Estrogen receptor beta dependent attenuation of cytokine-induced cyclooxygenase-2 by androgens in human brain vascular smooth muscle cells and rat mesenteric arteries

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Abstract

Androgens may provide protective effects in the vasculature under pathophysiological conditions. Our past studies have shown that dihydrotestosterone (DHT) decreases expression of cyclooxygenase-2 (COX-2) during cytokine, endotoxin, or hypoxic stimulation in human vascular smooth muscle cells, in an androgen receptor (AR)-independent fashion. Classically DHT is regarded as a pure AR agonist; however, it can be endogenously metabolized to 5 -androstane-3 , 17 -diol (3 -diol), which has recently been shown to be a selective estrogen receptor (ER) agonist. Therefore, we hypothesized that DHT's anti-inflammatory properties following cytokine stimulation are mediated through ER . Using primary human brain vascular smooth muscle cells (HBVSMC), we tested whether DHT's effect on IL-1 induced COX-2 expression was mediated via AR or ER . The metabolism of DHT to 3 -diol is a viable pathway in HBVSMC since mRNA for enzymes necessary for the synthesis and metabolism of 3 -diol [3alpha-hydroxysteroid dehydrogenase (HSD), 3 -HSD, 17 -HSD, CYP7B1] was detected. In addition, the expression of AR, ER , and ER mRNA was detected. When applied to HBVSMC, DHT (10 nM; 18 h) attenuated IL-1 -induced increases in COX-2 protein expression. The AR antagonist bicalutamide did not block DHT's ability to reduce COX-2. Both the non-selective estrogen receptor antagonist ICI 182,780 (1 μM) and the selective ER antagonist PHTPP (1 μM) inhibited the effect of DHT, suggesting that DHT actions are ER -mediated. In HBVSMC and in rat mesenteric arteries, 3 -diol, similar to DHT, reduced cytokine-induced COX-2 levels. In conclusion, DHT appears to be protective against the progression of vascular inflammation through metabolism to 3 -diol and activation of ER .

Keywords

1. Introduction

The cerebral vasculature plays a central role in the pathogenesis of cardiovascular diseases, such as stroke [1], and in the initiation of inflammation after cerebral ischemia, which is a key determinant in stroke outcome [1,2]. Following ischemia, cytokine-induced activation of the transcription factor nuclear factor kappa B (NF B) leads to an increase in the production of pro-inflammatory mediators, such as cyclooxygenase-2 (COX-2) [3,4]. The induction of pro-inflammatory mediators can be influenced by a number of endocrine factors and we

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previously reported that the potent androgen receptor (AR) agonist, dihydrotestosterone (DHT), can decrease levels of COX-2 following cytokine administration or hypoxia with glucose deprivation treatment. We further demonstrated that these responses occur independent of AR stimulation [5,6] since they could not be blocked by co-treatment with an AR antagonist.

The mechanism(s) for sex steroid influences on vascular inflammation is complex, given the numerous molecular pathways that these hormones can utilize to alter gene transcription and cellular responses. Both androgens and estrogens have been shown to have anti-inflammatory effects [5,7-9], although androgens have been reported to have some pro-inflammatory effects as well [10,11]. Classically, testosterone can activate the AR directly or indirectly through its conversion by 5 alpha-reductase to the more potent androgen, DHT [12,13]. Alternatively, testosterone can be metabolized to 17 -estradiol (E2) by the aromatase enzyme [14] and subsequently activate estrogen receptor alpha (ER) or beta (ER) [15,16]. A third and less well explored pathway for androgen action is through the conversion of DHT to 5 -androstane-3 , 17 -diol (3 -diol), a known selective ER agonist [16-18]. This metabolic pathway occurs by the action of the enzymes 3beta-hydroxysteroid dehydrogenase (3 -HSD), 3alpha-hydroxysteroid dehydrogenase (3 -HSD) or 17beta-hydroxysteroid dehydrogenase (17 -HSD) [18-22]. 3 -Diol is further converted to inactive metabolites by the enzyme CYP7B1 [18,23]. Since blood vessels contain AR, ER , ER , 5 reductase, aromatase, and 3 -HSD [24-30], 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 E2, 3 -diol has recently been shown to reduce expression of inflammatory markers in human umbilical vein endothelial cells [31]. Therefore, we have hypothesized that during cytokine stimulation, DHT decreases expression of the pro-inflammatory mediators via metabolism to 3 -diol and subsequent activation of ER in human brain vascular smooth muscle cells (HBVSMC). Additional experiments were performed in rodent peripheral arteries to determine if 3 -diol (*ex vivo*) was able to attenuate inflammation-induced COX-2 in an intact artery. COX-2 was investigated as a marker for vascular inflammation because of past studies demonstrating that COX-2 inhibition can decrease infarct size in experimental models of stroke [32] and because we have previously shown that in human coronary artery smooth muscle cells, DHT decreases cytokine-induced COX-2 expression via an AR-independent mechanism [5].

2. Experimental

2.1. Primary vascular smooth muscle cell culture and hormone/drug treatment

Primary brain vascular smooth muscle cells (HBVSMC) isolated from two donors were used in this study. HBVSMC from a 20-year-old male donor (Lot #ACBRI 405) were purchased from Cell Systems Corporation (Kirkland, WA) and HBVSMC isolated from a fetal donor (Lot #2733) were purchased from ScienCell Research Laboratories (Carlsbad, CA). Both sets of cells were grown under similar conditions: 5% CO_2, room air atmosphere at 37 °C, in Medium 231 (Invitrogen Corporation, Carlsbad, CA) supplemented with smooth muscle growth supplement (ScienCell) and 2% FBS.

2.1.1. Experimental protocol for fetal HBVSMC—In a previous study we demonstrated that DHT reduces hypoxia plus glucose deprivation-induced COX-2 expression via an unidentified AR-independent mechanism in HBVSMC harvested from a fetal donor [6]. In the current study we extended our investigations of the previous study [6] using cryo-preserved cells with the same lot number to determine if DHT's effect during cytokine stimulation is AR or ER mediated. Hormone/drug treatments were performed on

fetal cells at 80–90% confluency and at passage 7 in hormone free media. HBVSMC (passage 7) still expressed the smooth muscle-specific proteins -actin and smoothelin (data not shown). For the experimental protocol, cells were pre-treated for 1 h with vehicle (0.001% ethanol), the AR antagonist bicalutamide (BIC, 1 μM), or the non-selective ER antagonist ICI 182,780 (ICI, 1 μM; Tocris Bioscience; dissolved in ethanol), followed by 18 h of co-treatment with either vehicle (0.001% ethanol) or DHT (10 nM) then 6 h of IL-1 (5 ng/ml). A 6 h time point for COX-2 induction, a 10 nM DHT dose and a 1 μM antagonist dose were selected for these studies based on dose response curves from our previous studies [5,6].

2.1.2. Experimental protocol for adult HBVSMC—In the experiments involving the adult HBVSMCs the hormone/drug treatments were performed on cells at 80–85% confluency and at passage 9 in hormone free media. HBVSMC at passage 9 still expressed the smooth muscle-specific proteins -actin and smoothelin (data not shown). Cells were treated with DHT (10 nM), 3 -diol (10 nM) or vehicle (0.001% ethanol) for 18 h followed by interleukin-1beta (IL-1 , 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 vehicle (0.001% ethanol) or the selective ER 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), DHT (10 nM), or 3 -diol (10 nM), then 6 h of IL-1 (5 ng/ml).

2.2. Quantitative real-time RT-PCR

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was used to measure mRNA expression of gonadal steroid hormone receptors and steroid metabolizing enzymes in the adult male HBVSMCs treated for 6 h with hormone-free media, vehicle, or IL-1 (5 ng/ml). RNA was extracted using a standard phenol/chloroform/isoamyl procedure [33] 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 12 μl 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 relative concentrations of template in comparison to GAPDH. Absolute values in femtogram (fg) of template were calculated based on a standard curve generated by serial diluting the product formed by the respective primers prior to the GAPDH normalization. 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 °C annealing temperature was used for the 17 -HSD and 3 -HSD primer sets and a 55 °C annealing temperature was used for the ER 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. 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 relative concentration of

cDNA to GAPDH. Specificity was confirmed via thermal melting curve analysis which showed a single peak at the predicted melting temperature for each primer set.

2.3. Western blot

Levels of COX-2 protein were examined using standard immunoblotting methods, as previously described [5]. 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), AR (N20; epitope mapping at the N-terminus of AR of human origin) (1:500) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz CA and   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 (passage 9) and fetal HBVSMC (passage 7) 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).

2.4. Rat mesenteric artery isolation and hormone/drug treatment (ex vivo)

Animal protocols were approved by the Institutional Animal Care and Use Committee of Arizona State University under subcontract from the University of Arizona. Male Sprague–Dawley rats (250 g) used in this study were purchased from Charles River Laboratories (Portage, Michigan) and maintained in a temperature- and humidity-controlled environment within the Arizona State University Department of Animal Care and Technology (DACT). Animals were kept on 12:12 light dark cycle and food and water were available ad libitum. One week prior to euthanasia rats were transferred to a satellite animal facility at the University of Arizona COM-Phoenix campus in accordance with protocols approved by DACT and housed under the same conditions described above. The University of Arizona COM-Phoenix campus satellite facility is operated under the guidelines of the Arizona State University Institutional Animal Care and Use Committee and accredited by the American Association for Accreditation of Laboratory Animal Care.

2.4.1. Artery isolation—On the day of experiment rats were deeply anesthetized with isoflurane (5%), the abdomen was shaved and skin cleaned with betadine. Under aseptic conditions, a midline incision was made and the thoracic and abdominal cavities were exposed. Heparin (100 U/0.1 mL) was immediately injected into the right ventricle to circumvent clot formation. Following exsanguination via cardiac puncture, the small intestine (i.e. jejunum) and its associated mesentery were removed, rinsed in sterile saline and pinned in a Sylgard®-coated dissection dish (Dow Corning, Midland, MI) containing sterilized ice-cold physiological salt solution (PSS, mM): 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 0.4 NaH_2PO_4, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose aerated with 21% O_2, 6% CO_2, and balanced N_2. Making note not to stretch the vessels, segments of second and third order branches were carefully dissected and immediately transferred to a DMT (Danish

Myograph Technology, Aarhus, Denmark) stainless steel tissue bath that was pre-sterilized with ethanol solution and rinsed with sterile PSS. The DMT tissue bath served as an incubation chamber and arteries were gently secured using sterile nylon thread and bathed in fresh PSS bubbled with a 21% O_2, 5% CO_2, N_2 balance gas mixture. Following a 30 min equilibration, arteries were treated with VEH (PSS + 0.001% ethanol), IL-1 (10 ng/ml), or IL-1 (10 ng/ml) + 3 -diol (10 nM) for 3 h. In a separate experiment, artery segment incubations were conducted in the presence of VEH, lipopolysaccharide (LPS; 100 μg/ml) or LPS + 3 -diol for 3 h to investigate the effect of 3 -diol using another inflammatory stimulus. 1.5 h into the incubation the solution containing either the VEH or the treatments was rinsed and replaced with fresh PSS solution plus treatment(s) for an additional 1.5 h incubation. At the end of the 3 h incubations, arteries were removed, placed in an eppendorf tube and flash frozen on dry ice. Arteries were stored at −80 °C until further processed for protein analysis and Western blot.

2.4.2. Artery homogenization and western blot—Arteries were homogenized in tissue lysis buffer containing protease inhibitors and standard Western blot was performed (for details see Western blot protocol in cell section above). Membranes were incubated with a murine polyclonal COX-2 (1:500) antibody raised in rabbit (Cayman Chemical Cat #160126; Ann Arbor, MI) and actin (1:5000) mouse monoclonal antibody (Sigma Aldrich Corporation, St. Louis, MO) overnight at 4 °C in TPBS. Following TPBS washes, the membranes were incubated in Goat Anti-Mouse IR 800 Dye (1:15,000) secondary antibody (Li-COR) and/or Goat Anti-Rabbit IR 680 Dye (1:15,000) secondary antibody (Li-COR) for 1 h at room temperature. Similar to Western blot for cells, following additional TPBS washes, proteins were visualized and quantitated using an Odyssey Infrared Imager and data were analyzed using Odyssey V3.0 software (Li-COR).

2.5. Reagents and chemicals

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

2.6. 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). Data from Western blots were expressed as an optical density ratio relative to vehicle and normalized to the optical density values for actin bands. Multiple vehicles and treatment groups were included on the same gel and normalized to the first vehicle on the gel to account for variance. Normalizing to both a vehicle and a loading control limits variance between blots and allowed pooling of multiple blots from each experiment for comparisons. Each graph represents data obtained from 4 to 8 different membranes. Since the COX-2 bands present as doublets, both bands were analyzed together in a single imaging frame for each protein. Data from qRT-PCR studies are expressed as relative concentration of cDNA normalized to GAPDH and all measures were repeated a sufficient number of times for statistical analysis (n 7). All values are reported as means ± SEM. Unless otherwise noted, data were compared using one-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.

3. Results

3.1. Gonadal steroid receptors and steroid metabolizing enzyme mRNAs are expressed in HBVSMC

Expression of the necessary gonadal steroid receptors and steroid metabolizing enzymes for DHT metabolism/receptor activation was verified using qRT-PCR in adult HBVSMC grown in hormone-free media. The size of the amplified cDNA was confirmed by 2% agarose gel electrophoresis for each primer set. Messenger RNAs for AR, ERα, ERβ, 3α-HSD, 3β-HSD, 17β-HSD, and CYP7B1 were detected in varying amounts in adult HBVSMC (Table 2).

3.2. AR and ERβ expression is not altered by inflammatory stimuli

To determine if the expression of AR and ERβ mRNA is altered in response to inflammatory stimuli, adult HBVSMC were treated for 6 h with vehicle or IL-1β (5 ng/ml) and mRNA levels were measured by qRT-PCR. Expression of AR and ERβ mRNA was not altered by cytokine treatment (Fig. 1A). Since AR activation has previously been shown to increase the expression of the AR [34], we demonstrated that the AR is fully functional our HBVSM primary cell culture model. Cells were treated with vehicle (VEH) or DHT (10 nM; 6 h) and AR protein levels measured. DHT treatment increased AR protein expression in HBVSMC. Fig. 1B illustrates a representative blot for AR and the loading control β actin. Rat testis lysate served as a positive control for AR. Fig. 1C represents the calculated intensity ratio for AR bands from the representative blot in Fig. 1B ($n = 3$; $p < 0.05$).

3.3. Effect of DHT on COX-2 during IL-1β-stimulation is mediated via ERβ, not the androgen receptor, in HBVSMC

We have previously shown that DHT decreases cytokine-induced COX-2 expression in human coronary artery vascular smooth muscle cells and in HBVSMC exposed to hypoxia with glucose deprivation. Both effects were found to be AR-independent [5,6]. To test if DHT's effect to reduce cytokine-induced COX-2 expression in HBVSMC is also AR-independent and to further determine if DHT's effect is estrogen receptor mediated, fetal HBVSMC were pre-treated for 1 h with vehicle or the AR antagonist BIC (1 μM) or the non-subtype selective ER antagonist ICI 182,780 (ICI, 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. One-way ANOVA revealed that IL-1β significantly increased COX-2 protein expression compared to vehicle ($p < 0.05$; Fig. 2). Furthermore, DHT significantly decreased IL-1β-induced increases in COX-2 protein ($p < 0.05$ vs. IL-1β; Fig. 2). DHT's effect was not blocked by the AR antagonist BIC (Fig. 2), but was blocked by the ER antagonist ICI. Antagonist treatments without DHT did not have an effect on COX-2 levels.

3.4. DHT inhibited cytokine-induced increases in COX-2 via an estrogen receptor β-dependent mechanism in HBVSMC

To determine if DHT's anti-inflammatory actions are ERβ-dependent, adult 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. One-way ANOVA revealed that IL-1β significantly increased COX-2 protein expression compared to vehicle ($p < 0.05$; Fig. 3) in the adult cells, as we observed in the fetal cells. Furthermore, again DHT significantly reduced IL-1β-induced increases in COX-2 protein ($p < 0.05$ vs. IL-1β; Fig. 3). DHT's effect was reversed by co-treatment with the selective ERβ antagonist PHTPP ($p < 0.01$ vs. IL-1β + DHT; Fig. 3).

3.5. DHT and 3β-diol inhibited cytokine-induced increases in COX-2 in HBVSMC

To determine if DHT's anti-inflammatory actions could be mimicked by 3 -diol, HBVSMC were treated with vehicle, DHT (10 nM), or 3 -diol (10 nM) for 18 h followed by vehicle (VEH) or IL-1 (0.2, 1, or 5 ng/ml; 6 h) in continued presence of hormone. One-way ANOVA showed that both DHT and 3 -diol significantly reduced the effect of the 5 ng/ml or 1 ng/ml doses of IL-1 to increase COX-2 protein ($p < 0.05$ vs. IL-1 ; Fig. 4). The lowest dose of IL-1 (0.2 ng/ml) did not increase COX-2 expression above vehicle levels (data not shown).

3.6. 3β-Diol inhibited cytokine- and endotoxin-induced increases in COX-2 in rat mesenteric arteries

To assess whether the effects of 3 -diol on COX-2 persist in another experimental vascular model, arteries from rat mesenteric arteries were aseptically isolated and incubated *ex vivo* for 3 h with either IL-1 (cytokine; $n = 1$) or LPS (endotoxin; $n = 3$) in the absence or presence of 3 diol. Fig. 7A illustrates COX-2 protein expression in vehicle (VEH; 0.001% ethanol), IL-1 (10 ng/ml) or IL-1 10 ng/ml + 3 -diol (10 nM). IL-1 increased COX-2 protein expression and 3 -diol attenuated this effect. Fig. 5B illustrates COX-2 protein expression in arteries incubated with vehicle (PSS), the endotoxin lipopolysaccharide (LPS; 100 μg/ml), or LPS + 3 -diol. Fig. 5C illustrates quantitative data for LPS treatments. One way ANOVA showed LPS increased COX-2 protein levels ($p < 0.05$ vs. VEH). Increases in endotoxin-induced COX-2 were attenuated in the presence of 3 -diol ([#]$P < 0.05$ vs. LPS).

4. Discussion

The goal of this study was to determine the mechanism underlying DHT's anti-inflammatory effects in brain vascular smooth muscle cells. Using COX-2 protein expression as a marker for vascular inflammation, we examined the effects of the non-aromatizable androgen, DHT, and its metabolite 3 -diol, on COX-2 protein expression in human brain vascular smooth muscle cells. Furthermore, we examined the effects of 3 -diol *ex vivo* on cytokine and endotoxin induced COX-2 expression using an isolated rat artery model. The results of these studies support our hypothesis that during cytokine stimulation, DHT decreases COX-2 expression via metabolism to 3 -diol and subsequent activation of ER .

Androgens have been shown to have cardioprotective and anti-inflammatory actions. The cardioprotective effects of androgens include inhibition of hypoxic pulmonary hypertension [35] and decreased infarct size after cerebral ischemia in rodents [36-40]. In addition, low testosterone levels predict increased risk of stroke [41] and poorer outcome after stroke in men [42]. Androgens have been shown to reduce inflammation in a variety of cells/tissues including human endothelial [7,43-45] and vascular smooth muscle cells [5]. For instance, we have recently reported that DHT can inhibit both cytokine-induced and hypoxia plus glucose deprivation-induced COX-2 levels in human vascular smooth muscle cells [5,6]. Androgen treatment *in vivo* has also been shown to decrease inflammatory markers in androgen deficient men [46]. Thus, these data indicate that androgens might protect against vascular inflammation in androgen deficient men already predisposed to cardiovascular events. Since androgen levels decline with age [47], androgen deficiency is not uncommon in aged men. Since our studies and studies by others demonstrate the presence of ER in vascular tissues, targeting the 3 -diol/ER pathway may be a more direct approach to therapeutically reduce vascular inflammation compared to DHT treatment because activation of the AR receptor pathway results in higher risk for cancer in hormone sensitive target organs, such as prostate tissue.

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 [48,49] and human endothelial cells [10,11,50]. These apparent contradictory findings may be explained by a differential effect of androgens in the absence and presence of inflammation. In our studies, androgens increase vascular inflammation via an AR-dependent mechanism under physiological conditions, but decrease vascular inflammation via an AR-independent mechanism during pathophysiological conditions [5,48]. Since we have found androgens to be protective under pathophysiological conditions [6] but detrimental under healthy physiological conditions [5], many of the studies in healthy young rodents may not yield similar results unless some form of inflammation was induced. Additional differences may be explained by the dose of androgens used. Androgens appear to be protective at low doses, but detrimental at high doses, as has been shown with damage following cerebral ischemia [37] and after induced-inflammation in endothelial cells [7,10,11]. The results obtained in the current studies should be physiologically relevant since low doses of DHT and 3 -diol were used. Future studies will be aimed at determining the nature of the molecular switch(s) that reverses DHT actions from pro- to anti-inflammatory in vascular smooth muscle cells.

The results of our current studies point to ER as a novel receptor pathway utilized by DHT during cytokine stimulation. Since DHT can be converted to 3 -diol by the enzymes 3 -HSD, 3 -HSD and 17 -HSD [18-22], we sought to determine if these enzymes are also found in HBVSMC. Our data show that primary HBVSMC express the gonadal steroid receptors AR, ER , ER , as well as enzymes involved in 3 -diol metabolism. This is consistent with previous studies showing that blood vessels contain AR, ER , ER , and 3 -HSD [24-27,29,30]. Additionally, we found that AR and ER mRNA expression was not altered by cytokine treatment in our human cell model. Results of our studies also show that both DHT and 3 -diol decrease cytokine-induced COX-2 expression and that DHT's effects are likely mediated via ER . Since 3 -diol can be synthesized from DHT by several enzymes, for which specific inhibitors are not available, we were unable to block 3 -diol synthesis directly. However, we were able to show that an ER selective antagonist can block DHT's ability to inhibit cytokine-induced COX-2 protein expression. These data are supported by a recent study by Norata et al. [31] showing that 3 -diol has anti-inflammatory actions in human umbilical vein endothelial cells and mouse aorta, and that these effects are also mediated by ER [31].

Many studies have shown that ER activation has a variety of cardioprotective and anti-inflammatory effects. Cardioprotective effects of ER activation include protection against cerebral ischemia in rodents [51,52], protection against myocardial ischemia in mice [53-57], and protection against *in vitro* ischemia in mouse brain endothelial cells [58]. ER activation, like androgens, has been shown to have anti-inflammatory effects in a variety of cells/tissues including rat vascular smooth muscle cells [9] and human endothelial cells [31]. A recent study by Saijo et al. identified a mechanism for ER -mediated reduction of inflammatory markers in microglia that may help explain the effects we show in vascular cells. They found that 5 -androstene-3 ,17 -diol (Adiol), a metabolite of the adrenal androgen, dehydroepiandrosterone, suppresses inflammatory genes by binding to ER and recruiting C-terminal binding protein (CtBP), a co-repressor that binds to activator protein-1 dependent promoters, thereby repressing expression of pro-inflammatory genes [59]. Future studies are needed to determine if 3 -diol is similarly capable of directing CtBP recruitment by ER or if there are other co-regulatory proteins that are selectively activated by 3 -diol binding to ER . Taken together, these studies show that ER activation has similar protective effects to androgens, and support the theory that some of the beneficial effects of androgens may occur through activation of ER .

Aside from ER 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 [60,61]. DHT can also increase intracellular calcium via an unidentified membrane-bound receptor [62,63]. Furthermore, several authors have also hypothesized that a membrane bound AR may exist that is not blocked by classical AR antagonists [62-65]. Whether these alternative pathways for androgen action may also contribute to DHT's anti-inflammatory effects is yet to be determined and are beyond the scope of the current study.

To investigate the mechanism of DHT's anti-inflammatory effect we used a unique primary human vascular cell model. Although potentially more clinically relevant compared to rodent studies, we acknowledge that the use of cultured vascular cells does have certain limitations. First, cultured vascular smooth muscle cells may lose their smooth muscle phenotype with later passages [66]. Furthermore, it is also recognized that receptor protein levels for androgens and cytokines may also change with multiple passages in culture. To minimize the risk of changes in culture, all experiments were performed after a small number of passages (7 or 9) and at the time of experiments, HBVSMC still expressed the smooth muscle cell markers smoothelin and alpha actin. A second possible limitation is that each cell type originated from a single donor. However, this is likely not an issue since in our current study (and in other past studies) we utilized vascular smooth muscle cells from various male donors. Additionally, we found that DHT's anti-inflammatory effect was ER-mediated in both fetal and adult cells. We have also found that 3 -diol reduces IL-1 -induced expression of COX-2 in both smooth muscle cells and freshly isolated rat arteries. Thus, we have provided evidence that the DHT/3 -diol/ER pathway may be utilized at different points in development (fetal and adult) and that 3 -diol has anti-inflammatory effects in both the human and rat vasculature. Furthermore, our previous studies in primary human coronary vascular smooth muscle cells, originating from a different donor, also showed that DHT attenuated increases in COX-2 levels following IL-1 stimulation via an AR-independent mechanism [5]. These findings leave open the possibility that this pathway could be active in other vascular beds, an idea which is supported by our data in rat mesenteric arteries and by Norata et al. [31] who showed a similar effect in human umbilical vein endothelial cells [31]. We have also recently reported that DHT decreases hypoxia plus glucose deprivation-induced COX-2 in HBVSMC via an AR-independent mechanism [6]. Whether or not the DHT/3 -diol/ER pathway is responsible for this effect is yet to be determined.

Our findings demonstrate that DHT decreases cytokine-induced COX-2 expression via an ER -dependent mechanism. These data suggest that this effect of DHT may likely involve the metabolism of DHT to the ER agonist 3 -diol (Fig. 6). This is supported by data demonstrating that (1) HBVSMC express ER and the necessary enzymes to convert DHT to 3 -diol, (2) 3 -diol mimics the anti-inflammatory effect of DHT, and (3) DHT's effect to reduce cytokine-induced inflammation is ER dependent. Thus, we have identified an ER -dependent anti-inflammatory effect for DHT in the human brain vascular cells. A deeper understanding of how androgens modulate inflammation could provide insight into more effective approaches to manage the progression of vascular diseases that eventually lead to devastating end points, such as heart attack or stroke.

Acknowledgments

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Abbreviations

3 -HSD	3 alpha hydroxysteroid dehydrogenase
3 -diol	5 -androstane-3 ,17 -diol
3 -HSD	3 beta hydroxysteroid dehydrogenase
17 -HSD	17 beta hydroxysteroid dehydrogenase
Adiol	5 -androstene-3 ,17 -diol
AR	androgen receptor
COX-2	cyclooxygenase-2
DHT	dihydrotestosterone

| E2	17 -estradiol
ER	estrogen receptor alpha
ER	estrogen receptor beta
HBVSMC	human brain vascular smooth muscle cells
ICI	ICI 182,780
IL-1	interleukin-1 beta
LPS	lipopolysaccharide
NF B	nuclear factor kappa B
PHTPP	4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol
PSS	physiological salt solution
qRT-PCR	quantitative real-time reverse transcriptase polymerase chain reaction
VCAM-1	vascular cell adhesion molecule-1
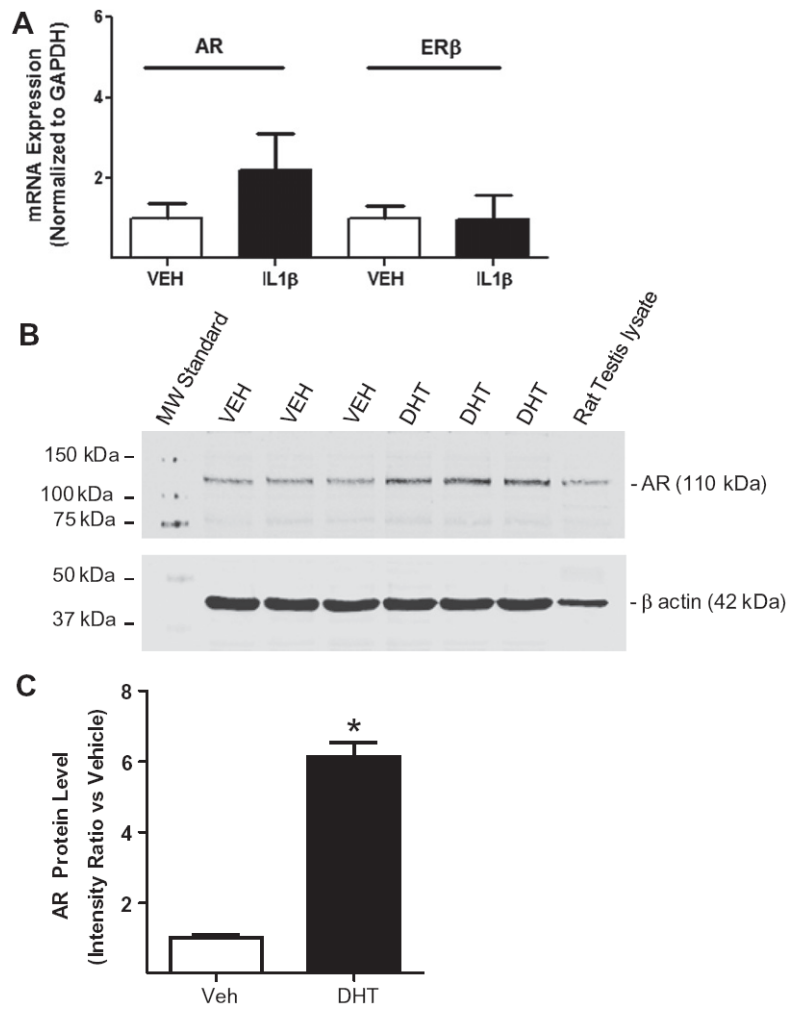
Fig. 1.
AR and ER expression is not altered by inflammatory stimuli. (A) Graph showing AR and ER mRNA expression level in adult human brain vascular smooth muscle cells (HBVSMC) treated for 6 h with vehicle (VEH) or interleukin-1 beta (IL-1 ; 5 ng/ml) using qRT-PCR. Each bar represents the mean ± SEM for 7–8 determinations. (B) Representative blot of androgen receptor (AR) expression in adult HBVSMC treated with VEH or DHT (10 nM) for 6 h. Rat testis lysate served as a positive control and beta actin verified equal amounts of total protein loaded in each lane. (C) Graph showing AR protein expression in adult HBVSMC expressed as an intensity ratio vs. vehicle. *$P < 0.05$ vs. VEH ($n = 3$ per group).
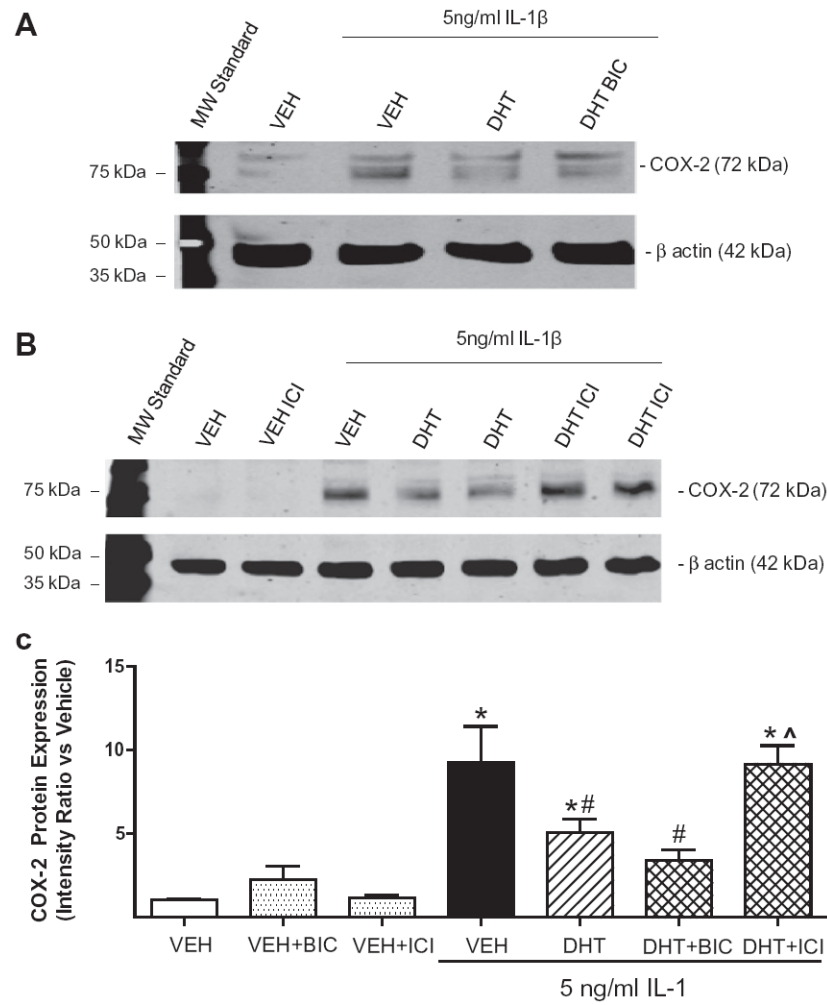
Fig. 2.
Effect of DHT on COX-2 during IL-1 -stimulation is androgen receptor independent/ER-dependent in HBVSMC. Cyclooxygenase-2 (COX-2) protein expression was assessed in fetal human brain vascular smooth muscle cells (HBVSMC) pre-treated for 1 h with vehicle (VEH), the androgen receptor antagonist bicalutamide (BIC; 1 μM), or the non-selective estrogen receptor antagonist ICI 182,780 (ICI, 1 μM), followed by VEH or dihydrotestosterone (DHT, 10 nM) for 18 h, then exposed to interleukin-1 beta (IL-1 ; 5 ng/ml; 6 h) in the continued presence of hormone/antagonist. (A) and (B) Representative blots for COX-2. C: Data analysis for COX-2 levels corrected for loading with beta actin and normalized to vehicle (VEH). *$P < 0.01$ vs. VEH, #$P < 0.05$ vs. IL-1 , #$P < 0.05$ vs. IL-1 , ˆ$P < 0.05$ vs. IL-1 + DHT (n 4 per group).
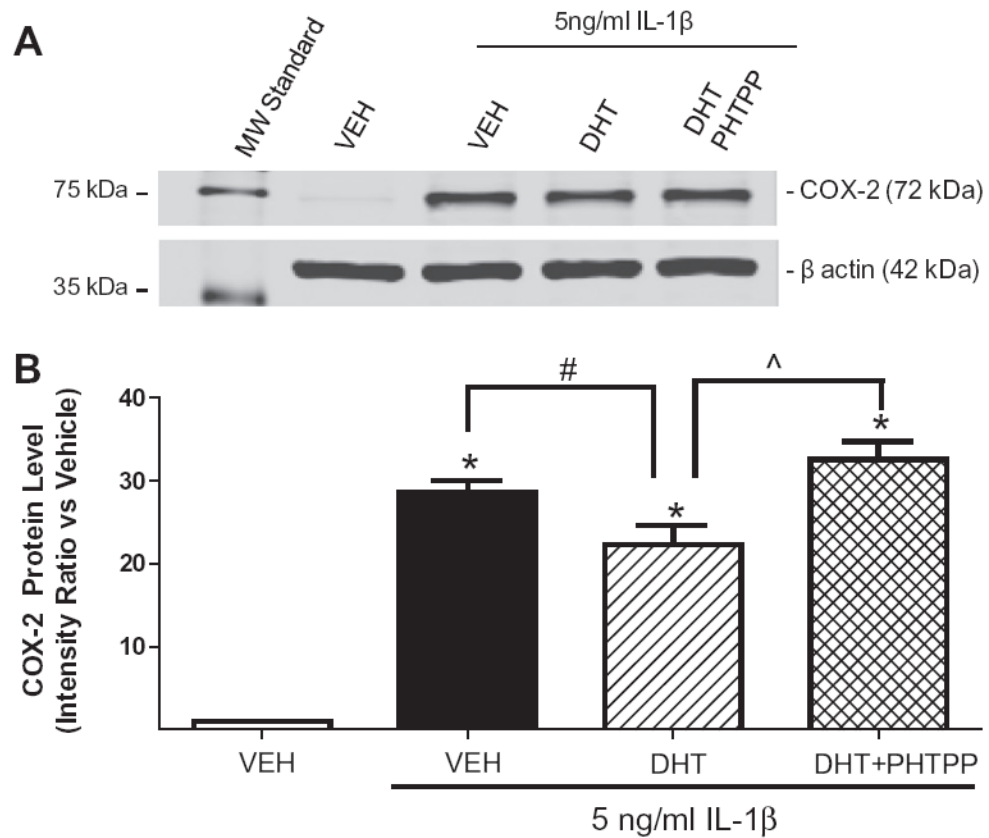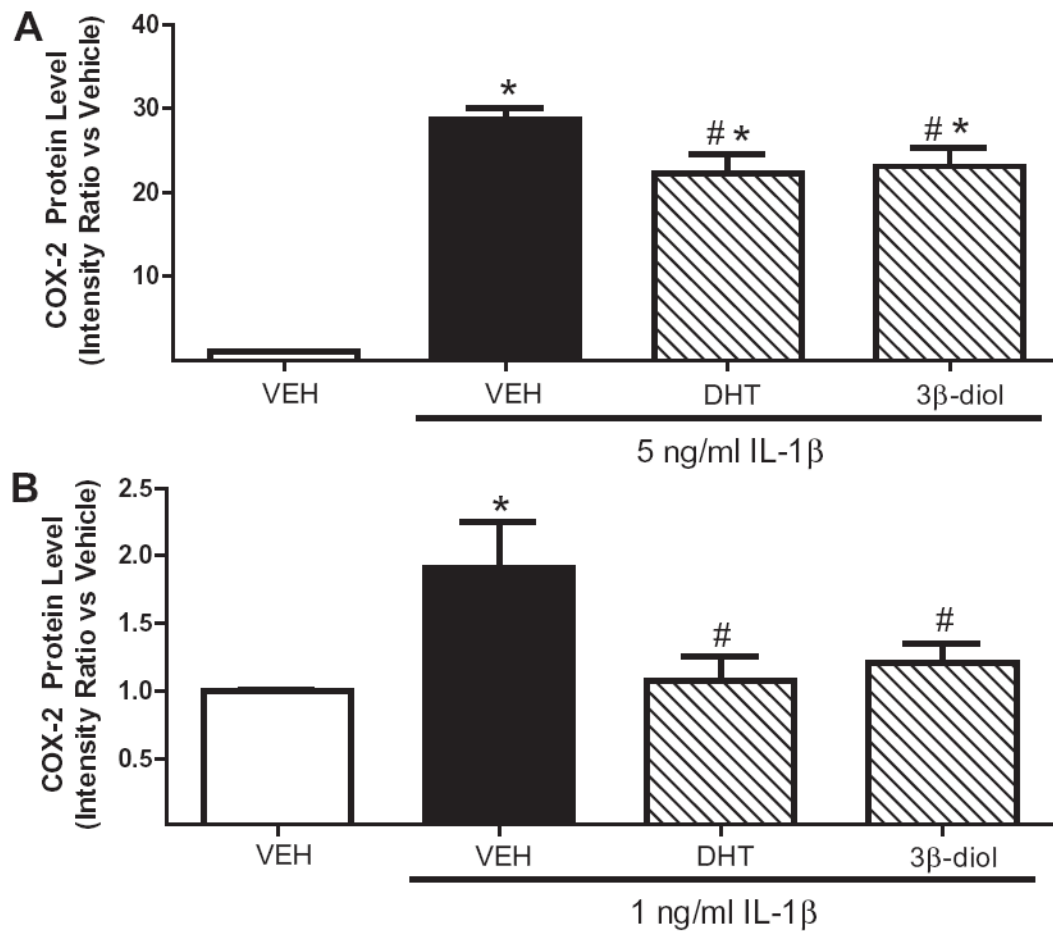
Fig. 3.
DHT inhibited cytokine-induced increases in COX-2 via an estrogen receptor -dependent mechanism in HBVSMC. Cyclooxygenase-2 (COX-2) protein expression was assessed in adult human brain vascular smooth muscle cells (HBVSMC) pre-treated for 1 h with vehicle (VEH) or the ER antagonist, PHTPP (1 μM), followed by VEH or dihydrotestosterone (DHT; 10 nM) for 18 h, then exposed to interleukin-1 beta (IL-1 ; 5 ng/ml; 6 h) in the continued presence of hormone/antagonist. (A) Representative blot for COX-2. B: Graph showing COX-2 levels after treatments. *$P < 0.001$ vs. VEH, #$P < 0.05$ vs. IL-1 , ^$P < 0.001$ vs. IL-1 + DHT (n 10 per group).

Fig. 4.
DHT and 3 -diol inhibited cytokine-induced increases in COX-2 in HBVSMC. (A)
Cyclooxygenase-2 (COX-2) protein expression was assessed in adult human brain vascular
smooth muscle cells (HBVSMC) treated with vehicle (VEH), dihydrotestosterone (DHT; 10
nM), or 3 -diol (10 nM) for 18 h, then exposed to interleukin-1 beta (IL-1 ; 5 ng/ml; 6 h) in
the continued presence of hormone. *$P < 0.001$ vs. VEH, [#]$P < 0.05$ vs. IL-1 (*n* 10 per
group). (B) COX-2 protein expression was assessed in adult HBVSMC treated with VEH,
DHT (10 nM), or 3 -diol (10 nM) for 18 h, then exposed to a low dose of IL-1 (1 ng/ml; 6
h) in the continued presence of hormone. *$P < 0.01$ vs. VEH, [#]$P < 0.01$ vs. IL-1 (*n* 10 per
group).

A Cytokine:

COX-2 (72 kDa)

β actin (42 kDa)

B Endotoxin:

COX-2 (72 kDa)
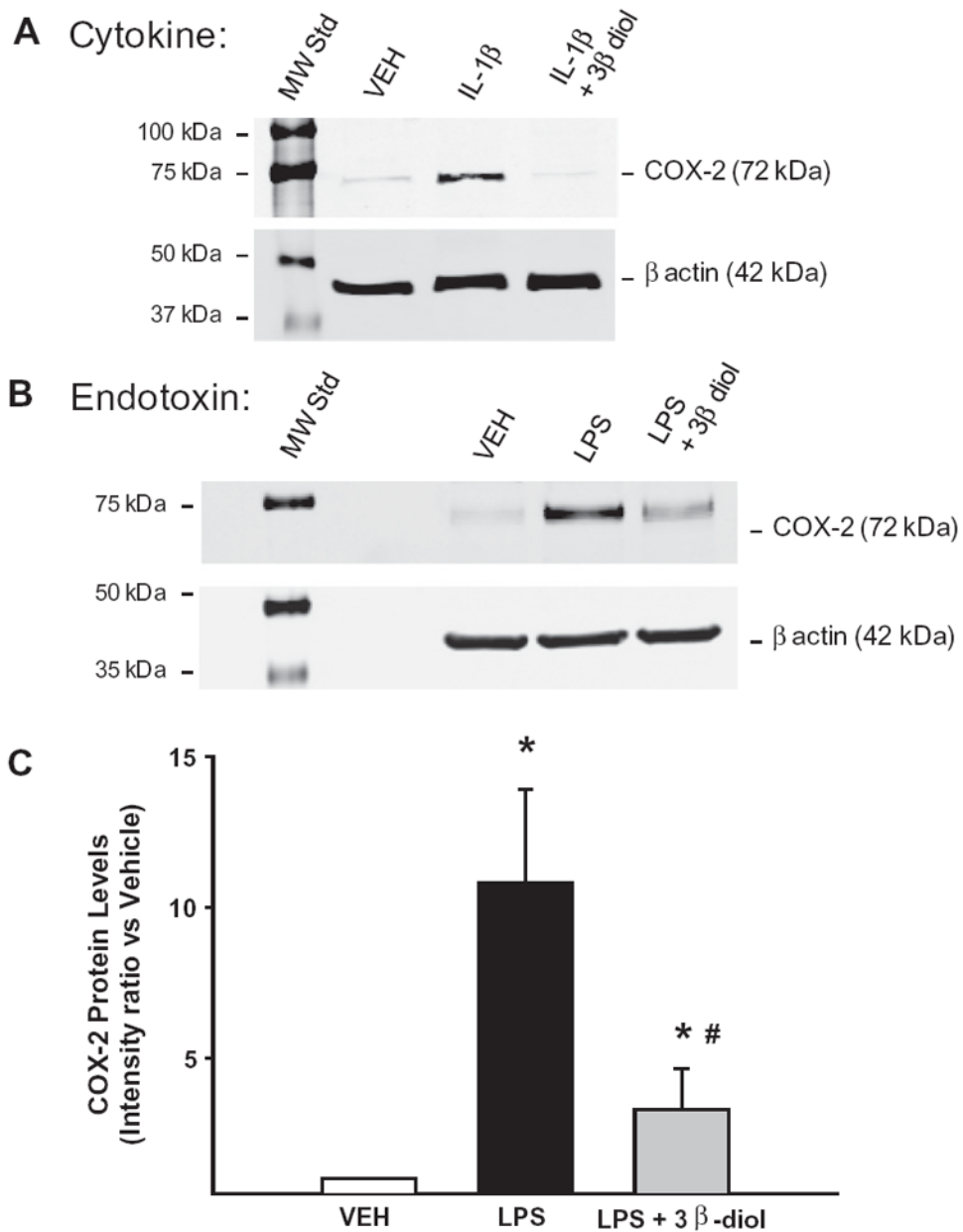
β actin (42 kDa)

C

Fig. 5.
3 -Diol inhibited cytokine- and endotoxin-induced increases in COX-2 in rat mesenteric arteries. Cyclooxygenase-2 (COX-2) protein levels were assessed in isolated mesenteric arteries treated *ex vivo* with either cytokine or endotoxin in the absence or presence of 3 -diol. Panel A illustrates the Western blot for COX-2 expression for the cytokine experiment: vehicle (VEH), interleukin-1 beta (IL-1 ; 10 ng/ml), or IL-1 (10 ng/ml) plus 3 -diol (10 nM). Panel B illustrates the LPS experiment: VEH, the endotoxin lipopolysaccharide (LPS; 100 μg/ml), or LPS + 3 -diol. Actin served as a loading control. Panel C: Graph showing COX-2 levels after LPS treatment. *P < 0.05 vs. VEH, #P < 0.05 vs. LPS (n = 3).
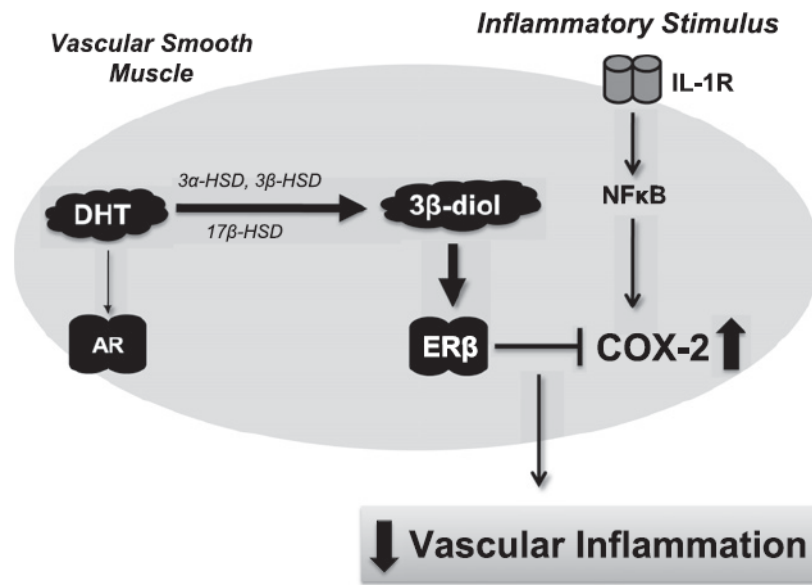
Fig. 6.
Schematic diagram of proposed mechanism for DHT's anti-inflammatory actions in vascular tissues. In our proposed model, during interleukin-1 beta (IL-1) induced inflammation, dihydrotestosterone (DHT) reduces cyclooxygenase-2 (COX-2) expression via metabolism to 3 beta-diol (3 -diol) by the enzymes 3 -hydroxysteroid dehydrogenase (HSD), 3 -HSD, and/or 17 -HSD in vascular smooth muscle cells (thick arrow). 3 -Diol then activates estrogen receptor beta (ER) and down regulates COX-2 expression. Although DHT has the ability to bind with AR, it does not appear to down regulate COX-2 expression via this mechanism (thin arrow). AR (androgen receptor), IL-1R (interleukin-1 receptor), NF B (nuclear factor kappa B).

Table 1

Primer sequences used for quantitative real time reverse transcriptase polymerase chain reaction.

Target gene	Forward primer	Reverse primer	Product size
AR	5 -AGTGGATGGGCTGAAAAA-3	5 -GGGTGTGGAAATAGATGGG-3	305 bp
ER	5 -AGGGAAAGTAGGGCAGAAA-3'	5 -ACGCTGGGAAATGAAGAA-3	172 bp
ER	5 -AAGAATATCTCTGTGTCAAGGCCATG-3	5 -GGCAATCACCCAAACCAAAG-3	143 bp
3 -HSD	5 -TGGGGTTGTGGTCCTGGCCA-3	5 -CCACACGCAGGGCCTTCTGG-3	229 bp
3 -HSD	5 -CAGCCAGGCATGGCCGACTC-3	5 -CTGCTGCCACCTCATGGGCC-3	235 bp
17 HSD	5 -AGAACCTGCTCTCCGCGCCT-3	5 -TCCATTGGGGCCCCTCCTCC-3	305 bp
CYP7B1	5 - ATGGCAGCAGTGCGTGACGA-3'	5 -CGCACACAGTAGTCCCCGGT-3	221 bp
GAPDH	5 -GCCTTCTCCATGGTGGTGAA-3	5 -GGTCGGGAGTCAACGGATTT-3	309 bp

Primer sequences used for qRT-PCR and product sizes in base pairs are listed for androgen receptor AR), estrogen receptors alpha (ER) and beta (ER), 3 -hydroxysteroid dehydrogenase (HSD), 3 -hydroxysteroid dehydrogenase (3 -HSD), 3 - hydroxysteroid dehydrogenase (3 -HSD) and 17 -hydroxysteroid dehydrogenase (17 -HSD); CYP7B1 (3 -diol inactivation enzyme), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 2

Gonadal steroid receptors and steroid metabolizing enzyme mRNAs are expressed in HBVSMC.

Target gene	mRNA concentration (fg)
AR	0.7593 ± 0.3215
ER	0.0006 ± 0.0001
ER	0.0005 ± 0.0000
3 -HSD	1.1490 ± 0.1035
3 -HSD	0.0006 ± 0.0001
17 -HSD	0.1024 ± 0.0131
CYP7B1	0.0999 ± 0.0116

Messenger RNA expression of hormone receptors androgen receptor (AR), estrogen receptor alpha (ER), and estrogen receptor beta (ER) and steroid metabolism enzymes 3 -hydroxysteroid dehydrogenase (HSD), 3 -HSD, 17 -HSD, and CYP7B1 were assessed in adult male human brain vascular smooth muscle cells (HBVSMC) treated for 6 h with hormone-free media using qRT-PCR. Each data point represents the mean ± SEM of 7–8 determinations.