

**Androgen Receptor Expression in
Human Coronary Vascular Smooth Muscle During
Cytokine, Angiotensin II or Hypoxic Exposure**

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Dedication

Dedicated to my daughters, Jaden and Joule, who I hope will be inspired to pursue scientific endeavors of their own.

Acknowledgements

I would like to acknowledge the following people in the Gonzales laboratory for providing me with technical advice and support:

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I wish them all success as they pursue their own careers in medicine, in biomedical research, or in allied fields. Thank you!

Abstract

An increasing body of evidence suggests that androgens may exert beneficial effects against the development and progression of vascular inflammation during pathological conditions. Our previous data have shown that the potent androgen receptor (AR) agonist, dihydrotestosterone (DHT), attenuates inflammation-induced vascular cell adhesion molecule-1 (VCAM-1) and cyclooxygenase-2 (COX-2) in human primary vascular smooth muscle (VSM) cells. Although this response was not blocked by the AR antagonist bicalutamide, it is not known if AR expression is altered during an inflammatory insult in VSM. The goal of this study was to investigate the effects of a variety of inflammatory stimuli: Angiotensin II (more recently recognized as a mediator of inflammation in the blood vessel wall (Ruiz-Ortega, et al. 2000) (Alvarez, et al. 2004)), hypoxia, and interleukin-1 beta (IL-1 β ; cytokine) on AR expression in human VSM cells. Since DHT's effect in the presence of an inflammatory stimulus is AR independent, we hypothesized levels of AR are decreased favoring less of an androgenic contribution during pro-inflammatory conditions. We initially confirmed that human VSM expresses AR and levels of the receptor are increased following androgen treatment in the absence of an

inflammatory stimulus. We further demonstrated that in addition to the full-length AR 110 kDa band detected via anti-AR-N20, we also detected a band migrating near 45 kDa in human VSM that is not present in rat testis lysate. Recent studies describe a variant form of the AR called AR45 expressed in human heart but not in rat (Ahrens-Fath, et al. 2005) (Weiss, Faus and Haendler 2007). Although we did not determine whether the bands migrating near 45 kDa were AR45 or possibly an endogenous break down product of the full-length AR (110 kDa) we did however observe expression of the lower migrating band during conditions of inflammation that may be cardioprotective. In the presence of an inflammatory stimulus we demonstrated that there was a consistent trend for IL-1 β and angiotensin II (ang II) to decrease AR expression in human coronary artery VSM. Using an *in vivo* global ischemic model of inflammation, AR was robustly decreased following a 20- and 30-minute occlusion and 21 hr reperfusion in rat pial arteries. However, unlike ang II, cytokine, or ischemia, *in vitro* hypoxic exposure in human VSM cells increased the lower migrating band density (45 kDa) and had no effect on band density at 110 kDa. In summary, our results confirm that levels of the classic AR (and possibly the novel AR45 variant form) are present in human VSM.

Additionally levels of AR may be altered under conditions of inflammation in human VSM cells and following ischemia/reperfusion in rodent cerebral arteries. We conclude that the AR independent attenuation of COX-2 and VCAM-1 by DHT following an inflammatory insult may be due in part to a decrease of AR levels in the blood vessel wall.

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Abbreviations

AR – androgen receptor

Ang II – Angiotensin II

AR45 – androgen receptor 45

COX-2 – cyclooxygenase-2

CVD – cardiovascular disease

d H₂O – distilled water

DHT – dihydrotestosterone

ER – estrogen receptor

FBS – fetal bovine serum

IL-1 β – interleukin-1 beta

iNOS – inducible nitric oxide synthase

NF- κ B – nuclear factor kappa B

PBS – phosphate buffered solution

PC – positive control (rat testis lysate)

PCR – polymerase chain reaction

TxA₂ – thromboxane A₂

TNF- α – tumor necrosis factor-alpha

TPBS – 1% tween in phosphate buffered solution

VCAM-1 – vascular cell adhesion molecule -1

VSM – vascular smooth muscle

WM – molecular weight standard

Introduction

Clinical Significance: Cardio-and cerebrovascular disease are the first and 4th leading causes of morbidity and mortality in the United States respectively (Kochanek, et al. 2011). There are several risk factors that are common to cerebrovascular and cardiovascular disease (CVD). These include smoking, hypertension, diabetes, obesity, sedentary lifestyle, and gender. To date, aged matched men continue to have a higher incidence of heart disease and stroke compared to post-menopausal women (Appelros, Stegmayr and Terent 2009); however women exhibit a worse outcome. Appelros et al. (2009) suggest that the increased severity of outcome in women may be due to the higher prevalence of thromboembolic source. Because sex differences are observed in both the incidence and outcome of stroke and heart disease, further studies are necessary to address the role of gonadal sex steroid hormones and their receptors on the pathophysiology of cerebrovascular and cardiovascular disease.

The focus of this study was to investigate the effect of various inducers of inflammation: cytokine (IL-1 β), hypoxia, or angiotensin II (recently recognized to be pro-inflammatory in the vasculature (Ruiz-Ortega, et al. 2000) (Alvarez, et al. 2004)), on androgen receptor (AR)

levels in primary human coronary artery vascular smooth muscle (VSM) cells. Because vascular inflammation plays a key role in the pathogenesis of heart disease and stroke (Danesh, et al. 2004) (Libby 2008) (Roman, Shanker, et al. 2003) (Shah, et al. 2009) investigations focusing on inflammation, hormones, and their receptors in the blood vessel wall are merited.

Role of sex steroids and their receptors in the blood vessel

wall: Experimental studies have shown that estrogens, specifically 17-beta estradiol (for simplicity 17-beta estradiol will be referred to as estrogen throughout the remainder of the paper), are generally anti-inflammatory and cardioprotective. Two isoforms of estrogen receptor (ER) have been described in the literature, ER- α and ER- β ; along with a more recently described membrane bound estrogen receptor gpER (formerly called GPR30). ER- α and ER- β are nuclear receptors and gpER is a seven-transmembrane G-coupled protein receptor. When stimulated, ERs can lead to genomic signaling or the rapid, non-genomic signaling, both leading to nitric oxide production (Meyer and Barton 2009). Nitric oxide production leads to vasodilation, resulting in reduced blood pressure which is associated with improved cardiovascular health (Ebrahim, et al. 2011).

The blood vessel wall is a target for the compounds that act on ER- α and ER- β to modulate vascular function. In rodents, ER- α is expressed in many tissues with studies suggesting higher levels of expression in the ovaries, heart, and liver, while ER- β is expressed mostly in the prostate and lung (Yu, et al. 2006) (Couse, et al. 1997). Studies have also demonstrated these receptors, specifically ER- α , in both the endothelium and VSM. (Dan, Cheung and Moore 2003) (Stirone, Duckles and Krause 2005).

Several studies have shown that ER- α and ER- β play a role in cerebrovascular and cardiovascular inflammation and disease. ER- α stimulation has been shown to be anti-inflammatory and protective against cerebrovascular and cardiovascular disease. For example, estrogen, acting through non-genomic ER- α activity, was found to decrease both basal and IL-1 β induced inflammation by inhibiting the pro-inflammatory transcription factor, nuclear factor kappa B (NF- κ B), in cultured rat brain endothelial cells (Galea, et al. 2002). Estrogen was also found to decrease IL-1 β induction of COX-2 *in-vivo* in ovariectomized rats by reducing transcription activity of NF- κ B (Ospina, et al. 2004). That estrogen and its alpha receptor reduce activation and transcription of NF- κ B and its resulting inflammation is

likely a key mechanism by which estrogen elicits cardio- and cerebro-protective effects. ER- β expression in the cardiovascular system has been shown to be deleterious. In fact, deceased women that expressed ER- β in coronary artery intimal cells had increased coronary artery calcification and atherosclerosis regardless of age (Christian, et al. 2006).

The role of androgens and AR in CVD and inflammation is more complex and is amenable to further investigation. AR has been detected in rat cerebral capillaries (Ohtsuki, et al. 2005) and arteries (Gonzales, Duckles and Krause 2009). AR expression was also detected in human brain microvascular endothelial cells and in human coronary artery VSM cells (Osterlund, et al. 2010). Testosterone, which acts on AR, has shown both pro-inflammatory and pro-atherogenic effects as well as anti-inflammatory and cardioprotective effects. For example, testosterone has been found to increase thromboxane synthase, an enzyme that converts metabolites of arachadonic acid to thromboxane A₂ (potent vasoconstrictor), in middle cerebral arteries of orchietomized male rats (Gonzales, et al. 2005). Similarly, increased expression of thromboxane A₂ and platelet aggregation was observed when exogenous testosterone was given to

16 healthy male volunteers (Choi and McLaughlin 2007). Additionally, testosterone was found to increase endotoxin-induced COX-2 and iNOS in cerebral VSM and endothelial cells of male rats in both *in-vivo* and *ex-vivo* conditions (Razmazra, Krause and Duckles 2005). The non-aromatizable and more potent AR agonist and testosterone metabolite, dihydrotestosterone (DHT), has been found to increase the levels of the pro-inflammatory enzymes inducible nitric oxide synthase (iNOS) and COX-2 in cerebral arteries of orchietomized male rats ***in the absence of inflammation*** (Gonzales, Duckles and Krause 2009). This effect was attenuated by the AR inhibitor flutamide, indicating that DHT's actions are AR dependent in the absence of inflammation. Similarly, DHT was found to increase COX-2 in cultured human coronary artery VSM cells, and this effect was also attenuated with the AR antagonist bicalutamide (Osterlund, Handa and Gonzales 2010). ***In the presence of inflammation or pathological conditions*** some studies support an anti-inflammatory role for androgens. In clinical studies testosterone administration to hypogonadal men reduces circulating tumor necrosis factor-alpha (TNF- α) and IL-1 β (Malkin, et al. 2004) and another study (English, et al. 2000) demonstrated that men with existing coronary artery disease had lower circulating testosterone

compared to healthy men of the same age. Testosterone administration to cultured human aortic endothelial cells exhibited decreased expression of TNF- α induced TNF- α and VCAM-1 and this response was AR dependent (Hatakeyama, et al. 2002). Osterlund et al. (2010) and Zuloaga and Gonzales (2011) have shown that the potent AR agonist, DHT, decreases cytokine-, endotoxin-, and hypoxia plus glucose deprivation-induced mediators such as VCAM-1 and COX-2 in human primary VSM cells. Although none of these studies determined if levels of the AR were altered in the experimental or test subjects, there is conflicting evidence for the involvement of AR stimulation in DHT's anti-inflammatory response.

DHT has been classically considered as the final physiological active product acting on AR; however, it has recently been recognized that metabolites of DHT exist and one of these metabolites can have estrogenic activity primarily acting on ER- β and not AR or ER- α (Morali, et al. 1994) (Weihua, et al. 2002) (Kuiper, et al. 1997). However estrogen has been shown to be anti-inflammatory by acting on ER- α (Dan et al. 2003) (Stirone et al. 2005) (Galea et al. 2002)(Ospina et al. 2004), while the expression of ER- β in human coronary intimal has been shown to be associated with early coronary

artery calcification and atherosclerosis (Christian, et al. 2006), pathological changes that are highly influenced by vascular inflammation (Danesh, et al. 2004) (Libby 2008) (Roman, Shanker, et al. 2003) (Shah, et al. 2009). Thus, DHT's effect to reduce cytokine, endotoxin or hypoxic induction of pro-inflammatory mediators independently of AR in VSM is unlikely to be mediated via the conversion to estrogenic compounds acting on ER- β , but may involve changes in the levels of AR under pathophysiological conditions.

In addition to the classic AR, a new functional splice variant of AR has recently been discovered in human tissue, AR45 (Ahrens-Fath, et al. 2005). In the same study, AR45 was found to be expressed primarily in the heart and minimally expressed in brain and testicular tissue in contrast to the full length AR which is ubiquitously in the body but more highly concentrated in testicular tissue. This study also found that AR45 inhibits AR transcriptional activity by binding to AR in a dominant negative interaction. This suggests a potential cardio-protective role for AR45 and a possible mechanism by which androgens may modulate inflammation independent of AR stimulation. It has been shown that the gene sequence of the alternate splice variant in AR45 has been highly conserved in mammals, with the exception of

mouse and rat (Weiss, Faus and Haendler 2007), suggesting a significant evolutionary importance for AR45. In mouse and rat, Weiss et al. (2007) showed that a stop codon exists in the genetic sequence making it unlikely that they express the protein.

Because DHT's effects were AR independent in the presence of inflammation, we wanted to evaluate whether this AR independence involves altering levels of AR under pathophysiological conditions. We **hypothesized** that exposure to pro-inflammatory mediators will decrease the levels of androgen receptor (AR) in human coronary VSM cells. The pro-inflammatory mediators that were tested in this study include IL-1 β , angiotensin II, and hypoxia. IL-1 β acts on IL-1 receptors which leads to activation of NF- κ B, a transcription factor for many cytokines and enzymes in the inflammatory cascade including COX-2, TNF- α , iNOS, IL-1 β , VEGF, and ICAM-1 (Barnes and Karin 1997). Angiotensin II, a classical upregulator of blood pressure through direct vasoconstriction and through the induction of aldosterone, has also been shown to activate NF- κ B directly through Angiotensin 1 (AT1) and Angiotensin 2 (AT2) receptors (Ruiz-Ortega, et al. 2000). Hypoxia on the other hand leads to the activation of hypoxia inducible factor (HIF). HIF leads to the upregulation of toll-

like receptors (Kuhlicke, et al. 2007) which activate NF- κ B after being activated by endotoxins such as lipopolysaccharide (LPS). We therefore tested the following specific aims:

1. Confirm the presence of AR expression in the human coronary VSM cells.
2. Determine if AR expression is altered by the potent AR agonist, dihydrotestosterone (DHT).
3. Determine the effect of the angiotensin II, cytokine IL-1 β or hypoxia on AR expression.
4. Determine if AR expression is altered following transient ischemia and reperfusion in rodent cerebral arteries.

Research Materials and Methods

Cell Culture

Primary (non-immortalized) cells from a 20 year old male were obtained from Cascade Biologics in Portland, OR and grown in media containing VSM growth supplements (Cascade Biologics) and 5% FBS. Cells were incubated at 37C in a 5% CO₂ – 95% room air atmosphere. Once the primary cells replicated to approximately 80% confluence (Fig. 1), the growth media was removed and a trypsin solution was added to detach cells from the flask surface. A trypsin neutralizer was then added, cells collected, trypsin/neutralizer solution removed and cells resuspended in fresh media. Each initial flask of cells was split into four new flasks and the process repeated when cells reached confluency. Cells were grown through 6 to 9 passages in order to minimize differentiation of the cell lines. At the nth passage, the cells were placed in charcoal stripped fetal bovine serum (5%FBS) from Cocalico Biologicals in Reamstown, PA and exposed to experimental conditions (Table 1). Following treatments cells were isolated (detailed protocol below), homogenized and assayed for protein content and prepared for western blot.

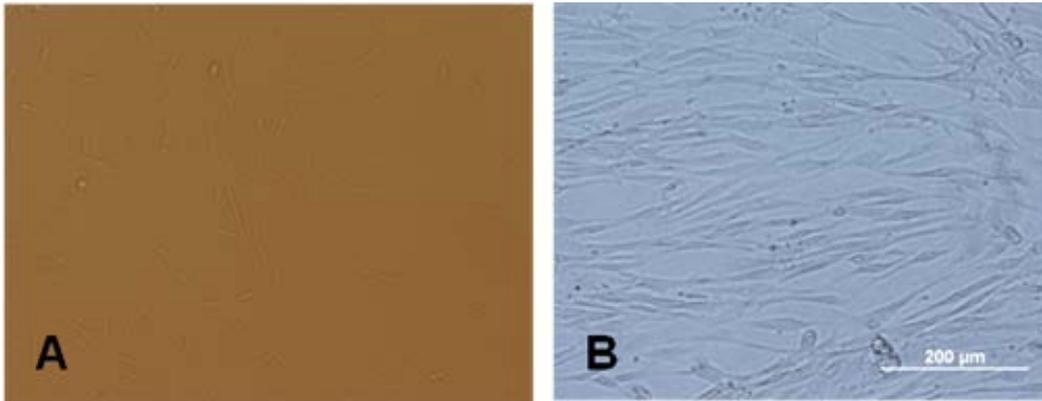


Figure 1. Representative images using bright field microscopy of primary human coronary artery vascular smooth muscle (VSM) cells. **Panel A** represents cells at 30% confluency and **panel B** represents cells at 85% confluency, a state at which cells were treated with hormone or drug.

Table 1.

	Exposure	Time
DHT	10nM, 50nM, or vehicle	chronic
IL-1β	5ng/ml or vehicle	12 hours
Ang II	100nM or vehicle	18 hours
Hypoxia	0.5% oxygen or vehicle	6 hours

Global ischemia and reperfusion

Following bilateral carotid occlusion or sham operation, rats were recovered for 21 hours (reperfusion) at which point they were deeply anesthetized and whole brains removed. Next pial arteries were dissected and collected for homogenization and isolation.

Cell isolation and homogenization

The FBS and treatment media was removed from the flasks containing the cultured cells. Flasks containing adherent human cells were washed with ice-cold phosphate buffered solution (PBS) containing 100 μ M sodium orthovanadate. The flasks were then mechanically scraped in ice-cold PBS to separate the cultured cells from the flask surface. The cell/PBS solution was then extracted and centrifuged at 4C, 800 Xg for 30 minutes in a Thermo Scientific Legend refrigerated centrifuge. The supernatant was then removed and discarded without disrupting the pelleted cells. The pellet was then resuspended in a fresh protease inhibitor cocktail solution containing DTT, pepstatin, leupeptin, aprotinin, and PMSF. Next cells were homogenized and briefly sonicated over ice in order to lyse the cell membranes. The solution of lysed cells and protease inhibitor was again centrifuged at

4C for 30 min at high speed. The supernatant containing the soluble cellular contents was collected and saved while the precipitate containing lipid bilayer and other insoluble cellular membranes was discarded.

Total protein analysis

Protein standards were created from a known protein stock solution (bovine serum albumin). The unknown cellular lysate samples were diluted to a 1:10 ratio in distilled water (d H₂O). A protein binding reagent was created from a BCA™ Protein Assay Kit from Thermo Fisher Scientific and added to the known standards and unknown protein solutions in a 96 well plate. The 96 well plate was then incubated for 20 minutes at 37C and afterward placed into a MultiSkan Spectrum photoanalyzer system using SkanIt RE software (Thermo Fisher Scientific, Waltham, MA) to determine total protein content in each sample.

Western blot

1.5 mm thick, 10 well western gels were prepared by solidifying a lower gel mixture of 5.82 mL d H₂O, 3.18 mL acrylamide: bis 40%, 3 mL lower gel buffer (7.5%), 9 μ L tetramethylethylenediamine (TEMED), and 60 mL 10% ammonium persulfate (APS) in d H₂O followed by an upper gel mixture of 2.4 mL d H₂O, 1 mL upper gel buffer (7.5%), 522 μ L acrylamide: bis 40%, 8 μ L TEMED, and 40 μ L 10% APS in d H₂O. The sample solutions of known total protein content were mixed with predetermined amounts of d H₂O, sample buffer (Invitrogen) and 10% 2-mercaptoethanol. The sample and sample buffer solution were incubated in boiling water for 5 minutes and then briefly and gently centrifuged. Prepared samples of equal amounts of protein (mg/ml) for each sample were loaded in the appropriate gel wells and then gels were electrophoresed using a Bio Rad™ electrophoresis apparatus using Invitrogen Novex® Tris-Glycine SDS Running Buffer solution. A Li-Cor two color molecular weight standard was also loaded as well as rat testes lysate as a positive control (PC) in order to mark the bands of interest. The electrophoresis proceeded at 125 volts for approximately 1 hour. The gel was removed from the box and placed in 1% tween + PBS (TPBS)

for a quick rinse and then soaked for 5 minutes in Invitrogen Novex Tris-Glycine Transfer Buffer solution. The gel was then sandwiched next to a Bio Rad nitrocellulose membrane enclosed in Bio Rad transmembrane filter paper and sponges. The sandwich was placed back into a Bio Rad box containing transfer buffer solution (Invitrogen). Protein transfer from the gel to the membrane proceeded at 0.25 amps for 1 hour. When complete, the membrane was quickly rinsed in TPBS, and then incubated for 30 minutes at room temperature in TPBS with 3% powdered milk as a blocking solution. The blocking solution was then removed by rinsing in TPBS 5 times x 5 minutes. The membrane was then incubated overnight at 4C in rabbit anti-AR (N20) sc-816 primary antibody (Santa Cruz) 1:500 and mouse anti- β -actin or GAPDH primary antibody (Santa Cruz) 1:5000 and 1:15,000 respectively in TPBS. The primary antibody solution was then removed from the membrane and the membrane is then washed 5 x 5 minutes TPBS. The membrane was incubated for 1 hour at room temperature with a green fluorescent anti-rabbit secondary antibody (Invitrogen) 1:15000 and red fluorescent anti-mouse secondary antibody (Invitrogen) 1:15000 in 3% blocking buffer solution.

Following incubation, the secondary antibody solution was removed and the membrane was again washed 5 x 5 minutes in TPBS.

Band Analysis

Following antibody exposure, membranes were analyzed on a Li-Cor Odyssey quantitative infra-red imager. Molecular weight markers and positive controls were used to identify bands of interest, and the optical density of each band of interest was obtained. The optical density for the protein of interest was normalized to the optical density for B-actin, which served as a loading control. The results are reported as band intensity normalized to β -actin (Fig. 3-6).

Statistical Analysis

Statistical significance was performed by using a two-tailed distribution Student's T test with equal variance.

Results

AR is present in human coronary VSM. In agreement with our past studies we detected the presence of AR in our human VSM cell culture model using western blot (Fig. 2) and polymerase chain reaction (PCR) (Zuloaga and Gonzales 2011, unpublished observation). A band was detected at the predicted 110 kDa which corresponds to the classical full length AR. The same 110 kDa was also confirmed in rat testis lysate that served as a positive control. In addition, a band at 45 kDa was also detected in the human VSM lysate that was not detected in the rat testis lysate. This band may correspond to the AR45 described by Ahrens-Fath et al. (2005) and Weiss et al. (2007).

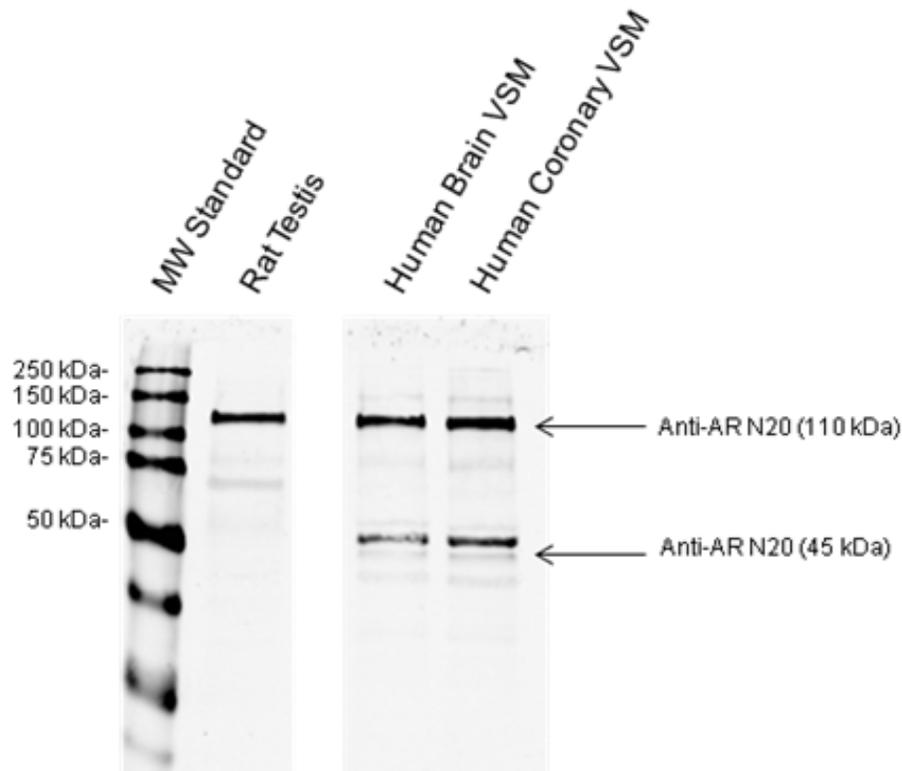
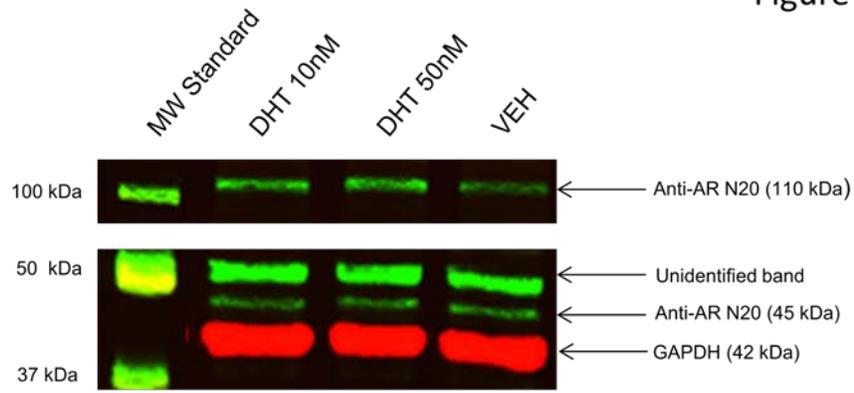


Figure 2. Androgen receptor (AR) expression in rat testis lysate and human brain and coronary vascular smooth muscle (VSM) (*borrowed as unpublished observational data from Gonzales et al 2011*). Anti-AR N20 was used to probe for AR using western blot. A molecular weight (MW) standard was used as a marker to assess band migration in tissue and cell lysate. The band detected by anti-AR N20 at 110 kDa signifies the classical reported AR in both rat testis and human brain and coronary VSM cell lysate. In human VSM cell lysate, additional bands were also detected at approximately 45 kDa. Bands at 45 kDa may correspond to the bands for the AR45 splice variant initially described in human heart tissue by Ahrens-Fath et al. 2005 and Weiss et al. 2007 or may be endogenous break down products of full length AR. There were no bands at 45 kDa in the rat testis lysate. Weiss et al (2007) described that the strongly conserved AR45 amino acid sequence is not found in mouse and rat due to the presence of a stop codon in the conserved amino acid sequence.

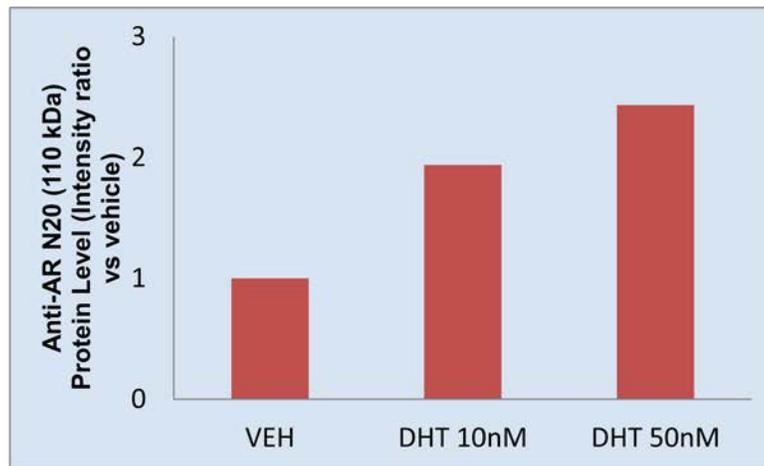
Dihydrotestosterone (DHT) increased AR protein levels in human coronary artery VSM. To show that our cells expressed functional AR, human coronary artery VSM cells were chronically treated with vehicle or DHT (10nM or 50nM). DHT treatment increased band density at 110 kDa (classic AR) and at 45 kDa (putative AR45) in a dose dependent manner (Fig. 3).

Figure 3

A



B



C

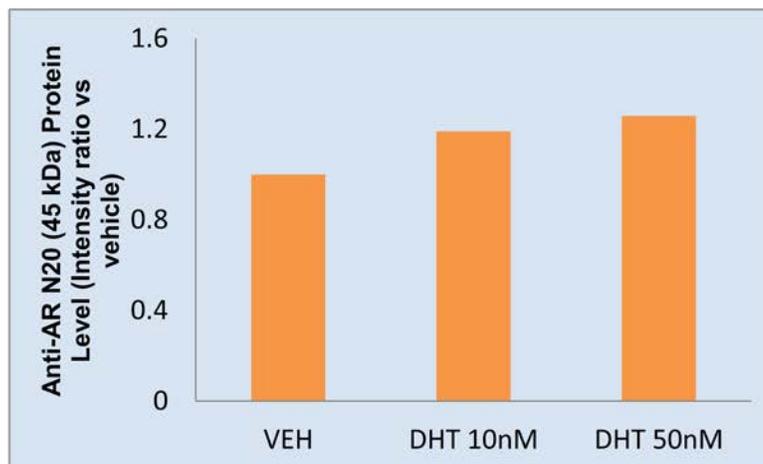


Figure 3. The effect of dihydrotestosterone (DHT) on androgen receptor expression (AR) in levels in human coronary vascular smooth muscle cells. **A:** Representative western blot of human coronary artery VSM cells exposed to DHT (10 nM or 50 nM) or vehicle (VEH) for 12 hours. A band detected at 110 kDa using anti-AR N20 correlates to the classical AR. In addition, bands were also detected at or near 45 kDa which may be AR45 or a endogenous break down product of the full length AR labeled as an unidentified band in the panel A. **B:** Optical density analysis of the AR 110 kDa bands from vehicle (VEH), DHT (10 nM) and DHT (50 nM) treated groups. **C:** Optical density analysis of the 45 kDa bands from vehicle (VEH), DHT (10 nM) and DHT (50 nM) treated groups. n=1 for all sample groups.

IL-1 β appears to decrease AR protein levels in human coronary artery VSM. IL-1 β treatment decreased AR by 26% and putative AR45 by 38%; n=1 compared to vehicle; n=2 (Fig. 4). Due to n=1 for IL-1 β treatment, a statistical analysis could not be performed on this result.

Figure 4

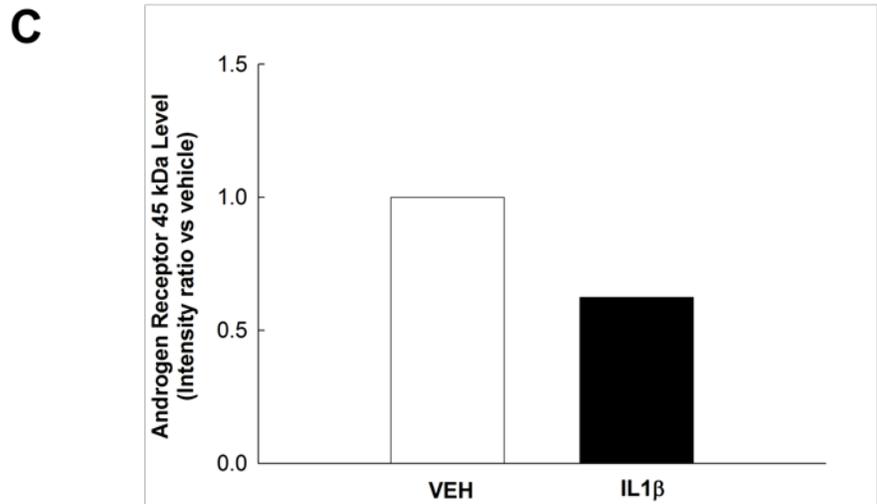
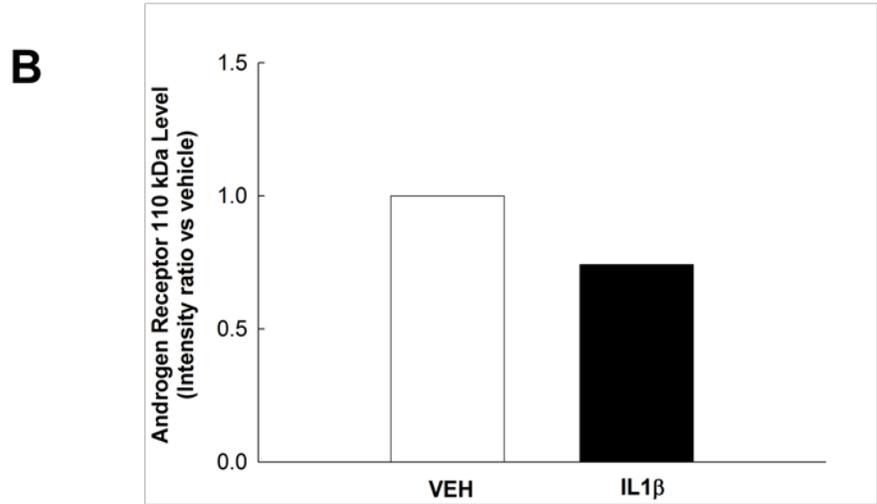
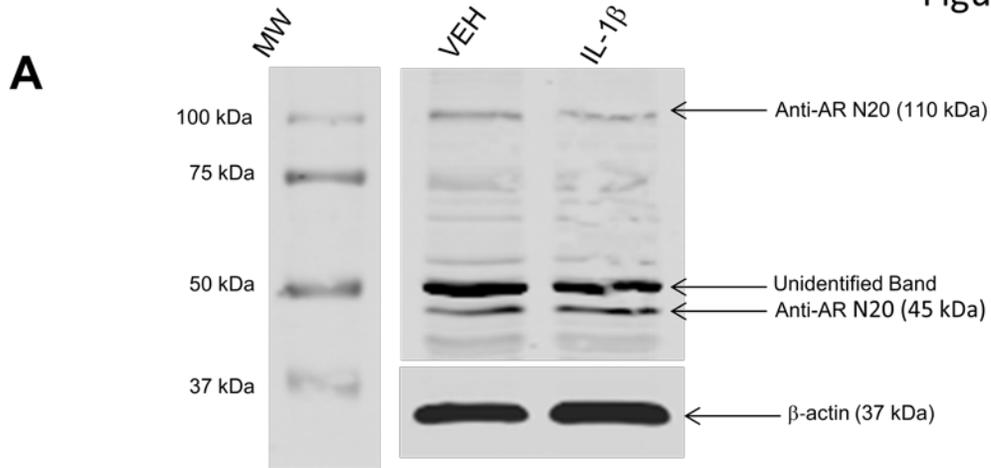


Figure 4. The effect of cytokine treatment on androgen receptor (AR) expression in human coronary vascular smooth muscle cells. **A:** Western blot of human coronary VSM cells exposed to IL-1 β (5 ng/mL) or vehicle (VEH) for 12 hours. **B:** Analysis of AR optical density from IL-1 β treated cells referenced to vehicle. **C:** Western blot analysis of the 45 kDa anti-AR N20 (AR45) referenced to vehicle. Vehicle (n=2); IL-1 β (n=1)

Angiotensin II decreased AR protein levels in human coronary artery VSM. Ang II treatment decreased AR by 41% ($p=0.026$) and putative AR45 by 10% ($p=0.95$) compared to vehicle; $n=3$. The change seen in putative AR45 was not statistically significant (Fig. 5).

Figure 5

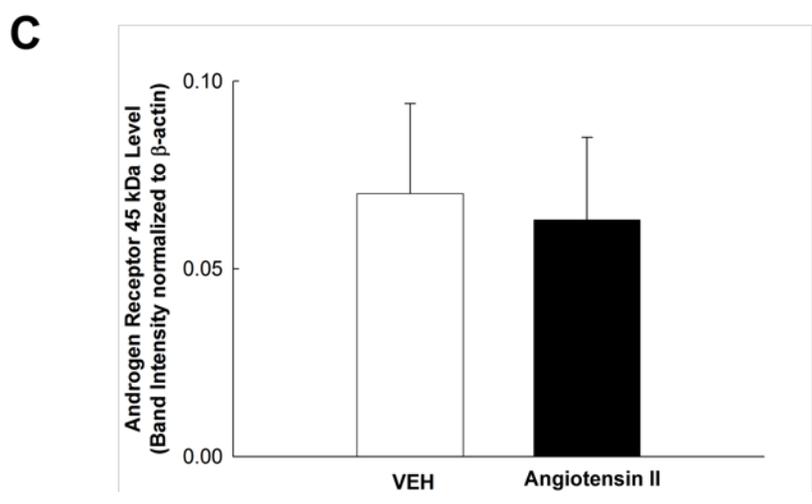
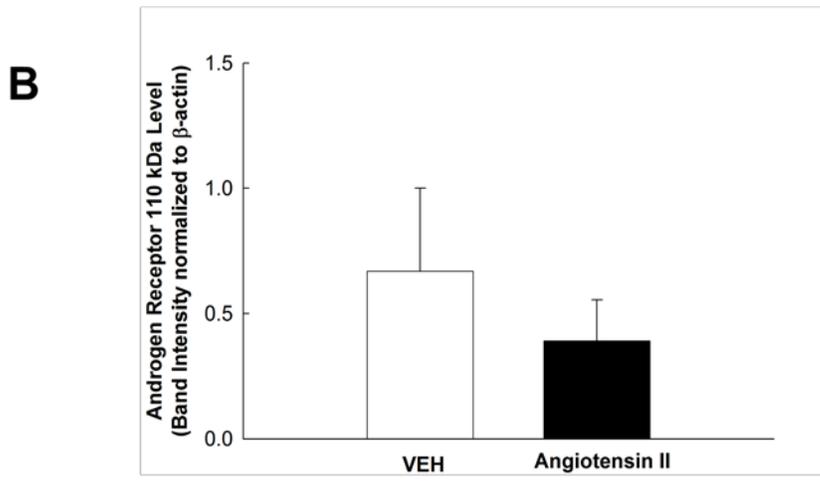
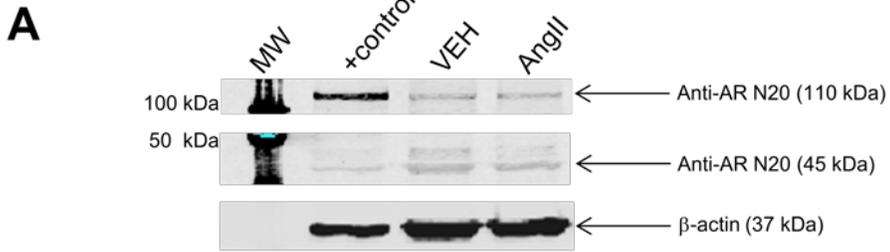


Figure 5. The effect of angiotensin II on androgen receptor (AR) levels in human coronary vascular smooth muscle cells. **A:** Representative western blot of human coronary VSM treated with angiotensin II (100 nM) or vehicle (VEH) for 18 hours. Positive control (+ control). **B:** Analysis of AR optical density compared to VEH. Vehicle (n=3); AngII (n=5) **C:** Analysis of the 45 kDa anti-AR N20 (AR45) optical density compared to VEH. Vehicle (n=5); AngII (n=8)

Hypoxia did not change AR protein levels in human coronary artery VSM. Although there was a trend for an increase in AR by 18% compared to vehicle (n=3, p=0.52), the difference was not statistically significant. However, levels for the AR45-like band were increased by 16% compared to normoxic (vehicle) treated cells (n=3, p=0.078) (Fig. 6).

Figure 6

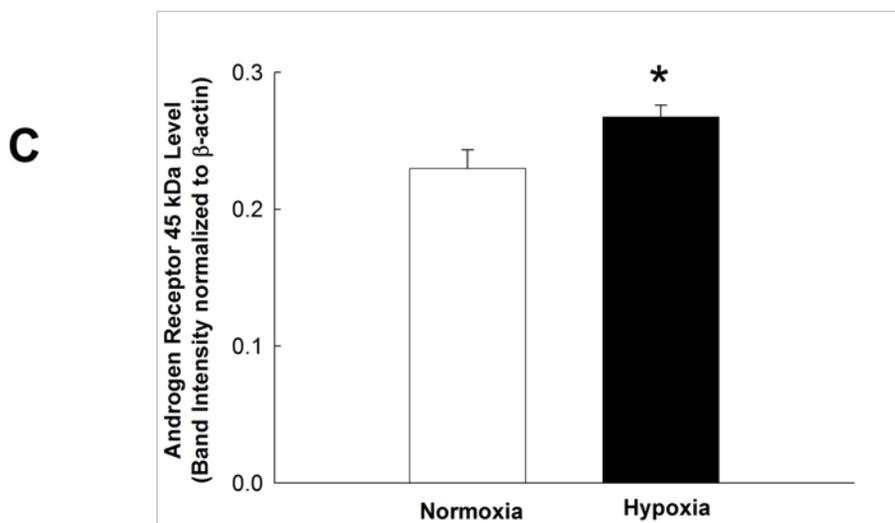
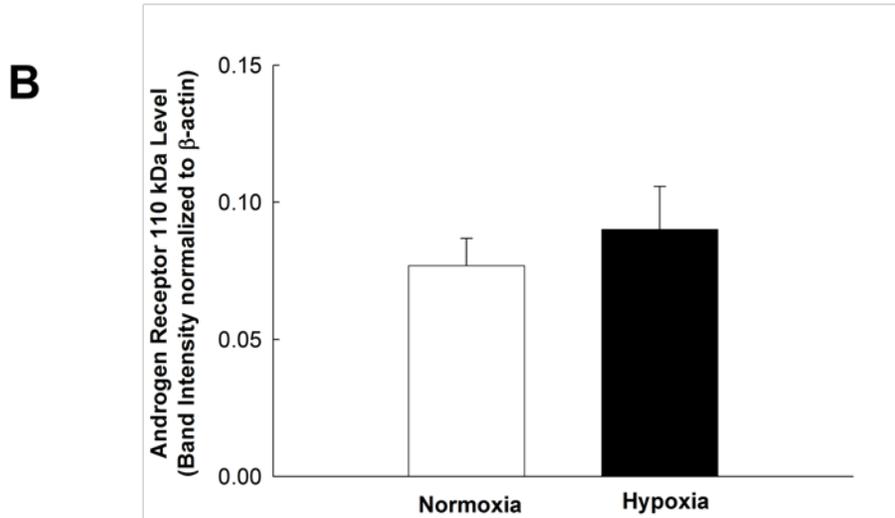
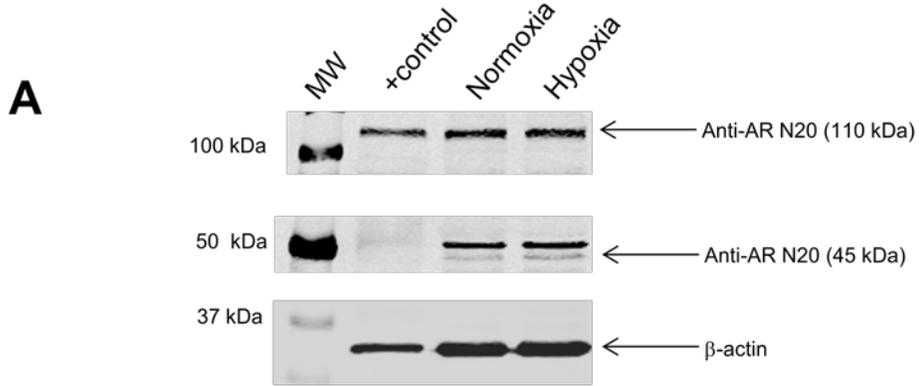


Figure 6. The effect of hypoxia on androgen receptor (AR) in human coronary vascular smooth muscle cells. **A:** Western blot of human coronary VSM exposed to hypoxia or normoxia for 6 hours. Positive control (+ control). **B:** Analysis of AR optical density in normoxic and hypoxic treated groups. **C:** Optical density of the 45kDa in normoxic and hypoxic treated groups. n=3 for all groups.

Androgen receptor was decreased following global ischemia and reperfusion injury (in vivo) in isolated rat pial arteries. AR was present in pial arteries from sham operated rats but was robustly decreased in subjects exposed to global ischemia (Fig. 7).

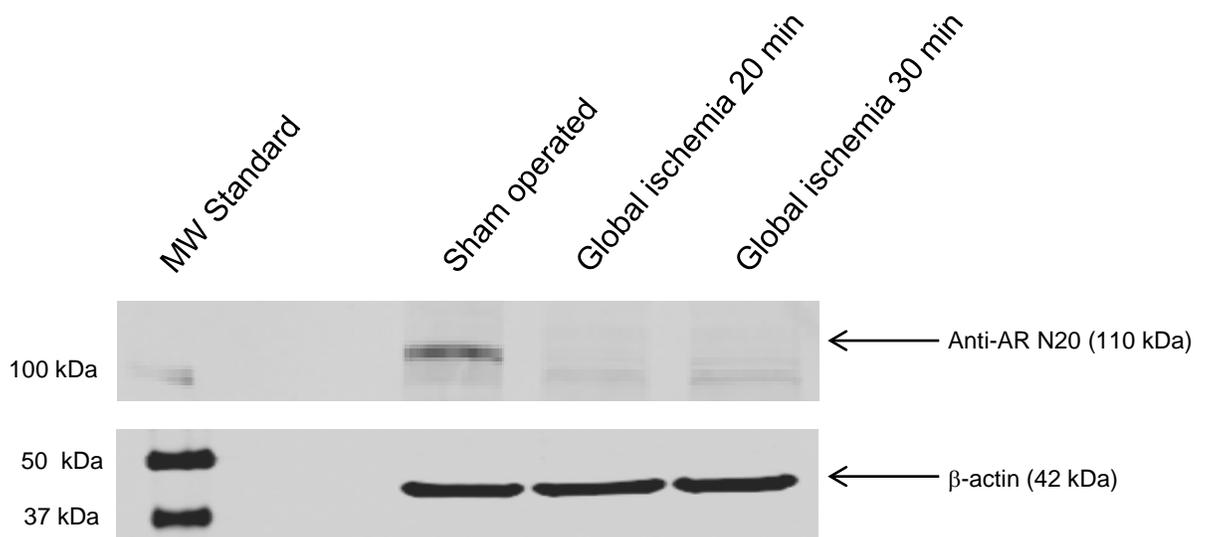


Figure 7. Androgen receptor (AR) expression in pial arteries isolated from rats that underwent global ischemia (bilateral carotid occlusion for 20 or 30 min) and reperfusion injury or sham operation. AR was present in pial arteries from sham operated rats but was decreased in rats subjected to global ischemia.

Discussion

The purpose of this study was to determine the effect of angiotensin II (ang II), hypoxia, or cytokine on AR expression in human VSM cells.

We hypothesized that exposure to ang II, hypoxia, or IL-1 β will decrease the levels of AR in human coronary VSM cells. In the presence of an inflammatory stimulus we demonstrated that there was a consistent trend for IL-1 β and ang II to decrease AR expression in VSM. The decrease in AR following ang II was statistically significant ($p=0.026$), however the $n=1$ sample size for IL-1 β did not allow for statistical analysis. Unlike ang II and cytokine treatments, *in vitro* hypoxic exposure in human VSM cells had no effect on AR.

A hypothesis to explain this discrepancy lies in the signaling mechanisms in the inflammatory mediators that were studied. IL-1 β acts on IL-1 receptors which directly leads to activation of NF- κ B and ang II has recently been shown to activate NF- κ B directly through ang II receptors (Ruiz-Ortega, et al. 2000). Hypoxia on the other hand leads to the activation of hypoxia inducible factor (HIF). HIF leads to the upregulation of toll-like receptors (Kuhlicke, et al. 2007) which activate NF- κ B after being activated by endotoxins such as

lipopolysaccharide (LPS). Activation of toll-like receptors also leads to the recruitment of neutrophils and lymphocytes that release cytokines. These cytokines act on their receptors to activate NF- κ B. Thus, hypoxia might not activate NF- κ B in sterilized cell cultures that lack endotoxins or circulating immune cells. HIF has been shown to activate NF- κ B in cultured cells (Koong, Chen and Giaccia 1994) but this requires lower levels of oxygenation (0.02%) than the levels required to activate HIF or than what was achieved in our study (0.5%) (Gale and Maxwell 2010).

An additional goal of this study was to understand and detect a functional splice variant of AR called AR45, recently discovered by Ahrens-Fath, et al. (2005). Our data further demonstrated that in addition to the full-length AR 110 kDa band detected via anti-AR-N20, a band migrating near 45 kDa in human VSM was observed that is not present in rat testis lysate. Analysis of this band, presumed to be AR45, showed that AR45 band density also increased following exposure to DHT in a dose dependent manner. AR45 levels were also decreased by IL-1 β and ang II. In the case of IL-1 β , AR45 was decreased in a greater proportion than AR (n=1). In the case of ang II, AR45 was decreased in a much lower proportion (n=8) than was AR

(n=5) and it is possible that the ratio of AR45 to AR was increased. In the case of hypoxia, the level of AR45 increased while the level of AR was unchanged (n=3), also increasing the ratio of AR45 to AR.

Increasing the ratio of AR45 to AR would theoretically be anti-inflammatory and hence anti-atherogenic. When activated by DHT, AR45 inhibits activity of AR in a dominant negative interaction (Ahrens-Fath, et al. 2005). Inhibition of pro-inflammatory and pro-atherogenic AR in cardiovascular system would be an evolutionary survival mechanism. The genetic sequence of AR45 has been highly conserved through many genus and species indicating its evolutionary importance (Weiss, Faus and Haendler 2007).

To date, studies that explore the effect of inflammation on AR -- or the role of AR45 in inflammation or cardiovascular health -- have not been published. Ang II (1-10 μ M for 48 hours) has been shown increase cell proliferation and to *increase* AR expression in prostate cancer cells (Hoshino, et al. 2011), however AR in these cells may be mutated or have altered signaling mechanisms (Miyoshi, et al. 2000) (Taplin, et al. 1995). It is undetermined if healthy prostate cells respond in the same way to ang II, or if the effects of ang II vary according to concentration and time (1-10mM for 48 hours versus

100mM for 18 hours in our study). Unpublished data by Georgi, et. al. (2011) also showed that ang II *increased* AR in cultured human coronary VSM ($p < 0.05$) which is contradictory to the current hypothesis and our data. Ang II has recently been shown to increase NF- κ B induced VCAM-1 as well as P and E-selectin expression *in-vivo* in rat endothelial cells (Alvarez, et al. 2004). Ang II has also been shown to increase interleukins 6 and 8, as well as TNF- α by NF- κ B transcription in cultured rat vascular smooth muscle cells (Ruiz-Ortega, et al. 2000). However, neither our study nor the Georgi, et al. study confirmed the induction of pro-inflammatory mediators with ang II. Ruiz-Ortega, et al. showed that ang II activation of NF- κ B can be inhibited by compounds such as ceramides (lipid molecules that are highly concentrated in cell membranes), a wide variety of structurally diverse antioxidants, and tyrosine kinase inhibitors. It is possible that this is not an exhaustive list and perhaps subtle differences may change the effect of ang II on inflammation in cell culture and isolation in laboratory experiments, and hence the level of AR.

Conclusions

Based on the results of our study, we confirm that AR is present in human coronary artery VSM cells and is appropriately upregulated by DHT. We further demonstrated that pro-inflammatory mediators do reduce the level of AR. IL-1 β and ang II decreased the level of AR. With the exception of IL-1 β (n=1) the level of AR45 was statistically unchanged or increased, increasing the ratio of AR45 to AR. As AR45 is activated by DHT it inhibits the action of AR, which has been shown to be pro-inflammatory. It may be this mechanism by which DHT *attenuates* the expression of inflammatory induced transcripts of NF- κ B under existing pathology, as seen by Osterlund, et al. (2010). This may also be the mechanism by which testosterone decreased circulating TNF- α and IL-1 β in hypogonadal men as observed by Malkin et al. (2004), and by which low testosterone is associated with a higher incidence of CVD (English, et al. 2000).

Cell culture vs human studies: The use of cell cultures to perform studies has many advantages including greater control of unmeasured variables and greater ability to investigate the biochemical pathways that underlie complex physiology in animals including humans.

However, *in vitro* studies using cultured cells does not precisely replicate physiology in an intact animal or human. For example, vascular smooth muscle cells in an intact animal are covered and shielded from circulating sex steroid hormones, peptide hormones, and cytokines by a single layer of endothelial cells. Due to this, expression of receptors for sex steroids, cytokines, and ang II may differ in cultured smooth muscle cells compared to cells in an intact animal. Additionally, levels of these receptors may change as they are passaged over several replication cycles. *In vivo* studies are difficult, especially in humans. Although mouse and rat do not express AR45, it would be worthwhile, to perform *in vivo* experimentation in which rats are exposed to ang II or IL-1 β and subsequently analyzed for AR expression in vascular tissue.

Future Directions

Further work would be desirable to prove that AR45 is present in VSM cells using PCR of RNA. The RNA primer sequence and RNA sequence for AR45 has been published (Ahrens-Fath, et al. 2005) and entered into Primer BLAST. Quantification of RNA by PCR could be used to confirm the response of AR and AR45 to pro-inflammatory mediators of inflammation. We observed a band migrating at 45 kDa with the anti-AR N20 antibody which was consistently seen in human cell lysate but not seen in rat tissue lysate, consistent with observations from Weiss, et al., (2007). Use of the anti-AR N20 antibody has been demonstrated by several researchers (Chen and Sawyers 2002) (Chen, et al. 2005) (Miyamoto, et al. 2007). However, the anti-AR N20 antibody binds to the N-terminal region of AR (Santa Cruz Biotechnology), while AR45 differs from AR in that exon 1 is missing and replaced with a novel 7 amino acid N-terminal sequence (Ahrens-Fath, et al. 2005). This would explain why the presumed density of the AR45 band in figures 2-6 is much lighter than the AR density even though AR45 is reported to be heavily expressed in cardiac tissue while AR is not. Nevertheless it is difficult to definitively conclude that the band at 45 kDa is in fact the protein of interest. Quantitative PCR of AR45 and AR RNA might

yield more conclusive data. Alternatively, an anti-AR antibody that binds to the C-terminal region, which is the same in both AR45 and AR, could also be used for future western blot studies.

Future studies on the effects of inflammation on AR and/or AR45 may also benefit from the inclusion of COX-2 or other indicators of inflammation in order to show that inflammation was indeed induced. This may be especially useful in studies involving ang II as conflicting studies now exist. Studies involving NF- κ B DNA binding activity in conjunction with pro-inflammatory mediators and their effect on AR expression can be used to confirm the pathway described by Ruiz-Ortega, et al. 2000, and inhibitors of NF- κ B employed to help determine if the effect of ang II or IL-1 β in reducing AR is indeed mediated through NF- κ B.

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