R. Williams et al., 2002), potentially helping to maintain the fibrous cap of atherosclerotic plaques (C. J. Malkin et al., 2003). Accordingly, castration of male rabbits has been shown to increase aortic atheroma by 100%, an effect which is inhibited by T replacement (P. Alexandersen et al., 1999). T replacement has also been used in clinical settings to relieve symptoms of angina (K. M. English et al., 2000a) and has also been show to decrease IL-1β and TNFα levels in men (G. D. Norata et al., 2006). Although many of these studies used the aromatizable androgen, T, in their experimental design I used the more potent AR agonist, DHT and noted similar findings. Thus, these data, along with my present data, suggest that androgen therapy might protect against vascular inflammation in men already predisposed to cardiovascular events.

By contrast to the beneficial effects of androgens, androgenic effects on inflammation remain controversial since some studies have shown pro-inflammatory effects of androgens (A. K. Death et al., 2004; A. Razmara et al., 2005; R. J. Gonzales et al., 2009), as well as detrimental effects of androgens on lesions size following cerebral ischemia (T. Hawk et al., 1998; J. Cheng et al., 2007). For example, the effects of androgens on outcome after cerebral ischemia are both age and dose-dependent. DHT was found to be protective at a low physiological dose or in aged male rodents, but detrimental if administered at a high dose or in young male rodents (J. Cheng et al., 2008; M. Uchida et al., 2009). The present data

suggest a similar story for vascular smooth muscle cells where vascular inflammatory actions of androgens may also be condition-dependent, with differing effects in the absence and presence of induced inflammation.

In summary, I have shown that DHT increases COX-2 protein levels in the absence of induced inflammation via an AR-dependent mechanism, but attenuates IL-1β-induced increases in COX-2 levels via an AR-independent mechanism in primary human coronary artery HCASMC. The findings suggest that DHT may be pro-inflammatory by augmenting COX-2 levels under physiological conditions, but anti-inflammatory by attenuating COX-2 under pathophysiological conditions. Surprisingly, the effects of DHT on COX-2 levels did not translate to changes in PGE_2 production although other COX-2 derived end-products (prostacyclin, $PGF_{2\alpha}$ or PGD_2) may prove to be androgen targets. Lastly, I found that HCASMC are more responsive (i.e. increases in COX-2) to IL-1β or LPS stimulation than vascular endothelial cells. Investigations of mechanisms associated with inflammatory effects on human vascular smooth muscle cells have been largely ignored in the literature in favor of the more commonly studied vascular endothelial cells. Thus, I have identified human vascular smooth muscle cells as potentially important targets for androgens in the vascular response to inflammation. This is of particular importance because investigating the mechanisms by which androgens modulate vascular inflammation may lead to better therapeutic targets for cardiovascular diseases such as atherosclerosis, myocardial infarction, and stroke.

Figures and Legends

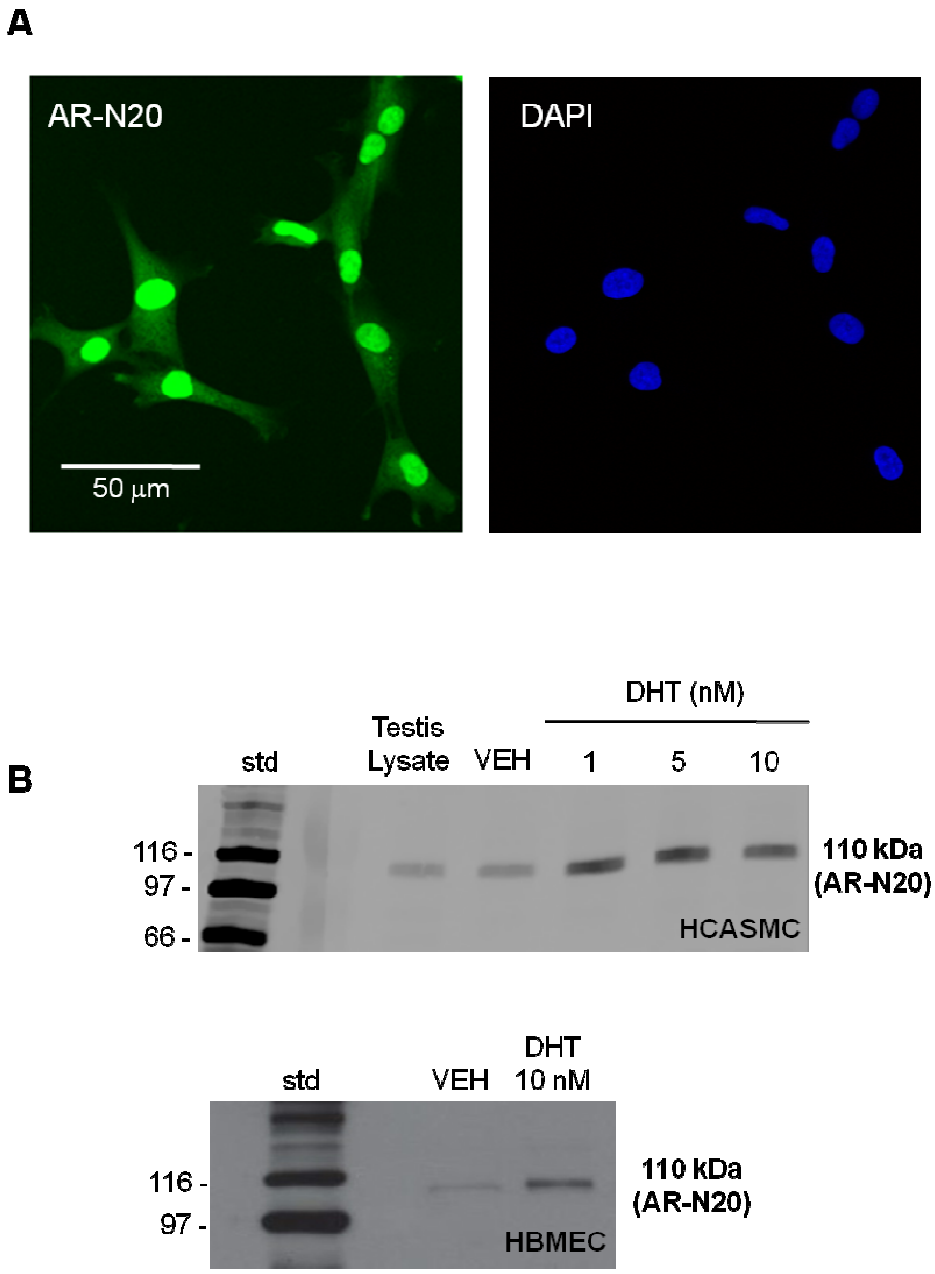


Figure 3.1. Androgen receptors (AR) are present in HCASMC and HBMEC. A) AR antibody and fluorescent secondary labeling detected the presence of AR in the cytoplasm and nucleus of HCASMC (green). DAPI was used to verify nuclear boundaries (blue). B) Representative Western blot of whole cell homogenates from DHT (6 h; 1, 5, or 10 nM) and vehicle (VEH) treated HCASMC (top) and HBMEC (bottom) labeled with AR antibody. Mouse testis lysate served as positive control. Scale bar = 50 microns.

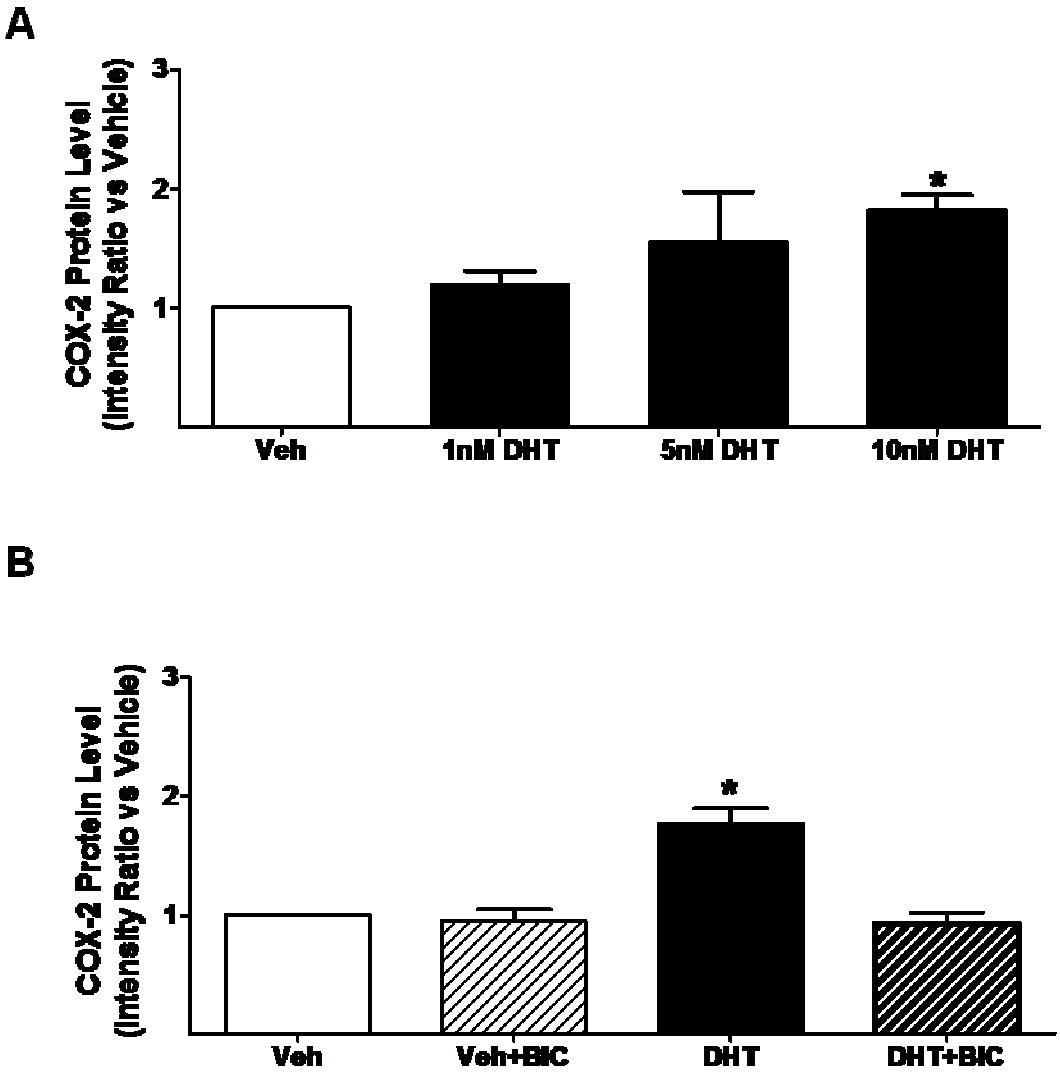


Figure 3.2. DHT increased COX-2 levels and this response was AR-dependent. A) Western blot analysis of COX-2 levels in response to 6 h treatment with vehicle or DHT (1, 5 or 10 nM). B) Western analysis of COX-2 levels in vehicle (Veh), Veh +bicalutamide (Bic; 1μM), DHT (10 nM) and DHT+Bic treated HCASMC. *P<0.001 vs. vehicle (Veh n=9; Veh+Bic n=9; DHT n=18; DHT+Bic n=9).

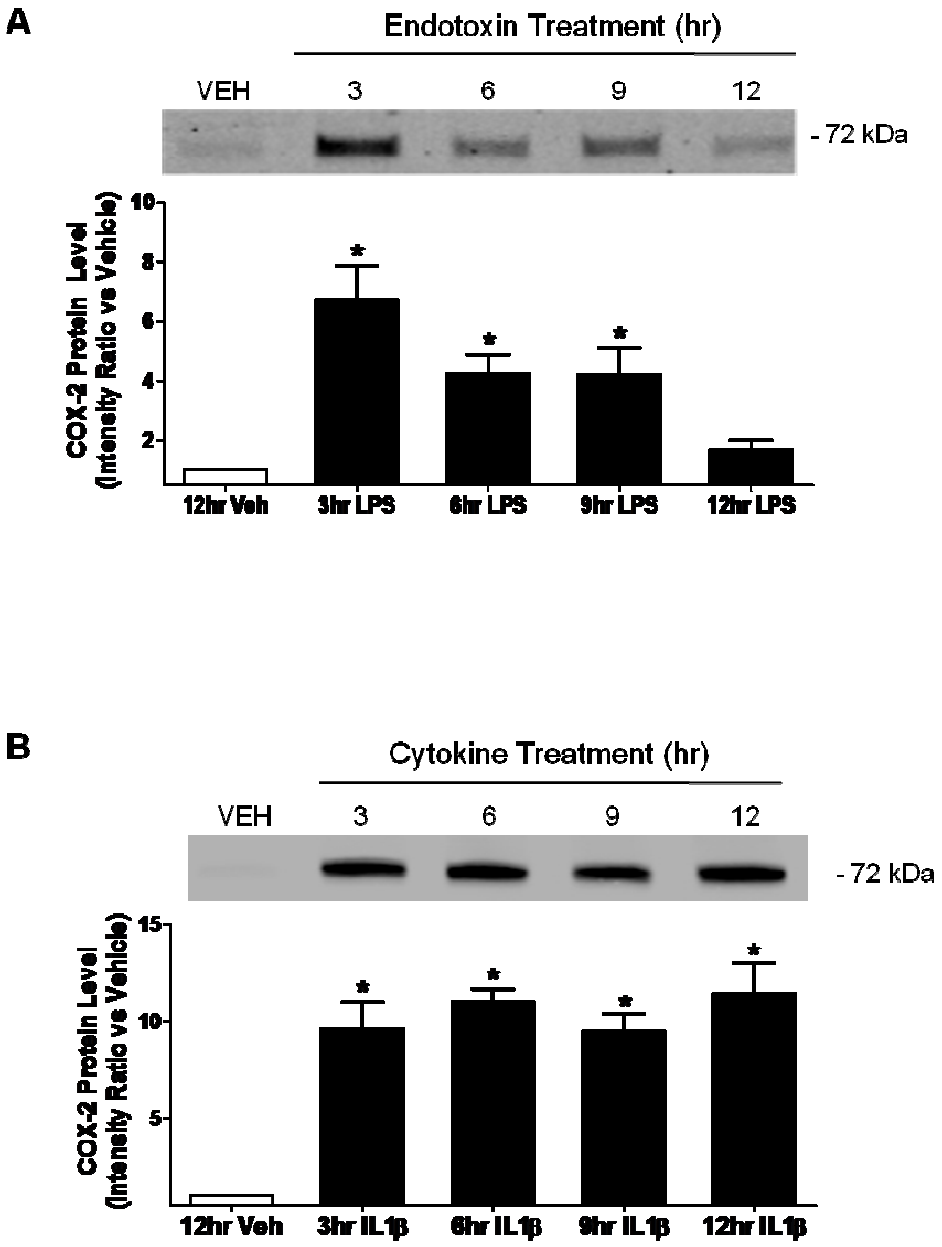


Figure 3.3. LPS or IL-1B increased COX-2 levels in HCASMC. A) Top Insert: Representative Western blot of COX-2 protein levels in HCASMC treated with vehicle (12 h) or LPS (100 µg/ml; 3, 6, 9, 12 h). Western analysis of a time course for COX-2 protein levels following LPS treatment (100 µg/ml) at 3 h (n=3), 6 h (n=9), and 9 h (n=3), and 12 h (n=3). B) Top Insert: Representative Western blot of COX-2 protein levels in HCASMC treated with vehicle (12 h) or IL-1β (3, 6, 9, 12 h). Western analysis of a time course for COX-2 levels following IL-1β treatment (5 ng/ml) at 3 h (n=3), 6 h (n=14), 9 h (n=3), and 12 h (n=3). *P<0.01 vs. 12 h vehicle.

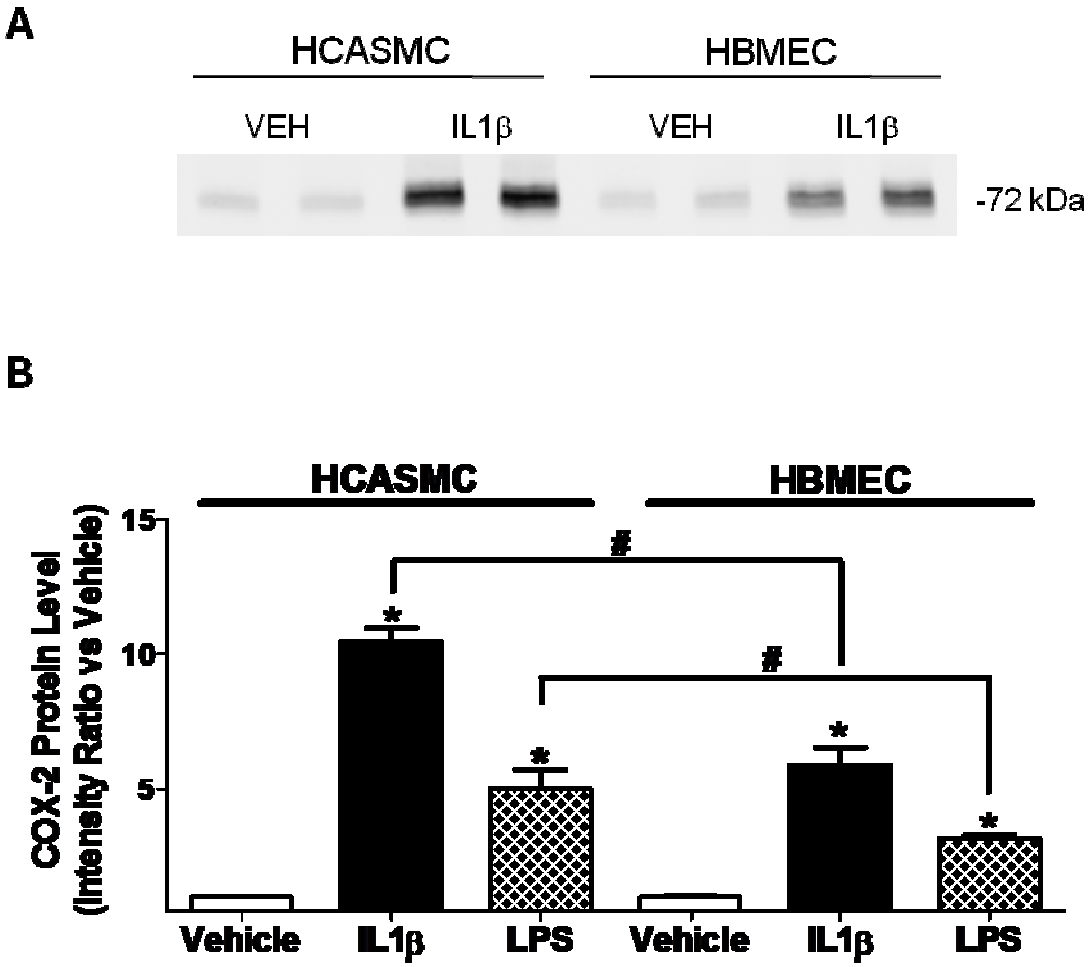


Figure 3.4. COX-2 protein levels in response to cytokine stimulation or endotoxin in HCASMC and HBMEC. A) Representative Western blot of COX-2 protein levels in HCASMC (n=4) and HBMEC (n=4) treated for 6 h with vehicle or IL-1β (5 ng/ml). B) Western analysis of COX-2 following 6 h treatment with IL-1β (5 ng/ml) or LPS (100 μg/ml) in HCASMC and HBMEC. *P<0.001 vs. vehicle. (n=4 per group).

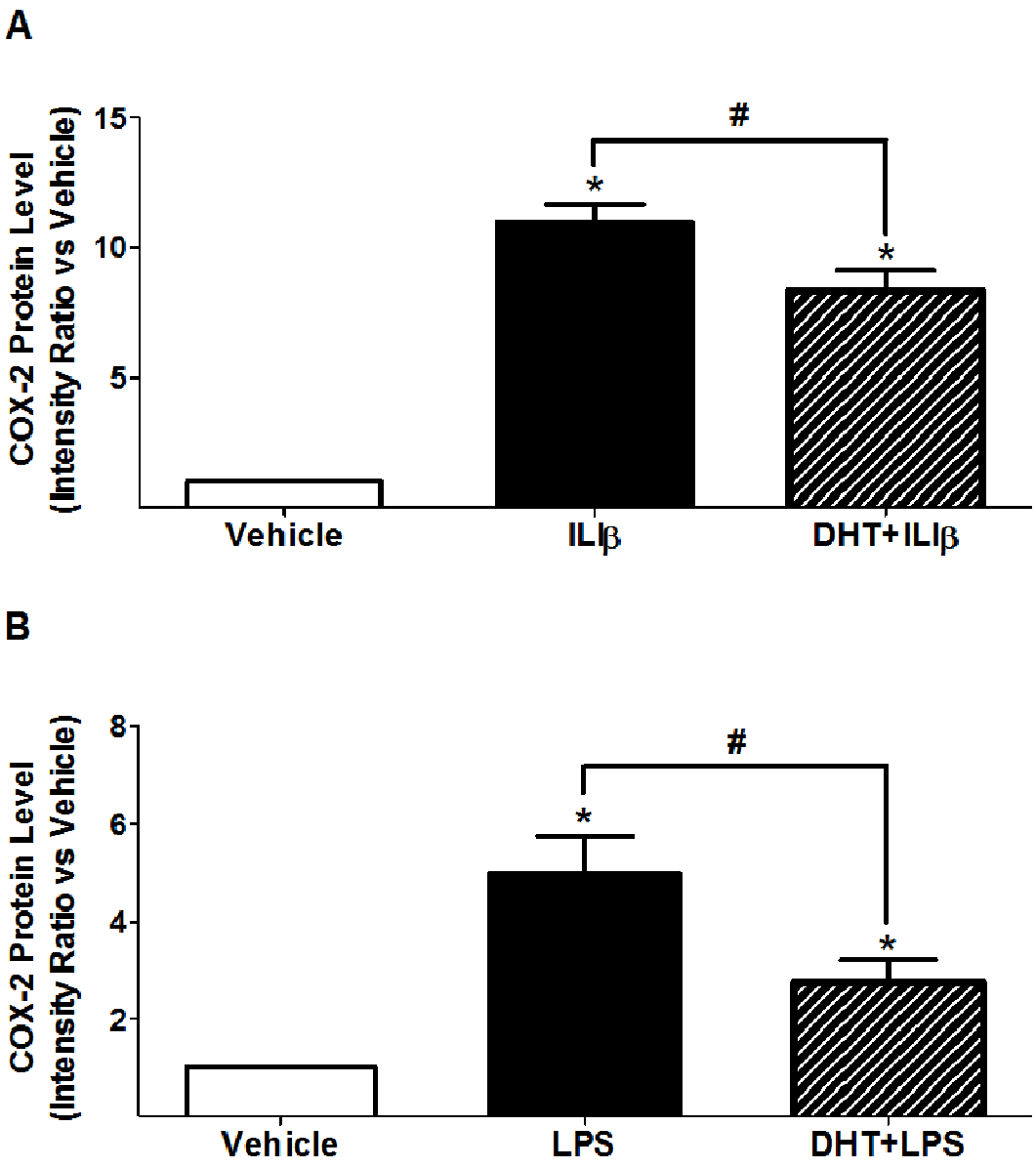


Figure 3.5. DHT attenuated cytokine and endotoxin induced increases in COX-2. A) Western analysis of COX-2 levels in response to 6 h treatment with vehicle, IL-1β (5 ng/ml), or IL-1β+DHT (5 ng/ml and 10 nM respectively) in HCASMC. B) Western analysis of COX-2 levels in response to 6 h treatment with vehicle, LPS (100 µg/ml) or LPS+DHT (100 µg/ml and 10 nM respectively) in HCASMC. *P<0.01 vs. vehicle (Veh n=18, IL-1β n=14, IL-1β+DHT n=17, LPS n=6, LPS+DHT n=4).

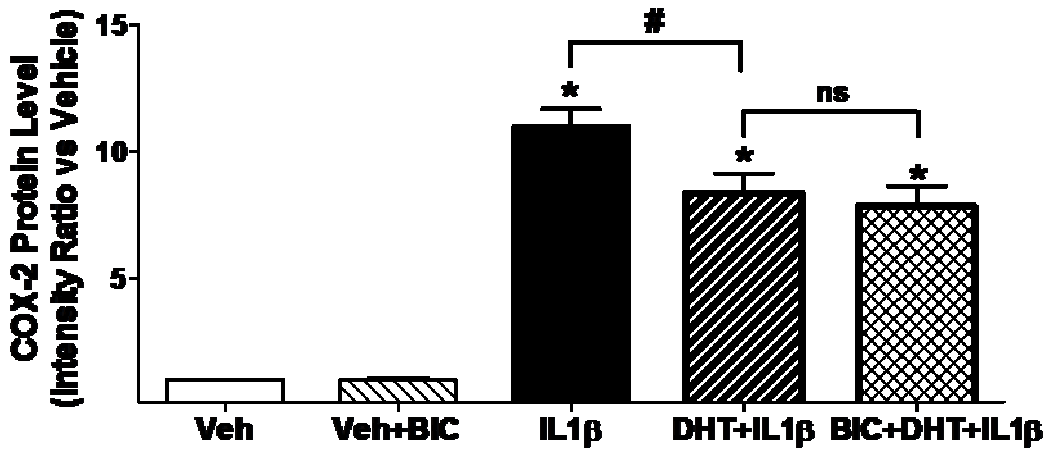


Figure 3.6. DHT attenuated cytokine induced increases in COX-2 via an AR-independent mechanism**.** Western analysis of COX-2 levels in response to 6 h treatment with vehicle, vehicle + bicalutamide (1 μM), IL-1β (5 ng/ml), IL-1β+DHT (5 ng/ml and 10 nM respectively), or IL-1β+DHT+bicalutamide (5 ng/ml, 10 nM, 1 μM respectively) in HCASMC. *P<0.01. (Veh n=18, Veh+Bic n= 9, IL-1β n=14, IL-1β+DHT n=17, IL-1β+DHT+Bic n=9).

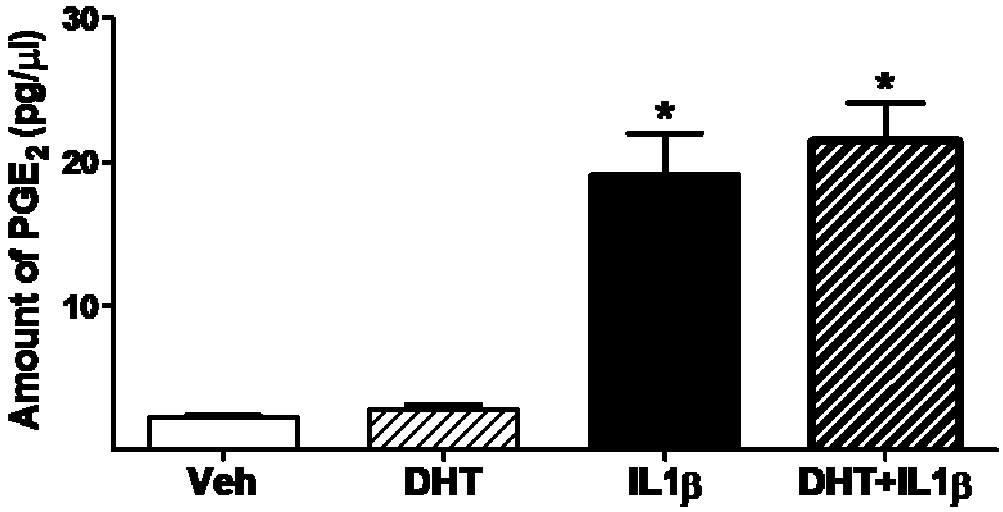


Figure 3.7. DHT did not alter PGE_2 production**.** ELISA analysis of PGE_2 production following 6 h treatment with vehicle, DHT (10 nM), IL-1β (5 ng/ml), or IL-1β+DHT (5 ng/ml and 10 nM respectively) in HCASMC. *P<0.001. (Veh n=3, DHT n=6, IL-1β n=3, IL-1β+DHT n=3).

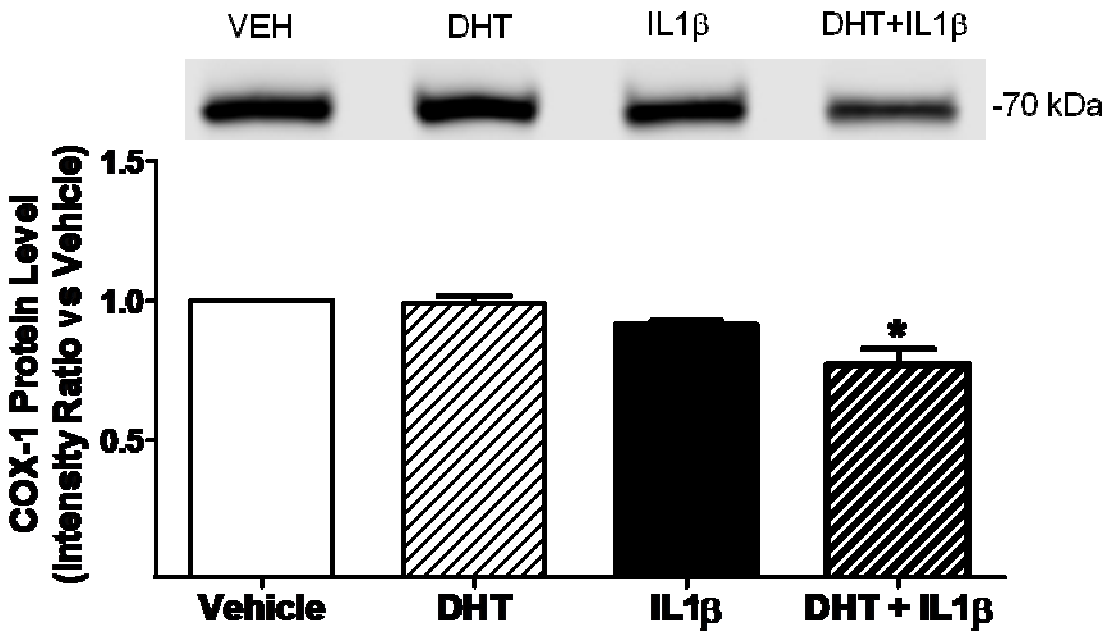


Figure 3.8. COX-1 levels are not increased by DHT. Top insert: Representative western blot of COX-1 levels in HCASMC treated for 6 h with vehicle, DHT (10 nM), IL-1β (5 ng/ml) or IL-1β + DHT. Bottom graph: Western analysis of COX-1 levels in HCASMC following 6 h treatment with vehicle, DHT (10 nM), IL-1β (5 ng/ml), or IL-1β+DHT (5 ng/ml and 10 nM respectively). *P<0.05. (n=4 per group).

CHAPTER 4: DIHYDROTESTOSTERONE ATTENUATES HIF-1ALPHA AND CYCLOOXYGENASE-2 IN CEREBRAL ARTERIES DURING HYPOXIA OR HYPOXIA WITH GLUCOSE DEPRIVATION

Abstract

My previous studies demonstrate that dihydrotestosterone (DHT) attenuates cytokine-induced cyclooxygenase-2 (COX-2) in coronary vascular smooth muscle. Since hypoxia inducible factor-1alpha (HIF-1α) activation can lead to production of COX-2, this study determined the influence of DHT on HIF-1α and COX-2 following hypoxia or hypoxia with glucose deprivation (HGD) in the cerebral vasculature. COX-2 and HIF-1α levels were assessed via Western blot and HIF-1α activation was indirectly measured via a DNA binding assay. Experiments were performed using cerebral arteries isolated from castrated male rats treated *in vivo* with placebo or DHT (45 mg/21 day pellets; 18 days) followed by hypoxic exposure *ex vivo* (1% O_2) or using cerebral arteries isolated from castrated male rats treated *ex vivo* with vehicle or DHT (10 or 100 nM) then exposed to hypoxia *ex vivo* (1% O_2). Additional studies were performed on primary human brain vascular smooth muscle cells treated with DHT (10 nM) or vehicle then exposed to hypoxia or HGD. Under normoxic conditions, DHT increased COX-2 ($p<0.05$) but had no effect on HIF-1α protein levels or

activation suggesting that the effect of DHT to increase COX-2 under normoxic conditions is independent of HIF-1α. Following hypoxia or HGD, HIF-1α and COX-2 levels were increased ($p<0.05$); this response was blunted by DHT ($p<0.05$) and not reversed by androgen receptor blockade. Hypoxia-induced HIF-1α DNA-binding was also attenuated by DHT ($p<0.001$). These results demonstrate that upregulation of COX-2 and HIF-1α in response to hypoxia is suppressed by DHT via a mechanism independent of androgen receptor stimulation.

Key Words

androgen, hypoxia inducible factor, inflammation, vascular smooth muscle

Background

Despite the greater incidence of stroke in men compared to age-matched premenopausal women (M. Writing Group et al., 2009) and women's poorer outcomes following stroke (R. E. Petrea et al., 2009), clinical studies regarding the effects of sex steroids on cerebral vascular pathophysiology remain a limited area of investigation. However, experimental research has shown that gonadal steroids modulate vascular inflammatory responses during pathological conditions (G. D. Norata et al., 2006; L. Sunday et al., 2006; R. J. Gonzales et al., 2009; K. L. Osterlund et al., 2010). This is of great interest because the cerebral vasculature plays a central role in the pathogenesis of cardiovascular diseases, such as stroke (G. J. del Zoppo and T. Mabuchi, 2003), and in the initiation of inflammation after cerebral ischemia which is a key determinant in stroke outcome (H. C. A. Emsley and P. J. Tyrrell, 2002; G. J. del Zoppo and T. Mabuchi, 2003). Following ischemia, inflammation is initiated by cytokine-induced activation of transcription factors such as nuclear factor kappa B (NFκB) and hypoxia-inducible factor 1- alpha (HIF-1α), leading to increased production of pro-inflammatory mediators, such as inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) (Q. Wang et al., 2007; A. Brooks et al., 2010). HIF-1α plays a particularly important role in cerebral ischemia because it is activated both by cytokines as a result of inflammation, and by low oxygen levels as a result of reduced blood flow (T. Hellwig-Burgel et al., 2005).

While most research has focused on the protective effects of estrogens, the role androgens play in vascular inflammation is not well understood. Both androgens and estrogens are generally credited with anti-inflammatory effects (C. J. Malkin et al., 2003; G. D. Norata et al., 2006; D. Xing et al., 2007; K. L. Osterlund et al., 2010), although some pro-inflammatory effects have been reported for androgens (J. A. McCrohon et al., 1999; A. K. Death et al., 2004). Furthermore, 3β-diol, an estrogen receptor β agonist derived from the potent androgen dihydrotestosterone (DHT), has been shown to reduce levels of inflammatory markers in rhesus monkey vascular smooth muscle cells and human umbilical vein endothelial cells (R. G. Mishra et al., 2006; G. D. Norata et al., 2010). In addition, the androgen, dehydroepiandrosterone, has been shown to decrease HIF-1α accumulation during hypoxia in human pulmonary smooth muscle cells (A. Dessouroux et al., 2008). Clinically, testosterone replacement in androgen-deficient men has been shown to reduce inflammatory cytokines (C. J. Malkin et al., 2004). Furthermore, lower testosterone levels predict increased incidence of stroke in older men, even after adjusting for conventional risk factors for cardiovascular disease (B. B. Yeap et al., 2009). In experimental models of stroke, estrogen has been consistently shown to decrease infarct size, while androgens have been shown to decrease infarct size at low doses, but increase damage at high doses (T. Hawk et al., 1998; M. Uchida et al., 2009). It is possible that these differences in inflammatory markers, stroke incidence, and ischemic outcome are due in part to gonadal steroid regulation of vascular inflammation.

The goal of this study was to determine if the potent androgen receptor agonist, DHT, can influence the vascular inflammatory response during hypoxia. Using both *in vitro* hypoxia and *in vitro* hypoxia with glucose deprivation (HGD, model of some of the cellular insults that occur during ischemia) as well as a hypoxic *ex vivo* intact artery model, this study focused on the effects of DHT on the inflammatory mediator COX-2 during hypoxia and HGD. I chose COX-2 as my marker for vascular inflammation because COX-2 has been shown to be particularly important in cerebral ischemia since COX-2 inhibition can decrease infarct size in experimental models of stroke (K. Sugimoto and C. Iadecola, 2003). Using two model systems, primary human brain vascular smooth muscle cells and isolated rat pial vessels, I examined the hypothesis that DHT would decrease levels of HIF-1α and its transcriptional target, COX-2, during hypoxia or HGD.

Materials and Methods

Animals

Experimental and surgical protocols were approved by the Institutional Animal Care and Use Committees of the University of California, Irvine, and Arizona State University (under subcontract from the University of Arizona). Male Wistar rats (3 mo) used in this study were purchased from Charles Rivers.

Chronic In vivo Dihydrotestosterone Treatment

Intact male rats were anesthetized with isoflurane (1.5%) and gonadectomies performed under aseptic conditions. Immediately following testes removal, placebo or 5α-androstan-17β-ol-3-one (DHT; 45mg/21day) pellets (Innovative Research of America Inc, Sarasota, FL) were implanted subcutaneously and the incision secured with stainless steel staples. Post-surgery rats received a single injection (i.m.) of penicillin (penicillin G benzathine/penicillin G procaine, 30,000 U) and the closure was treated with a topical triple antibiotic ointment. Rats were returned to their home cage and maintained under a 12 hr:12 hr light/dark cycle with fresh water, food, and bedding for recovery. Eighteen days following pellet implants, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and prepared for cerebral vessel isolation. The thoracic cavity was surgically opened and a syringe and needle (21 gauge) was used to puncture the right ventricle for blood collection. Blood samples were immediately processed and serum was stored at -80°C until DHT levels were measured via ELISA (limit of detection of 6 pg/ml; Alpha Diagnostics, San Antonio, TX). Next, while the heart was still beating, heparin (100 U) was immediately injected into the right ventricle and allowed to circulate before the animal was exsanguinated and decapitated. Prior to removing the brain, the entire head was dipped in betadine, wiped with an alcohol pad, and placed in a sterile field. After removal, the brain was rinsed in ice-cold, sterile phosphate buffered saline (PBS) and placed in a sterile Sylgard-coated dissection dish containing PBS on ice. Maintaining a sterile field, pial

artery segments (pial vessels including middle cerebral artery and basilar artery) were dissected microscopically and placed in 12 well plates (vessels from 1 animal per well) containing ice-cold Dulbecco's modified Eagles medium (DMEM) one ice. After collection vessels were transferred to pre-warmed wells containing a 1:1 mixture of DMEM and hormone free Medium 231 (Cascade Biologics; Portland OR). Following a 30 to 40 minute equilibration in an incubator (5% CO_2; 37^{o}C) vessels were transferred to new wells containing fresh pre-warmed media and incubated either in a plexi-glass chamber (BioSpherix; Lacona, NY) supplied with either 21% O_2, 5% CO_2, N_2 balance (normoxic conditions) or 1% O_2, 5% CO_2 and N_2 balance (hypoxic conditions). The percentage of O_2 was monitored using a compact oxygen sensor (ProOx 110; BioSpherix)

Ex vivo Dihydrotestosterone Treatment of Rat Pial Arteries

Male rats, 2-weeks post gonadectomy, were anesthetized with a lethal dose of pentobarbital, exsanguinated and decapitated. Using aseptic technique as described above, brains were removed and placed in ice-cold, sterile PBS. Cerebral arteries (middle cerebral artery, circle of Willis, and basilar) were dissected as described above and then transferred to a 1:1 mixture of DMEM and hormone free Medium 231 (Cascade Biologics). Vessels were placed in an a 5% CO_2 incubator maintained at 37°C and equilibrated for approximately 30 to 40 minutes. Next, vessels were promptly transferred to fresh DMEM: Medium 231 containing DHT (10 or 100 nM) or vehicle (0.001% ethanol) prewarmed to 37°C and either placed in a small hypoxic chamber gassed with 1% O_2, 5% CO_2,

N_2 balance at 37°C or in a separate normoxic chamber gassed with 21% O_2, 5% CO_2, N_2 balance at 37°C. Tissue segments were incubated for 1 h for the HIF-1α DNA binding protocol and 6 h for the HIF-1α protein analysis studies. As described above the level of O_2 (%) was monitored using a compact oxygen sensor.

Tissue Preparation Following Hypoxic Exposure

Following all incubations, vessels were immediately removed from the chamber/incubator, placed in ice-cold lysis buffer and prepared for Western blot or placed in ice-cold nuclear extraction buffer (Active Motif, Carlsbad, CA) and prepared for HIF-1α DNA binding analysis. If feasible, lysates were stored at -80°C until used. For HIF-1α DNA binding studies, the nuclear fraction was isolated according to the manufacturer's instructions and the success of the isolation was confirmed via Western blot using anti-histone-1 (Santa Cruz Biotechnology; Santa Cruz, CA) (data not shown). Protein content for both the ELISA and Western blot was determined by the bicinchoninic acid assay (Pierce, Rockford, IL).

HIF-1α DNA Binding Assay

To assess HIF-1α DNA binding, nuclear lysates or nuclear lysates isolated from pial arteries pre-exposed to cobalt chloride ($CoCl_2$; positive control), were examined using the TransAM HIF-1α immunoassay kit (Active Motif). To initiate the DNA binding assay protocol, lysates (10 µg per well) were added to 96-well

plates labeled with an immobilized oligonucleotide containing the hypoxia response element consensus sequence from the *EPO* gene (5′-TACGTGCT-3′). The presence of HIF was detected following 1 h incubation using an anti-HIF-1α antibody. Secondary horseradish peroxidase antibody and developing solution exposure completed the reaction. Absorbance values as a result of the colorimetric reaction were measured within 5 min using a MultiSkan Spectrum and SkanIt RE software (450 nm wavelength; Thermo Fischer Scientific, Waltham, MA).

Cell Culture and Hormone Treatment

Primary human brain vascular smooth muscle cells (HBVSMC) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and received cryopreserved at passage 1. Cells were futher passaged and grown in a 5% CO_2 incubator/room air at 37˚C in smooth muscle growth medium (SMGM) with Smooth Muscle Growth Supplement containing 2% fetal bovine serum (FBS; ScienCell). Hormone/drug treatments were performed on cells when they reached 80 to 85% confluency and at passage 5 or 6. Hormone/drug treatments were carried out in hormone-free media supplemented with Charcoal Stripped FBS (Invitrogen Corporation, Carlsbad, CA). A 6 h time point for COX-2 induction by an inflammatory stimulus (hypoxia or hypoxia with glucose deprivation) and a 10 nM DHT dose were selected for these studies to investigate the effects of DHT treatment on COX-2 protein levels. Both the time point and the dose were selected based on results from my previous studies

showing that COX-2 protein levels increase 6 h after initiation of an inflammatory stimulus (endotoxin/cytokine) and that 10 nM DHT can inhibit this effect in human coronary artery vascular smooth muscle cells (K. L. Osterlund et al., 2010). Depending on the experiment, cells were treated with DHT (10 nM) or vehicle (0.001% ethanol) for 18 h followed by hypoxia (1% O_2; 6 h) or hypoxia with glucose deprivation (1% O_2; 6 h) using a plexi-glass hypoxic chamber (BioSpherix). For hypoxia with glucose deprivation experiments, normal growth media was replaced with DMEM without glucose (Invitrogen) and then immediately placed in the hypoxic chamber. In a separate set of experiments, cells were pre-treated for 1 h with the androgen receptor (AR)-antagonist bicalutamide (1μM; dissolved in DMSO) followed by 18 h of co-treatment with either vehicle (0.001% ethanol + 0.01% DMSO) or DHT, then 6 h of oxygen-glucose deprivation. A bicalutamide dose of 1μM is effective for AR antagonism in my *in vitro* model as previously shown in my earlier studies (K. L. Osterlund et al., 2010).

Immunocytochemistry

Human Brain Vascular Smooth Muscle Cells:

Cells were plated on sterilized glass cover slips and grown in a 5% CO_2 incubator/room air at 37˚C in SMGM with Smooth Muscle Growth Supplement containing 2% FBS (ScienCell). When cells reached approximately 60% confluency they were divided into groups and culture medium was replaced with IL-1β (5 ng/ml) or $CoCl_2$ (100 μM). Some cells did not receive treatment and

were used to verify the presence of smooth muscle alpha actin and smoothelin. Following incubation or treatment cells were fixed (4% formaldehyde), washed in filtered PBS (pH 7.4), and permeabilized in methanol (-20^{o}C). Next cells were incubated in 2% BSA/PBS to block non-specific binding then incubated with COX-2 (1:200) monoclonal antibody (Cayman Chemical, Ann Arbor, MI), alpha-smooth muscle actin (1:200) monoclonal antibody (Sigma-Aldrich, St. Louis, MO), or smoothelin (1:200) polyclonal antibody (Santa Cruz Biotechnology) in PBS containing 2% BSA over night at 4^{o}C. Next cells were washed in PBS (4 x 5 min), incubated with Alexa Fluor 555 and Alexa Fluor 488 (1:6000; Invitrogen) for 1 h in PBS containing 2% BSA and washed in PBS (4 x 5 min). Cover slips were dabbed for excess PBS and mounted on glass slides using mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) to label nuclei. Cells were visualized using a Zeiss 710 confocal microscope (Carl Zeiss International, Germany). Binding specificity of the secondary antibody was determined in some slides by omitting the primary antibody incubation step. In slides not receiving primary antibody no fluorescence was detected (data not shown).

Rat Pial Arteries:

Using a dissection microscope, pial arteries were dissected from whole brain isolated from intact rats deeply anesthetized with isoflurane vapor (5%/air mixture) and treated with heparin (10 to 15 units/kg) prior to exsanguination. Arteries collected in sterile 24 well plates containing 1:1 mixture of DMEM and SMGM (ScienCell) at room temperature. Next vessels were transferred to fresh

wells containing fresh media and allowed a 30 min equilibration as 37^{o}C. Following the equilibration vessels were treated with $CoCl_2$ (100 μM) and incubated for 3 h. After treatment with $CoCl_2$, arteries were cut into very small segments (approximately 500 μm) and transferred to eppendorf tubes containing filtered PBS containing sodium nitroprusside (SNP; 100 μM) to maximally dilate arteries. Arteries were washed with PBS/SNP for 3 min on a rotator to remove any blood from inside the lumen. Next vessel segments were fixed in 4% formaldehyde for 30 min at room temperature, washed three times (10 min) in PBS then permeabilized with methanol (-20^{o}C; 5 min). Vessels were then washed with 2% bovine serum albumin (BSA) for 5 min and blocked with fresh BSA (2%) for 1 h. Tissues were incubated in HIF-1α (1:500) rabbit polyclonal antibody (Thermo Fisher Scientific, Waltham, MA) over night at 4^{o}C. After incubation, vessel segments were washed (3 x 10 min with filtered 2% BSA) and incubated with a fluorescent secondary antibodies (1:6000; Alexa Fluor 488; Invitrogen) for 1 h at room temperature followed by a final wash (3 x 10 min with filtered BSA) before mounting. Using fine forceps, small pial segments were arranged on microscope slides that were cover slipped with VectaShield mounting medium (Vector Laboratories) then sealed with fingernail polish. Images were obtained using a Carl Zeiss LSM 710 confocal microscope (Carl Zeiss International).

Western Blot

Levels of HIF-1α and COX-2 protein were examined using standard immunoblotting methods, as previously described (K. L. Osterlund et al., 2010). Briefly, cells/pial arteries were homogenized in lysis buffer and total protein content of whole cell lysate was determined. Next, samples were diluted in Tris-Glycine SDS sample buffer (Invitrogen) and heated to boiling for 5 min. Two color fluorescent standard (LI-COR Biosciences, Lincoln, NE) and diluted samples were loaded into 7.5% Smart gels (LI-COR). Proteins were separated via SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes and non-specific binding was blocked by incubation at room temperature for 30 min in PBS containing 1% Tween (TPBS) and 3% nonfat dried milk (Carnation, Nestle, Wilkes-Barre, PA). Membranes were incubated in COX-2 (1:1000) mouse monoclonal antibody (Cayman Chemical, Ann Arbor, MI), HIF-1α (1:1000) rabbit polyclonal antibody (Thermo Fisher Scientific), and β actin (1:5,000) mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO) overnight at 4˚C in TPBS. Following TPBS washes, the membranes were incubated in Goat Anti-Mouse IR 800 Dye or Goat Anti-Rabbit IR 680 Dye secondary antibodies (LI-COR) for 1 h at room temperature. COX-2 antibody specificity was verified with lipopolysaccharide and phorbol myistate acetate stimulated mouse macrophage cell lysate (Raw-264.7, Santa Cruz Biotechnology, Santa Cruz, CA), which is a positive control for COX-2 protein (data not shown). HIF-1α antibody specificity was verified with cobalt chloride stimulated HBVSMC, which is a positive control for HIF-1α protein (Figure 4.6B).

Following additional TPBS washes, proteins were visualized using an Odyssey Infrared Imager and data was analyzed using Odyssey V3.0 software (LI-COR).

Reagents

All reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise noted.

Statistical Analysis

For data from Western analysis, samples from each treatment were run on the same blot for direct comparison and treatments were repeated for statistical analysis ($n \geq 4$). Data from Western blots are expressed as an optical density ratio relative to vehicle and normalized to the optical density values for β actin bands (for human cells) or GAPDH bands (for rodent tissue). Multiple vehicles were run on the same gel and normalized to the first vehicle on the gel so that variance within vehicle groups was accounted for. Each gel contained at least one vehicle for comparison to the treatment groups. This method of normalizing to both a vehicle and loading control limits variance between blots since the fluorescent detection system is very sensitive and each figure represents data obtained from 4-8 different gels. For HIF-1α DNA binding assay data, values are reported as optical density at 450 nm wavelength. All values are reported as means ± SEM. Data were compared using analysis of variance (ANOVA), and group means were compared using post hoc tests (Student-Newmans-Keuls; Prism Software, Irvine, CA). A level of $p<0.05$ was considered significant.

Results

Dihydrotestosterone treatment in vivo increased blood serum DHT levels in gonadectomized rats.

Gonadectomy resulted in a decrease in DHT serum levels (91±10 pg/ml) compared to values measured in intact male rats (941±32 pg/ml). In contrast, gonadectomized rats receiving replacement DHT pellets had comparable DHT serum levels (1089±39 pg/ml) that were not significantly different from the DHT serum levels measured in intact males.

Chronic in vivo DHT treatment increases COX-2 under normoxic conditions and inhibits hypoxia-induced COX-2 levels following hypoxic exposure in rat pial arteries.

To determine the effect of long-term DHT treatment on COX-2 protein levels following hypoxia, rat pial arteries were isolated from gonadectomized male rats treated *in vivo* with DHT (45mg pellet, 18 days) or placebo (PLB) followed by 6 h of normoxia (21% O_2) or hypoxia (1% O_2) *ex vivo* in continued presence of hormone (Figure 4.1). Figure 4.1A illustrates a representative Western blot of COX-2 levels from each group tested and figure 4.1B illustrates the data analysis. Under simulated normoxic conditions COX-2 protein levels were increased in pial arteries following DHT administration *in vivo* compared to arteries isolated from the placebo treated rats (PLB). In addition, hypoxic exposure also resulted in an increase in COX-2 protein levels in rat pial arteries. DHT administration *in vivo* blocked hypoxia-induced increases in COX-2 protein

levels. Vessels from rats treated with DHT and then exposed to hypoxia *ex vivo* had similar COX-2 protein levels to vessels from placebo treated rats that were exposed to normoxia (Figure 4.1B).

***Ex vivo DHT treatment increases COX-2 under normoxic conditions and decreases COX-2 following hypoxia in rat pial arteries*.**

To simplify the complex steroid metabolism *in vivo,* a set of experiments were designed to determine the effect of *ex vivo* DHT treatment on COX-2 protein levels following hypoxia. Pial arteries were isolated from gonadectomized male rats treated *ex vivo* with DHT (10 and 100 nM) or vehicle (VEH) followed by 6 h of normoxia (21% O_2) or hypoxia (1% O_2) *ex vivo* in continued presence of hormone. Figure 4.2A illustrates a representative Western blot of COX-2 levels from each group tested and figure 4.2B illustrates the data analysis. The higher dose of DHT (100 nM) administered *ex vivo* increased COX-2 levels under normoxic conditions compared to vehicle treated pial arteries. During hypoxia, COX-2 was significantly increased compared to the normoxic vehicle control. This increase in hypoxia-induced COX-2 levels was significantly blunted by DHT 100 nM but not 10 nM in rat pial arteries.

DHT blunted hypoxia-induced nuclear HIF-1α activation in rat pial arteries.

To determine the effect of DHT treatment on HIF-1α activation, rat pial arteries were treated *ex vivo* with vehicle or DHT (10 nM; 6 h) or isolated from gonadectomized rats treated *in vivo* with vehicle or DHT (45mg pellet; 18 days)

and then exposed to *ex vivo* to normoxia (21% O2, 1 h) or hypoxia (1%O_2; 1 h) in the continued presence of hormone. Under normoxic conditions, HIF-1α DNA-binding was below the level of detection in vessels treated with vehicle or DHT (Figure 4.3A and B). Hypoxia increased HIF-1α DNA binding as predicted. However, DHT treatment in the presence of hypoxia, both *ex vivo* and *in vivo*, decreased HIF-1α DNA-binding compared to hypoxia alone.

COX-2 is expressed in HBVSMC following cytokine stimulation.

Before I assessed the effects of DHT and hypoxia on COX-2 levels in the human brain vascular smooth muscle cell (HBVSMC) model, I first assessed the induction of COX-2 using a known stimulus of the proinflammatory mediator via immunocytochemistry. I confirmed the induction of COX-2 (red) in response to IL-1β (5 ng/ml; 3h) shown in figure 4.4A. Also shown in figure 4.4A are panels of nuclei labeling with DAPI (blue) and anti-β-actin (green) to demonstrate nuclear/cell borders. In separate experiments, markers for vascular smooth muscle were also assessed (Figure 4.4B). HBVSMC at passage 6 expressed both α smooth muscle actin (green) and smoothelin (red) favoring a smooth muscle cell phenotype. In addition, these cells also expressed AR (data not shown).

DHT differentially modulates COX-2 during normoxia and hypoxia in HBVSMC.

To determine the effect of DHT treatment on COX-2 protein levels during normoxic and hypoxic conditions, HBVSMC were treated with vehicle or DHT (10 nM; 18 h) followed by 6 h of normoxia (21% O_2) or hypoxia (1% O_2) in continued presence of hormone (Figure 4.5). A representative Western blot for COX-2 in all the treatment groups is illustrated in figure 4.5A. Figure 4.5B represents the data analysis for COX-2 protein levels in HBVSMC. Under normoxic conditions, DHT increased COX-2 protein levels in HBVSMC. Similarly, hypoxia also increased COX-2 protein levels in HBVSMC. DHT blocked the effect of hypoxia to increase COX-2 protein levels since COX-2 levels were not significantly different between the normoxia vehicle group and the DHT hypoxia group (ANOVA).

DHT blunted hypoxia-induced increases in HIF-1α protein in HBVSMC.

In order to visualize localization of HIF-1α protein in the cerebral vasculature, rat pial arteries and HBVSMC cells were labeled with anti-HIF-1α antibody and assessed via confocal microscopy. HIF-1α localization was confirmed in rat cerebral arteries (Figure 4.6A) and HBVSMC (Figure 4.6B) following $CoCl_2$ induction *in vitro*. Labeling with anti-HIF-1α was detected in the nuclear fraction of both the endothelial (left panel) and vascular smooth muscle (right panel) layer (Figure 6A). In order to determine the effect of DHT treatment on hypoxia-induced HIF-1α in HBVSMC, cells were treated with vehicle or DHT

(10 nM, 18 h) followed by normoxia (21%O_2; 6 h) or hypoxia (1%O_2; 6 h) in the continued presence of hormone or vehicle and changes in protein levels were measured via Western blot. Under normoxic conditions, DHT treatment did not alter HIF-1α levels compared to vehicle (Figure 4.6C). However, as predicted, HIF-1α protein levels were increased following hypoxia, this increase in HIF-1α protein is likely due to increased HIF-1α stabilization due to inhibition of oxygen-sensitive hydroxylases that normally phosphorylate HIF and target it for degradation by the proteosome. This hypoxic-induced increase in HIF-1α levels were blunted in the presence of DHT. $CoCl_2$ is reported as the positive indicator for HIF-1α stabilization using Western blot.

DHT blunted HGD-induced increases in COX-2 and HIF-1a levels in HBVSMC via an AR-independent mechanism.

In order to more closely model some of the cellular insults that occur during ischemic stroke, such as reduced oxygen and nutrient availability due to reduced blood flow, hypoxia with glucose deprivation (HGD) was used to determine the effects of DHT on COX-2 (Figure 4.7A) and HIF-1α (Figure 4.7B) levels during hypoxic stress. In addition, the AR antagonist bicalutamide was used to determine AR involvement. HBVSMC were treated with vehicle or DHT (10 nM; 18 h) in the absence or presence of the AR antagonist bicalutamide (1μM; 1 h pretreatment + 18 h co-treatment with DHT) followed by HGD (1%O_2; 6 h) in the continued presence of hormone. Bicalutamide alone had no effect on COX-2 or HIF-1α protein levels. HGD increased both COX-2 and HIF-1α protein

levels as expected. During HGD, DHT decreased COX-2 and HIF-1α protein levels compared to HGD alone. Interestingly, the AR antagonist bicalutamide did not block the effect of DHT on COX-2 or HIF-1α protein levels.

Discussion

The goal of this study was to assess the effects of DHT on vascular inflammation during hypoxic stress in the cerebral vasculature. Using COX-2 as marker for vascular inflammation, I determined the effects of the potent androgen, DHT, on COX-2 levels during hypoxia in rat pial arteries and primary human brain vascular smooth muscle cells, human cells were also exposed to hypoxia with glucose deprivation to more closely model some of the cellular insults that occur during ischemia. I found that DHT increased COX-2 levels under normoxic conditions, but decreased COX-2 levels during hypoxic conditions or following *in vitro* ischemia. Furthermore, since HIF-1α is a transcription factor controlling COX-2 levels and it is stabilized/ activated during hypoxia or ischemia, I tested the hypothesis that changes in HIF-1α activation or levels would correlate with changes in COX-2 levels. My results demonstrate that, like COX-2 levels, HIF-1α activation and protein levels were reduced by DHT treatment during hypoxia or HGD, whereas under normoxic conditions, DHT did not alter HIF-1α. Using the HGD model, I also demonstrated that DHT's effects on COX-2 and HIF-1α levels are AR-independent. Thus, it appears that during conditions of hypoxic stress DHT has an anti-inflammatory effect that may possibly be mediated via HIF-1α, but not via activation of the AR.

To my knowledge, this is the first report of DHT's effects on vascular inflammation during hypoxia or hypoxia with glucose deprivation in the cerebral vasculature. Previously, I have shown that under normal physiological conditions DHT increases COX-2 levels in both rat pial arteries (R. J. Gonzales et al., 2009) and human coronary artery smooth muscle cells via an AR-dependent mechanism (K. L. Osterlund et al., 2010). The current data also support a pro-inflammatory role for DHT under normal physiological conditions, and add to the current literature by identifying human brain vascular smooth muscle as a target for androgens. In contrast, I have also previously shown that during cytokine-induced inflammation DHT decreases COX-2 protein levels via an AR-independent mechanism (K. L. Osterlund et al., 2010). My *in vitro* data point to vascular smooth muscle cells as important mediators of this response to DHT, however, it is likely that the endothelial cells also play an important role in the response I observed in the rat pial arteries. For instance, Norata et. al. has shown that DHT also attenuates proinflammatory markers during cytokine-induced inflammation in human endothelial cells (G. D. Norata et al., 2006). The current data show similar patterns during the pathophysiological conditions of hypoxic stress in both rodent pial arteries and human brain vascular smooth muscle cells. Interestingly, the dose of DHT needed to attenuate COX-2 or HIF-1α levels was higher in the rat pial vessels (100 nM) compared to the human vascular smooth muscle cells (10 nM). Whether this difference in sensitivity to DHT is due to species differences, culture differences, or the presence of

endothelial cells is not known, but would be interesting to pursue in future investigations.

Coupling my current findings with previous results, it appears that DHT may be pro-inflammatory under normal physiological conditions, but anti-inflammatory under a variety of pathophysiological conditions. An intriguing explanation for this paradox is that DHT is working through different molecular mechanisms during normal compared to pathophysiological conditions. I have identified two different pathways that are candidates for these differences.

First, the current data show that DHT only affects HIF-1α levels under hypoxia or hypoxia with glucose deprivation, not under normoxia. Therefore, it is possible that DHT has differing actions during normoxia and hypoxia due to interactions with the HIF-1α pathway. Aside from being a transcription factor in the pathway leading to COX-2 levels, HIF-1α also participates in considerable cross-talk with the COX-2 pathway. For example, under normoxia the COX-2 end product PGE_2 activates its EP1 receptor to increase HIF-1α levels in human embryonic kidney cells (R. Ji et al., 2010). During hypoxia, HIF-1α binds to the toll-like receptor 4 (TLR4) promoter causing upregulation of TLR4. This enhances the response of macrophages to the endotoxin lipopolysaccharide (LPS), resulting in increased COX-2 (K. So Young et al., 2010). Furthermore, LPS and hypoxia increased HIF-1α stabilization in equine digital vein endothelial cells and the effects of the two stimuli in combination were more than additive (A. Brooks et al., 2010). Therefore, it appears that hypoxia may enhance susceptibility to subsequent inflammatory signals and vice versa. Whether the

NFκB pathway plays a role in this response is still debatable. In RAW264.7 cells, hypoxia-induced COX-2 levels was blocked by HIF-1α inhibition, but not by NFκB inhibition (Y.-N. Liu et al., 2009). However, this response was shown to be NFκB-dependent in human vascular endothelial cells (J. F. Schmedtje et al., 1997). A detailed examination of human pulmonary artery smooth muscle cells showed that hypoxia increased NFκB activity and that NFκB inhibition could reduce HIF-1α. Furthermore, mutation of the NFκB binding site in the HIF-1α promoter prevented hypoxia-induced HIF-1α transcription (R. S. BelAiba et al., 2007). Therefore, it seems possible that DHT could be reducing COX-2 levels during hypoxia through changes in HIF-1α activation directly or through changes in NFκB activity. Previous studies have shown that DHT can alter NFκB activity in rat pial arteries under normoxia (R. J. Gonzales et al., 2009), but this has yet to be tested under hypoxic conditions. Since there is considerable cross-talk between HIF-1α and COX-2, DHT could be indirectly decreasing HIF-1α levels via decreases in COX-2 or vice versa, alternatively, DHT could be directly suppressing both HIF-1α and COX-2 levels via independent mechanisms. Future studies are planned to address some of these hypotheses which are currently beyond the scope of this study.

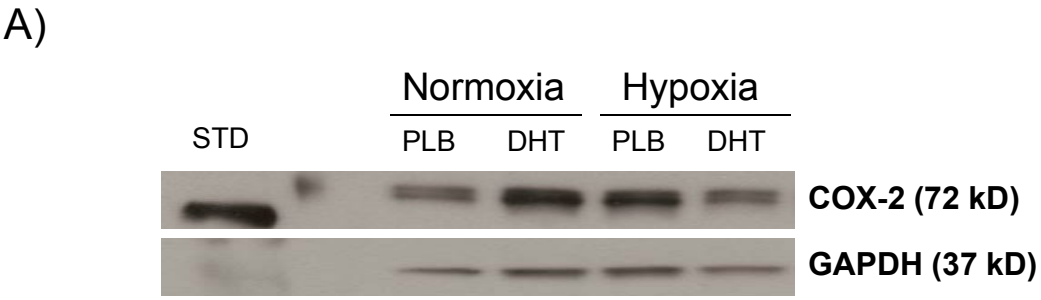
A second explanation for DHT's paradoxical actions is AR involvement. My previous and current results support such a hypothesis since, during normal conditions DHT's effects on COX-2 are AR-dependent (i.e. they can be blocked by treatment with the AR antagonist, bicalutamide); however, during cytokine-induced inflammation or hypoxia with glucose deprivation DHT's effects are AR-

independent. Investigating the effects of androgens on vascular inflammation is complicated by the fact that there are several potential receptor pathways that androgens can activate. Using the classically described mechanism, testosterone can activate the androgen receptor directly or following its conversion by 5 alpha-reductase to the more potent androgen, dihydrotestosterone (DHT) (R. J. Handa et al., 1987; E. D. Lephart et al., 2001). Alternatively, testosterone can be metabolized to estradiol by the aromatase enzyme (C. E. Roselli et al., 1985) and subsequently activate estrogen receptor alpha (ERα) or beta (ERβ) (G. G. Kuiper et al., 1997; G. G. Kuiper et al., 1998). A third and less explored pathway for androgen action is through the conversion of DHT to 5α-androstane-3β,17β-diol (3β-diol), an ERβ agonist (G. G. Kuiper et al., 1996; G. G. Kuiper et al., 1997; Z. Weihua et al., 2002), by the enzymes 3beta-hydroxysteroid dehydrogenase (3β-HSD), 3alpha- hydroxysteroid dehydrogenase (3α-HSD) and 17beta-hydroxysteroid dehydrogenase (17β-HSD)(Y. Jin and T. M. Penning, 2001; Z. Weihua et al., 2002; A. Gangloff et al., 2003; S. Torn et al., 2003; S. Steckelbroeck et al., 2004). Blood vessels contain AR, ERα, ERβ, 5α-R, aromatase, and 3β-HSD (G. D. Snyder et al., 2002; Y. Nakamura et al., 2005; R. J. Gonzales et al., 2007; H.-C. Shih et al., 2008), therefore, the presence of these enzymes and receptors allows for potential androgenic and estrogenic effects which can influence vascular inflammation through a variety of pathways. My current data point to ERβ as a possible alternative receptor pathway for DHT's action during pathophysiological conditions. This is a particularly interesting theory because it has recently been

shown that 3β-diol has anti-inflammatory actions in human umbilical vein endothelial cells and mouse aorta (G. D. Norata et al., 2010).

In summary, I have shown that during hypoxia or hypoxia with glucose deprivation DHT decreased cerebral vascular inflammation by decreasing both COX-2 and HIF-1α levels, likely via an AR-independent mechanism. This is of particular importance because it has been shown that men with low testosterone levels are at increased risk for coronary artery disease, hypertension, and stroke (K. M. English et al., 2000b; B. B. Yeap et al., 2009). Physiologically, circulating androgen levels decrease as men age (H. A. Feldman et al., 2002); therefore, it is important to understand the impact of this decline on vascular function and health. Because very little is known about the role of androgens in diseases such as stroke, I investigated the actions of androgens on cerebrovascular inflammation during pathophysiological conditions of hypoxia or hypoxia with glucose deprivation. A better understanding of how androgens modulate inflammation could potentially provide insight into more effective approaches to manage the progression of vascular diseases in men, which can eventually lead to devastating consequences, such as heart attack or stroke.

Figures and Legends



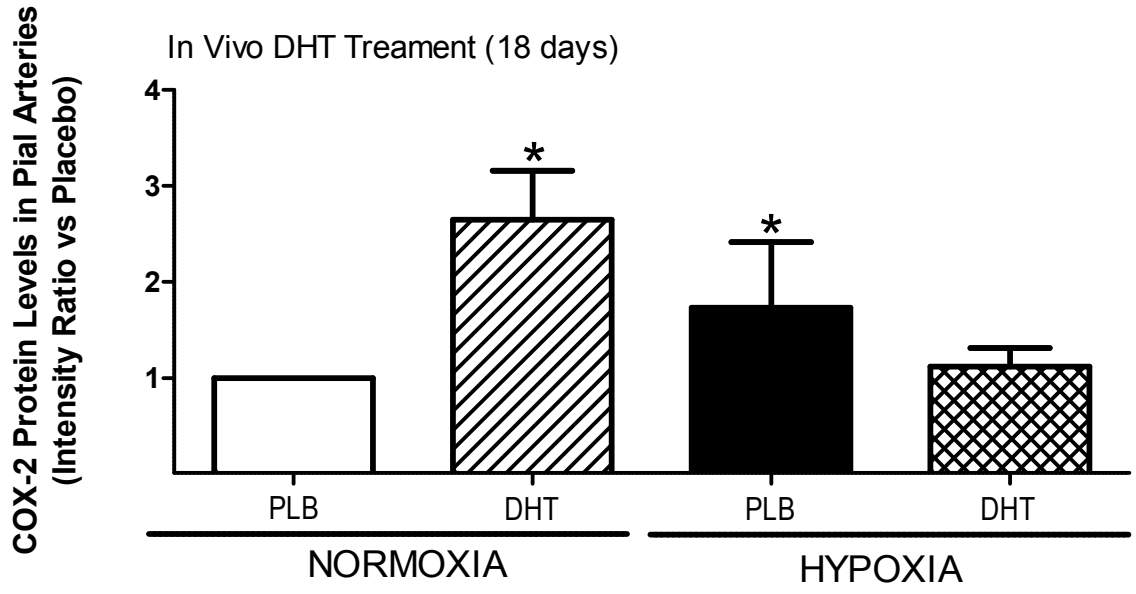


Figure 4.1. *Chronic in vivo DHT treatment increases COX-2 under normoxic conditions and inhibits hypoxia-induced COX-2 levels following hypoxic exposure in rat pial arteries*. COX-2 levels following normoxic and hypoxic exposure *ex vivo* (1% O_2; 6 h) in pial arteries isolated from castrated male rats treated *in vivo* with placebo (PLB) or DHT (45mg pellet; 18 days). Panel A: Representative blot for COX-2 protein levels in pial artery lysates. GAPDH was used as a loading control and a standard molecular weight marker (STD) was loaded on the left hand side of blot. Panel B: Data analysis of mean intensity ratios of DHT treated groups compared to PLB following normoxic or hypoxic exposure. Each bar represents the mean +/- SEM of n≥ 5 per group. *P<0.05 vs. Veh.

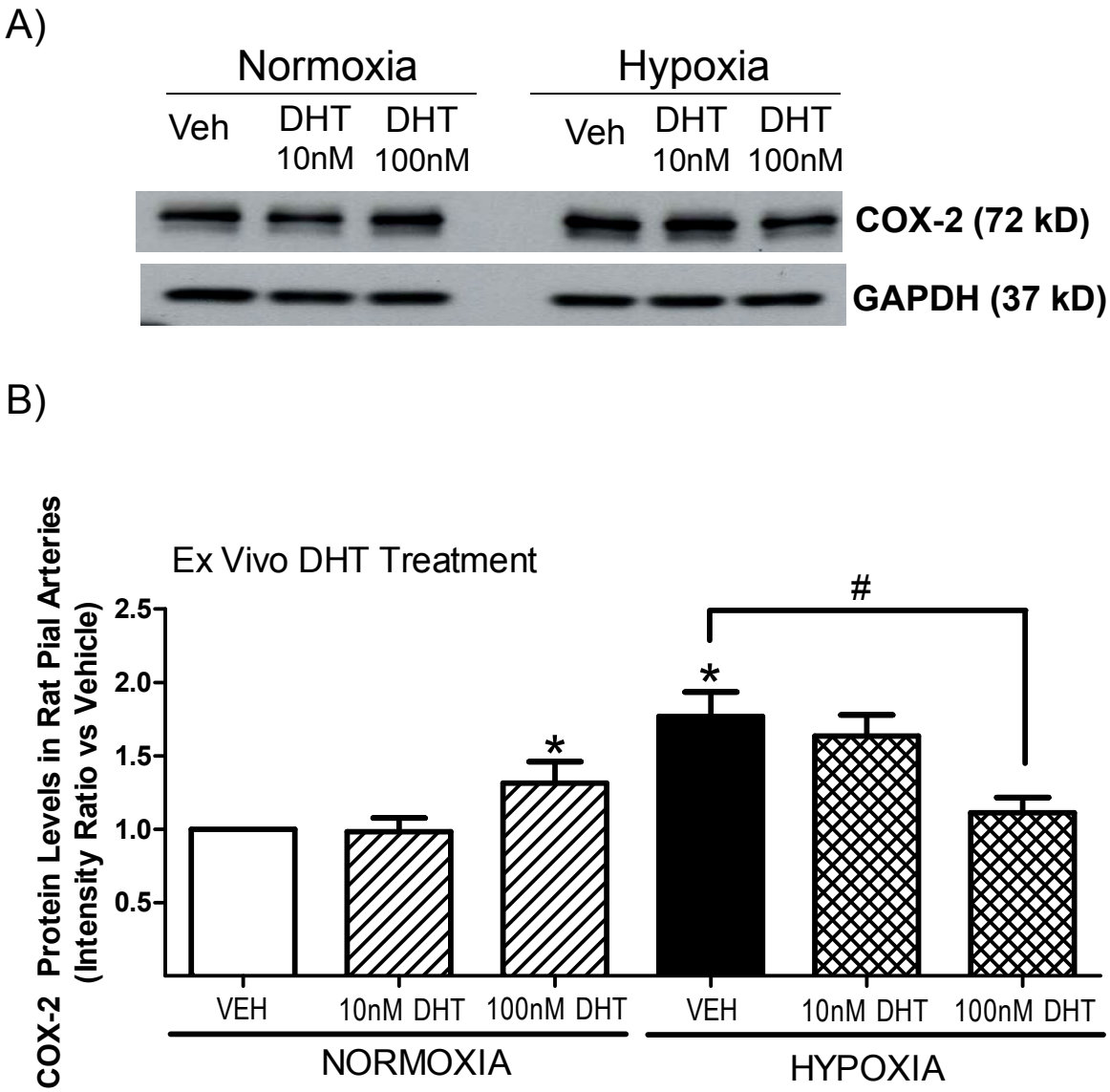
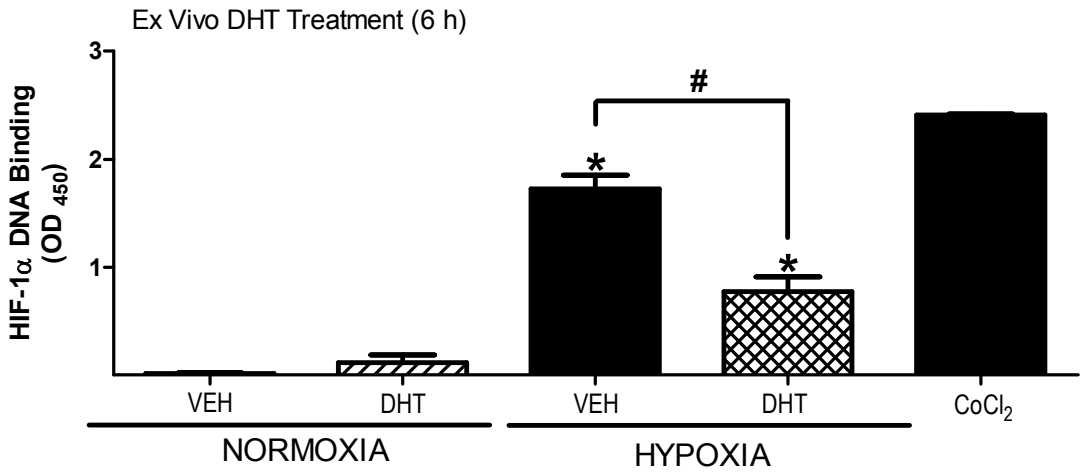


Figure 4.2. *Ex vivo DHT treatment increases COX-2 under normoxic conditions and decreases COX-2 following hypoxia in rat pial arteries*. COX-2 levels in pial arteries, dissected from castrated male rats, treated with DHT (10 or 100 nM; 6 h) or vehicle (Veh) *ex vivo* followed by exposure to normoxia or hypoxia *ex vivo* (1% O_2; 6 h). A: Representative blot for COX-2 and GAPDH (loading control) protein levels. B: Data analysis of COX-2 mean intensity ratio compared to vehicle (VEH). Each bar represents the mean +/- SEM of n≥ 5 per group.*P<0.05 vs. Normoxia Veh; #P<0.05 vs. Hypoxia Veh.

A)



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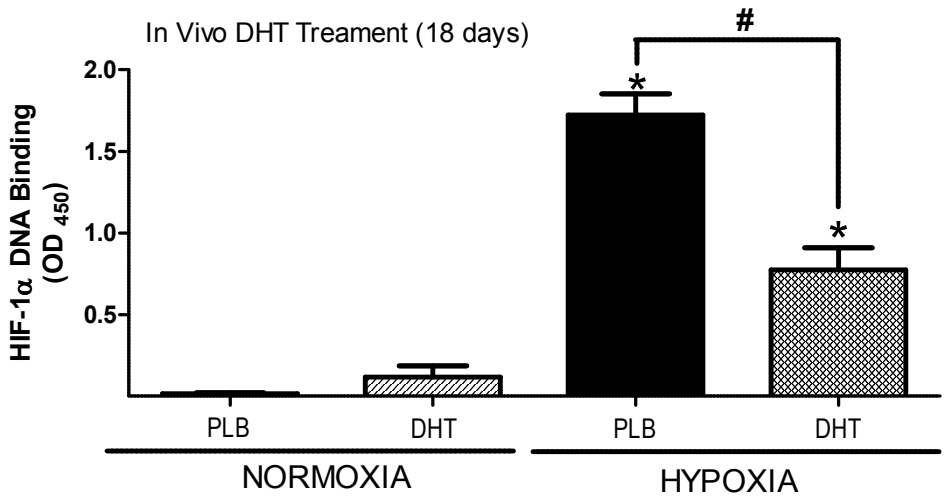
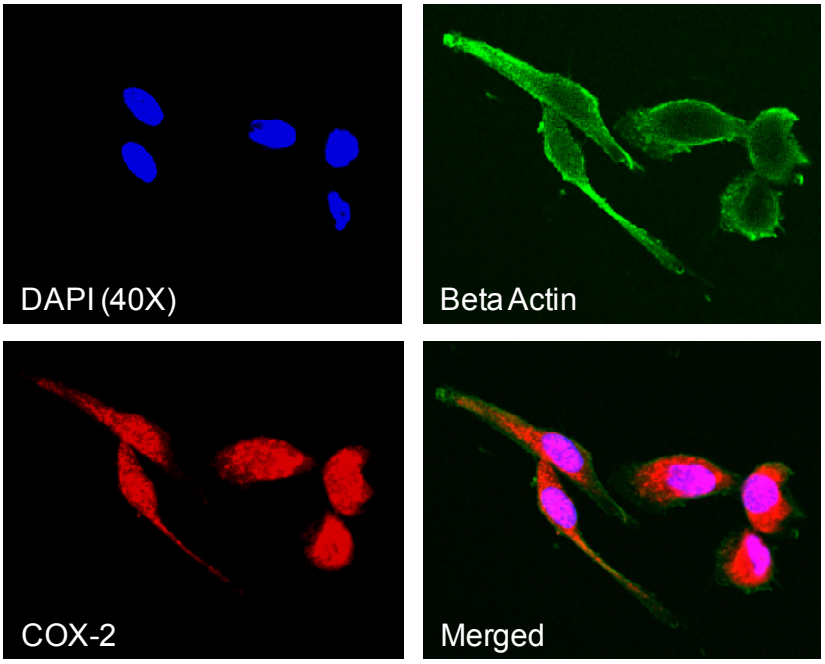


Figure 4.3. *DHT blunted hypoxia-induced nuclear HIF-1α activation in rat pial arteries.* HIF-1α DNA binding was assessed in pial artery nuclear isolates. Panel A: HIF-1α activation in nuclear isolates from castrated rat pial arteries pretreated *ex vivo* with vehicle or DHT (100 nM; 6 h) followed by exposure to normoxia or hypoxia (1% O_2; 1 h). A small population of arteries were stimulated with cobalt chloride ($CoCl_2$), which inhibits the hydroxylases that normally target HIF-1α for degradation by the proteosome leading to HIF-1α stabilization, and used as a positive indicator of HIF-1α activation. Panel B: Assessment of HIF-1α activation in nuclear isolates from pial arteries isolated from castrated male rats treated *in vivo* with vehicle (Veh) or DHT (45mg pellet, 18 days) followed by exposure to hypoxia *ex vivo* (1% O_2; 1 h). Each bar represents the mean +/- SEM of n≥ 4 per group. *P<0.001 vs. normoxia VEH; #P<0.001 vs. Hypoxia Veh.

A)



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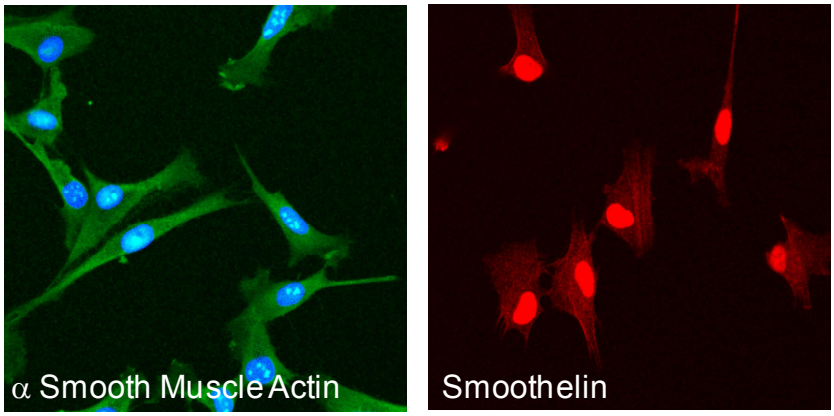


Figure 4.4. *COX-2 is expressed in HBVSMC following IL-1β stimulation.* Panel A: Human brain vascular smooth muscle cells (HBVSMC) were stimulated with IL-1β (5 ng/ml; 3 h) and immunocytochemistry verified cytokine-induced COX-2 (red) levels. Beta actin is shown in green and DAPI (blue) was used as a nuclear marker. Panel B: HBVSMC labeled with either alpha smooth muscle actin (green) or smoothelin (red) antibodies to verify levels of vascular smooth muscle cell markers.

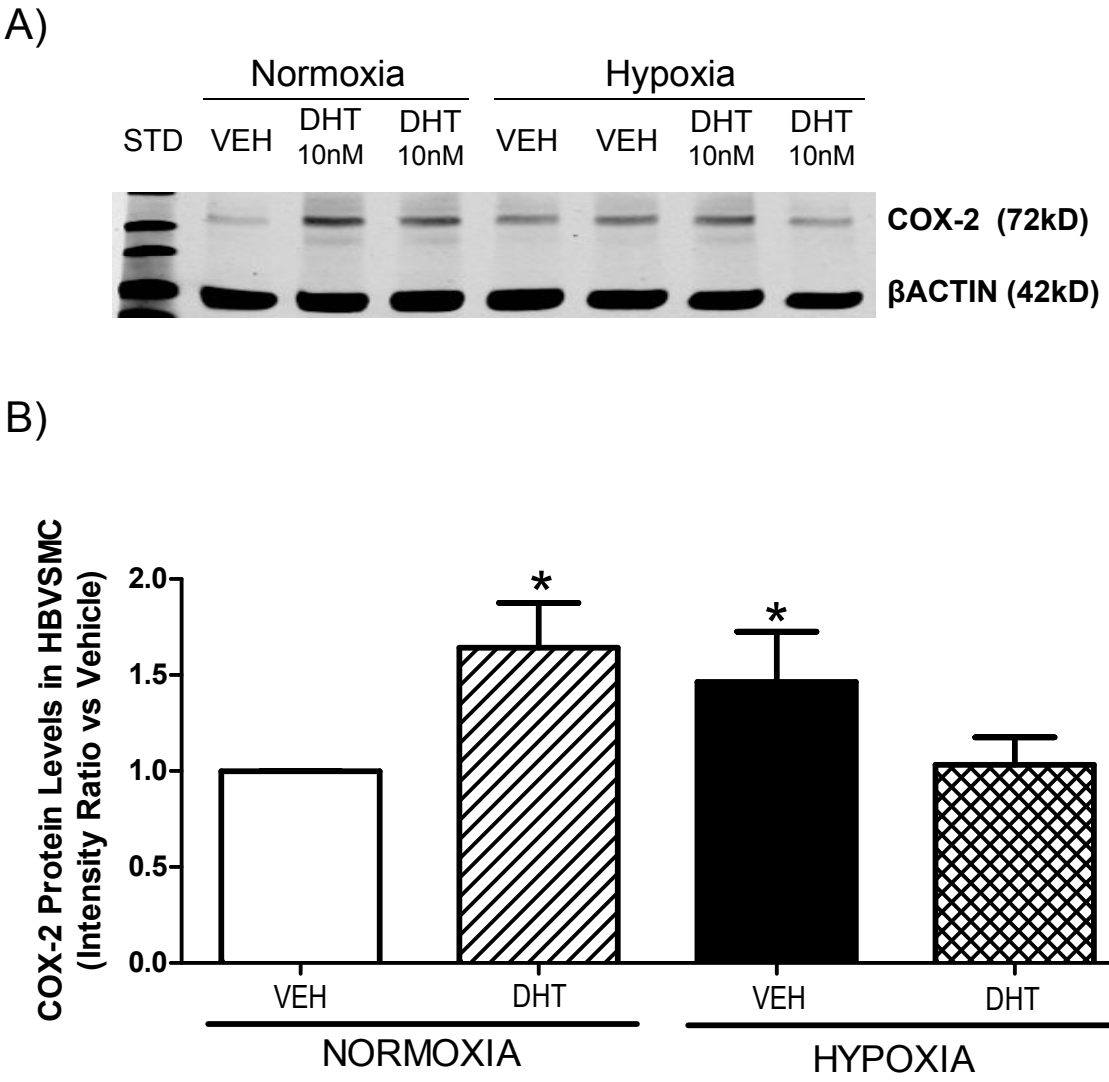


Figure 4.5. *DHT differentially modulates COX-2 during normoxia and hypoxia in human HBVSMC.* COX-2 protein levels was assessed in cells pretreated *in vitro* with Veh or DHT (10 nM; 18h) then exposed *in vitro* to normoxia or hypoxia (1% O_2; 6 h). Panel A: Representative blot for COX-2 protein levels in all groups. Beta actin served as a loading control. Panel B: Data analysis of COX-2 mean intensity ratio compared to vehicle (VEH). Each bar represents the mean +/- SEM of n≥ 9 per group. *P<0.05 vs. Veh.

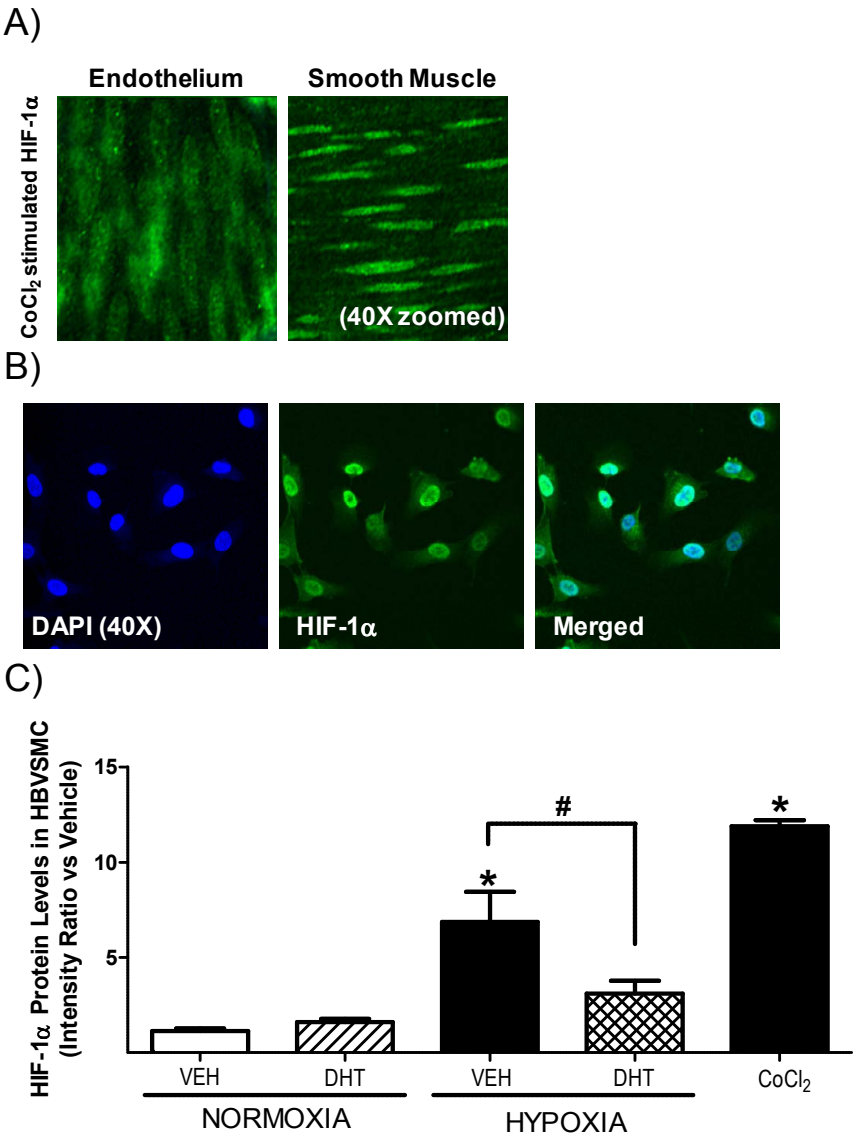


Figure 4.6. *DHT blunted hypoxia-induced increases in HIF-1α protein in HBVSMC.* HIF-1α localization via immunocytochemistry in an intact rat pial artery (panel A) and HBVSMC (panel B) stimulated with cobalt chloride ($CoCl_2$; 3h). Panel A represents $CoCl_2$ induced HIF-1α in endothelial (left panel) and vascular smooth muscle (right panel) layers. Labeling was more pronounced in the nuclear fraction as evidenced by the orientation of each cell type in the blood vessel wall. The endothelial nuclei are oriented in the direction of blood flow and the vascular smooth muscle oriented perpendicular to blood flow. Panel B represents $CoCl_2$ induced HIF-1α protein localization in HBVSMC. Panel C: HIF-1α protein levels were assessed via Western blot in HBVSMC pretreated *in vitro* with Veh or DHT (10 nM; 18h), then exposed to normoxia or hypoxia (1% O_2, 6 h). $CoCl_2$, which inhibits the hydroxylases that normally target HIF-1α for degradation by the proteosome leading to HIF-1α stabilization, was used as a positive control. Each bar represents the mean +/- SEM of n≥ 6 per group.*P<0.01 vs. Normoxia Veh; #P<0.01 vs. Hypoxia Veh.

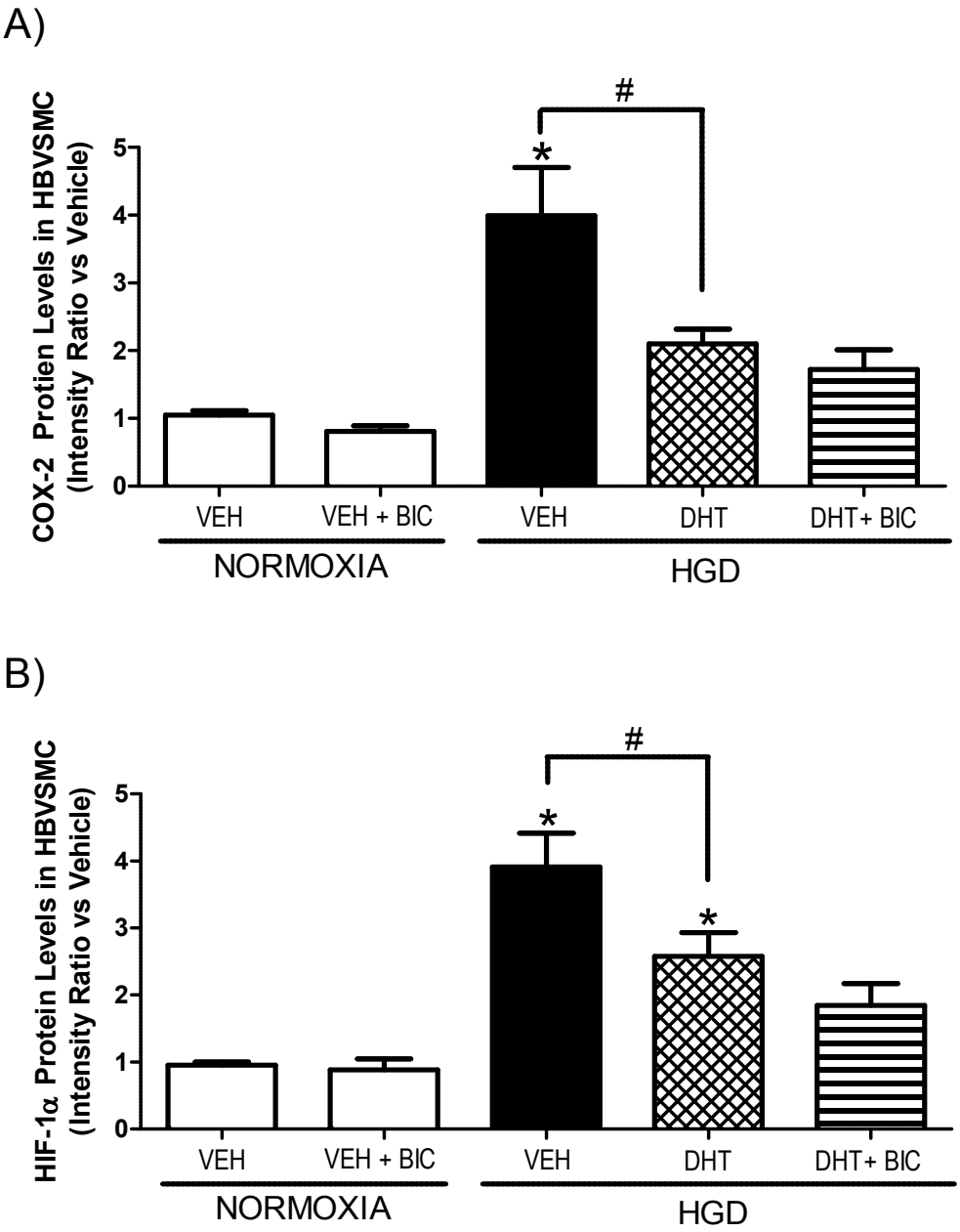


Figure 4.7. *DHT blunted HGD-induced COX-2 and HIF-1α levels HBVSMC via an AR-independent mechanism.* COX-2 and HIF-1α protein levels were assessed in HBVSMC pretreated *in vitro* with Veh, DHT (10 nM) or DHT + bicalutamide (androgen receptor antagonist, 1μM) for 18h, then exposed to normoxia or hypoixa with glucose deprivation (1% O_2; 6 h). Panel A: COX-2 Western analysis results; each bar represents the mean +/- SEM of n≥ 9 per group. *P<0.001 vs. Veh, #P<0.001 vs. HGD Veh. Panel B: HIF-1α Western analysis results; each bar represents the mean +/- SEM of n≥ 6 per group. *P<0.01 vs. Veh, #P<0.01 vs. HGD Veh.

CHAPTER 5: INFLAMMATION-INDUCED TLR4 EXPRESSION AND REACTIVE OXYGEN SPECIES ARE ATTENUATED BY DIHYDROTESTOSTERONE IN HUMAN PRIMARY VASCULAR SMOOTH MUSCLE CELLS

Abstract

Dihydrotestosterone (DHT) has been shown to attenuate endotoxin and cytokine- induced cyclooxygenase-2 (COX-2) in human vascular smooth muscle (VSM) cells. COX-2 plays a role in vascular inflammation and has been implicated as a source of reactive oxygen species (ROS). In rats, low doses of androgens have been shown to be protective during cerebral ischemia and to decrease oxidative stress in vascular tissues. Because toll-like receptor 4 (TLR4) has been implicated in injury-induced oxidative stress, I hypothesized that chronic DHT treatment will decrease injury induced stress by attenuating TLR4 expression and decreasing ROS following endotoxin or hypoxia with glucose deprivation (HGD) in primary human VSM cells. TLR4 localization was detected via confocal microscopy, TLR4 protein levels were measured via western blot, and ROS generation was measured using the indicator dye carboxy-H_2DCFDA. Endotoxin, hypoxia, and HGD all increased TLR4 expression compared to controls. In contrast, endotoxin and HGD-induced TLR4 expression was attenuated in the presence of DHT (10 nM). In the HGD studies, DHT's effect on TLR4 was androgen receptor independent. Similar to the TLR4 studies, cytokine-induced ROS production was blunted by DHT. Thus in

conditions of injury induced stress, androgens may confer protection from vascular injury in part by attenuating both TLR4 expression and ROS production.

Key Words

Vascular smooth muscle, interleukin-1 beta, lipopolysaccharide, inflammation, androgen, toll-like receptor 4, ischemia

Introduction

Vascular inflammation and oxidative stress contribute substantially to the formation and progression of cardiovascular disease. Epidemiological data report that the overall risk for heart disease and stroke is highest in men but the severity of outcome is greatest in women (R. E. Petrea et al., 2009; M. Writing Group et al., 2009). Experimental studies provide evidence that these sex differences may be attributed to endogenous gonadal steroids. In general estrogen has been shown to have positive effects on the blood vessel wall and therefore be protective against cardiovascular disease. On the other hand, the role for androgens in vascular disease is more complex based on mixed results especially as it pertains to vascular inflammation. For example, in human umbilical vein endothelial cells, the potent androgen dihydrotestosterone (DHT) was shown to increased expression of vascular cell adhesion molecule (VCAM) in one study (A. K. Death et al., 2004), but decrease expression VCAM in another study using the same cell type but a lower dose of DHT (G. D. Norata et al., 2006). The goal of this study was to assess the modulation of dihydrotestosterone (DHT) on the innate immune receptor Toll-like receptor 4 (TLR4) and downstream reactive oxygen species (ROS) production following inflammation-induced injury in a human vascular smooth muscle (VSM) cell model.

Vascular inflammation is important in the pathology and outcome of heart attack and stroke since ischemia evokes a strong inflammatory response characterized by activation and release of cytokines, chemokines, adhesion molecules, and proteolytic enzymes that exacerbate tissue damage (G. J. del Zoppo and T. Mabuchi, 2003; M. Nedergaard et al., 2003). The regulation of inflammation is

under the control of several transcription factors including the transcription factor nuclear factor kappa B (NFκB). NFκB can be activated through the endotoxin signaling receptor, Toll-like receptor (TLR) or interleukin-1 receptor (IL-1R). Both receptors share a homologous cytoplasmic signaling Toll/IL-1R (TIR) domain that when activated initiates a signaling cascade culminating in activation of NFκB and transcription of pro-inflammatory genes (S. M. Dauphinee and A. Karsan, 2006). TLRs have mainly been shown to be expressed in immune cells, including monocytes, macrophages, dendritic cells and B cells. However, expression of the TLR family member, TLR4, has also been reported in non-immune cells such as coronary vascular smooth muscle (J. Oyama et al., 2004; L. L. Stoll et al., 2004) and endothelial cells (J. Pugin et al., 1993; E. Faure et al., 2000; S. M. Dauphinee and A. Karsan, 2006). The presence of TLR4 in VSM and endothelium suggest a role for TLR4 in vascular disease.

Activation of TLR4, results in increased inflammation, oxidative stress, and ischemic damage. TLR4 induces expression of a variety of pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules and pro-inflammatory enzymes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), via activation of NFκB (S. M. Dauphinee and A. Karsan, 2006). These increases in COX-2 and iNOS lead to increased oxidative stress due to the peroxidase activity of COX-2 and peroxynitrite formation during NO production. Oxidative stress is a major contributor to the progression of atherosclerosis (G. Zalba et al., 2007). Evidence suggests that TLR4 plays role in pathology of cerebrovascular disease. For example, symptomatic carotid artery

plaques have increased TLR4 expression compared to asymptomatic plaques (A. Katsargyris et al., 2011). In addition, TLR4 mRNA and protein significantly increase after intracerebral hemorrhage or cerebral ischemia in rodents (L. W. Weiyu Teng, Weishuang Xue, and Chao Guan, 2009; K. Hyakkoku et al., 2010), and these increases in TLR4 correlate with increased damage. Taken together, these studies suggest that TLR4 may be a critical regulator of inflammation and oxidative stress both during endotoxin-induced inflammation and during ischemic injury.

Androgens may provide protection against cardiovascular disease due to their anti-inflammatory and anti-oxidant actions. Anti-inflammatory effects of androgens have been demonstrated in both humans and experimental animal models. In androgen deficient men, testosterone replacement decreases cytokine levels (C. J. Malkin et al., 2004; S. Y. Kalinchenko et al., 2010a). In TNFα-stimulated human umbilical vein endothelial cells, DHT (potent endogenous androgen receptor agonist) has been shown to decrease a variety of inflammatory proteins including COX-2 and TLR4 (G. D. Norata et al., 2006). I recently demonstrated that DHT attenuated endotoxin-induced COX-2 levels in human coronary VSM cells (K. L. Osterlund et al., 2010). I further observed that DHT decreased COX-2 levels in human brain VSM cells following hypoxia with glucose deprivation (which models so of the cellular insults that occur during ischemia; unpublished data). This suggests that DHT has the ability to suppress pro-inflammatory mediators following an inflammatory stimulus or hypoxic/ischemic stress. Androgens, and DHT in particular, are reported to be potent anti-oxidants both *in vitro* and *in vivo*. In mouse embryonic stem cells, hydrogen peroxide-induced ROS generation, lipid peroxide

formation, and DNA fragmentation were inhibited by pretreatment with DHT (M. N. Lee et al., 2008). Castration has also been shown to induce oxidative stress in rat prostate epithelium, while androgen replacement partially reversed this oxidative stress in castrates (N. N. Tam et al., 2003). These studies suggest that androgens can be protective by reducing inflammation and oxidative stress.

Because TLR4 is implicated in oxidative stress associated with pathogenic endotoxin-induced inflammation and, more recently, implicated in injury-induced inflammation in the absence of endotoxin (T. V. Arumugam et al., 2009), I hypothesized that DHT treatment will decrease inflammation and oxidative stress by attenuating TLR4 expression and decreasing ROS following endotoxin or hypoxia with glucose deprivation in primary human VSM cells.

Materials and Methods

Animals

Experimental and surgical protocols were approved by the Institutional Animal Care and Use Committee of the University of Arizona and Arizona State University. Adult male *Long Evans* rats were bred in house at the Arizona State University animal care facility. Rats were housed under a 12:12-h light-dark cycle with food and water available ad libitum.

Immunohistochemical Labeling for TLR4 in Pial Arteries

Animals were deeply anesthetized with isoflurane vapor (5%/air mixture), and the thoracic cavity exposed. Following injection with heparin (10 to 15 units/kg)

animals were exsanguinated and whole brain was removed. Using a dissecting microscope, pial arteries were dissected and collected in filtered phosphate buffered saline (PBS) containing sodium nitroprusside (SNP; 100 μM) to maximally dilate arteries. Arteries were cut into small segments (2mm in length), transferred to eppendorf tubes, and washed with PBS/SNP for 10 min on a rotator to remove any blood from inside the lumen. Next vessel segments were fixed in 4% formaldehyde for 30 min at room temperature, washed (3 x 10 min) in PBS then permeabilized with methanol (-20°C; 5 min). Vessels were then washed with 2% bovine serum albumin (BSA) for 5 min and blocked with fresh BSA (2%) for 1 h at room temperature. Tissues were incubated in primary antibodies specific for either alpha actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or TLR4 H80 (1:500; Santa Cruz Biotechnology) overnight at 4°C. After incubation, vessels were washed (3 x 10 min) and incubated with Alexa Fluor 555 anti-mouse or Alexa Fluor 480 anti-rabbit secondary antibodies (Invitrogen; 1:6,000) either overnight at 4°C or 1 h at room temperature followed by a final wash (3 x 10 min) in PBS before mounting. Using forceps, small pial artery segments were arranged on microscope slides that were cover slipped with VectaShield mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to label nuclei, then sealed with fingernail polish. Images were obtained using a Carl Zeiss LSM 710 confocal microscope (Carl Zeiss International, Germany). In control staining conditions, vessels were incubated with the secondary antibody alone (data not shown).

Cell Culture and Hormone/Drug Treatment:

Primary human coronary artery VSM cells (Lot # 4C1284) were purchased from Invitrogen Corporation (Carlsbad, CA) for use in TLR4 Western blot experiments. Primary human coronary artery VSM (Lot # RI443) were purchased for Cell Systems Corporation (Kirkland, WA) for use in the TLR4 immunocytochemistry experiment. Primary human brain VSM cells (Lot # 2733) were purchased from ScienCell Research Laboratories (Carlsbad, CA). Cells were grown in a 5% CO_2 incubator at 37˚C, in Medium 231 (Invitrogen) supplemented with smooth muscle growth supplement (ScienCell) and 2% FBS (brain VSM cells) or 5% FBS (coronary VSM cells).

Hormone/drug treatments were performed on cells at 80 to 90% confluency and at passage 5 or 6. Cell treatments were carried out in hormone-free media supplemented with Charcoal Stripped FBS (Invitrogen). A 6 h time point for TLR4 induction and a 10 nM DHT dose were selected for studies to investigate the effects of DHT treatment on TLR4 protein expression based on my previous studies (K. L. Osterlund et al., 2010).

Immunocytochemical Labeling for TLR4 in Human Brain VSM Cells:

Immunocytochemistry was used to verify the expression of TLR4 in human coronary VSM cells. Cells were plated on glass cover slips pre-coated with poly-L-lysine and treated with vehicle or LPS (6 h, 100 µg/ml) under sterile conditions. Cells were fixed (4% formaldehyde), washed in PBS (pH 7.4), and permeabilized in methanol (-20°C). Cells were first incubated in 2% BSA in PBS to block non-specific

binding, incubated with TLR4 H-80 (1:500) rabbit polyclonal antibody (Santa Cruz Biotechnology) in PBS containing 2% BSA for 1 h, washed in PBS (5x5 min), incubated with incubated with Alexa Fluor 555 anti-rabbit secondary antibodie (Invitrogen; 1:6,000) for 1 h in PBS containing 2% BSA and washed in PBS (5x5 min). Coverslips were mounted on glass slides using mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to label nuclei. Cells were visualized using a Zeiss 710 confocal microscope (Carl Zeiss International). Omission of the primary antibody removed fluorescence signal and was used to determine specificity of binding (data not shown). At passage 11, human coronary VSM cells still expressed the smooth muscle-specific protein α-Actin (not shown).

Nuclear Isolation:

Nuclear isolation was performed using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

Western Blot for TLR4:

Human coronary artery VSM cells were treated with DHT (10 nM) or vehicle (0.001% ethanol) for 6 h in the presence of vehicle (100 µl PBS) or lipopolysaccharide from *Escherichia coli* (LPS 100 µg/ml). Human brain VSM cells were treated with DHT (10 nM) or vehicle (0.001% ethanol) for 18 h followed by 6 h of one of the following treatments in the continued presence of hormone: 1) normoxia (21% O_2), 2) hypoxia (1% O_2), or 3) hypoxia with glucose deprivation

(HGD; 1% O_2 in glucose-free media). In a separate set of experiments, brain VSM cells were pre-treated for 1 h with the AR-antagonist bicalutamide (1 μM; dissolved in DMSO) or vehicle (0.01% DMSO) followed by 18 h of co-treatment with either vehicle (0.001% ethanol) or DHT (10 nM) then followed by 6 h of HGD (1% O_2 in glucose-free media) or normoxia. A bicalutamide dose of 1 μM was chosen based on previous studies (K. L. Osterlund et al., 2010). TLR4 protein expression was examined using standard immunoblotting methods, as previously described (K. L. Osterlund et al., 2010). Briefly, cells were homogenized in lysis buffer and total protein content of whole cell lysate was determined. Next, samples were diluted in Tris-Glycine SDS sample buffer (Invitrogen) and boiled for 5 min. Two color fluorescent standard (LI-COR Biosciences, Lincoln, NE) and diluted samples were loaded into 7.5% Smart gels (LI-COR). Proteins were separated via SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes and non-specific binding was blocked by incubation at room temperature for 30 min in PBS containing 1% Tween (TPBS) and 3% dried milk. Membranes were incubated in TLR4 H-80 (1:200) rabbit polyclonal antibody (Santa Cruz) and β-actin (1:5000) mouse monoclonal antibody (Sigma) overnight at 4˚C in TPBS. Following TPBS washes, the membranes were incubated in Goat Anti-Mouse IR 800 Dye or Goat Anti-Rabbit IR 680 Dye secondary antibodies (1:15,000; LI-COR) for 1 h at room temperature. TLR4 antibody specificity was verified with LPS stimulated Raw-264.7 (mouse macrophage) cell lysate (Santa Cruz), which is a positive control for TLR4 protein (data not shown). Nuclear extract was verified using a polyclonal antibody for the nuclear protein lamin (1:1000, Cell Signaling).

Following additional TPBS washes, proteins were visualized using an Odyssey Infrared Imager and data was analyzed using Odyssey V3.0 software (LI-COR).

Measurement of Reactive Oxygen Species

Human coronary artery VSM cells were plated in 24-well cell culture dish at passage 8. Cells were treated with vehicle (0.001% ethanol), or DHT (10 nM) for 18 h in Medium 231 containing 5% charcoal stripped FBS (Invitrogen). For the detection of ROS, specifically hydrogen peroxide and superoxide free radical a stock solution of 5-(and-6-)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Invitrogen; 5 mM), was prepared in ethanol. Following 18 h of hormone treatment, cells were incubated at 37°C for 30 min in supplemented phenol red-free media (CSC Medium + 5% charcoal stripped FBS, Cell Systems Corporation, Kirkland, WA) containing 30μM carboxy-H_2DCFDA in the presence or absence of interleukin-1 beta (IL-1β; 5 ng/ml). Next, cells were washed with PBS once to remove the excess probe and replaced with phenol red-free/FBS-free media (DMEM/F12, Invitrogen) in preparation for fluorescence scanning. Fluorescent intensity was measured on a Tecan Safire2 multi-well fluorescence plate reader (Tecan Austria GmbH, Salzburg, Austria) using 495 nm excitation and 525 nm emission settings.

Reagents:

All reagents were purchased from Sigma Aldrich Corporation (St. Louis, Missouri) unless otherwise noted.

Statistical Analysis:

Samples from each treatment were run on the same Western blot or 24-well plate (ROS measurement) for direct comparison and treatments were repeated for statistical analysis (n=4-24). Data from Western blots are expressed as an optical density ratio relative to vehicle. ROS measurements are expressed as fluorescence intensity. All values are reported as means ± SEM. Data were compared using analysis of variance (ANOVA), and differences were compared using post hoc tests (Student-Newman-Keuls; Prism Software, Irvine, CA). A level of $p<0.05$ was considered significant.

Results

TLR4 is Localized in the Endothelium an VSM of Rat Pial Arteries.

Representative confocal images are shown in Figure 5.1. TLR4 immunoreactivity was detected in the endothelial (panel H) and smooth muscle (panel I) layer of both large (>100 μm, panel D-F) and small (<100 μm; panel A-C) rat pial arteries.

TLR4 is Expressed in Human VSM Cells.

Using immunocytochemistry, TLR4 immunoreactivity was detected in human coronary VSM cells. Representative confocal images cells treated with vehicle (top panel) or 100μg/ml LPS (bottom panel) are shown in Figure 5.2A. Western blot revealed the presence of TLR4 in coronary VSM cell lysates. The anti-TLR4 H80

revealed bands migrating at ~95kDa, while anti-lamin showed bands at ~70kDa (Figure 5.2B). Lamin was used to verify nuclear isolation methods.

Endotoxin, Hypoxia, and HGD Increase TLR4 Expression in Human VSM Cells.

TLR4 protein expression was examined under a variety of pathophysiological conditions. Human VSM cells were treated with vehicle under normoxia (21% O_2), endotoxin (LPS, 100 μg/ml), hypoxia (1% O_2), or HGD (1% O_2 in glucose-free media), an *in vitro* model for some of the cellular insults that occur during ischemia, for 6 h. All three pathophysiological conditions increased TLR4 expression compared to vehicle normoxia ($p<0.001$; Figure 5.3).

DHT Decreases TLR4 Expression Following Endotoxin Exposure.

To determine the effect of DHT on TLR4 expression following endotoxin-induced inflammation, human coronary artery VSM cells were treated with vehicle or DHT (10 nM) in the presence or absence of LPS (100 μg/ml). LPS caused a significant increase in TLR4 compared to vehicle ($p<0.001$), and DHT attenuated this increase ($p<0.01$ vs. LPS; Figure 5.4).

DHT Decreases TLR4 Expression Following HGD via an AR-Independent Mechanism.

To determine the effect of DHT on TLR4 expression following ischemia-like conditions, human brain VSM cells were treated with vehicle or DHT (10 nM) for 18 h, followed by 6 h of normoxia (21% O_2) or HGD (1% O_2 in glucose-free media) in

the continued presence of hormone. Furthermore, to determine if DHT attenuates ischemia-induced increases in TLR4 via androgen receptor (AR) activation, additional studies were conducted in the presence of the AR antagonist bicalutamide (1 μM). HGD caused a significant increase in TLR4 compared to vehicle normoxia ($p<0.001$), and DHT attenuated this response ($p<0.01$ vs. HGD). DHT's attenuation of TLR4 expression in presence of LPS was not blocked by co-treatment with bicalutamide (Figure 5.5).

DHT Decreases Cytokine-Induced Oxidative Stress.

Reactive oxygen species (ROS) production was assessed in primary human coronary artery VSM cells pretreated with vehicle or DHT (10 nM) for 18 h, then exposed to the cytokine IL-1β (5 ng/ml, 30 min) in the presence of the ROS-indicator dye carboxy-H_2DCFDA. IL-1β increased ROS production compared to vehicle ($p<0.01$), and DHT blocked this response (Figure 5.6).

Discussion

The goal of this study was to determine if DHT treatment decreases oxidative stress by attenuating both TLR4 expression and ROS in VSM. I examined the effects of the non-aromatizable androgen DHT on TLR4 expression and oxidative stress in human VSM cells under conditions of endotoxin-induced inflammation and HGD. My results demonstrate that: 1) TLR4 is expressed in human VSM cells and both the endothelial and smooth muscle cell layer of rat pial arteries, 2) endotoxin, hypoxia, and HGD all increased TLR4 expression compared to vehicle/normoxic

controls, 3) DHT inhibited endotoxin-induced and HGD-induced TLR4 expression, 4) DHT's effect during HGD is androgen-receptor independent, and 5) DHT attenuates cytokine-induced ROS production. A summary diagram is presented in Figure 5.7.

In this study, I used primary culture of human VSM cells to investigate the effect of DHT on TLR4 expression and ROS production. The use of cultured cells can provide certain limitations. For example, in this study, each cell type originated from a single donor. In the current study I found that DHT reduced TLR4 expression in both coronary and brain VSM cells, which originated from different donors, thus minimizing the concern of using cells isolated from a single donor. Furthermore, I have also observed that DHT attenuates increases in another pro-inflammatory mediator COX-2 following cytokine stimulation via an AR-independent mechanism in human brain VSM cells isolated from a different donor (unpublished data) and have previously reported this AR-independent effect on COX-2 levels in human coronary VSM cells (K. L. Osterlund et al., 2010). A second limitation of using vascular cultured cells is that their properties change over time. For example, smooth muscle cells in culture are no longer contractile. To minimize the risk of changes in culture, all experiments were performed after a small number of passages (5-6). At the time of experiments (passages 5-6) the cells in this study expressed the smooth muscle cell markers smoothelin and alpha actin (data not shown), as well as the gonadal steroid receptors AR, ERα, ERβ (unpublished data).

To my knowledge this is the first report of hypoxia or HGD's effects on TLR4 expression in VSM cells. In contrast, LPS has been shown to increase TLR4 expression in human aortic VSM cells (F. Y. Lin et al., 2006; H. Li et al., 2007; Y. H.-

J. Heo S-K, Noh E-K, Park W-H, Park S-D, 2008). Furthermore, this increase in TLR4 was dependent on NADPH oxidase (an enzyme responsible for superoxide/ROS production), providing further evidence for that TLR4 activation is associated with increased oxidative stress. In contrast to my finding that hypoxia increased TLR4 expression in human VSM cells, one report in endothelial cells found that hypoxia increased ROS and decreased TLR4 expression (I. Ishida et al., 2002). However, a more recent study in rat cardiac microvascular endothelial cells showed that hypoxia with reoxygenation increased TLR4, NFκB, interleukin-6, and tumor necrosis factor alpha and that administration of a TLR4-neutralizing antibody could block these effects of hypoxia (Z. Zhang et al., 2011) . Furthermore, in macrophages hypoxia has been shown to increase TLR4 expression via activation of the transcription factor hypoxia inducible factor 1 alpha (HIF-1α) (K. So Young et al., 2010). In further support of this idea, in endothelial cells LPS has been shown to increase HIF-1α stabilization (A. Brooks et al., 2010). Thus, it appears that in most cases hypoxia has been shown to upregulate TLR4 expression, in a fashion consistent with what I show in the current study.

My data also show, for the first time, that HGD, and *in vitro* model for some aspects of ischemia, increases TLR4 expression in human VSM cells. Although, data in VSM cells had previously been lacking, in whole brain TLR4 mRNA and protein have been shown to significantly increase after intracerebral hemorrhage or cerebral ischemia induced by middle cerebral artery occlusion (MCAO, *in vivo* stroke model) in rodents (L. W. Weiyu Teng, Weishuang Xue, and Chao Guan, 2009; K. Hyakkoku et al., 2010; X. K. Tu et al., 2010). Furthermore, TLR4 deficient mice

have lower infarct volumes, better neurological and behavioral test outcomes, less brain inflammation, and decreased NFκB activation after MCAO than controls (J. R. Caso et al., 2007; J. R. Caso et al., 2008; F. Hua et al., 2009; K. Hyakkoku et al., 2010). In addition, TLR4 deficient mice also have lower levels of malondialdehyde (cytotoxic product of membrane lipid peroxidation caused by ROS) after MCAO (J. R. Caso et al., 2007; J. R. Caso et al., 2008). This further supports the idea that TLR4 activation increases ROS production. It has also been shown that oxidative stress upregulates TLR4 expression in rat and human cerebral endothelial cells (P. Nagyoszi et al., 2010). Using IL-1β, a ligand for the IL-1 receptor that contains a homologous cytosolic signaling domain to TLR4 and therefore activates the same pathway as TLR4, I also show that ROS production is increased following stimulation of this pathway, further strengthening the link between the TLR4 signaling pathway and oxidative stress.

To my knowledge this is the first report of DHT's effects on TLR4 expression or oxidative stress in VSM cells, although, a similar effect of DHT to reduce TLR4 expression following LPS or tumor necrosis factor alpha-induced inflammation has been reported in human endothelial cells (G. D. Norata et al., 2006). In mouse macrophages, testosterone treatment has been shown to decrease both TLR4 expression and sensitivity (J. A. Rettew et al., 2008). DHT has also been shown to reduce oxidative stress in other cell types. As previously mentioned, in mouse embryonic stem cells, hydrogen peroxide-induced ROS generation, lipid peroxide formation, and DNA fragmentation were inhibited by pretreatment with DHT (M. N. Lee et al., 2008). Castration has also been shown to induce oxidative stress in rat

prostate epithelium, while androgen replacement partially reversed this oxidative stress in castrates (N. N. Tam et al., 2003). Furthermore, DHT has also been shown to increase antioxidant levels and reduce the levels of the cytokine tumor necrosis factor-alpha in a rat model of arthritis (K. Ganesan et al., 2008). In human umbilical vein endothelial cells, DHT has been shown to protect against hydrogen peroxide-induced apoptosis (Z. R. Xu et al., 2010). In male rats, DHT inhibited oxidative-stress-induced platelet aggregation (S. Li et al., 2007) . These studies support an anti-inflammatory, anti-oxidant role of DHT.

There are several potential mechanisms by which DHT may alter TLR4 expression. Negative regulation of TLR4 signaling has been shown to occur in the vasculature via several androgen-sensitive proteins including, phosphatidylinositol 3-kinase (PI3K), the anti-apoptotic zinc-finger protein A20, fas-associated death domain (FADD), or the chemokine receptor CXCR4 (S. M. Dauphinee and A. Karsan, 2006). All of these proteins/pathways have been shown to be regulated by androgens. For example, in macrophages testosterone-induced apoptosis has been shown to be FADD-dependent (L. Jin et al., 2006). A20 mRNA expression has been shown to be up-regulated by DHT (10 nM) in human prostate cancer cells (O. Golovko et al., 2005). In human aortic endothelial cells, testosterone stimulated rapid phosphorylation of Akt in a time- and dose-dependent manner via PI3K. Furthermore, co-immunoprecipitation assays revealed a testosterone-dependent interaction between AR and the p85alpha subunit of PI3K (J. Yu et al., 2010). And finally, the synthetic androgen (R1881) has been shown to upregulate CXCR4 in prostate cancer cells (J. Cai et al., 2010). These results provide evidence for just

some of the potential mechanisms by which androgens may downregulate TLR4 expression.

DHT's anti-inflammatory effects do not appear to be androgen-receptor mediated in human VSM cells. My current finding shows that DHT reduces HGD-induced TLR4 expression via an AR-independent mechanism, since the AR antagonist bicalutamide had no effect. I have previously observed that DHT also attenuates cytokine (K. L. Osterlund et al., 2010), endotoxin (K. L. Osterlund et al., 2010), hypoxic, and HGD-induced COX-2 expression via an AR-independent mechanism in human VSM cells (unpublished data). Others have also demonstrated AR-independent actions of androgens, for example DHT can activate the sex hormone binding globulin receptor (N. Fortunati et al., 1996; A. M. Nakhla and W. Rosner, 1996) and can increase intracellular calcium via an unidentified membrane-bound receptor (W. P. Benten et al., 1999b; W. P. Benten et al., 1999a). Furthermore, several authors have also hypothesized that a membrane bound AR may exist that is not blocked by classical AR antagonists (E. F. Konoplya and E. H. Popoff, 1992; W. P. Benten et al., 1999b; W. P. Benten et al., 1999a; C. A. Heinlein and C. Chang, 2002). Since DHT cannot be aromatized to estradiol like testosterone can, it is frequently used to explore the effects of androgens mediated by the androgen receptor. Using the classically described mechanism of action, testosterone can activate the androgen receptor (AR) directly or following its conversion by 5 alpha-reductase to the more potent androgen, dihydrotestosterone (DHT) (R. J. Handa et al., 1987; E. D. Lephart et al., 2001). Alternatively, testosterone can be metabolized to estradiol by the aromatase enzyme (C. E.

Roselli et al., 1985) and subsequently activate estrogen receptor alpha (ERα) or beta (ERβ) (G. G. Kuiper et al., 1997; G. G. Kuiper et al., 1998). A third and less well explored pathway for androgen action is through the conversion of DHT to 5α-androstane-3β,17β-diol (3β-diol), an ERβ agonist (G. G. Kuiper et al., 1996; G. G. Kuiper et al., 1997; Z. Weihua et al., 2002), by the enzymes 3beta-hydroxysteroid dehydrogenase (3β-HSD), 3alpha- hydroxysteroid dehydrogenase (3α-HSD) and 17beta-hydroxysteroid dehydrogenase (17β-HSD)(Y. Jin and T. M. Penning, 2001; Z. Weihua et al., 2002; A. Gangloff et al., 2003; S. Torn et al., 2003; S. Steckelbroeck et al., 2004). Blood vessels contain AR, ERβ, and 3β-HSD (G. D. Snyder et al., 2002; Y. Nakamura et al., 2005; R. J. Gonzales et al., 2007; H.-C. Shih et al., 2008), as do human VSM cells (unpublished data), thus allowing for the possibility that DHT is acting via ERβ during pathophysiological conditions. In support of this theory, it has recently been shown that 3β-diol has anti-inflammatory actions in human umbilical vein endothelial cells and mouse aorta (G. D. Norata et al., 2010). I intend to pursue this DHT/ERβ pathway in future studies.

In summary, I have shown that DHT reduces TLR4 expression and oxidative stress during inflammation and hypoxia with glucose deprivation in human VSM cells. These findings demonstrate that androgens may provide protection against inflammation in VSM under a variety of pathophysiological conditions in part by decreasing TLR4 expression and subsequent ROS production. This is of particular importance because understanding the mechanisms by which androgens influence vascular inflammation may contribute to new therapeutic methods for the treatment

of cardiovascular diseases such as atherosclerosis, myocardial infarction, and stroke.

Figures and Legends

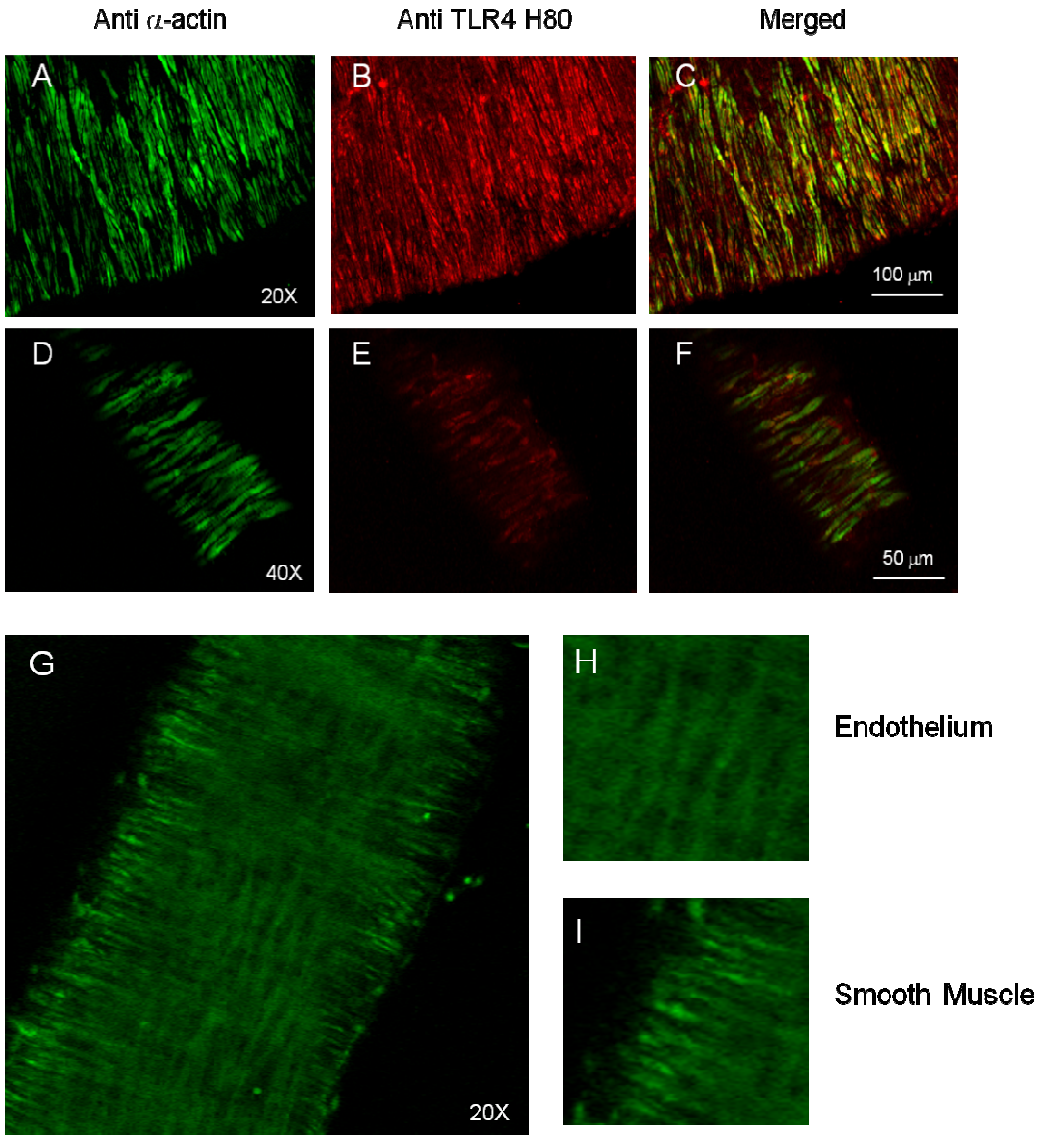


Figure 5.1. ***TLR4 is Localized in the Endothelium and VSM of Rat Pial Arteries.*** Confocal microscopy revealed the presence and localization of TLR4 in larger and small rat pial arteries (A to C ≥ 100 μm; D to F ≤ 100 μm). Fluorescent labeling for anti-TLR4 H80 (RED) are shown in panels B and E and anti alpha actin (GREEN) are shown in panels A and D. Merged images are illustrated in panels C and F. Confocal laser scanning simultaneously captured both the endothelial and vascular smooth muscle layer in a rat pial artery segment (approx. 250 μm dia.; panel F). Panel G represents an enlarged image of the endothelial layer (blood vessel lumen) and panel G represents the smooth muscle layer (edge of vessel wall).

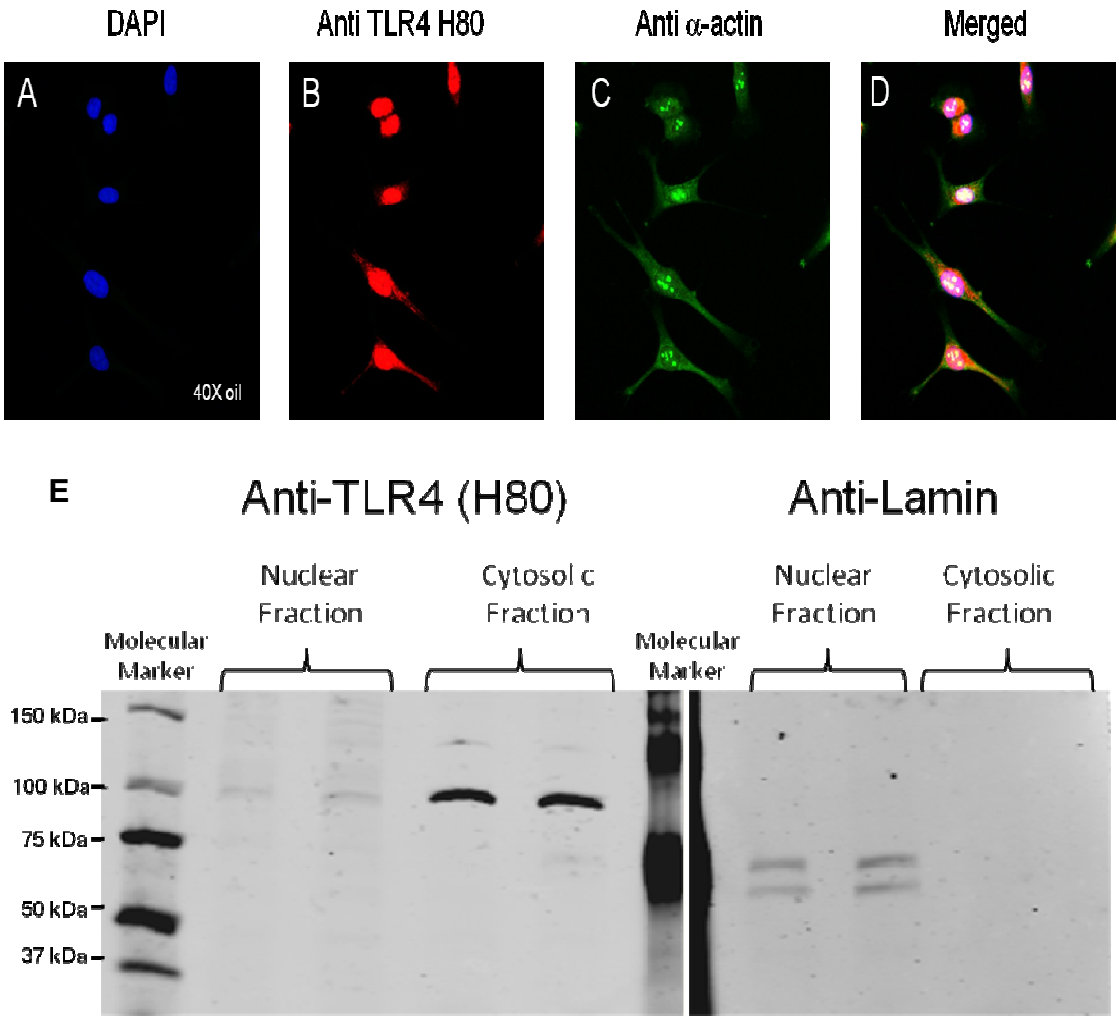


Figure 5.2. ***Localization of TLR4 immunoreactivity in primary human coronary VSM cells (passage 11)***. Cells were imaged using confocal microscopy. Cells were stimulated with vehicle (top row) or lipopolysaccharide (LPS; 100 µg/ml) for 6 hours (bottom row; panel A). Nuclei labeled with 4',6-diamidino-2-phenylindole (DAPI; BLUE), TLR4-ir (GREEN), and merged images are shown in panel A.. Panel B shows a representative Western blot of nuclear and cytosolic fraction human brain VSM cell lysate in which the left side was exposed to Anti-TLR4 H80 resulting in bands migrating ~95kDa. The right side of the blot was incubated in Anti-lamin immunoreactivity and bands were detected at ~70kDa. HBVSM= Human brain VSM cells.

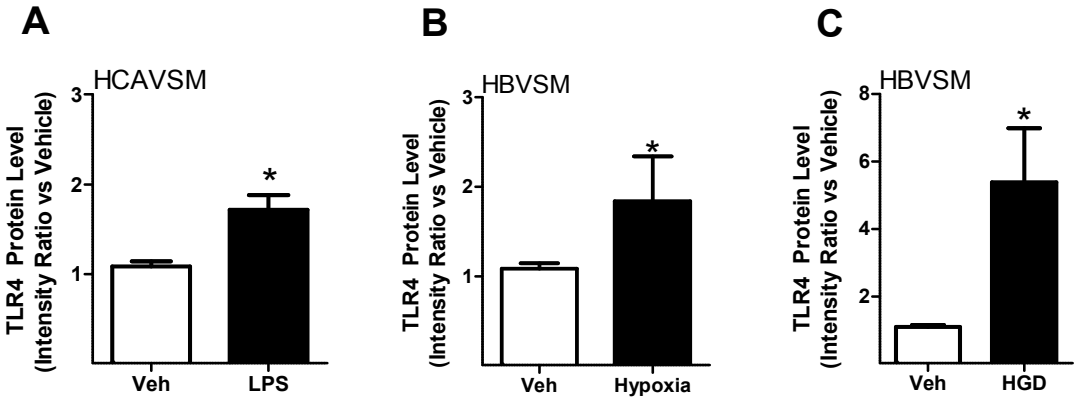


Figure 5.3. ***Endotoxin, Hypoxia, and HGD Increase TLR4 Expression in Human VSM Cells.*** TLR4 protein expression was assessed in primary human VSM cells from brain (hypoxia & HGD study) or coronary artery (LPS study). Cells were exposed to 6 h of the endotoxin LPS (100 μg/ml), hypoxia (1% O_2), hypoxis with glucose deprivation (HGD). *P<0.05 vs. normoxia Veh (n≥3 per group).

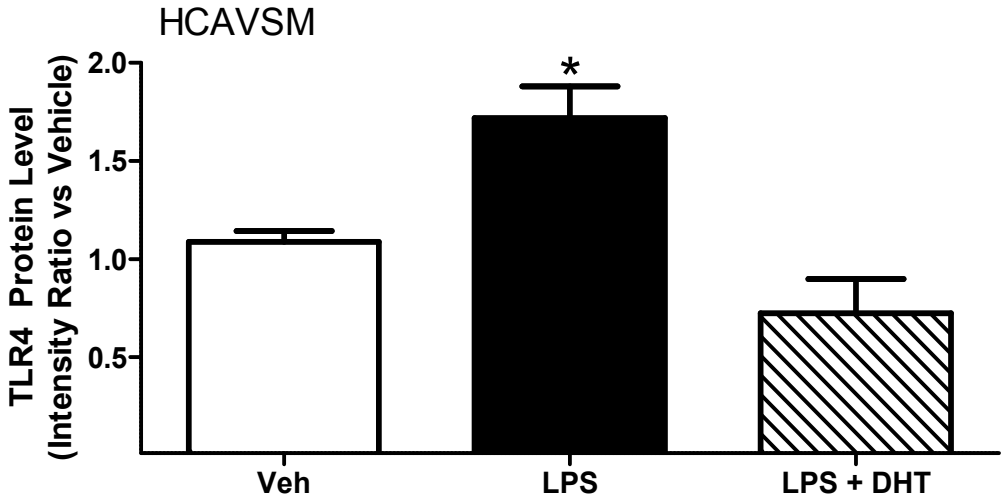


Figure 5.4. ***DHT decreases TLR4 Expression Following Endotoxin Exposure.*** TLR4 protein expression was assessed in primary human coronary artery VSM cells treated with Veh or DHT (10 nM; 6 h), in the absence or presence of the endotoxin LPS (100 μg/ml, 6 h). *P<0.001 vs. Veh (n≥ 3 per group). HCAVSM = human coronary artery VSM cells.

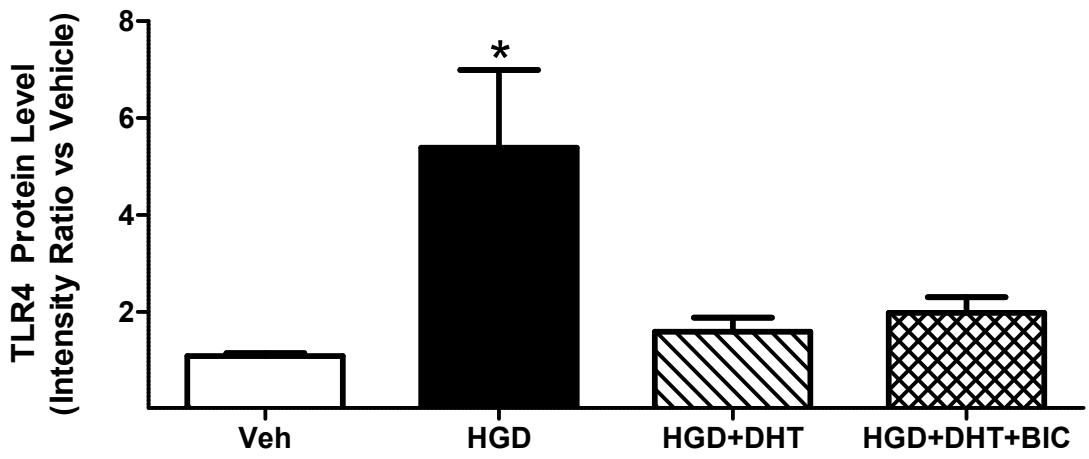


Figure 5.5. ***DHT decreases TLR4 Expression Following HGD via an AR-Independent Mechanism.*** TLR4 protein expression was assessed in primary human brain VSM cells pretreated with Veh, DHT (10 nM) or DHT + Bicalutamide (1 μM) for 18 h, then exposed to hypoxia with glucose deprivation (HGD, 1% O_2; 6 h). *P<0.001 vs. Veh (n≥ 5 per group). HBVSM = human brain VSM cells.

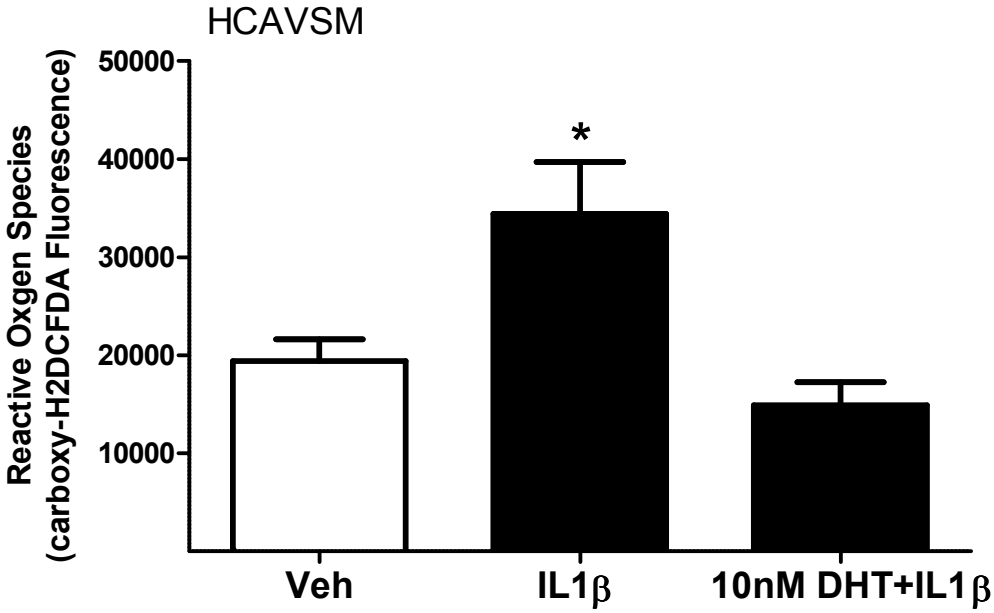


Figure 5.6. ***DHT Decreases Cytokine-Induced Oxidative Stress.*** ROS production was assessed in primary human coronary artery VSM cells pretreated with Vehicle or DHT (10 nM) for 18 h, then exposed to IL-1β (5 ng/ml, 30min) in the presence of the ROS-indicator dye carboxy-H2DCFDA. *P<0.01 vs. Veh (n≥ 7 per group). HCAVSM = human coronary artery VSM cells.

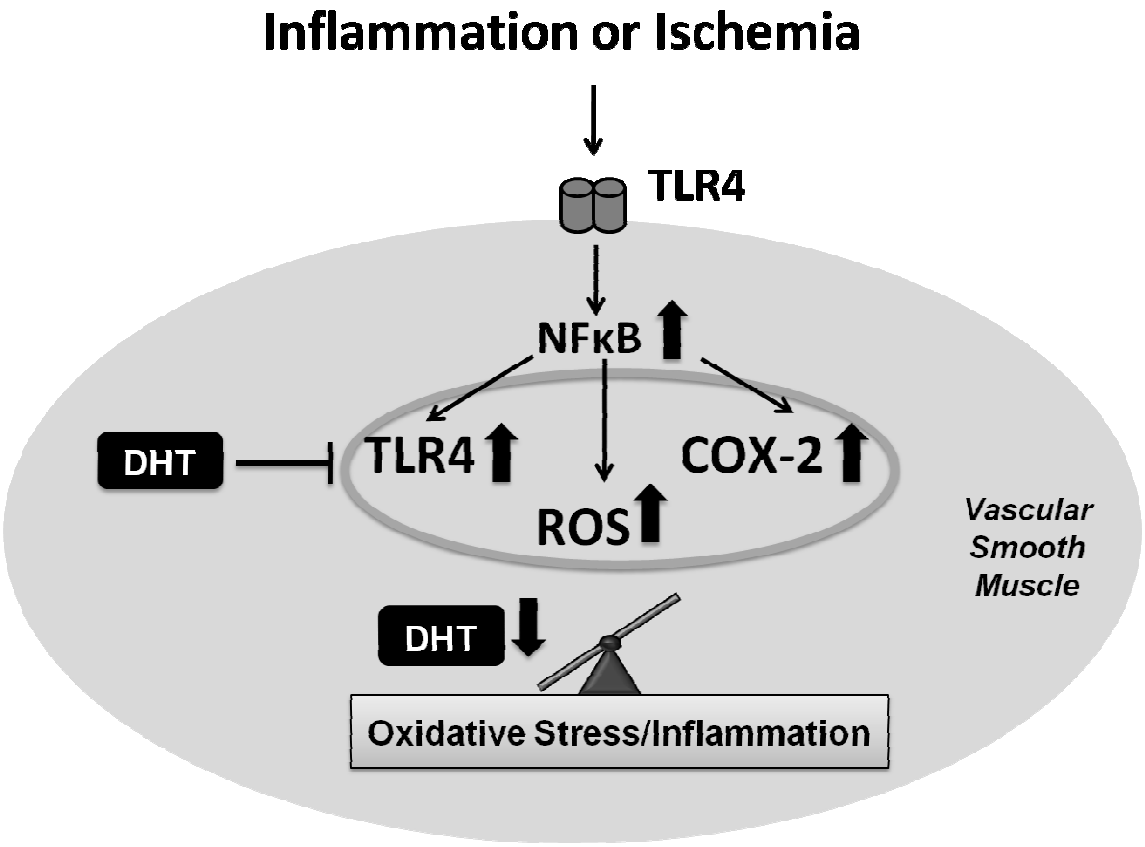


Figure 5.7. ***Summary Diagram of DHT's Effects During Inflammation and Ischemia.*** During inflammation or ischemia TLR4 activation leads to increased activation of NFκB resulting in increased production of COX-2, TLR4, and ROS. DHT inhibits COX-2, TLR4 and ROS production thus reducing inflammation and oxidative stress in the vasculature.

CHAPTER 6: ANTI-INFLAMMATORY EFFECT OF DIHYDROTESTOSTERONE DURING CYTOKINE-INDUCED INFLAMMATION IN HUMAN BRAIN VASCULAR SMOOTH MUSCLE CELLS IS MEDIATED THROUGH ESTROGEN RECEPTOR BETA

Abstract

The existence of sex differences in cardiovascular disease suggests a role for gonadal steroids in modulating vascular inflammation. Vascular inflammation has been shown to be an early and critical event in the pathogenesis of many cardiovascular diseases (i.e. atherosclerosis and ischemia reperfusion injury). Sex steroids (especially estradiol) have been implicated in alleviating vascular inflammation in experimental animal models. Additionally, my past studies have shown that dihydrotestosterone (DHT) treatment decreases expression of cyclooxygenase-2 (COX-2) during cytokine-induced inflammation and this response is androgen receptor (AR)-independent. Since DHT can be converted to 3β-diol, an estrogen receptor (ER) β-selective agonist, I hypothesized that DHT would decrease IL-1β induced COX-2 expression in primary human brain vascular smooth muscle cells (HBVSMC) via conversion to 3β-diol and subsequent activation of ERβ. Expression of mRNA was detected in HBVSMC for the gonadal steroid receptors AR, ERα, and ERβ. Furthermore, mRNA was also detected for steroid metabolizing enzymes necessary for conversion of DHT to 3β-diol [3alpha-hydroxysteroid

dehydrogenase (HSD), 3β-HSD, 17β-HSD, and for the inactivation of 3β-diol (CYP7B1)]. Treatment (18 h) with either DHT (10 nM) or its metabolite 3β-diol (10 nM) reduced IL-1β-induced increases in COX-2 expression. Pre-treatment with the AR antagonist bicalutamide (1 μM) did not block the effect of DHT. Both the non-selective ER antagonist ICI 182,780 (1 μM) and the selective ERβ antagonist PHTPP (1 μM) inhibited the effect of DHT, suggesting that DHT actions are ERβ-mediated. Therefore, DHT appears to be protective against cerebrovascular inflammation via conversion to 3β-diol and subsequent activation of ERβ.

Key Words

Vascular smooth muscle, interleukin-1 beta, estrogen receptor beta, inflammation, androgen

Introduction

The cerebral vasculature plays a central role in the pathogenesis of cardiovascular diseases, such as stroke (G. J. del Zoppo and T. Mabuchi, 2003), and in the initiation of inflammation after cerebral ischemia which is a key determinant in stroke outcome (H. C. A. Emsley and P. J. Tyrrell, 2002; G. J. del Zoppo and T. Mabuchi, 2003). Following ischemia, inflammation is initiated by cytokine-induced activation the transcription factor nuclear factor kappa B (NFκB) leading to increases production of pro-inflammatory mediators, such as inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) (Q. Wang et al., 2007; A. Brooks et al., 2010). I previously reported that the potent androgen receptor agonist, dihydrotestosterone (DHT), can suppress levels of COX-2 following endotoxin or cytokine stimulation. I further demonstrated that this response is independent of androgen receptor stimulation (K. L. Osterlund et al., 2010).

Investigating the effects of sex steroids on vascular inflammation is complicated by the numerous potential receptor pathways that these compounds can utilize to alter gene transcription and cellular responses. Both androgens and estrogens have been shown to have anti-inflammatory effects (C. J. Malkin et al., 2003; G. D. Norata et al., 2006; D. Xing et al., 2007; K. L. Osterlund et al., 2010), although androgens have been reported to have some pro-inflammatory effects as well (J. A. McCrohon et al., 1999; A. K. Death et al., 2004). Using the classically described mechanism, testosterone can activate the androgen receptor (AR) directly or following its conversion by 5 alpha-reductase to the more potent androgen, dihydrotestosterone (DHT) (R. J. Handa et al., 1987; E. D. Lephart et al., 2001).

Alternatively, testosterone can be metabolized to estradiol by the aromatase enzyme (C. E. Roselli et al., 1985) and subsequently activate estrogen receptor alpha (ERα) or beta (ERβ) (G. G. Kuiper et al., 1997; G. G. Kuiper et al., 1998). A third and less well explored pathway for androgen action is through the conversion of DHT to 5α-androstane-3β,17β-diol (3β-diol), an ERβ agonist (G. G. Kuiper et al., 1996; G. G. Kuiper et al., 1997; Z. Weihua et al., 2002), by the enzymes 3beta-hydroxysteroid dehydrogenase (3β-HSD), 3alpha- hydroxysteroid dehydrogenase (3α-HSD) or 17beta-hydroxysteroid dehydrogenase (17β-HSD)(Y. Jin and T. M. Penning, 2001; Z. Weihua et al., 2002; A. Gangloff et al., 2003; S. Torn et al., 2003; S. Steckelbroeck et al., 2004). 3β-diol is converted to inactive metabolites by the enzyme CYP7B1 (M. Sundin et al., 1987; Z. Weihua et al., 2002). Since blood vessels contain AR, ERα, ERβ, 5α-R, aromatase, and 3β-HSD (T. C. Register and M. R. Adams, 1998; G. D. Snyder et al., 2002; Y. Nakamura et al., 2005; R. J. Gonzales et al., 2007; H.-C. Shih et al., 2008), the expression of these enzymes and receptors allows for any of the potential receptor mediated effects which can influence vascular inflammation.

Similar to testosterone and estradiol, 3β-diol has recently been shown to reduce expression of inflammatory markers in human umbilical vein endothelial cells (G. D. Norata et al., 2010). Therefore, I have hypothesized that during cytokine-induced inflammation, DHT decreases the pro-inflammatory mediator COX-2 via metabolism to 3β-diol and subsequent activation of ERβ in human brain vascular smooth muscle cells (HBVSMC). I chose COX-2 as my marker for vascular inflammation because COX-2 has been shown to be particularly important in

cerebral ischemia since COX-2 inhibition can decrease infarct size in experimental models of stroke (K. Sugimoto and C. Iadecola, 2003) and because I have previously shown that in human coronary artery vascular smooth muscle cells, DHT decreases cytokine-induced COX-2 protein expression via an AR-independent mechanism (K. L. Osterlund et al., 2010).

Materials and Methods

Cell Culture and Hormone/Drug Treatment:

Primary HBVSMC, isolated from a 20 year old male donor (Lot #ACBRI 405), were purchased from Cell Systems Corporation (Kirkland, WA). Primary HBVSMC isolated from fetal cells (Lot# 2733) were purchased from ScienCell Research Laboratories (Carlsbad, CA). Cells were grown in 5% CO_2, 95% room air atmosphere at 37˚C, in Medium 231 (Invitrogen Corporation, Carlsbad, CA) supplemented with smooth muscle growth supplement (ScienCell) and 2% FBS.

Hormone/drug treatments were performed on cells at 80 to 90% confluency and at passage 8 or 9. At passage 9, HBVSMC still expressed the smooth muscle-specific proteins α-Actin and smoothelin (data not shown). Cell treatments were carried out in hormone-free media supplemented with Charcoal Stripped FBS (Invitrogen). A 6 h time point for COX-2 induction and a 10 nM DHT dose were selected for these studies based on my previous studies (K. L. Osterlund et al., 2010). Cells were treated with DHT (10 nM), 3β-diol (10 nM) or vehicle (0.001% ethanol) for 18 h followed by interleukin-1beta (IL-1β, 1 ng/ml or 5 ng/ml); for an additional 6 h. In a separate set of experiments, cells were pre-treated for 1 h with

the AR-antagonist bicalutamide (BIC, 1 μM, Tocris Bioscience, Ellisville, MO; dissolved in DMSO), the non-selective ER antagonist ICI 182,780 (ICI, 1 μM; Tocris Bioscience; dissolved in ethanol), or the selective ERβ antagonist 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP, 1 μM; Tocris Bioscience; dissolved in ethanol) followed by 18 h of co-treatment with either vehicle (0.001% ethanol + 0.01% DMSO for BIC study; 0.001% ethanol for PHTPP or ICI studies), DHT (10 nM), or 3β-diol (10 nM), then 6 h of IL-1β (5 ng/ml). Antagonist doses of 1 μM were chosen based on results from previous studies (K. L. Osterlund et al., 2010).

Quantitative Real-Time RT-PCR

Quantitative real-time reverse transcriptase polymerase chain reaction (QPCR) was used to measure mRNA expression of gonadal steroid hormone receptors and steroid metabolizing enzymes in adult male cells treated with hormone-free media for 6 h. RNA was extracted using a standard phenol/chloroform/isoamyl procedure (R. Ribaudo et al., 2001) and purity and concentration was confirmed spectrophotometrically using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE). RNA (1 μg) was reverse-transcribed using iSCRIPT (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The resulting cDNA was quantified using the fluorescent detection reagent Quant-iT OliGreen ssDNA Reagent (Invitrogen, Carlsbad, CA). The quantity of cDNA in each PCR reaction was normalized based on the fluorescent quantification, and real-time RT-PCR was performed on 0.34 pg of template using a

LightCycler 480 (Roche Diagnostics Incorporated, Indianapolis, IN). Each reaction included 12µl of SYBR green master mix (Roche Diagnostics Incorporated), 0.5 µl forward primer, 0.5 µl reverse primer, and 2 µl of template or water (control). Data are reported as absolute femtograms (fg) template based on a standard curve generated by serial diluting the product formed by the respective primers. Primer sequences and product sizes (base pairs) are listed in Table 1. The reaction involved an initial melting step at 95 °C for 10 min followed by 50 cycles of 95 °C (denature) for 10 s, 60 °C (annealing) for 10 s, and 72 °C (elongation) for 6 s. A modification of this protocol with a 65° annealing temperature was used for the 17β-HSD and 3α-HSD primer sets and a 55°C annealing temperature was used for the ERβ primer set. Samples were assayed alongside a cDNA standard curve for each primer to determine the absolute cDNA concentration present. For standard curve generation, cDNA was diluted to a stock concentration of 10 pg/ml then serially diluted from 1 pg/ml to 0.0001 fg/ml in PCR grade sterile water to generate eight working standards for each primer pair. The size of the amplified cDNA was confirmed by 2% agarose gel electrophoresis (Figure 6.2A). Negative controls, where water was used in place of template, were used in all experiments. Levels of mRNA expression for each sample were determined by comparison to the standard curve and reported as the absolute concentration of cDNA. Specificity was confirmed via thermal melting curve analysis which showed a single peak at the predicted melting temperature for each primer set.

Western Blot:

Levels of COX-2 protein were examined using standard immunoblotting methods, as previously described (K. L. Osterlund et al., 2010). Briefly, cells were homogenized in lysis buffer and total protein content of whole cell lysate was determined. Next, samples were diluted in Tris-Glycine SDS sample buffer (Invitrogen) and boiled for 5 min. Two color fluorescent standard (LI-COR Biosciences, Lincoln, NE) and diluted samples were loaded into 7.5% Smart gels (LI-COR). Proteins were separated via SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes and non-specific binding was blocked by incubation at room temperature for 30 min in PBS containing 1% Tween (TPBS) and 3% non-fat dried milk. Membranes were incubated with COX-2 (1:1000) mouse monoclonal antibody (Cayman Chemical, Ann Arbor, MI) and β actin (1:5000) mouse monoclonal antibody (Sigma Aldrich Corporation, St. Louis, MO) overnight at 4˚C in TPBS. Following TPBS washes, the membranes were next incubated in Goat Anti-Mouse IR 800 Dye (1:15,000) secondary antibody (LI-COR) for 1 h at room temperature. COX-2 antibody specificity was verified with LPS stimulated Raw-264.7 (mouse macrophage) cell lysate (Santa Cruz Biotechnology, Santa Cruz, CA), which is a positive control for COX-2 protein. Vascular smooth muscle phenotype was confirmed (data not shown) in adult male and fetal HBVSMC at passage 9 with anti-alpha actin monoclonal antibody (1:500, Santa Cruz Biotechnology) and anti-smoothelin polyclonal antibody (1:500, Santa Cruz Biotechnology). Following additional TPBS washes, proteins were visualized

and quantitated using an Odyssey Infrared Imager and data was analyzed using Odyssey V3.0 software (LI-COR).

Reagents:

All reagents were purchased from Sigma Aldrich Corporation (St. Louis, MO) unless otherwise noted.

Statistical Analysis:

Samples from each treatment group were run on the same Western blot to allow for direct comparison. For each treatment, individual measures were repeated to achieve an appropriate number for statistical analysis (n=4-17). Data from QPCR studies are expressed as fg of cDNA and all measures were repeated a sufficient number of times for statistical analysis (n=7-8). Data from Western blots are expressed as an optical density ratio relative to vehicle. All values are reported as means ± SEM. Unless otherwise noted, data were compared using 1 way analysis of variance (ANOVA) across treatment groups using Prism Software (Irvine, CA), and when indicated, differences were compared post hoc using Student Newman-Keuls test. A level of $p<0.05$ was considered significant.

Results

Effects of DHT on COX-2 During IL-1β-Induced Inflammation are AR-Independent/ER-dependent.

I have previously shown that DHT decreases cytokine-induced COX-2 expression in human coronary artery vascular smooth muscle cells via an AR-independent mechanism (K. L. Osterlund et al., 2010). To confirm this effect of DHT treatment in HBVSMC and to test AR and ER-dependence, HBVSMC were pre-treated for 1 h with vehicle, the AR antagonist bicalutamide (1 μM), or the non-selective ER antagonist ICI 182,780 (1 μM), then treated with vehicle or DHT (10 nM; 18 h) followed by vehicle or IL-1β (5 ng/ml; 6 h) in continued presence of hormone/antagonist. DHT decreased IL-1β-induced increases in COX-2 protein ($p<0.05$ vs. IL-1β; Figures 1 and 2). DHT's effect was not blocked by the AR antagonist bicalutamide (Figure 6.1), but was blocked by the non-selective ER antagonist ICI 182,780 ($p<0.05$ vs. IL-1β+DHT; Figure 6.2). Neither antagonist had an effect on COX-2 levels alone.

Gonadal Steroid Receptors and Steroid Metabolizing Enzyme mRNAs are Expressed in HBVSMC.

To confirm expression of necessary gonadal steroid receptors and steroid metabolizing enzymes for DHT metabolism/receptor activation in HBVSMC, HBVSMC were grown in hormone-free media and mRNA levels were measured by QPCR. The size of the amplified cDNA was confirmed by 2% agarose gel electrophoresis for each primer set (Figure 6.3A). Expression of mRNA for AR,

ERα, ERβ, 3α-HSD, 3β-HSD, 17β-HSD, and CYP7B1 was detected in adult HBVSMC. AR expression was higher than ERα or ERβ expression ($p<0.05$; Figure 6.3B), 3α-HSD expression was higher than 3β-HSD, 17β-HSD, or CYP7B1 expression ($p<0.05$; Figure 6.3C).

DHT and 3β-diol Inhibited Moderate and Low Dose Cytokine-Induced Increases in COX-2.

In order to determine if DHT's anti-inflammatory actions could be mimicked by 3β-diol, adult male HBVSMC were treated with vehicle, DHT (10 nM), or 3β-diol (10 nM) for 18 h followed by vehicle or IL-1β (5 ng/ml; 6 h) in continued presence of hormone. Both DHT and 3β-diol decreased IL-1β-induced increases in COX-2 protein ($p<0.05$ vs. IL-1β; Figure 6.4A). To determine if DHT/3β-diol could inhibit low levels of cytokine-induced inflammation, HBVSMC were treated with vehicle, DHT (10 nM; 18 h), or 3β-diol (10 nM; 18 h) followed by vehicle or a low dose of IL-1β (1 ng/ml; 6 h) in the continued presence of hormone. The lower (1ng/ml) dose of IL-1β caused a significantly smaller increase in COX-2 expression compared to the higher (5 ng/ml) dose ($p<0.001$, two-way ANOVA), but still increased COX-2 expression compared to vehicle treated cells ($p<0.01$ vs. VEH). Both DHT ($p<0.01$ vs. IL-1β) and 3β-diol ($p<0.01$ vs. ILI-1β) prevented low dose IL-1β-induced increases in COX-2 protein (Figure 6.4B). The lowest dose of IL-1β (0.2 ng/ml) did not increase COX-2 expression above vehicle levels (data not shown).

DHT Inhibited Cytokine-Induced Increases in COX-2 via an ERβ-Dependent Mechanism.

In order to determine if DHT's anti-inflammatory actions are ERβ-dependent, adult male HBVSMC were pre-treated for 1 h with vehicle or the selective ERβ antagonist PHTPP (1 μM), then treated with vehicle or DHT (10 nM; 18 h) followed by vehicle or IL-1β (5 ng/ml; 6 h) in continued presence of hormone/antagonist. DHT decreased IL-1β-induced increases in COX-2 protein ($p<0.05$ vs. IL-1β; Figure 6.5). DHT's effect was blocked by the selective ERβ antagonist PHTPP ($p<0.01$ vs. IL-1β+DHT).

Discussion

The goal of this study was to determine if DHT's anti-inflammatory effects are mediated via ERβ. Using COX-2 protein expression as a marker for vascular inflammation, I examined the effects of the non-aromatizable androgen, DHT, and its metabolite 3β-diol, on COX-2 protein expression in HBVSMC. The results of these studies show that: 1) DHT decreases cytokine-induced COX-2 expression via an AR-independent/ER-dependent mechanism in HBVSMC. 2) Primary cultured HBVSMC express AR, ERα, ERβ, and the enzymes necessary for 3β-diol metabolism (3α-HSD, 3β-HSD, 17β-HSD, and CYP7B1). 4) Both DHT and its metabolite 3β-diol attenuate moderate (5 ng/ml) and low dose (1 ng/ml) IL-1β-induced COX-2 expression. 5) DHT attenuates cytokine-induced COX-2 expression via ERβ-dependent mechanism. Taken together, these data support my hypothesis

that during cytokine-induced inflammation in HBVSMC, DHT decreases COX-2 via metabolism to 3β-diol and subsequent activation of ERβ.

In addition to my recent finding that DHT can inhibit cytokine-induced COX-2 production in human coronary artery smooth muscle cells (K. L. Osterlund et al., 2010), androgens have been shown to have other cardioprotective, anti-inflammatory, and anti-oxidant effects. Cardioprotective effects of androgens include inhibition of hypoxic pulmonary hypertension (M. Oka et al., 2007) and decreased infarct size after cerebral ischemia in rodents (Y. Pan et al., 2005; J. Cheng et al., 2008; Z. K. Li et al., 2008; M. Uchida et al., 2009; J. Cheng et al., 2010). In addition, low testosterone levels predict increased incidence of stroke (B. B. Yeap et al., 2009) and poorer outcome after stroke in men (L. L. Jeppesen et al., 1996). Androgens have been shown to reduce inflammation in a variety of cells/tissues including human monocytes (Z. G. Li et al., 1993), human macrophages (M. P. Corcoran et al., 2010), mouse macrophages (D. A. Padgett and R. M. Loria, 1998; J. A. Rettew et al., 2008; Y. Qiu et al., 2010), human prostate cancer cells (E. T. Keller et al., 1996), human endothelial cells (H. Hatakeyama et al., 2002; T. K. Mukherjee et al., 2002; G. D. Norata et al., 2006; H. Jin et al., 2009), and human coronary artery vascular smooth muscle cells (K. L. Osterlund et al., 2010). Androgen treatment *in vivo* has also been shown to decrease inflammatory markers in androgen deficient men (C. J. Malkin et al., 2004), men with autoimmune rheumatic conditions (A. Bizzarro et al., 1987; M. Cutolo et al., 1991), men with metabolic syndrome (S. Y. Kalinchenko et al., 2010a; S. Y. Kalinchenko et al., 2010b), and rabbits fed a high cholesterol diet (Y. Qiu et al., 2010). Androgens have

also been shown to have anti-oxidant effects in rat ventral prostate (N. N. Tam et al., 2003) and human endothelial cells (Z. R. Xu et al., 2010). Thus, these data indicate that androgen therapy might protect against vascular inflammation in androgen deficient men already predisposed to cardiovascular events. Since androgen levels decline with age (B. B. Yeap, 2008), androgen deficiency is not uncommon in older men Therefore, my data suggest that targeting the 3β-diol/ERβ pathway may be a more direct approach to reduce vascular inflammation. This could be accomplished with 3β-diol treatment, treatment with another ERβ selective agonist such asdiarylpropionitrile or inhibition of CYP7B1, the enzyme that converts 3β-diol to inactive metabolites (M. Sundin et al., 1987; Z. Weihua et al., 2002).

The results of my current studies point to ERβ as an alternate receptor pathway utilized by DHT during cytokine-induced inflammation. Since DHT can be converted to 3β-diol by the enzymes 3α-HSD, 3β-HSD and 17β-HSD (Y. Jin and T. M. Penning, 2001; Z. Weihua et al., 2002; A. Gangloff et al., 2003; S. Torn et al., 2003; S. Steckelbroeck et al., 2004), I sought to determine if these enzymes are also found in HBVSMC. My data show that primary HBVSMC express the gonadal steroid receptors AR, ERα, ERβ, as well as enzymes involved in 3β-diol metabolism, 3α-HSD, 3β-HSD, 17β-HSD, and CYP7B1. This is consistent with previous studies showing that blood vessels contain AR, ERα, ERβ, and 3β-HSD (G. D. Snyder et al., 2002; Y. Nakamura et al., 2005; R. J. Gonzales et al., 2007; H.-C. Shih et al., 2008). Results of my studies also show that both DHT and 3β-diol decrease cytokine-induced COX-2 expression and that DHT's effects are mediated via ERβ since an ERβ selective antagonist can block the actions of DHT. These data are supported

by a recent study by Norata et al (2010) showing that 3β-diol has anti-inflammatory actions in human umbilical vein endothelial cells and mouse aorta, and that these effects are also mediated by ERβ (G. D. Norata et al., 2010).

Many studies have shown that ERβ activation, like androgen treatment, has a variety of cardioprotective and anti-inflammatory effects. Cardioprotective effects of ERβ activation include protection against vascular injury in mice (M. D. Iafrati et al., 1997), prevention of L-glutamate-induced increases in blood pressure in male rats (S. Gingerich and T. L. Krukoff, 2006), prevention of aldosterone/high salt intake-induced increases in blood pressure, cardiac mass, and cardiac myocyte hypertrophy in rats (P. A. Arias-Loza et al., 2007), prevention of neointima formation in mice (Y. D. Krom et al., 2007), protection against damage in a rat carotid injury model (S. Makela et al., 1999), protection against cerebral ischemia in rodents (H. V. Carswell et al., 2004; N. R. Miller et al., 2005), protection against myocardial ischemia in mice (T. Pelzer et al., 2005; I. Nikolic et al., 2007; M. Wang et al., 2008; N. D. Vornehm et al., 2009; M. Wang et al., 2009), and protection against *in vitro* ischemia in mouse brain endothelial cells (J. Guo et al., 2010). In humans, ERβ gene polymorphisms have been associated with higher systolic blood pressure (S. Ogawa et al., 2000; J. A. Ellis et al., 2004) and increased risk of myocardial infarction (K. M. Rexrode et al., 2007; S. Domingues-Montanari et al., 2008). A similar phenotype is observed in ERβ deficient mice, which develop both systolic and diastolic hypertension as they age (Y. Zhu et al., 2002). ERβ activation, like androgens, has been shown to have anti-inflammatory effects in a variety of cells/tissues such as rodent microglial (A. E. Baker et al., 2004; J. A. Smith et al.,

2011), mouse brain (C. M. Brown et al., 2010), motorneurons (A. Das et al., 2011), astrocytes (D. K. Lewis et al., 2008), rat vascular smooth muscle cells (D. Xing et al., 2007), and human endothelial cells (G. D. Norata et al., 2010). Taken together, these studies show that ERβ activation has similar protective effects to androgens, and support the theory that some of the beneficial effects of androgens may occur through activation of ERβ.

Aside from ERβ activation, other AR-independent mechanisms for androgen action have been proposed for DHT's anti-inflammatory actions. DHT can activate the sex hormone binding globulin receptor to increase cyclic AMP and protein kinase A (N. Fortunati et al., 1996; A. M. Nakhla and W. Rosner, 1996). DHT can also increase intracellular calcium via an unidentified membrane-bound receptor (W. P. Benten et al., 1999b; W. P. Benten et al., 1999a). Furthermore, several authors have also hypothesized that a membrane bound AR may exist that is not blocked by classical AR antagonists (E. F. Konoplya and E. H. Popoff, 1992; W. P. Benten et al., 1999b; W. P. Benten et al., 1999a; C. A. Heinlein and C. Chang, 2002). Whether these alternative pathways for androgen action may also contribute to DHT's anti-inflammatory effects is yet to be determined.

In this study, I used primary human cultured vascular smooth muscle cells to investigate the mechanism of DHT's anti-inflammatory effect. Although more clinically relevant compared to rodent studies, the use of cultured cells does provide certain limitations. First, each cell type originated from a single donor. However, my previous studies in primary human coronary artery vascular smooth muscle cells originating from a different donor also showed that DHT attenuated increases in

COX-2 levels following IL-1β stimulation via an AR-independent mechanism (K. L. Osterlund et al., 2010). Furthermore, my findings match those of Norata et al (2010) who showed that 3β-diol decreased cytokine-induced vascular inflammation via an ERβ-dependent mechanism in human umbilical vein endothelial cells (G. D. Norata et al., 2010). Taken together these studies minimize the concern of using cells isolated from a single donor. The second major limitation of using cultured vascular smooth muscle cells is that their properties change over time. For example, smooth muscle cells quickly lose their contractile properties(J. Thyberg et al., 1997). It is possible that receptor levels for androgens and cytokines may also change in culture. To minimize the risk of changes in culture, all experiments were performed after a small number of passages (8 or 9). Furthermore, at the time of experiments (passages 8-9) the cells still expressed the smooth muscle cell markers smoothelin and alpha actin (data not shown).

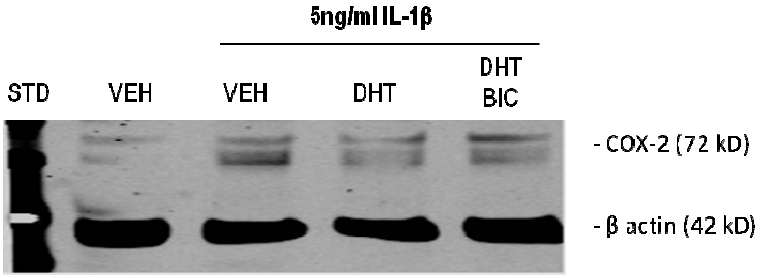
In summary, I have shown that DHT decreases cytokine-induced COX-2 expression via an ERβ-dependent mechanism that likely involves the metabolism of DHT to the ERβ agonist 3β-diol. This is supported by my finding that HBVSMC express ERβ and the necessary enzymes to convert DHT to 3β-diol and by my finding that 3β-diol mimics the anti-inflammatory effect of DHT (Figure 6.6). Thus, I have identified an ERβ-dependent anti-inflammatory effect for DHT in HBVSMC. A deeper understanding of how androgens modulate inflammation could potentially provide insight into more effective approaches to manage the progression of vascular diseases which eventually lead to devastating end points, such as heart attack or stroke.

Figures and Legends

Target Gene	Forward Primer	Reverse Primer	Product Size
Androgen Receptor	5'-AGTGGATGGGCTGAAAAA-3'	5'- GGGTGTGGAAATAGATGGG-3'	305 bp
Estrogen Receptor α	5'- AGGGAAAGTAGGGCAGAAA-3'	5'- ACGCTGGGAAATGAAGAA-3'	172 bp
Estrogen Receptor β	5'-AAGAATATCTCTGTGTCAAGGCCATG-'3	5'-GGCAATCACCCAAACCAAAG-3'	143 bp
3α-HSD	5'-TGGGGTTGTGGTCCTGGCCA-3'	5'CCACACGCAGGGCCTTCTGG-3'	229 bp
3β-HSD	5'- CAGCCAGGCATGGCCGACTC-3'	5' CTGCTGCCACCTCATGGGCC-3'	235 bp
17β-HSD	5'-AGAACCTGCTCTCCGCGCCT-3'	5'- TCCATTGGGGCCCCTCCTCC-3'	305 bp
CYP7B1	5'- ATGGCAGCAGTGCGTGACGA-3'	5'- CGCACACAGTAGTCCCCGGT-3'	221 bp

Table 6.1. ***Primer sequences used for quantitative real time reverse transcriptase polymerase chain reaction.*** Primer sequences used for QPCR and product sizes in base pairs are listed for androgen receptor, estrogen receptors alpha and beta, 3alpha-hydroxysteroid dehydrogenase (HSD), 3beta-HSD, 17beta-HSD, and CYP7B1.

A)



B)

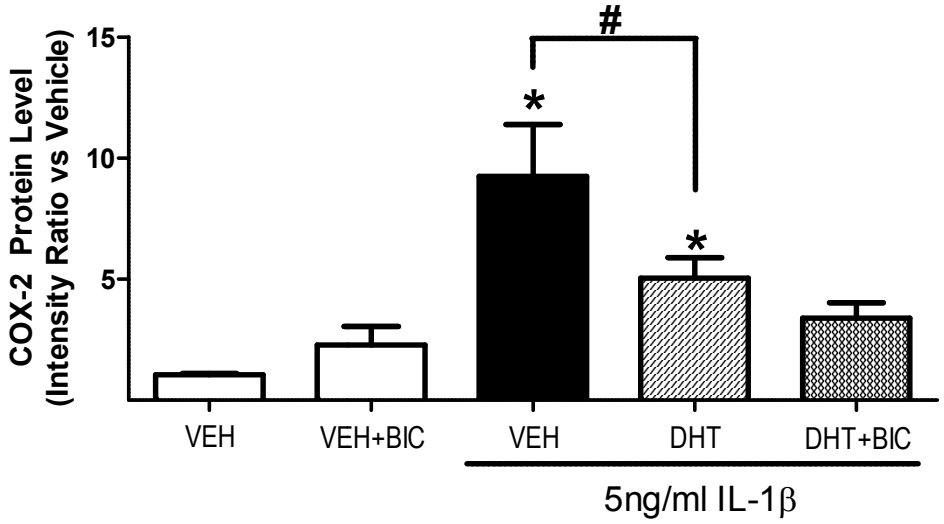
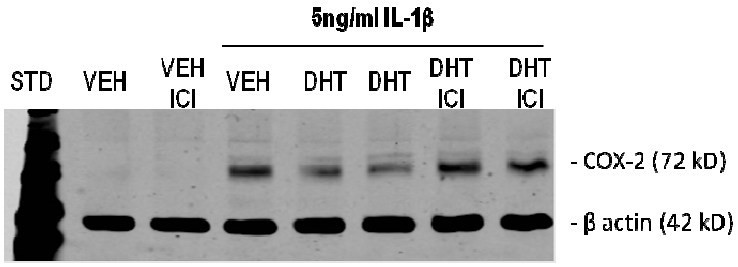


Figure 6.1. ***Effects of DHT on IL-1β-Induced COX-2 are Androgen Receptor-Independent.*** COX-2 protein expression was assessed in fetal HBVSMC pre-treated for 1 h with vehicle (VEH) or bicalutamide (BIC, 1 μM) followed by VEH or dihydrotestosterone (DHT, 10 nM) for 18 h, then exposed to IL-1β (5 ng/ml; 6 h) in the continued presence of hormone/antagonist. A: Representative blot for COX-2. B: COX-2 levels after bicalutamide treatment. *P<0.01 vs. VEH, #P<0.001 vs. IL-1β (n= 4-17 per group).

A)



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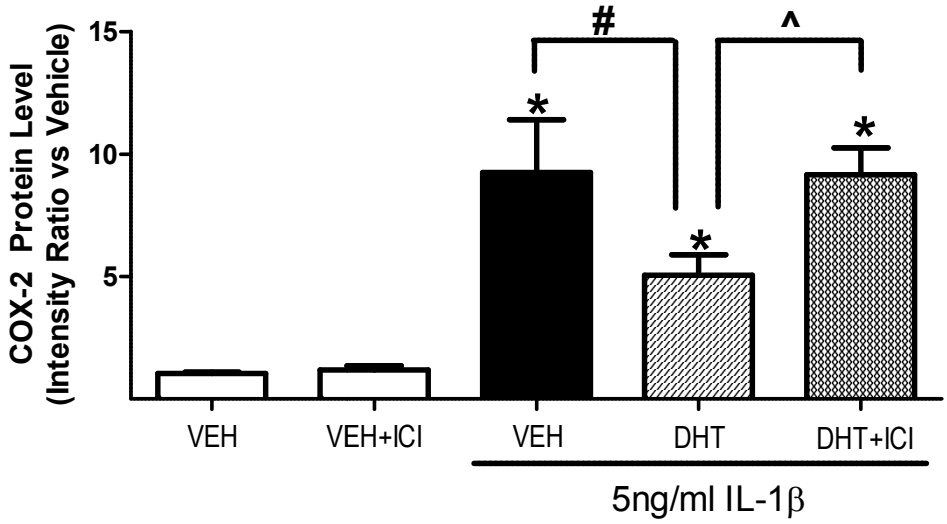


Figure 6.2. ***Effects of DHT on IL-1β-Induced COX-2 are Estrogen Receptor Dependent.*** COX-2 protein expression was assessed in fetal HBVSMC pre-treated for 1 h with vehicle (VEH) or ICI 182,780 (ICI, 1 μM), followed by VEH or dihydrotestosterone (DHT, 10 nM) for 18 h, then exposed to IL-1β (5 ng/ml; 6 h) in the continued presence of hormone/antagonist. A: Representative blot for COX-2. B: COX-2 levels after ICI treatment. *P<0.01 vs. VEH, #P<0.05 vs. IL-1β, ^P<0.05 vs. IL-1β+DHT (n=6-17 per group).

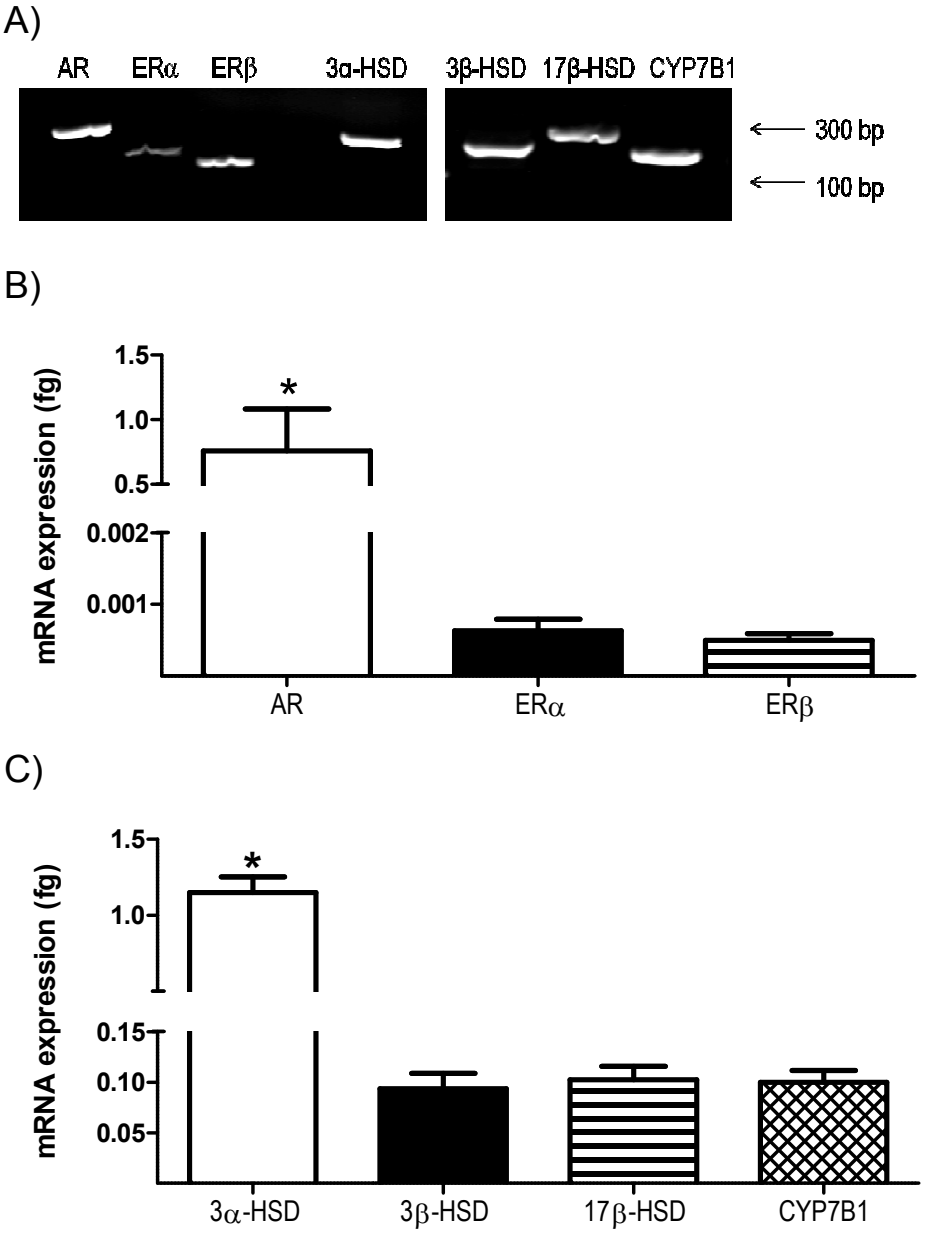
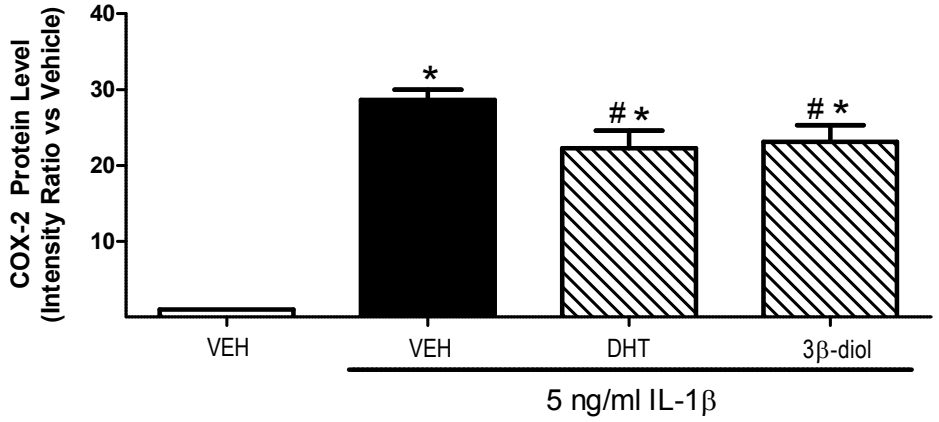


Figure 6.3. ***Gonadal Steroid Receptors and Steroid Metabolizing Enzyme mRNAs are Expressed in HBVSMC.*** A) Representative images of QPCR products run on a 2% agarose gel. B) Hormone receptors AR, ERα, and ERβ mRNA expression was assessed in adult male HBVSMC treated for 6 h with hormone-free media. *P<0.05 vs. ERα or ERβ (n=7-8 per group). C) Steroid metabolism enzymes 3α-HSD, 3β-HSD, 17β-HSD, and CYP7B1 mRNA expression was assessed in adult male HBVSMC treated for 6 h with hormone-free media. *P<0.05 vs. 3β-HSD, 17-β-HSD, or CYP7B1 (n= 7-8 per group).

A)



B)

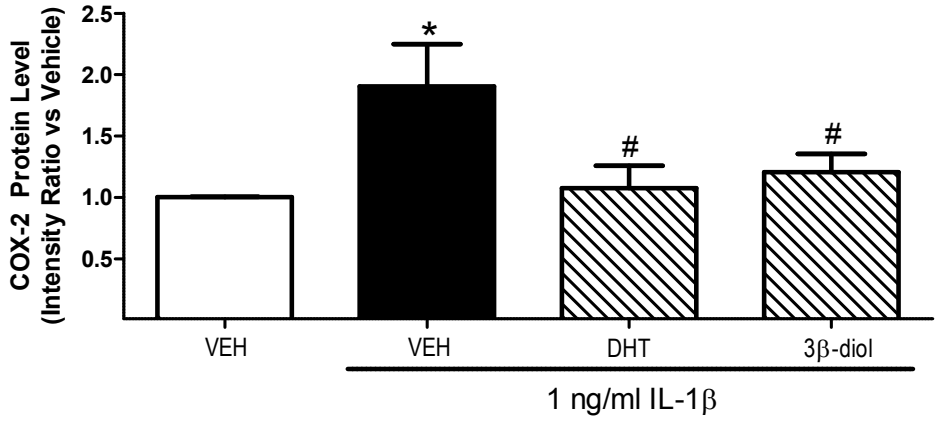


Figure 6.4. ***DHT and 3β-diol Inhibited Moderate and Low Dose Cytokine-Induced Increases in COX-2.*** A: COX-2 protein expression was assessed in adult male HBVSMC treated with VEH, DHT (10 nM), or 3β-diol (10 nM) for 18 h, then exposed to IL-1β (5 ng/ml; 6 h) in the continued presence of hormone. *P<0.001 vs. VEH, #P<0.05 vs. IL-1β (n= 10-13 per group). B: COX-2 protein expression was assessed in adult male HBVSMC treated with VEH, DHT (10 nM), or 3β-diol (10 nM) for 18 h, then exposed to a low dose of IL-1β (1 ng/ml; 6 h) in the continued presence of hormone. *P<0.01 vs. VEH, #P<0.01 vs. IL-1β (n= 4-10 per group).

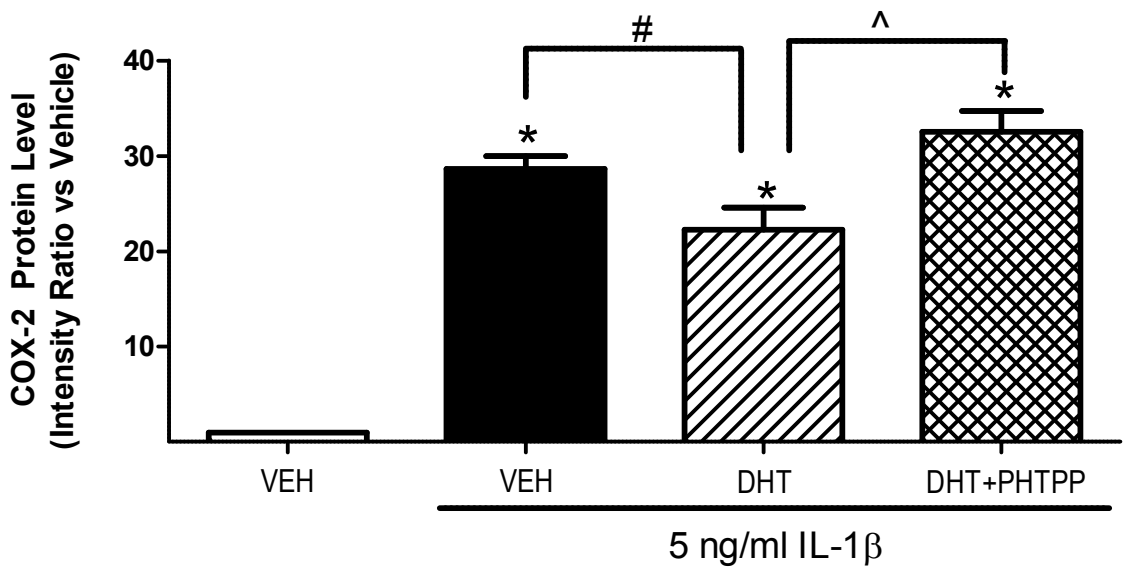


Figure 6.5. ***DHT Inhibited Cytokine-Induced Increases in COX-2 via an ERβ-Dependent Mechanism.*** COX-2 protein expression was assessed in adult male HBVSMC pre-treated for 1 h with vehicle (VEH) or the ERβ antagonist, PHTPP (1 μM), followed by VEH or DHT (10 nM) for 18 h, then exposed to IL-1β (5 ng/ml; 6 h) in the continued presence of hormone/antagonist. *P<0.001 vs. VEH, #P<0.05 vs. IL-1β, ^P<0.001 vs. IL-1β+DHT (n= 10-13 per group).

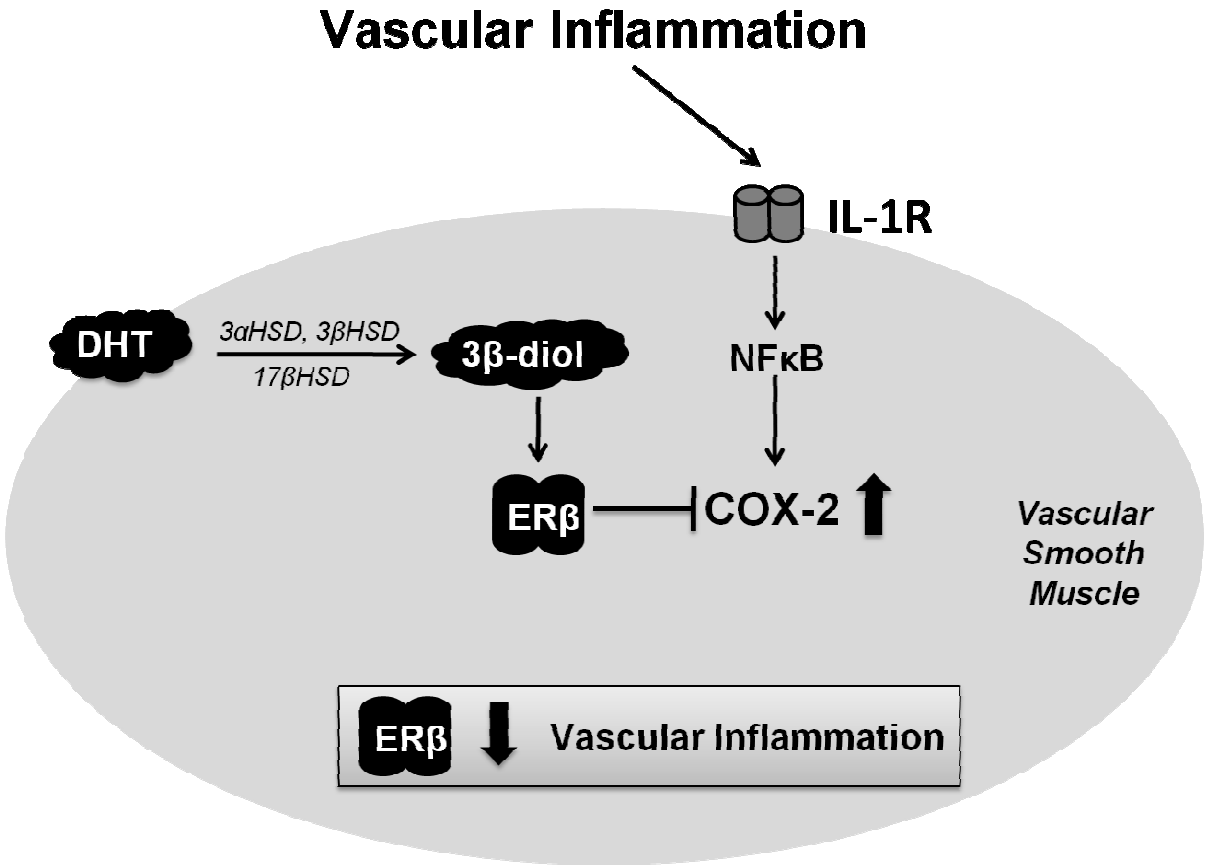


Figure 6.6. Schematic Diagram of Proposed Mechanism for DHT's Anti-Inflammatory Actions. In our proposed model, during interleukin-1 beta (IL-1β) induced inflammation, dihydrotestosterone (DHT) reduces cyclooxygenase-2 (COX-2) expression via metabolism to 3 beta-diol (3β-diol) by the enzymes 3α-hydroxysteroid dehydrogenase (HSD), 3β-HSD, and/or 17β-HSD in vascular smooth muscle cells. 3β-diol then activates estrogen receptor beta (ERβ) and downregulates COX-2 expression. This pathway can be inhibited by the enzyme CYP7B1, which converts 3β-diol to its inactive metabolites. IL-1R (interleukin-1 receptor), NFκB (nuclear factor kappa B).

CHAPTER 7: DISCUSSION

The overall goal of this dissertation was to determine the mechanisms by which androgens can influence the vascular inflammatory response under both physiological and pathophysiological conditions. The hypotheses tested were 1) dihydrotestosterone (DHT) increases levels of vascular inflammatory mediators under physiological conditions via androgen receptor (AR) activation, 2) DHT decreases levels of vascular inflammatory mediators under pathophysiological conditions via an AR-independent mechanism, and 3) DHT's anti-inflammatory effects during vascular inflammation are estrogen receptor beta (ERβ)-mediated.

In support of my first hypothesis, I found that DHT increased cyclooxygenase-2 (COX-2) expression under normoxia/normal conditions in human coronary artery vascular smooth muscle (VSM) cells, human brain VSM cells, and rat pial arteries. In human coronary VSM cells, I showed that this effect was AR-dependent. In support of my second hypothesis, I found that DHT decreased cytokine-, endotoxin-, hypoxia-, or hypoxia with glucose deprivation (HGD)-induced COX-2 expression in human VSM cells. In addition, DHT also decreased hypoxia-induced COX-2 and hypoxia-inducible factor-1 alpha (HIF-1α) expression in rat pial arteries, hypoxia and HGD-induced HIF-1α in human brain VSM cells, endotoxin and HGD-induced toll-like receptor 4 (TLR4) expression in human VSM cells, and cytokine-induced oxidative stress in human VSM cells. I found that DHT's effect on cytokine-induced COX-2 expression was AR-independent in human coronary and brain VSM cells,

and that DHT's effects on HGD-induced COX-2 and TLR4 expression were AR-independent in human brain VSM cells. In support of my third hypothesis, I found that DHT decreased cytokine-induced COX-2 expression in human brain VSM cells via an ERβ-dependent mechanism, likely via metabolism to 3β-diol.

Androgens appear to be pro-inflammatory during normal, physiological conditions via activation of AR. Support for this hypothesis can be found in the present studies, as well as in the literature. In chapter 3, I show that in human coronary VSM cells, but not human brain endothelial cells, DHT increases COX-2 expression under basal, un-stimulated conditions. In chapter 4, I also show that DHT increases COX-2 under normoxia in both rat pial arteries and human brain VSM cells. Previous data also shows that DHT has pro-inflammatory effects in the absence of induced inflammation or injury. DHT treatment, *in vivo* and *ex vivo*, increases nuclear factor kappa B (NFκB)-DNA binding activity, and increases COX-2 and inducible nitric oxide synthase (iNOS) protein levels in the absence of induced inflammation via an AR-independent mechanism in rat cerebral arteries (R. J. Gonzales et al., 2009). Taken together, the data point to an AR-mediated, pro-inflammatory role for androgens under normal, physiological conditions.

Androgens appear to be anti-inflammatory during pathophysiological conditions, via an AR-independent mechanism. In chapter 3, I demonstrated that DHT could inhibit cytokine- or endotoxin-induced COX-2 expression when co-administered for 6 h. This effect was not blocked by the AR antagonist bicalutamide. In chapter 4, I found that DHT prevented or attenuated hypoxia-induced COX-2 and HIF-1α expression in human VSM cells and rat pial arteries. I also showed that DHT

attenuated HGD-induced COX-2 and HIF-1α expression in human brain VSM. This effect was not blocked by the AR antagonist bicalutamide. In chapter 5, I showed that DHT inhibited endotoxin- or HGD-induced TLR4 expression in human brain VSM cells. Again, the effects of DHT during HGD were not inhibited by bicalutamide. Finally, in chapter 6, I show that DHT inhibited cytokine-induced COX-2 expression in fetal and adult human brain VSM cells, while bicalutamide had no effect. Taken together, my studies show that under pathophysiological conditions of cytokine-, endotoxin-, hypoxia, or HGD DHT decreases vascular inflammation, likely via an AR-independent mechanism.

DHT's anti-inflammatory effects appear to be at least partially mediated by the DHT metabolite 3β-diol and ERβ signaling. In chapter 6, I show that DHT's effect to attenuate cytokine-induced COX-2 expression in brain VSM cells cannot be blocked with an AR antagonist, but can be blocked with either a non-selective ER antagonist, or a selective ERβ antagonist. Additionally I found that DHT's anti-inflammatory effects could be mimicked by its metabolite 3β-diol, an ERβ agonist. Using QPCR, I also showed that human brain VSM cells express AR, ERβ, and ERα as well as the enzymes necessary to metabolize DHT to 3β-diol (3α-HSD, 3β-HSD and 17β-HSD). My data is supported by a recent study from Norata et al (2010), who also found that 3β-diol, via ERβ activation, could attenuate cytokine-induced adhesion molecules, monocyte chemoattractant protein, and interleukin-6 in human umbilical vein endothelial cells. They also found that 3β-diol decreased endotoxin-induced interleukin-6, and adhesion molecule expression in male mouse aorta (G. D. Norata et al., 2010). Furthermore, *in vitro* treatment with 3β-diol has been shown to

downregulate thromboxane receptor expression in vascular smooth muscle, which was blocked by pretreatment with an ERβ antagonist (R. G. Mishra et al., 2006). These studies provide evidence that 3β-diol, acting via ERβ, has anti-inflammatory effects in the vasculature. In addition, my study also shows that human brain VSM cells have the enzymes necessary to convert DHT to 3β-diol and that DHT's anti-inflammatory effect is ERβ-mediated.

Based on my studies, I hypothesize that DHT has at least two separate pathways that it can utilize to influence vascular inflammation. Under normal physiological conditions, DHT activates the AR and causes an increase in pro-inflammatory mediators such as COX-2. Conversely, under pathophysiological conditions, such as endotoxin, cytokine, hypoxia, or HGD, DHT is converted to 3β-diol, which subsequently activates ERβ causing a decrease in pro-inflammatory mediators such as COX-2. Based on this theory, low dose androgen treatment would be beneficial for men with existing cardiovascular disease or inflammatory illness. Since humans have generally started to develop atherosclerosis by adulthood, this theory would support the use of androgen replacement therapy or ERβ agonists in virtually any androgen-deficient man. Since androgen levels decline with age (B. B. Yeap, 2008), this represents a large proportion of the male population. Clinical data supports this hypothesis. In men, low androgen levels predict increased incidence of stroke (B. B. Yeap et al., 2009), aortic aneurysm (B. B. Yeap et al., 2010), hypercholesterolemia, diabetes, coronary heart disease (K. M. English et al., 2000b; A. Ponholzer et al., 2010) and hypertension (K. M. English et al., 1997). Furthermore, low androgen levels are also associated with poorer

outcome after stroke (L. L. Jeppesen et al., 1996), increased vascular mortality (C. J. Malkin et al., 2010), increased arterial stiffness (M. Yaron et al., 2009), increased inflammation (V. Kupelian et al., 2010), adverse lipid profile, high levels of fibrinogen, insulin, and pro-coaguable factors (K. M. English et al., 1997). T replacement has also been used in clinical settings to relieve symptoms of angina (K. M. English et al., 2000a) and to reduce inflammation (C. J. Malkin et al., 2004). These data indicate that androgen therapy may be beneficial in aging men. However, my data suggest that targeting the 3β-diol/ERβ pathway may be a more direct approach to reduce vascular inflammation. This could be accomplished with 3β-diol treatment, treatment with another ERβ selective agonist such as diarylpropionitrile (DPN), or inhibition of CYP7B1, the enzyme that converts 3β-diol to inactive metabolites (M. Sundin et al., 1987; Z. Weihua et al., 2002).

The majority of my studies were performed using cultured cells, which can present a number of limitations that need to be addressed. First, each cell type originated from a single donor. However, the human coronary VSM cells using in chapter 3 were from a different donor than those from chapter 5. Also, the human brain VSM cells used in chapter 4 were from a different donor than those used in chapter 5 and chapter 6. Therefore, I have found anti-inflammatory effects of DHT in vascular smooth muscle cells from 4 different donors. In addition, my work is supported by studies that have found similar results in rodent tissue (chapter 4) (R. J. Gonzales et al., 2009) and human endothelial cells (G. D. Norata et al., 2006; G. D. Norata et al., 2010).

A second concern with using cultured cells, and smooth muscle cells in particular, is that properties can change in culture. For example, smooth muscle cells lose their contractile properties in culture. For this reason, experiments were performed at the smallest number of passages possible to minimize this risk. Furthermore, I confirmed that each VSM cell type was still expressing the VSM cell specific proteins alpha-actin and smoothelin, and that my endothelial cells still expressed the endothelial cell specific protein Von Willebrand Factor, at same number of passages in which the experiments were performed. Furthermore, in chapter 2 I confirmed that human coronary VSM cells expressed AR at passage 6, and in chapter 6 I confirmed that human brain VSM cells they expressed AR, ERα, ERβ, 3β-HSD, 17β-HSD, and CYP7B1 at passage 9. Expression of these hormone receptors and hormone metabolism enzymes provides evidence that these cells still expressed all the necessary proteins for gonadal steroid receptor activation and metabolism of DHT. I believe the benefits of using a more clinically relevant human cell model outweigh the risk for changes in culture and inter-donor variability for the mechanistic questions answered by my studies. However, *in vivo* studies in rodents will be necessary to confirm that DHT's anti-inflammatory effects in culture translate to beneficial physiological changes in live animals.

In order to provide further support for my theory that DHT's anti-inflammatory effects occur via ERβ activation, there are several future studies I would like to conduct. First, I would like provide evidence that human brain VSM cells can produce 3β-diol from DHT. In order to do this, I would treat human brain VSM cells with vehicle or DHT (10nM, 100nM, or 1μM) for 18 h and then measure 3β-diol in the

media using liquid chromatography mass spectrometry (LCMS). With increasing concentrations of DHT the cells should produce increasing amounts of 3β-diol. Next, I would like to repeat my DHT and 3β-diol studies outlined in chapter 6 in human brain endothelial cells, as well as the LCMS experiment, in order to determine if endothelial cells also have the ability to convert DHT to 3β-diol, and if DHT has ERβ/3β-diol mediated anti-inflammatory effects in human brain endothelial cells. I would then like to repeat these studies *ex vivo* in rat pial arteries to determine the net effect of DHT/ 3β-diol when both VSM cells and endothelial cells are present. To further clarify the role of the AR, I would compare the effects of DHT and 3β-diol on cytokine-induced COX-2 expression in freshly isolated pial arteries from testicular feminization mutation (TFM) rats that lack functional ARs and wild type male rats. I predict that both DHT and 3β-diol would decrease cytokine-induced COX-2 expression in wild type and TFM rats, but that an ERβ antagonist would block these effects. These studies would provide support for my hypothesis that DHT's anti-inflammatory effects are 3β-diol/ERβ –mediated and AR-independent in the cerebral vasculature.

For my second set of experiments, I would like to determine the mechanism by which ERβ activation inhibits cytokine-induced COX-2 expression. To do this I would use a COX-2 promoter/luciferase reporter gene assay. Interleukin-1 beta (IL-1β) stimulation would be used to increase COX-2 transcription. IL-1 receptor activation leads increased activation of NFκB, which binds to an NFκB binding site in the COX-2 promoter causing increased transcription of COX-2 (C. Cao et al., 1997; J. S. Michael et al., 1999). Human brain VSM cells would be transfected with a

vector containing the COX-2 promoter driving a luciferase reporter gene or a control vector. The transfected cells will be pre-treated (18 h) with vehicle, DHT or 3β-diol and challenged with IL-1β (6 h) in the continued presence of hormone. These experiments would be repeated using the AR antagonist bicalutamide to determine AR involvement and the ERβ specific-antagonist PHTPP to determine ERβ involvement. I predict that DHT and 3β-diol would attenuate COX-2 transcriptional activity compared to vehicle following IL-1β stimulation, via an AR-independent, ERβ-dependent mechanism. These findings would provide molecular evidence to support the hypothesis that DHT decreases cytokine-induced COX-2 expression via conversion to 3β-diol and subsequent ERβ-mediated suppression of COX-2 transcription.

Finally, to determine if DHT's anti-inflammatory effects via ERβ activation have an effect *in vivo*, I would like to compare the effects of low-dose *in vivo* DHT treatment on infarct size and COX-2 expression in middle cerebral arteries isolated from ERβ deficient mice and wild type mice that have undergone middle cerebral artery occlusion (MCAO; *in vivo* stroke model) or endotoxin (LPS) injection. These studies would also be repeated in TFM rats and wild type rats. I predict that DHT would reduce infarct size and pial vessel COX-2 expression in the wild type rodents and TFM rats, but not in the ERβ-deficient mice. In addition basilar arteries (the largest pial vessel) would be isolated from each of these rodents for length-tension measurements using a wire myograph system. I predict that the vessels from rodents that were subjected to LPS challenge or MCAO would have increased COX-2 expression and thus would have a greater contractile response to COX-2

inhibition. I predict that vessels from DHT treated wild type rodents and TFM rats would have a reduced contractile response to COX-2 inhibition compared to vehicle treated wild type rodents or DHT treated ERβ deficient mice. These studies would provide support for my hypothesis that low dose DHT is anti-inflammatory and protective against ischemia and inflammation via an ERβ-mediated mechanism.

In conclusion, my data support my hypotheses that DHT increases levels of vascular inflammatory mediators under physiological conditions via AR activation, DHT decreases levels of vascular inflammatory mediators under pathophysiological conditions via an AR-independent mechanism, and that DHT's anti-inflammatory effects during inflammation are ERβ-mediated. These studies contribute to the field of vascular inflammation in two important ways. First, I have identified ERβ activation as an important anti-inflammatory pathway for androgens in VSM. This finding is particularly intriguing because estrogens have almost exclusively been shown to have protective effects in the vasculature, while the effects of androgens have been contradictory, with many animal studies shown detrimental effects, and but much of the clinical data showing protective effects. The discrepancy appears to be largely dose and condition dependent. The subjects of animal studies are generally young healthy rodents, while the subjects of clinical studies are generally middle aged to elderly men with declining or already deficient androgen levels likely with significant amounts of atherosclerosis. Since I have found androgens only to be protective under pathophysiological conditions, many of the studies in healthy young rodents may not yield similar results unless some form of inflammatory or ischemic insult was induced. Furthermore, doses of androgens administered in

human studies are more likely to be the lowest physiological dose, while rodent studies and many cell culture studies tend to use slightly higher doses to maximize effects. For instance, one study in human umbilical vein endothelial cells used 400nM DHT and found pro-inflammatory effects of DHT, while another used a very low 0.1 nM dose and anti-inflammatory effects of DHT in the same cell type (A. K. Death et al., 2004). Additionally, using a rodent model of cerebral ischemia, Uchida et al. found that low doses of either T or DHT were protective against ischemia induced brain damage, while higher doses were actually detrimental (M. Uchida et al., 2009). Therefore, both experimental conditions and dose are of upmost importance when examining the effects of androgens on vascular inflammation. The results obtained in my studies should be physiologically relevant since a low dose of DHT was used.

The second important contribution to the field I have made is demonstrating the presence of a variety of steroid metabolizing enzymes in human VSM cells. This data emphasized the importance of local *de novo* synthesis of gonadal steroids in human vasculature. Many studies focus on plasma hormone levels, but the presence of a variety of gonadal steroid metabolizing enzymes within VSM cells allows for both ER and AR mediated effects of androgens, as I have demonstrated.

I believe that due to DHT's anti-inflammatory and anti-oxidant effects that I have observed in the vasculature, that low-dose androgen therapy or ERβ agonists may be beneficial in aging, androgen deficient men with atherosclerosis to prevent rupture of plaques by reducing oxidative stress and inflammation. In addition, these protective effects of DHT/ERβ agonist treatment may also be beneficial in reducing

damage following stroke. Due to the negative cardiovascular effects of androgen deficiency, men at risk for cardiovascular events, such as a heart attack or stroke, should have their androgen levels tested. If androgen levels are low, these men could be placed on low dose androgen or ERβ agonist therapy to prevent plaque rupture or reduce secondary brain or heart injury following cerebral or cardiac ischemia. This preventative measure could reduce the frequency and severity of ischemic events in aging men.

REFERENCES

Abe T, Kunz A, Shimamura M, Zhou P, Anrather J, Iadecola C (2008) The neuroprotective effect of prostaglandin E2 EP1 receptor inhibition has a wide therapeutic window, is sustained in time and is not sexually dimorphic. J Cereb Blood Flow Metab 29:66-72.

Adams MR, Register TC, Golden DL, Wagner JD, Williams JK (1997) Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. Arterioscler Thromb Vasc Biol 17:217-221.

Adams MR, Kaplan JR, Manuck SB, Koritnik DR, Parks JS, Wolfe MS, Clarkson TB (1990) Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys. Lack of an effect of added progesterone. Arteriosclerosis 10:1051-1057.

Ahmad AS, Yun YT, Ahmad M, Maruyama T, Dore S (2008) Selective blockade of PGE2 EP1 receptor protects brain against experimental ischemia and excitotoxicity, and hippocampal slice cultures against oxygen-glucose deprivation. Neurotox Res 14:343-351.

Ahmad M, Zhang Y, Liu H, Rose ME, Graham SH (2009) Prolonged opportunity for neuroprotection in experimental stroke with selective blockade of cyclooxygenase-2 activity. Brain Res 1279:168-173.

Ahmad M, Ahmad AS, Zhuang H, Maruyama T, Narumiya S, Doré S (2007) Stimulation of prostaglandin E2-EP3 receptors exacerbates stroke and excitotoxic injury. Journal of Neuroimmunology 184:172-179.

Ahrens-Fath I, Politz O, Geserick C, Haendler B (2005) Androgen receptor function is modulated by the tissue-specific AR45 variant. FEBS J 272:74-84.

Al Zubair K, Razak A, Bexis S, Docherty JR (2005) Relaxations to oestrogen receptor subtype selective agonists in rat and mouse arteries. Eur J Pharmacol 513:101-108.

Alexandersen P, Haarbo J, Byrjalsen I, Lawaetz H, Christiansen C (1999) Natural androgens inhibit male atherosclerosis: a study in castrated, cholesterol-fed rabbits. Circ Res 84:813-819.

Alkayed NJ, Murphy SJ, Traystman RJ, Hurn PD, Miller VM (2000) Neuroprotective effects of female gonadal steroids in reproductively senescent female rats. Stroke 31:161-168.

Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, Hurn PD (1998) Gender-linked brain injury in experimental stroke. Stroke 29:159-165; discussion 166.

Arias-Loza PA, Hu K, Dienesch C, Mehlich AM, Konig S, Jazbutyte V, Neyses L, Hegele-Hartung C, Heinrich Fritzemeier K, Pelzer T (2007) Both estrogen receptor subtypes, alpha and beta, attenuate cardiovascular remodeling in aldosterone salt-treated rats. Hypertension 50:432-438.

Arumugam TV, Okun E, Tang SC, Thundyil J, Taylor SM, Woodruff TM (2009) Toll-like receptors in ischemia-reperfusion injury. Shock 32:4-16.

Baker AE, Brautigam VM, Watters JJ (2004) Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor beta. Endocrinology 145:5021-5032.

Bamford J, Sandercock P, Dennis M, Burn J, Warlow C (1990) A prospective study of acute cerebrovascular disease in the community: the Oxfordshire Community Stroke Project--1981-86. 2. Incidence, case fatality rates and overall outcome at one year of cerebral infarction, primary intracerebral and subarachnoid haemorrhage. J Neurol Neurosurg Psychiatry 53:16-22.

BelAiba RS, Bonello S, Zahringer C, Schmidt S, Hess J, Kietzmann T, Gorlach A (2007) Hypoxia Up-Regulates Hypoxia-Inducible Factor-1{alpha} Transcription by Involving Phosphatidylinositol 3-Kinase and Nuclear Factor {kappa}B in Pulmonary Artery Smooth Muscle Cells. Mol Biol Cell 18:4691-4697.

Benhamou Y, Favre J, Musette P, Renet S, Thuillez C, Richard V, Tamion F (2009) Toll-like receptors 4 contribute to endothelial injury and inflammation in hemorrhagic shock in mice. Crit Care Med 37:1724-1728.

Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC (2010) Molecular cell biology of androgen receptor signalling. Int J Biochem Cell Biol 42:813-827.

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

Benten WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, Mossmann H, Wunderlich F (1999b) Functional testosterone receptors in plasma membranes of T cells. FASEB J 13:123-133.

Bernardi A, Frozza RL, Horn AP, Campos MM, Calixto JB, Salbego C, Pohlmann AR, Guterres SS, Battastini AM (2010) Protective effects of indomethacin-loaded nanocapsules against oxygen-glucose deprivation in organotypic hippocampal slice cultures: involvement of neuroinflammation. Neurochem Int 57:629-636.

Betz AL, Yang GY, Davidson BL (1995) Attenuation of stroke size in rats using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist in brain. J Cereb Blood Flow Metab 15:547-551.

Bizzarro A, Valentini G, Di Martino G, DaPonte A, De Bellis A, Iacono G (1987) Influence of testosterone therapy on clinical and immunological features of autoimmune diseases associated with Klinefelter's syndrome. J Clin Endocrinol Metab 64:32-36.

Bkaily G, Pothier P, D'Orléans-Juste P, Simaan M, Jacques D, Jaalouk D, Belzile F, Hassan G, Boutin C, Haddad G, Neugebauer W (1997) The use of confocal microscopy in the investigation of cell structure and function in the heart, vascular endothelium and smooth muscle cells. Molecular and Cellular Biochemistry 172:171-194.

Bonello S, Zahringer C, BelAiba RS, Djordjevic T, Hess J, Michiels C, Kietzmann T, Gorlach A (2007) Reactive Oxygen Species Activate the HIF-1{alpha} Promoter Via a Functional NF{kappa}B Site. Arterioscler Thromb Vasc Biol 27:755-761.

Breedlove SM, Rosenzweig MR, Watson NV (2007) Biological Psychology, 5 Edition. Sunderland, Massachusetts: Sinaver Associates, Inc.

Brooks A, Menzies-Gow N, Bailey S, Cunningham F, Elliott J (2010) Endotoxin-induced HIF-1α stabilisation in equine endothelial cells: synergistic action with hypoxia. Inflammation Research.

Brown-Sequard CE (1889) The effects produced on man by subcutaneous injections of a liquid obtained from the testicles of animals. Lancet:105-107.

Brown CM, Mulcahey TA, Filipek NC, Wise PM (2010) Production of proinflammatory cytokines and chemokines during neuroinflammation: novel roles for estrogen receptors alpha and beta. Endocrinology 151:4916-4925.

Butenandt A, Hanisch G (1935a) About Testosterone. Conversion of Dehydro-androsterones into androstendiol and testosterone; a way for the structure assignment of testosterone from cholestrol. Hoppe Seylers Z Physiol Chem 237:89.

Butenandt A, Hanisch G (1935b) The conversion of dehydroandrosterone into androstenol-(17)-one-3 (testosterone); a method for the production of testosterone from cholesterol (preliminary communication) Chemische Berichte:1859-1862.

Cai J, Kandagatla P, Singareddy R, Kropinski A, Sheng S, Cher ML, Chinni SR (2010) Androgens Induce Functional CXCR4 through ERG Factor Expression in TMPRSS2-ERG Fusion-Positive Prostate Cancer Cells. Transl Oncol 3:195-203.

Cao C, Matsumura K, Watanabe Y (1997) Induction of cyclooxygenase-2 in the brain by cytokines. Ann N Y Acad Sci 813:307-309.

Carswell HV, Macrae IM, Gallagher L, Harrop E, Horsburgh KJ (2004) Neuroprotection by a selective estrogen receptor beta agonist in a mouse model of global ischemia. Am J Physiol Heart Circ Physiol 287:H1501-1504.

Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I (2007) Toll-Like Receptor 4 Is Involved in Brain Damage and Inflammation After Experimental Stroke. Circulation 115:1599-1608.

Caso JR, Pradillo JM, Hurtado O, Leza JC, Moro MA, Lizasoain I (2008) Toll-Like Receptor 4 Is Involved in Subacute Stress-Induced Neuroinflammation and in the Worsening of Experimental Stroke. Stroke 39:1314-1320.

Chen H, Kim GS, Okami N, Narasimhan P, Chan PH (2011) NADPH oxidase is involved in post-ischemic brain inflammation. Neurobiology of Disease In Press, Uncorrected Proof.

Cheng J, Alkayed NJ, Hurn PD (2007) Deleterious effects of dihydrotestosterone on cerebral ischemic injury. J Cereb Blood Flow Metab 27:1553-1562.

Cheng J, Uchida M, Zhang W, Grafe MR, Herson PS, Hurn PD (2010) Role of salt-induced kinase 1 in androgen neuroprotection against cerebral ischemia. J Cereb Blood Flow Metab.

Cheng J, Hu W, Toung TJ, Zhang Z, Parker SM, Roselli CE, Hurn PD (2008) Age-dependent effects of testosterone in experimental stroke. J Cereb Blood Flow Metab 29:486-494.

Cipollone F, Fazia M, Iezzi A, Ciabattoni G, Pini B, Cuccurullo C, Ucchino S, Spigonardo F, De Luca M, Prontera C, Chiarelli F, Cuccurullo F, Mezzetti A (2004) Balance between PGD synthase and PGE synthase is a major determinant of atherosclerotic plaque instability in humans. Arterioscler Thromb Vasc Biol 24:1259-1265.

Clarkson TB (2007) Estrogen effects on arteries vary with stage of reproductive life and extent of subclinical atherosclerosis progression. Menopause 14:373-384.

Clarkson TB, Anthony MS, Wagner JD (2001) A comparison of tibolone and conjugated equine estrogens effects on coronary artery atherosclerosis and bone density of postmenopausal monkeys. J Clin Endocrinol Metab 86:5396-5404.

Collaco-Moraes Y, Aspey B, Harrison M, de Belleroche J (1996) Cyclo-oxygenase-2 messenger RNA induction in focal cerebral ischemia. J Cereb Blood Flow Metab 16:1366-1372.

Corcoran MP, Meydani M, Lichtenstein AH, Schaefer EJ, Dillard A, Lamon-Fava S (2010) Sex hormone modulation of proinflammatory cytokine and C-reactive protein expression in macrophages from older men and postmenopausal women. J Endocrinol 206:217-224.

Cormac TT, Eoin PC (2009) The Role of NF-KB in Hypoxia-Induced Gene Expression. Annals of the New York Academy of Sciences 1177:178-184.

Costarella CE, Stallone JN, Rutecki GW, Whittier FC (1996) Testosterone causes direct relaxation of rat thoracic aorta. J Pharmacol Exp Ther 277:34-39.

Cruz MN, Douglas G, Gustafsson JA, Poston L, Kublickiene K (2006) Dilatory responses to estrogenic compounds in small femoral arteries of male and female estrogen receptor-beta knockout mice. Am J Physiol Heart Circ Physiol 290:H823-829.

Cutolo M, Balleari E, Giusti M, Intra E, Accardo S (1991) Androgen replacement therapy in male patients with rheumatoid arthritis. Arthritis Rheum 34:1-5.

Das A, Smith JA, Gibson C, Varma AK, Ray SK, Banik NL (2011) Estrogen receptor agonists and estrogen attenuate TNF-{alpha}-induced apoptosis in VSC4.1 motoneurons. J Endocrinol 208:171-182.

Dauphinee SM, Karsan A (2006) Lipopolysaccharide signaling in endothelial cells. Lab Invest 86:9-22.

Death AK, McGrath KCY, Sader MA, Nakhla S, Jessup W, Handelsman DJ, Celermajer DS (2004) Dihydrotestosterone Promotes Vascular Cell Adhesion Molecule-1 Expression in Male Human Endothelial Cells via a Nuclear Factor-{kappa}B-Dependent Pathway. Endocrinology 145:1889-1897.

Deenadayalu VP, White RE, Stallone JN, Gao X, Garcia AJ (2001) Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. Am J Physiol Heart Circ Physiol 281:H1720-1727.

del Zoppo GJ, Mabuchi T (2003) Cerebral Microvessel Responses to Focal Ischemia. J Cereb Blood Flow Metab 23:879-894.

Delcker A, Diener HC, Timmann D, Faustmann P (1993) The role of vertebral and internal carotid artery disease in the pathogenesis of vertebrobasilar transient ischemic attacks. Eur Arch Psychiatry Clin Neurosci 242:179-183.

Dessouroux A, Akwa Y, Baulieu EE (2008) DHEA decreases HIF-1[alpha] accumulation under hypoxia in human pulmonary artery cells: Potential role in the treatment of pulmonary arterial hypertension. The Journal of Steroid Biochemistry and Molecular Biology 109:81-89.

Domingues-Montanari S, Subirana I, Tomas M, Marrugat J, Senti M (2008) Association between ESR2 genetic variants and risk of myocardial infarction. Clin Chem 54:1183-1189.

Donzelli A, Braida D, Finardi A, Capurro V, Valsecchi AE, Colleoni M, Sala M (2010) Neuroprotective effects of genistein in mongolian gerbils: estrogen receptor-beta involvement. J Pharmacol Sci 114:158-167.

Du L, Bayir H, Lai Y, Zhang X, Kochanek PM, Watkins SC, Graham SH, Clark RS (2004) Innate gender-based proclivity in response to cytotoxicity and programmed cell death pathway. J Biol Chem 279:38563-38570.

Duckles SP, Krause DN (2007) Cerbrovascular effects of oestrogen: multiplicity of action. . Clinical and Experimental Pharmacology and Physiology 34:801-808.
Ehrentraut S, Frede S, Stapel H, Mengden T, Grohe C, Fandrey J, Meyer R, Baumgarten G (2007) Antagonism of lipopolysaccharide-induced blood pressure attenuation and vascular contractility. Arterioscler Thromb Vasc Biol 27:2170-2176.
Ellis JA, Infantino T, Harrap SB (2004) Sex-dependent association of blood pressure with oestrogen receptor genes ERalpha and ERbeta. J Hypertens 22:1127-1131.
Eltzschig HK, Carmeliet P (2011) Hypoxia and inflammation. N Engl J Med 364:656-665.
Emsley HC, Smith CJ, Georgiou RF, Vail A, Hopkins SJ, Rothwell NJ, Tyrrell PJ (2005) A randomised phase II study of interleukin-1 receptor antagonist in acute stroke patients. J Neurol Neurosurg Psychiatry 76:1366-1372.
Emsley HCA, Tyrrell PJ (2002) Inflammation and Infection in Clinical Stroke. J Cereb Blood Flow Metab 22:1399-1419.
English KM, Steeds R, Jones TH, Channer KS (1997) Testosterone and coronary heart disease: is there a link? QJM 90:787-791.
English KM, Steeds RP, Jones TH, Diver MJ, Channer KS (2000a) Low-dose transdermal testosterone therapy improves angina threshold in men with chronic stable angina: A randomized, double-blind, placebo-controlled study. Circulation 102:1906-1911.
English KM, Mandour O, Steeds RP, Diver MJ, Jones TH, Channer KS (2000b) Men with coronary artery disease have lower levels of androgens than men with normal coronary angiograms. Eur Heart J 21:890-894.
Er F, Michels G, Brandt MC, Khan I, Haase H, Eicks M, Lindner M, Hoppe UC (2007) Impact of testosterone on cardiac L-type calcium channels and Ca2+ sparks: acute actions antagonize chronic effects. Cell Calcium 41:467-477.
Fannon SA, Vidaver RM, Marts SA (2001) An abridged history of sex steroid hormone receptor action. J Appl Physiol 91:1854-1859.
Fathali N, Ostrowski RP, Lekic T, Jadhav V, Tong W, Tang J, Zhang JH (2010) Cyclooxygenase-2 inhibition provides lasting protection against neonatal hypoxic-ischemic brain injury. Crit Care Med 38:572-578.
Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, Polentarutti N, Muzio M, Arditi M (2000) Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. J Biol Chem 275:11058-11063.
Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB (2002) Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. J Clin Endocrinol Metab 87:589-598.
Florian M, Freiman A, Magder S (2004a) Treatment with 17-beta-estradiol reduces superoxide production in aorta of ovariectomized rats. Steroids 69:779-787.
Florian M, Lu Y, Angle M, Magder S (2004b) Estrogen induced changes in Akt-dependent activation of endothelial nitric oxide synthase and vasodilation. Steroids 69:637-645.
Fogal B, Hewett SJ (2008) Interleukin-1β: a bridge between inflammation and excitotoxicity? Journal of Neurochemistry 106:1-23.
Fogal B, Li J, Lobner D, McCullough LD, Hewett SJ (2007) System x(c)- activity and astrocytes are necessary for interleukin-1 beta-mediated hypoxic neuronal injury. J Neurosci 27:10094-10105.

Foradori CD, Weiser MJ, Handa RJ (2008) Non-genomic actions of androgens. Front Neuroendocrinol 29:169-181.

Fortunati N, Fissore F, Fazzari A, Becchis M, Comba A, Catalano MG, Berta L, Frairia R (1996) Sex steroid binding protein exerts a negative control on estradiol action in MCF-7 cells (human breast cancer) through cyclic adenosine 3',5'-monophosphate and protein kinase A. Endocrinology 137:686-692.

Foudi N, Louedec L, Cachina T, Brink C, Norel X (2009) Selective cyclooxygenase-2 inhibition directly increases human vascular reactivity to norepinephrine during acute inflammation. Cardiovascular Research 81:269-277.

Galea E, Santizo R, Feinstein DL, Adamsom P, Greenwood J, Koenig HM, Pelligrino DA (2002) Estrogen inhibits NF kappa B-dependent inflammation in brain endothelium without interfering with I kappa B degradation. Neuroreport 13:1469-1472.

Ganesan K, Tiwari M, Balachandran C, Manohar BM, Puvanakrishnan R (2008) Estrogen and testosterone attenuate extracellular matrix loss in collagen-induced arthritis in rats. Calcif Tissue Int 83:354-364.

Gangloff A, Shi R, Nahoum V, Lin SX (2003) Pseudo-symmetry of C19 steroids, alternative binding orientations, and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase. FASEB J 17:274-276.

Gatson JW, Kaur P, Singh M (2006) Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147:2028-2034.

Geary GG, Krause DN, Duckles SP (2000a) Estrogen reduces mouse cerebral artery tone through endothelial NOS- and cyclooxygenase-dependent mechanisms. Am J Physiol Heart Circ Physiol 279:H511-519.

Geary GG, Krause DN, Duckles SP (2000b) Gonadal hormones affect diameter of male rat cerebral arteries through endothelium-dependent mechanisms. Am J Physiol Heart Circ Physiol 279:H610-618.

George SJ (1998) Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis. Curr Opin Lipidol 9:413-423.

Gingerich S, Krukoff TL (2006) Estrogen in the paraventricular nucleus attenuates L-glutamate-induced increases in mean arterial pressure through estrogen receptor beta and NO. Hypertension 48:1130-1136.

Golovko O, Nazarova N, Tuohimaa P (2005) A20 gene expression is regulated by TNF, Vitamin D and androgen in prostate cancer cells. J Steroid Biochem Mol Biol 94:197-202.

Gonzales RJ, Krause DN, Duckles SP (2004) Testosterone suppresses endothelium-dependent dilation of rat middle cerebral arteries. Am J Physiol Heart Circ Physiol 286:H552-560.

Gonzales RJ, Duckles SP, Krause DN (2009) Dihydrotestosterone stimulates cerebrovascular inflammation through NFkappaB, modulating contractile function. J Cereb Blood Flow Metab 29:244-253.

Gonzales RJ, Ghaffari AA, Duckles SP, Krause DN (2005) Testosterone treatment increases thromboxane function in rat cerebral arteries. Am J Physiol Heart Circ Physiol 289:H578-585.

Gonzales RJ, Ansar S, Duckles SP, Krause DN (2007) Androgenic/estrogenic balance in the male rat cerebral circulation: metabolic enzymes and sex steroid receptors. J Cereb Blood Flow Metab 27:1841-1852.

González-Cadavid NF, Vernet D, Navarro AF, Rodriguez J, Swerdloff RS, Rajfer J (1993) Up-regulation of the levels of androgen receptor and its mRNA by androgens in smooth-muscle cells from rat penis. Molecular and Cellular Endocrinology 90:219-229.

Gordon KB, Macrae IM, Carswell HV (2005) Effects of 17beta-oestradiol on cerebral ischaemic damage and lipid peroxidation. Brain Res 1036:155-162.

Greco R, Mangione AS, Amantea D, Bagetta G, Nappi G, Tassorelli C (2011) IkappaB-alpha expression following transient focal cerebral ischemia is modulated by nitric oxide. Brain Res:145-151.

Grodstein F, Manson JE, Stampfer MJ (2006) Hormone therapy and coronary heart disease: the role of time since menopause and age at hormone initiation. J Womens Health (Larchmt) 15:35-44.

Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ (2000) A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. Ann Intern Med 133:933-941.

Guo J, Krause DN, Horne J, Weiss JH, Li X, Duckles SP (2010) Estrogen-receptor-mediated protection of cerebral endothelial cell viability and mitochondrial function after ischemic insult in vitro. J Cereb Blood Flow Metab 30:545-554.

Guo S, Stins M, Ning M, Lo EH (2010) Amelioration of inflammation and cytotoxicity by dipyridamole in brain endothelial cells. Cerebrovasc Dis 30:290-296.

Guo X, Razandi M, Pedram A, Kassab G, Levin ER (2005) Estrogen induces vascular wall dilation: mediation through kinase signaling to nitric oxide and estrogen receptors alpha and beta. J Biol Chem 280:19704-19710.

Hacke W, Schwab S, Horn M, Spranger M, De Georgia M, von Kummer R (1996) 'Malignant' middle cerebral artery territory infarction: clinical course and prognostic signs. Arch Neurol 53:309-315.

Handa RJ, Stadelman HL, Resko JA (1987) Effect of estrogen on androgen receptor dynamics in female rat pituitary. Endocrinology 121:84-89.

Hatakeyama H, Nishizawa M, Nakagawa A, Nakano S, Kigoshi T, Uchida K (2002) Testosterone inhibits tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells. FEBS Lett 530:129-132.

Hawk T, Zhang YQ, Rajakumar G, Day AL, Simpkins JW (1998) Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats. Brain Research 796:296-298.

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

Hellwig-Burgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W (2005) Review: Hypoxia-Inducible Factor-1 (HIF-1): A Novel Transcription Factor in Immune Reactions. Journal of Interferon & Cytokine Research 25:297-310.

Heo S-K YH-J, Noh E-K, Park W-H, Park S-D (2008) LPS induces inflammatory responses in human aortic vascular smooth muscle cells via Toll-like receptor 4 expression and nitric oxide production. Immunology Letters 120:57-64.

Hierholzer C, Harbrecht BG, Billiar TR, Tweardy DJ (2001) Hypoxia-inducible factor-1 activation and cyclo-oxygenase-2 induction are early reperfusion-independent inflammatory events in hemorrhagic shock. Arch Orthop Trauma Surg 121:219-222.

Higashida T, Peng C, Li J, Dornbos D, 3rd, Teng K, Li X, Kinni H, Guthikonda M, Ding Y (2011) Hypoxia-Inducible Factor-1alpha Contributes to Brain edema after Stroke by Regulating Aquaporins and Glycerol Distribution in Brain. Curr Neurovasc Res.

Horowitz A, Menice CB, Laporte R, Morgan KG (1996) Mechanisms of smooth muscle contraction. Physiol Rev 76:967-1003.

Hsu HJ, Hsu NC, Hu MC, Chung BC (2006) Steroidogenesis in zebrafish and mouse models. Mol Cell Endocrinol 248:160-163.

Hua F, Ma J, Ha T, Kelley JL, Kao RL, Schweitzer JB, Kalbfleisch JH, Williams DL, Li C (2009) Differential roles of TLR2 and TLR4 in acute focal cerebral ischemia/reperfusion injury in mice. Brain Res 1262:100-108.

Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E (1998) Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. JAMA 280:605-613.

Hulten LM, Levin M (2009) The role of hypoxia in atherosclerosis. Curr Opin Lipidol 20:409-414.

Hyakkoku K, Hamanaka J, Tsuruma K, Shimazawa M, Tanaka H, Uematsu S, Akira S, Inagaki N, Nagai H, Hara H (2010) Toll-like receptor 4 (TLR4), but not TLR3 or TLR9, knock-out mice have neuroprotective effects against focal cerebral ischemia. Neuroscience 171:258-267.

Iadecola C, Gorelick PB (2005) The Janus face of cyclooxygenase-2 in ischemic stroke: shifting toward downstream targets. Stroke 36:182-185.

Iafrati MD, Karas RH, Aronovitz M, Kim S, Sullivan TR, Jr., Lubahn DB, O'Donnell TF, Jr., Korach KS, Mendelsohn ME (1997) Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice. Nat Med 3:545-548.

Ii M, Hoshiga M, Negoro N, Fukui R, Nakakoji T, Kohbayashi E, Shibata N, Furutama D, Ishihara T, Hanafusa T, Losordo DW, Ohsawa N (2009) Adrenal androgen dehydroepiandrosterone sulfate inhibits vascular remodeling following arterial injury. Atherosclerosis 206:77-85.

Iliff JJ, Alkayed NJ (2009) Soluble Epoxide Hydrolase Inhibition: Targeting Multiple Mechanisms of Ischemic Brain Injury with a Single Agent. Future Neurol 4:179-199.

Ishida I, Kubo H, Suzuki S, Suzuki T, Akashi S, Inoue K, Maeda S, Kikuchi H, Sasaki H, Kondo T (2002) Hypoxia diminishes toll-like receptor 4 expression through reactive oxygen species generated by mitochondria in endothelial cells. J Immunol 169:2069-2075.

Jazbutyte V, Arias-Loza PA, Hu K, Widder J, Govindaraj V, von Poser-Klein C, Bauersachs J, Fritzemeier KH, Hegele-Hartung C, Neyses L, Ertl G, Pelzer T (2008) Ligand-dependent activation of ER{beta} lowers blood pressure and attenuates cardiac hypertrophy in ovariectomized spontaneously hypertensive rats. Cardiovasc Res 77:774-781.

Jensen EV (1962) On the mechanism of estrogen action. Perspect Biol Med 6:47-59.

Jeppesen LL, Jorgensen HS, Nakayama H, Raaschou HO, Olsen TS, Winther K (1996) Decreased serum testosterone in men with acute ischemic stroke. Arterioscler Thromb Vasc Biol 16:749-754.

Ji R, Chou C-L, Xu W, Chen X-B, Woodward DF, Regan JW (2010) EP1 Prostanoid Receptor Coupling to Gi/o Upregulates the Expression of Hypoxia-Inducible Factor-1a Through Activation of a Phosphoinositide-3 Kinase Signaling Pathway. Molecular Pharmacology 77:1025-1036.

Jin H, Qiu WB, Mei YF, Wang DM, Li YG, Tan XR (2009) Testosterone alleviates tumor necrosis factor-alpha-mediated tissue factor pathway inhibitor downregulation via suppression of nuclear factor-kappa B in endothelial cells. Asian J Androl 11:266-271.

Jin L, Ai X, Liu L, Wang Z, Cheng Y, Qiao Z (2006) Testosterone induces apoptosis via Fas/FasL-dependent pathway in bone marrow-derived macrophages. Methods Find Exp Clin Pharmacol 28:283-293.

Jin Y, Penning TM (2001) Steroid 5alpha-reductases and 3alpha-hydroxysteroid dehydrogenases: key enzymes in androgen metabolism. Best Pract Res Clin Endocrinol Metab 15:79-94.

Jones RD, Hugh Jones T, Channer KS (2004) The influence of testosterone upon vascular reactivity. Eur J Endocrinol 151:29-37.

Jones RD, Pugh PJ, Hall J, Channer KS, Jones TH (2003) Altered circulating hormone levels, endothelial function and vascular reactivity in the testicular feminised mouse. Eur J Endocrinol 148:111-120.

Jung Y-J, Isaacs JS, Lee S, Trepel J, Neckers L (2003) IL-1beta; mediated up-regulation of HIF-1alpha; via an NFkB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J:03-0329fje.

Kalinchenko SY, Tishova YA, Mskhalaya GJ, Gooren LJ, Giltay EJ, Saad F (2010a) Effects of Testosterone Supplementation on Markers of the Metabolic Syndrome and Inflammation in Hypogonadal Men with the Metabolic Syndrome: The Double-Blind Placebo-Controlled Moscow Study. Clin Endocrinol (Oxf).

Kalinchenko SY, Tishova YA, Mskhalaya GJ, Gooren LJG, Giltay EJ, Saad F (2010b) Effects of testosterone supplementation on markers of the metabolic syndrome and inflammation in hypogonadal men with the metabolic syndrome: the double-blinded placebo-controlled Moscow study. Clinical Endocrinology 73:602-612.

Karim R, Stanczyk FZ, Hodis HN, Cushman M, Lobo RA, Hwang J, Mack WJ (2010) Associations between markers of inflammation and physiological and pharmacological levels of circulating sex hormones in postmenopausal women. Menopause 17:785-790.

Katsargyris A, Theocharis SE, Tsiodras S, Giaginis K, Bastounis E, Klonaris C (2010) Enhanced TLR4 endothelial cell immunohistochemical expression in symptomatic carotid atherosclerotic plaques. Expert Opin Ther Targets 14:1-10.

Katsargyris A, Tsiodras S, Theocharis S, Giaginis K, Vasileiou I, Bakoyiannis C, Georgopoulos S, Bastounis E, Klonaris C (2011) Toll-like receptor 4 immunohistochemical expression is enhanced in macrophages of symptomatic carotid atherosclerotic plaques. Cerebrovasc Dis 31:29-36.

Kawano T, Anrather J, Zhou P, Park L, Wang G, Frys KA, Kunz A, Cho S, Orio M, Iadecola C (2006) Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. Nat Med 12:225-229.

Keller ET, Chang C, Ershler WB (1996) Inhibition of NFkappaB activity through maintenance of IkappaBalpha levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. J Biol Chem 271:26267-26275.

Kishimoto K, Li RC, Zhang J, Klaus JA, Kibler KK, Dore S, Koehler RC, Sapirstein A (2010) Cytosolic phospholipase A2 alpha amplifies early cyclooxygenase-2 expression, oxidative stress and MAP kinase phosphorylation after cerebral ischemia in mice. J Neuroinflammation 7:42.

Koerner IP, Zhang W, Cheng J, Parker S, Hurn PD, Alkayed NJ (2008) Soluble epoxide hydrolase: regulation by estrogen and role in the inflammatory response to cerebral ischemia. Front Biosci 13:2833-2841.

Konoplya EF, Popoff EH (1992) Identification of the classical androgen receptor in male rat liver and prostate cell plasma membranes. Int J Biochem 24:1979-1983.

Krause DN, Duckles SP, Pelligrino DA (2006) Influence of sex steroid hormones on cerebrovascular function. J Appl Physiol 101:1252-1261.

Krom YD, Pires NM, Jukema JW, de Vries MR, Frants RR, Havekes LM, van Dijk KW, Quax PH (2007) Inhibition of neointima formation by local delivery of estrogen receptor alpha and beta specific agonists. Cardiovasc Res 73:217-226.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925-5930.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863-870.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252-4263.

Kupelian V, Chiu GR, Araujo AB, Williams RE, Clark RV, McKinlay JB (2010) Association of sex hormones and C-reactive protein levels in men. Clin Endocrinol (Oxf) 72:527-533.

Lahm T, Crisostomo PR, Markel TA, Wang M, Wang Y, Tan J, Meldrum DR (2008) Selective estrogen receptor-alpha and estrogen receptor-beta agonists rapidly decrease pulmonary artery vasoconstriction by a nitric oxide-dependent mechanism. Am J Physiol Regul Integr Comp Physiol 295:R1486-1493.

Lang JT, McCullough LD (2008) Pathways to ischemic neuronal cell death: are sex differences relevant? J Transl Med 6:33.

Lee MN, Lee SH, Lee MY, Kim YH, Park JH, Ryu JM, Yun SP, Lee YJ, Kim MO, Park K, Han HJ (2008) Effect of dihydrotestosterone on mouse embryonic stem cells exposed to H2O2-induced oxidative stress. J Vet Sci 9:247-256.

Lee Y-S, Song YS, Giffard RG, Chan PH (2005) Biphasic role of nuclear factor-[kappa] B on cell survival and COX-2 expression in SOD1 Tg astrocytes after oxygen glucose deprivation. J Cereb Blood Flow Metab 26:1076-1088.

Lephart ED, Lund TD, Horvath TL (2001) Brain androgen and progesterone metabolizing enzymes: biosynthesis, distribution and function. Brain Res Brain Res Rev 37:25-37.

Lewis DK, Johnson AB, Stohlgren S, Harms A, Sohrabji F (2008) Effects of estrogen receptor agonists on regulation of the inflammatory response in astrocytes from young adult and middle-aged female rats. J Neuroimmunol 195:47-59.
Li H, He Y, Zhang J, Sun S, Sun B (2007) Lipopolysaccharide regulates toll-like receptor 4 expression in human aortic smooth muscle cells. Cell Biol Int 31:831-835.
Li S, Li X, Li J, Deng X, Li Y (2007) Inhibition of oxidative-stress-induced platelet aggregation by androgen at physiological levels via its receptor is associated with the reduction of thromboxane A2 release from platelets. Steroids 72:875-880.
Li ZG, Danis VA, Brooks PM (1993) Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro. Clin Exp Rheumatol 11:157-162.
Li ZK, Shen L, Ke H, Li F, Ni LM, Li QH (2008) Effects of androgen on the expression of brain aromatase cytopigment and nerve growth factor in neonatal rats with hypoxic-ischemic brain damage. Zhongguo Dang Dai Er Ke Za Zhi 10:441-446.
Lin FY, Chen YH, Tasi JS, Chen JW, Yang TL, Wang HJ, Li CY, Chen YL, Lin SJ (2006) Endotoxin induces toll-like receptor 4 expression in vascular smooth muscle cells via NADPH oxidase activation and mitogen-activated protein kinase signaling pathways. Arterioscler Thromb Vasc Biol 26:2630-2637.
Liu CJ, Lo JF, Kuo CH, Chu CH, Chen LM, Tsai FJ, Tsai CH, Tzang BS, Kuo WW, Huang CY (2009) Akt mediates 17beta-estradiol and/or estrogen receptor-alpha inhibition of LPS-induced tumor necresis factor-alpha expression and myocardial cell apoptosis by suppressing the JNK1/2-NFkappaB pathway. J Cell Mol Med 13:3655-3667.
Liu F, Li Z, Li J, Siegel C, Yuan R, McCullough LD (2009) Sex differences in caspase activation after stroke. Stroke 40:1842-1848.
Liu F, Lang J, Li J, Benashski SE, Siegel M, Xu Y, McCullough LD (2011) Sex Differences in the Response to Poly(ADP-ribose) Polymerase-1 Deletion and Caspase Inhibition After Stroke. Stroke 42:1090-1096.
Liu Y-N, Pan S-L, Liao C-H, Huang D-Y, Guh J-H, Peng C-Y, Chang Y-L, Teng C-M (2009) Evodiamine Represses Hypoxia-Induced Inflammatory Proteins Expression and Hypoxia-Inducible Factor 1[alpha] Accumulation in RAW264.7. Shock 32:263-269.
Loddick SA, Rothwell NJ (1996) Neuroprotective effects of human recombinant interleukin-1 receptor antagonist in focal cerebral ischaemia in the rat. J Cereb Blood Flow Metab 16:932-940.
Luksha L, Poston L, Gustafsson JA, Hultenby K, Kublickiene K (2006) The oestrogen receptor beta contributes to sex related differences in endothelial function of murine small arteries via EDHF. J Physiol 577:945-955.
Ma CX, Yin WN, Cai BW, He M, Wu J, Wang JY, Zeng Y, Ding JL, You C (2009) Activation of TLR4/NF-kappaB signaling pathway in early brain injury after subarachnoid hemorrhage. Neurol Res.
Ma R, Jiang SS, Cheng XM, Gong JB, Zhang QG, Lin QS (2010) [Membrane testosterone receptors in cultured vascular smooth muscle cells]. Zhonghua Nan Ke Xue 16:428-432.
Ma Y, Qiao X, Falone AE, Reslan OM, Sheppard SJ, Khalil RA (2010) Gender-specific reduction in contraction is associated with increased estrogen receptor expression in single vascular smooth muscle cells of female rat. Cell Physiol Biochem 26:457-470.

Maddahi A, Edvinsson L (2010) Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway. Journal of Neuroinflammation 7:14.
Mainwaring WI, Irving R (1970) The partial purification of a soluble androgen receptor. Biochem J 118:12P-13P.
Makela S, Savolainen H, Aavik E, Myllarniemi M, Strauss L, Taskinen E, Gustafsson JA, Hayry P (1999) Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors alpha and beta. Proc Natl Acad Sci U S A 96:7077-7082.
Malkin CJ, Pugh PJ, Jones RD, Jones TH, Channer KS (2003) Testosterone as a protective factor against atherosclerosis--immunomodulation and influence upon plaque development and stability. J Endocrinol 178:373-380.
Malkin CJ, Pugh PJ, Jones RD, Kapoor D, Channer KS, Jones TH (2004) The Effect of Testosterone Replacement on Endogenous Inflammatory Cytokines and Lipid Profiles in Hypogonadal Men. J Clin Endocrinol Metab 89:3313-3318.
Malkin CJ, Pugh PJ, Morris PD, Asif S, Jones TH, Channer KS (2010) Low serum testosterone and increased mortality in men with coronary heart disease. Heart 96:1821-1825.
Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the second decade. Cell 83:835-839.
Marrachelli VG, Miranda FJ, Centeno JM, Salom JB, Torregrosa G, Jover-Mengual T, Perez AM, Moro MA, Alborch E (2010) Role of NO-synthases and cyclooxygenases in the hyperreactivity of male rabbit carotid artery to testosterone under experimental diabetes. Pharmacol Res 61:62-70.
Martin D, Chinookoswong N, Miller G (1994) The interleukin-1 receptor antagonist (rhIL-1ra) protects against cerebral infarction in a rat model of hypoxia-ischemia. Exp Neurol 130:362-367.
Masferrer JL, Zweifel BS, Colburn SM, Ornberg RL, Salvemini D, Isakson P, Seibert K (1995) The Role of Cyclooxygenase-2 in Inflammation. Am J Ther 2:607-610.
Matsui H, Ihara Y, Fujio Y, Kunisada K, Akira S, Kishimoto T, Yamauchi-Takihara K (1999) Induction of interleukin (IL)-6 by hypoxia is mediated by nuclear factor (NF)-ÎºB and NF-IL6 in cardiac myocytes. Cardiovascular Research 42:104-112.
McCrohon JA, Jessup W, Handelsman DJ, Celermajer DS (1999) Androgen exposure increases human monocyte adhesion to vascular endothelium and endothelial cell expression of vascular cell adhesion molecule-1. Circulation 99:2317-2322.
McCullough L, Wu L, Haughey N, Liang X, Hand T, Wang Q, Breyer RM, Andreasson K (2004) Neuroprotective Function of the PGE2 EP2 Receptor in Cerebral Ischemia. J Neurosci 24:257-268.
McCullough LD, Alkayed NJ, Traystman RJ, Williams MJ, Hurn PD (2001) Postischemic estrogen reduces hypoperfusion and secondary ischemia after experimental stroke. Stroke 32:796-802.
McCullough LD, Zeng Z, Blizzard KK, Debchoudhury I, Hurn PD (2005) Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. J Cereb Blood Flow Metab 25:502-512.

McNeill AM, Kim N, Duckles SP, Krause DN, Kontos HA (1999) Chronic estrogen treatment increases levels of endothelial nitric oxide synthase protein in rat cerebral microvessels. Stroke 30:2186-2190.

McNeill AM, Zhang C, Stanczyk FZ, Duckles SP, Krause DN (2002) Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels: effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone. Stroke 33:1685-1691.

Mercurio F, Manning AM (1999) Multiple signals converging on NF-[kappa]B. Current Opinion in Cell Biology 11:226-232.

Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA (2001) Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J Med Chem 44:4230-4251.

Michael JS, Mark AD, Nicolas GB (1999) Interleukin-1 beta activates expression of cyclooxygenase-2 and inducible nitric oxide synthase in primary hippocampal neuronal culture: Platelet-activating factor as a preferential mediator of cyclooxygenase-2 expression. Journal of Neuroscience Research 58:593-598.

Miller NR, Jover T, Cohen HW, Zukin RS, Etgen AM (2005) Estrogen can act via estrogen receptor alpha and beta to protect hippocampal neurons against global ischemia-induced cell death. Endocrinology 146:3070-3079.

Miller WL (1988) Molecular biology of steroid hormone synthesis. Endocr Rev 9:295-318.

Mishra RG, Stanczyk FZ, Burry KA, Oparil S, Katzenellenbogen BS, Nealen ML, Katzenellenbogen JA, Hermsmeyer RK (2006) Metabolite ligands of estrogen receptor-{beta} reduce primate coronary hyperreactivity. Am J Physiol Heart Circ Physiol 290:H295-303.

Miyamoto O, Tamae K, Kasai H, Hirakawa H, Hayashida Y, Konishi R, Itano T (2003) Suppression of hyperemia and DNA oxidation by indomethacin in cerebral ischemia. Eur J Pharmacol 459:179-186.

Mizukami Y (2010) In vivo functions of GPR30/GPER-1, a membrane receptor for estrogen: from discovery to functions in vivo. Endocr J 57:101-107.

Momoi H, Ikomi F, Ohhashi T (2003) Estrogen-induced augmentation of endothelium-dependent nitric oxide-mediated vasodilation in isolated rat cerebral small arteries. Jpn J Physiol 53:193-203.

Montgomery S, Shaw L, Pantelides N, Taggart M, Austin C (2003) Acute effects of oestrogen receptor subtype-specific agonists on vascular contractility. Br J Pharmacol 139:1249-1253.

Mosselman S, Polman J, Dijkema R (1996) ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49-53.

Mukherjee TK, Dinh H, Chaudhuri G, Nathan L (2002) Testosterone attenuates expression of vascular cell adhesion molecule-1 by conversion to estradiol by aromatase in endothelial cells: Implications in atherosclerosis. Proceedings of the National Academy of Sciences of the United States of America 99:4055-4060.

Mulcahy NJ, Ross J, Rothwell NJ, Loddick SA (2003) Delayed administration of interleukin-1 receptor antagonist protects against transient cerebral ischaemia in the rat. Br J Pharmacol 140:471-476.

Nagyoszi P, Wilhelm I, Farkas AE, Fazakas C, Dung NT, Hasko J, Krizbai IA (2010) Expression and regulation of toll-like receptors in cerebral endothelial cells. Neurochem Int 57:556-564.

Nakamura Y, Suzuki T, Inoue T, Tazawa C, Moriya T, Saito H, Ishibashi T, Takahashi S, Yamada S, Sasano H (2005) 3beta-Hydroxysteroid Dehydrogenase in Human Aorta. Endocrine Journal 52:111-115.

Nakamura Y, Suzuki T, Miki Y, Tazawa C, Senzaki K, Moriya T, Saito H, Ishibashi T, Takahashi S, Yamada S, Sasano H (2004) Estrogen receptors in atherosclerotic human aorta: inhibition of human vascular smooth muscle cell proliferation by estrogens. Mol Cell Endocrinol 219:17-26.

Nakhla AM, Rosner W (1996) Stimulation of prostate cancer growth by androgens and estrogens through the intermediacy of sex hormone-binding globulin. Endocrinology 137:4126-4129.

Navarro-Dorado J, Orensanz LM, Recio P, Bustamante S, Benedito S, Martinez AC, Garcia-Sacristan A, Prieto D, Hernandez M (2008) Mechanisms involved in testosterone-induced vasodilatation in pig prostatic small arteries. Life Sci 83:569-573.

Nedergaard M, Ransom B, Goldman SA (2003) New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 26:523-530.

Nettleship JE, Jones TH, Channer KS, Jones RD (2007) Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in the Tfm mouse: an effect that is independent of the classic androgen receptor. Circulation 116:2427-2434.

Nikolic I, Liu D, Bell JA, Collins J, Steenbergen C, Murphy E (2007) Treatment with an estrogen receptor-beta-selective agonist is cardioprotective. J Mol Cell Cardiol 42:769-780.

Nizet V, Johnson RS (2009) Interdependence of hypoxic and innate immune responses. Nat Rev Immunol 9:609-617.

Norata GD, Cattaneo P, Poletti A, Catapano AL (2010) The androgen derivative 5alpha-androstane-3beta,17beta-diol inhibits tumor necrosis factor alpha and lipopolysaccharide induced inflammatory response in human endothelial cells and in mice aorta. Atherosclerosis 212:100-106.

Norata GD, Tibolla G, Seccomandi PM, Poletti A, Catapano AL (2006) Dihydrotestosterone Decreases Tumor Necrosis Factor-alpha and Lipopolysaccharide-Induced Inflammatory Response in Human Endothelial Cells. J Clin Endocrinol Metab 91:546-554.

Ogawa S, Emi M, Shiraki M, Hosoi T, Ouchi Y, Inoue S (2000) Association of estrogen receptor beta (ESR2) gene polymorphism with blood pressure. J Hum Genet 45:327-330.

Oka M, Karoor V, Homma N, Nagaoka T, Sakao E, Golembeski SM, Limbird J, Imamura M, Gebb SA, Fagan KA, McMurtry IF (2007) Dehydroepiandrosterone upregulates soluble guanylate cyclase and inhibits hypoxic pulmonary hypertension. Cardiovascular Research 74:377-387.

Oliver KM, Garvey JF, Ng CT, Veale DJ, Fearon U, Cummins EP, Taylor CT (2009) Hypoxia activates NF-kappaB-dependent gene expression through the canonical signaling pathway. Antioxid Redox Signal 11:2057-2064.

Ono H, Sasaki Y, Bamba E, Seki J, Giddings JC, Yamamoto J (2002) Cerebral thrombosis and microcirculation of the rat during the oestrous cycle and after ovariectomy. Clin Exp Pharmacol Physiol 29:73-78.

Orshal JM, Khalil RA (2004) Gender, sex hormones, and vascular tone. Am J Physiol Regul Integr Comp Physiol 286:R233-249.

Ospina JA, Duckles SP, Krause DN (2003) 17beta-estradiol decreases vascular tone in cerebral arteries by shifting COX-dependent vasoconstriction to vasodilation. Am J Physiol Heart Circ Physiol 285:H241-250.

Osterlund KL, Handa RJ, Gonzales RJ (2010) Dihydrotestosterone alters cyclooxygenase-2 levels in human coronary artery smooth muscle cells. Am J Physiol Endocrinol Metab 298:E838-845.

Otsui K, Inoue N, Kobayashi S, Shiraki R, Honjo T, Takahashi M, Hirata K, Kawashima S, Yokoyama M (2007) Enhanced expression of TLR4 in smooth muscle cells in human atherosclerotic coronary arteries. Heart Vessels 22:416-422.

Oyama J, Blais C, Jr., Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T (2004) Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. Circulation 109:784-789.

Padgett DA, Loria RM (1998) Endocrine regulation of murine macrophage function: effects of dehydroepiandrosterone, androstenediol, and androstenetriol. J Neuroimmunol 84:61-68.

Pan Y, Zhang H, Acharya AB, Patrick PH, Oliver D, Morley JE (2005) Effect of testosterone on functional recovery in a castrate male rat stroke model. Brain Research 1043:195-204.

Patrono C, Baigent C (2009) Low-Dose Aspirin, Coxibs, and other NSAIDS: A Clinical Mosaic Emerges. Mol Interv 9:31-39.

Pelligrino DA, Ye S, Tan F, Santizo RA, Feinstein DL, Wang Q (2000) Nitric-oxide-dependent pial arteriolar dilation in the female rat: effects of chronic estrogen depletion and repletion. Biochem Biophys Res Commun 269:165-171.

Pelzer T, Loza PA, Hu K, Bayer B, Dienesch C, Calvillo L, Couse JF, Korach KS, Neyses L, Ertl G (2005) Increased mortality and aggravation of heart failure in estrogen receptor-beta knockout mice after myocardial infarction. Circulation 111:1492-1498.

Petrea RE, Beiser AS, Seshadri S, Kelly-Hayes M, Kase CS, Wolf PA (2009) Gender Differences in Stroke Incidence and Poststroke Disability in the Framingham Heart Study. Stroke 40:1032-1037.

Ponholzer A, Madersbacher S, Rauchenwald M, Jungwirth S, Fischer P, Tragl KH (2010) Vascular risk factors and their association to serum androgen levels in a population-based cohort of 75-year-old men over 5 years: results of the VITA study. World J Urol 28:209-214.

Pugin J, Schurer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS (1993) Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A 90:2744-2748.

Pugsley MK, Tabrizchi R (2000) The vascular system. An overview of structure and function. J Pharmacol Toxicol Methods 44:333-340.

Qiu Y, Yanase T, Hu H, Tanaka T, Nishi Y, Liu M, Sueishi K, Sawamura T, Nawata H (2010) Dihydrotestosterone Suppresses Foam Cell Formation and Attenuates Atherosclerosis Development. Endocrinology 151:3307-3316.

Radwanska E (1993) The role of reproductive hormones in vascular disease and hypertension. Steroids 58:605-610.

Raffetto JD, Qiao X, Beauregard KG, Khalil RA (2010) Estrogen receptor-mediated enhancement of venous relaxation in female rat: implications in sex-related differences in varicose veins. J Vasc Surg 51:972-981.

Razmara A, Krause DN, Duckles SP (2005) Testosterone augments endotoxin-mediated cerebrovascular inflammation in male rats. Am J Physiol Heart Circ Physiol 289:H1843-1850.

Register TC, Adams MR (1998) Coronary artery and cultured aortic smooth muscle cells express mRNA for both the classical estrogen receptor and the newly described estrogen receptor beta. J Steroid Biochem Mol Biol 64:187-191.

Relton JK, Rothwell NJ (1992) Interleukin-1 receptor antagonist inhibits ischaemic and excitotoxic neuronal damage in the rat. Brain Res Bull 29:243-246.

Rembold CM (1992) Regulation of contraction and relaxation in arterial smooth muscle. Hypertension 20:129-137.

Rettew JA, Huet-Hudson YM, Marriott I (2008) Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biol Reprod 78:432-437.

Rexrode KM, Ridker PM, Hegener HH, Buring JE, Manson JE, Zee RY (2007) Polymorphisms and haplotypes of the estrogen receptor-beta gene (ESR2) and cardiovascular disease in men and women. Clin Chem 53:1749-1756.

Ribaudo R, Gilman M, Kingston RE, Choczynski P, Sacchi N (2001) Preparation of RNA from tissues and cells. Curr Protoc Immunol Chapter 10:Unit 10 11.

Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M (2008) NF-KB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1[agr]. Nature 453:807-811.

Roger VL et al. (2011) Heart Disease and Stroke Statistics--2011 Update: A Report From the American Heart Association. Circulation 123:e18-e209.

Roselli CE, Horton LE, Resko JA (1985) Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. Endocrinology 117:2471-2477.

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 288:321-333.

Ruzicka L, Wttstein A (1935) The crystalline production of the testicle hormone, testosterone (Androsten-3-ol-17-ol). Helvetica Chimica Acta:1264-1275.

Saleem S, Li RC, Wei G, Dore S (2007) Effects of EP1 receptor on cerebral blood flow in the middle cerebral artery occlusion model of stroke in mice. J Neurosci Res 85:2433-2440.

Salom JB, Burguete MC, Perez-Asensio FJ, Torregrosa G, Alborch E (2001) Relaxant effects of 17-beta-estradiol in cerebral arteries through Ca(2+) entry inhibition. J Cereb Blood Flow Metab 21:422-429.

Santizo RA, Anderson S, Ye S, Koenig HM, Pelligrino DA (2000) Effects of estrogen on leukocyte adhesion after transient forebrain ischemia. Stroke 31:2231-2235.
Saren P, Welgus HG, Kovanen PT (1996) TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. J Immunol 157:4159-4165.
Schmedtje JF, Ji Y-S, Liu WL, DuBois RN, Runge MS (1997) Hypoxia Induces Cyclooxygenase-2 via the NF-κB p65 Transcription Factor in Human Vascular Endothelial Cells. Journal of Biological Chemistry 272:601-608.
Schmidt BM, Gerdes D, Feuring M, Falkenstein E, Christ M, Wehling M (2000) Rapid, nongenomic steroid actions: A new age? Front Neuroendocrinol 21:57-94.
Seibert K, Masferrer J, Zhang Y, Gregory S, Olson G, Hauser S, Leahy K, Perkins W, Isakson P (1995) Mediation of inflammation by cyclooxygenase-2. Agents Actions Suppl 46:41-50.
Selvamani A, Sohrabji F (2010) Reproductive age modulates the impact of focal ischemia on the forebrain as well as the effects of estrogen treatment in female rats. Neurobiology of Aging 31:1618-1628.
Shih H-C, Lin C-L, Wu S-C, Kwan A-L, Hong Y-R, Howng S-L (2008) Upregulation of estrogen receptor alpha and mediation of 17beta-estradiol vasoprotective effects via estrogen receptor alpha in basilar arteries in rats after experimental subarachnoid hemorrhage. Journal of Neurosurgery 109:92-99.
Smith JA, Das A, Butler JT, Ray SK, Banik NL (2011) Estrogen or Estrogen Receptor Agonist Inhibits Lipopolysaccharide Induced Microglial Activation and Death. Neurochem Res:Epub ahead of print, DOI 10.1007/s11064-11010-10336-11067.
Snyder GD, Krishna UM, Falck JR, Spector AA (2002) Evidence for a membrane site of action for 14,15-EET on expression of aromatase in vascular smooth muscle. Am J Physiol Heart Circ Physiol 283:H1936-1942.
So Young K, Yong Jun C, Sun Myung J, Byung Ho L, Yi-Sook J, Joo Young L (2010) Hypoxic stress up-regulates the expression of Toll-like receptor 4 in macrophages via hypoxia-inducible factor. Immunology 129:516-524.
Sobey CG, Weiler JM, Boujaoude M, Woodman OL (2004) Effect of short-term phytoestrogen treatment in male rats on nitric oxide-mediated responses of carotid and cerebral arteries: comparison with 17beta-estradiol. J Pharmacol Exp Ther 310:135-140.
Somjen D, Kohen F, Gayer B, Knoll E, Many A, Stern N (2009) Dihydrotestosterone and estradiol-17[beta] mutually neutralize their inhibitory effects on human vascular smooth muscle cell growth in vitro. The Journal of Steroid Biochemistry and Molecular Biology 113:171-176.
Song YS, Kim M-S, Kim H-A, Jung B-I, Yang J, Narasimhan P, Kim GS, Jung JE, Park E-H, Chan PH (2010) Oxidative stress increases phosphorylation of I[kappa]B kinase-[alpha] by enhancing NF-[kappa]B-inducing kinase after transient focal cerebral ischemia. J Cereb Blood Flow Metab 30:1265-1274.
Stampfer MJ, Willett WC, Colditz GA, Rosner B, Speizer FE, Hennekens CH (1985) A prospective study of postmenopausal estrogen therapy and coronary heart disease. N Engl J Med 313:1044-1049.
Stanimirovic D, Shapiro A, Wong J, Hutchison J, Durkin J (1997) The induction of ICAM-1 in human cerebromicrovascular endothelial cells (HCEC) by ischemia-like conditions promotes enhanced neutrophil/HCEC adhesion. J Neuroimmunol 76:193-205.

Stasinopoulos I OBD, Bhujwalla ZM (2009) Inflammation, but not hypoxia, mediated HIF-1alpha activation depends on COX-2. Cancer Bio Ther 8:31-35.

Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. J Med Chem 43:4934-4947.

Steckelbroeck S, Jin Y, Gopishetty S, Oyesanmi B, Penning TM (2004) Human cytosolic 3alpha-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3beta-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action. J Biol Chem 279:10784-10795.

Stirone C, Boroujerdi A, Duckles SP, Krause DN (2005a) Estrogen receptor activation of phosphoinositide-3 kinase, akt, and nitric oxide signaling in cerebral blood vessels: rapid and long-term effects. Mol Pharmacol 67:105-113.

Stirone C, Duckles SP, Krause DN, Procaccio V (2005b) Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. Mol Pharmacol 68:959-965.

Stirone C, Chu Y, Sunday L, Duckles SP, Krause DN (2003) 17 Beta-estradiol increases endothelial nitric oxide synthase mRNA copy number in cerebral blood vessels: quantification by real-time polymerase chain reaction. Eur J Pharmacol 478:35-38.

Stoll LL, Denning GM, Li WG, Rice JB, Harrelson AL, Romig SA, Gunnlaugsson ST, Miller FJ, Jr., Weintraub NL (2004) Regulation of endotoxin-induced proinflammatory activation in human coronary artery cells: expression of functional membrane-bound CD14 by human coronary artery smooth muscle cells. J Immunol 173:1336-1343.

Strehlow K, Rotter S, Wassmann S, Adam O, Grohe C, Laufs K, Bohm M, Nickenig G (2003) Modulation of antioxidant enzyme expression and function by estrogen. Circ Res 93:170-177.

Sugimoto K, Iadecola C (2003) Delayed effect of administration of COX-2 inhibitor in mice with acute cerebral ischemia. Brain Res 960:273-276.

Sunday L, Tran MM, Krause DN, Duckles SP (2006) Estrogen and progestagens differentially modulate vascular proinflammatory factors. Am J Physiol Endocrinol Metab 291:E261-267.

Sunday L, Osuna C, Krause DN, Duckles SP (2007) Age alters cerebrovascular inflammation and effects of estrogen. Am J Physiol Heart Circ Physiol 292:H2333-2340.

Sundin M, Warner M, Haaparanta T, Gustafsson JA (1987) Isolation and catalytic activity of cytochrome P-450 from ventral prostate of control rats. J Biol Chem 262:12293-12297.

Tam NN, Gao Y, Leung YK, Ho SM (2003) Androgenic regulation of oxidative stress in the rat prostate: involvement of NAD(P)H oxidases and antioxidant defense machinery during prostatic involution and regrowth. Am J Pathol 163:2513-2522.

Tarkowski E, Rosengren L, Blomstrand C, Jensen C, Ekholm S, Tarkowski A (1999) Intrathecal expression of proteins regulating apoptosis in acute stroke. Stroke 30:321-327.

Tennant M, McGeachie JK (1990) Blood vessel structure and function: a brief update on recent advances. Aust N Z J Surg 60:747-753.

Tep-areenan P, Kendall DA, Randall MD (2002) Testosterone-induced vasorelaxation in the rat mesenteric arterial bed is mediated predominantly via potassium channels. Br J Pharmacol 135:735-740.

Thyberg J, Roy J, Tran PK, Blomgren K, Dumitrescu A, Hedin U (1997) Expression of caveolae on the surface of rat arterial smooth muscle cells is dependent on the phenotypic state of the cells. Lab Invest 77:93-101.

Ting P (1990) Indomethacin attenuates early postischemic vasogenic edema and cerebral injury. Adv Neurol 52:119-126.

Tipping PG, Hancock WW (1993) Production of tumor necrosis factor and interleukin-1 by macrophages from human atheromatous plaques. Am J Pathol 142:1721-1728.

Torn S, Nokelainen P, Kurkela R, Pulkka A, Menjivar M, Ghosh S, Coca-Prados M, Peltoketo H, Isomaa V, Vihko P (2003) Production, purification, and functional analysis of recombinant human and mouse 17beta-hydroxysteroid dehydrogenase type 7. Biochem Biophys Res Commun 305:37-45.

Toung TJK, Traystman RJ, Hurn PD, Miller VM (1998) Estrogen-Mediated Neuroprotection After Experimental Stroke in Male Rats • Editorial Comment. Stroke 29:1666-1670.

Traupe T, Stettler CD, Li H, Haas E, Bhattacharya I, Minotti R, Barton M (2007) Distinct roles of estrogen receptors alpha and beta mediating acute vasodilation of epicardial coronary arteries. Hypertension 49:1364-1370.

Tsatsanis C, Androulidaki A, Venihaki M, Margioris AN (2006) Signalling networks regulating cyclooxygenase-2. Int J Biochem Cell Biol 38:1654-1661.

Tu XK, Yang WZ, Shi SS, Wang CH, Zhang GL, Ni TR, Chen CM, Wang R, Jia JW, Song QM (2010) Spatio-temporal distribution of inflammatory reaction and expression of TLR2/4 signaling pathway in rat brain following permanent focal cerebral ischemia. Neurochem Res 35:1147-1155.

Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B, Roy AK (2000) Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. Mol Endocrinol 14:1162-1174.

Uchida M, Palmateer JM, Herson PS, Devries AC, Cheng J, Hurn PD (2009) Dose-dependent effects of androgens on outcome after focal cerebral ischemia in adult male mice. J Cereb Blood Flow Metab.

Vagnerova K, Liu K, Ardeshiri A, Cheng J, Murphy SJ, Hurn PD, Herson PS (2010) Poly (ADP-ribose) polymerase-1 initiated neuronal cell death pathway--do androgens matter? Neuroscience 166:476-481.

Vagnoni KE, Christiansen ND, Holyoak GR, Janowiak MA, Martin PH (1999) Cellular source in ewes of prostaglandin-endoperoxide synthase-2 in uterine arteries following stimulation with lipopolysaccharide. Biol Reprod 61:563-568.

vanÂ uden P, Kenneth NS, Rocha S (2008) Regulation of hypoxia-inducible factor-1α by NFKB. Biochem J 412:477-484.

Vasudevan N, Pfaff DW (2008) Non-genomic actions of estrogens and their interaction with genomic actions in the brain. Front Neuroendocrinol 29:238-257.

Vila N, Castillo J, Davalos A, Chamorro A (2000) Proinflammatory Cytokines and Early Neurological Worsening in Ischemic Stroke. Stroke 31:2325-2329.

Vinall PE, Simeone FA (1986) Effects of oxygen and glucose deprivation on vasoactivity in isolated bovine middle cerebral arteries. Stroke 17:970-975.

Vornehm ND, Wang M, Abarbanell A, Herrmann J, Weil B, Tan J, Wang Y, Kelly M, Meldrum DR (2009) Acute postischemic treatment with estrogen receptor-alpha agonist or estrogen receptor-beta agonist improves myocardial recovery. Surgery 146:145-154.

Wang M, Crisostomo PR, Markel T, Wang Y, Lillemoe KD, Meldrum DR (2008) Estrogen receptor beta mediates acute myocardial protection following ischemia. Surgery 144:233-238.

Wang M, Wang Y, Weil B, Abarbanell A, Herrmann J, Tan J, Kelly M, Meldrum DR (2009) Estrogen receptor beta mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia. Am J Physiol Regul Integr Comp Physiol 296:R972-978.

Wang Q, Tang XN, Yenari MA (2007) The inflammatory response in stroke. Journal of Neuroimmunology 184:53-68.

Wang W, Deng M, Liu X, Ai W, Tang Q, Hu J (2011) TLR4 Activation Induces Nontolerant Inflammatory Response in Endothelial Cells. Inflammation:Epub ahead of print, DOI 10.1007/s10753-10010-19258-10754.

Warner SJ, Friedman GB, Libby P (1989) Immune interferon inhibits proliferation and induces 2'-5'-oligoadenylate synthetase gene expression in human vascular smooth muscle cells. J Clin Invest 83:1174-1182.

Watanabe Y, Littleton-Kearney MT, Traystman RJ, Hurn PD (2001) Estrogen restores postischemic pial microvascular dilation. Am J Physiol Heart Circ Physiol 281:H155-160.

Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, Kim K, Sawyers CL (2010) Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc Natl Acad Sci U S A 107:16759-16765.

Weihua Z, Lathe R, Warner M, Gustafsson JA (2002) An endocrine pathway in the prostate, ERbeta, AR, 5alpha-androstane-3beta,17beta-diol, and CYP7B1, regulates prostate growth. Proc Natl Acad Sci U S A 99:13589-13594.

Weiser MJ, Foradori CD, Handa RJ (2008) Estrogen receptor beta in the brain: from form to function. Brain Res Rev 57:309-320.

Weiyu Teng LW, Weishuang Xue, and Chao Guan (2009) Activation of TLR4-Mediated NFκB Signaling in Hemorrhagic Brain in Rats. Mediators of Inflammation.

Williams MR, Ling S, Dawood T, Hashimura K, Dai A, Li H, Liu JP, Funder JW, Sudhir K, Komesaroff PA (2002) Dehydroepiandrosterone inhibits human vascular smooth muscle cell proliferation independent of ARs and ERs. J Clin Endocrinol Metab 87:176-181.

Writing Group M et al. (2009) Heart Disease and Stroke Statistics--2009 Update: A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 119:e21-181.

Xing D, Feng W, Miller AP, Weathington NM, Chen Y-F, Novak L, Blalock JE, Oparil S (2007) Estrogen modulates TNF-{alpha}-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-beta activation. Am J Physiol Heart Circ Physiol 292:H2607-2612.

Xu J, He L, Ahmed SH, Chen S-W, Goldberg MP, Beckman JS, Hsu CY, Iadecola C (2000) Oxygen-Glucose Deprivation Induces Inducible Nitric Oxide Synthase and

Nitrotyrosine Expression in Cerebral Endothelial Cells Editorial Comment. Stroke 31:1744-1751.

Xu ZR, Hu L, Cheng LF, Qian Y, Yang YM (2010) Dihydrotestosterone protects human vascular endothelial cells from H(2)O(2)-induced apoptosis through inhibition of caspase-3, caspase-9 and p38 MAPK. Eur J Pharmacol 643:254-259.

Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K (1995) Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. Stroke 26:676-680; discussion 681.

Yang GY, Zhao YJ, Davidson BL, Betz AL (1997) Overexpression of interleukin-1 receptor antagonist in the mouse brain reduces ischemic brain injury. Brain Res 751:181-188.

Yang GY, Liu XH, Kadoya C, Zhao YJ, Mao Y, Davidson BL, Betz AL (1998) Attenuation of ischemic inflammatory response in mouse brain using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist. J Cereb Blood Flow Metab 18:840-847.

Yang GY, Mao Y, Zhou LF, Ye W, Liu XH, Gong C, Lorris Betz A (1999) Attenuation of temporary focal cerebral ischemic injury in the mouse following transfection with interleukin-1 receptor antagonist. Brain Res Mol Brain Res 72:129-137.

Yang S-H, Perez E, Cutright J, Liu R, He Z, Day AL, Simpkins JW (2002) Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. J Appl Physiol 92:195-201.

Yang X, Coriolan D, Murthy V, Schultz K, Golenbock DT, Beasley D (2005) Proinflammatory phenotype of vascular smooth muscle cells: role of efficient Toll-like receptor 4 signaling. Am J Physiol Heart Circ Physiol 289:H1069-1076.

Yaron M, Greenman Y, Rosenfeld JB, Izkhakov E, Limor R, Osher E, Shenkerman G, Tordjman K, Stern N (2009) Effect of testosterone replacement therapy on arterial stiffness in older hypogonadal men. Eur J Endocrinol 160:839-846.

Yeap BB (2008) Are declining testosterone levels a major risk factor for ill-health in aging men[quest]. Int J Impot Res 21:24-36.

Yeap BB, Hyde Z, Norman PE, Chubb SA, Golledge J (2010) Associations of total testosterone, sex hormone-binding globulin, calculated free testosterone, and luteinizing hormone with prevalence of abdominal aortic aneurysm in older men. J Clin Endocrinol Metab 95:1123-1130.

Yeap BB, Hyde Z, Almeida OP, Norman PE, Chubb SAP, Jamrozik K, Flicker L, Hankey GJ (2009) Lower Testosterone Levels Predict Incident Stroke and Transient Ischemic Attack in Older Men. J Clin Endocrinol Metab 94:2353-2359.

Yeh SH, Ou LC, Gean PW, Hung JJ, Chang WC (2011) Selective Inhibition of Early-but Not Late-Expressed HIF-1alpha Is Neuroprotective in Rats after Focal Ischemic Brain Damage. Brain Pathol 21:249-262.

Young KC, Hussein SM, Dadiz R, deMello D, Devia C, Hehre D, Suguihara C (2010) Toll-like receptor 4-deficient mice are resistant to chronic hypoxia-induced pulmonary hypertension. Exp Lung Res 36:111-119.

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

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

Zalba G, Fortuno A, San Jose G, Moreno MU, Beloqui O, Diez J (2007) Oxidative stress, endothelial dysfunction and cerebrovascular disease. Cerebrovasc Dis 24 Suppl 1:24-29.

Zhang B, Subramanian S, Dziennis S, Jia J, Uchida M, Akiyoshi K, Migliati E, Lewis AD, Vandenbark AA, Offner H, Hurn PD (2010) Estradiol and G1 reduce infarct size and improve immunosuppression after experimental stroke. J Immunol 184:4087-4094.

Zhang W, Iliff JJ, Campbell CJ, Wang RK, Hurn PD, Alkayed NJ (2009) Role of soluble epoxide hydrolase in the sex-specific vascular response to cerebral ischemia. J Cereb Blood Flow Metab.

Zhang X, Wang LY, Jiang TY, Zhang HP, Dou Y, Zhao JH, Zhao H, Qiao ZD, Qiao JT (2002) Effects of testosterone and 17-beta-estradiol on TNF-alpha-induced E-selectin and VCAM-1 expression in endothelial cells. Analysis of the underlying receptor pathways. Life Sci 71:15-29.

Zhang X, Deguchi K, Yamashita T, Ohta Y, Shang J, Tian F, Liu N, Panin VL, Ikeda Y, Matsuura T, Abe K (2010) Temporal and spatial differences of multiple protein expression in the ischemic penumbra after transient MCAO in rats. Brain Res 1343:143-152.

Zhang Z, Li W, Sun D, Zhao L, Zhang R, Wang Y, Zhou X, Wang H, Cao F (2011) Toll-like receptor 4 signaling in dysfunction of cardiac microvascular endothelial cells under hypoxia/reoxygenation. Inflamm Res 60:37-45.

Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thoren P, Smithies O, Gustafsson JA, Mendelsohn ME (2002) Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science 295:505-508.

LIST OF ABBREVIATIONS

3α-HSD: 3 alpha-hydroxysteroid dehydrogenase

3β-diol: 5α-androstane-3β,17β-diol

3β-HSD: 3 beta-hydroxysteroid dehydrogenase

5α-R: 5 alpha reductase

17β-HSD: 17 beta-hydroxysteroid dehydrogenase

ACh: actylcholine

AIF: apoptosis inducing factor

AR: androgen receptor

ARE: androgen response element

BIC: bicalutamide

BK_{Ca}: large conductance calcium activated potassium channel

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

COX-1: cyclooxygenase-1

COX-2: cyclooxygase-2

DHEA: dehydroepiandrosterone

DHT: dihydrotestosterone (5α-androstan-17β-ol-3-one)

DPN: diarylpropionitrile

E: estrogen/estradiol

EDHF: endothelium derived hyperpolarizing factor

EETs: epoxyeicosatrienoic acids

ELAM: endothelial cell leukocyte adhesion molecule

eNOS: endothelial nitric oxide synthase

ER: estrogen receptor

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

ERE: estrogen response element

GABA: gamma amino butyric acid

GPCR: g protein coupled receptor

HBVSMC: human brain vascular smooth muscle cells

HBMEC: human brain microvascular endothelial cells

HCASMC: human coronary vascular smooth muscle cells

HGD: hypoxia with glucose deprivation

HIF-1α: hypoxia inducible factor 1 alpha

HRE: hormone response element

HUVEC: human umbilical vein endothelial cells

ICAM: inter-cellular adhesion molecule

ICI: ICI 182,780

IκB: Inhibitor of NFκB

IL-1β: interleukin-1beta

IL-1R: interleukin-1 receptor

IL-6: interleukin-6

iNOS: inducible nitric oxide synthase

IP_3: inositol 1,4,5-triphosphate

LPS: lipopolysaccharide

M3: muscarinic 3 acytlcholine receptor

MCAO: middle cerebral artery occlusion (*in vivo* stroke model)

MLCK: myosin light chain kinase

NADPH: nicotinamide adenine dinucleotide phosphate

NFκB: nuclear factor kappa B

NO: nitric oxide

NOS: nitric oxide synthase

NOX: nicotinamide adenine dinucleotide phosphate oxidase

OGD: oxygen glucose deprivation (*in vitro* stroke model)

OVX: ovariecotomy, ovariectomized

PARP: poly (ADP ribose) polymerase

PE: phenylephrine

PHTPP: 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol

PIP_2: phosphatidylinositol 4,5 bisphosphate

PLC: phospholipase C

PTT: 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol

ROS: reactive oxygen species

sEH: soluable epoxide hydrolase

T: testosterone

TIR: Toll/interleukin-1 receptor domain

TLR4: toll-like receptor 4

TNFα: tumor necrosis factor alpha

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

VGCC: voltage gated calcium channels

VSM: vascular smooth muscle