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# Dihydrotestosterone attenuates hypoxia inducible factor-1α and cyclooxygenase-2 in cerebral arteries during hypoxia or hypoxia with glucose deprivation

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**Zuloaga KL, Gonzales RJ.** Dihydrotestosterone attenuates hypoxia inducible factor-1α and cyclooxygenase-2 in cerebral arteries during hypoxia or hypoxia with glucose deprivation. *Am J Physiol Heart Circ Physiol* 301: H1882–H1890, 2011. First published August 19, 2011; doi:10.1152/ajpheart.00446.2011.—Dihydrotestosterone (DHT) attenuates cytokine-induced cyclooxygenase-2 (COX-2) in coronary vascular smooth muscle. Since hypoxia inducible factor-1α (HIF-1α) activation can lead to COX-2 production, 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 (18 days) followed by hypoxic exposure ex vivo (1% $O_2$), cerebral arteries isolated from castrated male rats treated ex vivo with vehicle or DHT (10 or 100 nM; 18 h) and then exposed to hypoxia ex vivo (1% $O_2$), or primary human brain vascular smooth muscle cells treated with DHT (10 nM; 6 h) or vehicle then exposed to hypoxia or HGD. Under normoxic conditions, DHT increased COX-2 (cells 51%; arteries ex vivo 31%; arteries in vivo 161%) but had no effect on HIF-1α. Following hypoxia or HGD, HIF-1α and COX-2 levels were increased; this response was blunted by DHT (cells HGD: −47% COX-2, −34% HIF-1α; cells hypoxia: −29% COX-2, −54% HIF-1α; arteries ex vivo: −37% COX-2; arteries in vivo: −35% COX-2) and not reversed by androgen receptor blockade. Hypoxia-induced HIF-1α DNA-binding was also attenuated by DHT (arteries ex vivo and in vivo: −55%). These results demonstrate that upregulation of COX-2 and HIF-1α in response to hypoxia is suppressed by DHT via an androgen receptor-independent mechanism.

androgen; inflammation; vascular smooth muscle

DESPITE THE GREATER INCIDENCE of stroke in men compared with age-matched premenopausal women (25a) and women's poorer outcomes following stroke (35), 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 (14, 32, 34, 43). This is of great interest because the cerebral vasculature plays a central role in the pathogenesis of cardiovascular diseases, such as stroke (7), and in the initiation of inflammation after cerebral ischemia, which is a key determinant in stroke outcome (7, 9). Following ischemia, inflammation is initiated by cytokine-induced activation of transcription factors such as nuclear factor-κB (NF-κB) and hypoxia-inducible factor 1-α (HIF-1α), leading to increased production of proinflammatory mediators, such as inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) (2, 46). 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 (17).

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 (26, 32, 34, 49), although some proinflammatory effects have been reported for androgens (6, 28). Furthermore, 5α-androstane-3β,17β-diol (3β-diol), an estrogen receptor (ER)-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 (29, 31). In addition, the androgen dehydroepiandrosterone has been shown to decrease HIF-1α accumulation during hypoxia in human pulmonary smooth muscle cells (8). Clinically, testosterone replacement in androgen-deficient men leads to a reduction in circulating inflammatory cytokines (27). Furthermore, lower testosterone levels predict increased incidence of stroke in older men, even after adjusting for conventional risk factors for cardiovascular disease (50). In experimental models of stroke, estrogen has been consistently shown to decrease infarct size, while androgens decrease infarct size at low doses but increase damage at high doses (16, 45). 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 (AR) agonist DHT can influence the vascular inflammatory response during hypoxia. Using both in vitro hypoxia and in vitro hypoxia with glucose deprivation (HGD) to model 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. We chose COX-2 as our 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 (42). Because others (4, 5, 20) have shown that HIF-1α induces transcription of COX-2 via interaction with the COX-2 promotor, using two model systems, primary human brain vascular smooth muscle cells (HBVSMC) and isolated rat pial vessels, we examined the hypothesis that DHT

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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 River.

*Chronic in vivo DHT treatment.* Intact male rats were anesthetized with isoflurane (1.5%), and gonadectomies were performed under aseptic conditions. Immediately following testes removal, placebo or 5α-androstan-17β-ol-3-one (DHT; 45 mg/21 day) pellets (Innovative Research of America, Sarasota, FL) were implanted subcutaneously, and the incision was secured with stainless steel staples. Postsurgery rats received a single injection (im) 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 12:12-h light-dark cycle with fresh water, food, and bedding for recovery. Eighteen days following pellet implants, animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and prepared for cerebral vessel isolation. A time point of ~3-wk of DHT treatment was selected based on past studies (14) demonstrating alterations in vascular function with this duration of hormone treatment. Additionally, animals were killed on day 18 to ensure sufficient DHT delivery from the 45-mg/21-day pellet. Following deep anesthesia, the thoracic cavity was surgically opened and a syringe and needle (21 gauge) were 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. Before the brain was removed, 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 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, circle of Willis, and basilar artery) were dissected microscopically and placed in 12-well plates (vessels from 1 animal per well) containing ice-cold DMEM. After collection, vessels were transferred to prewarmed wells containing a 1:1 mixture of DMEM and hormone free medium 231 (Cascade Biologics, Portland OR). Following a 30- to 40-min equilibration in an incubator (5% $CO_2$; 37°C), vessels were transferred to new wells containing fresh prewarmed 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% $CO_2$, $N_2$ balance (hypoxic conditions). The percentage of $O_2$ was monitored using a compact oxygen sensor (ProOx 110; BioSpherix)

*Ex vivo DHT treatment of rat pial arteries.* Male rats, 2 wk postgonadectomy, were anesthetized with a lethal dose of pentobarbital, exsanguinated, and decapitated. With the use of aseptic technique, brains were removed and placed in ice-cold, sterile PBS. Cerebral arteries (middle cerebral artery, circle of Willis, and basilar) were dissected and then transferred to a 1:1 mixture of DMEM and hormone-free medium 231 (Cascade Biologics). Next, vessels were placed in an a 5% $CO_2$ incubator maintained at 37°C and equilibrated for ~30 to 40 min and then promptly transferred to fresh DMEM: medium 231 containing DHT (10 or 100 nM; 6 h) 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 studies. As described above, the level of $O_2$ (%) was monitored using a compact oxygen sensor. An insult of 1% hypoxia was chosen based on a number of studies that demonstrated that this level of hypoxia is sufficient to stimulate proinflammatory gene expression, including COX-2 (mRNA and protein; Refs. 3, 5, 33).

*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. When it was necessary, 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 preexposed 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 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 with smooth muscle growth supplement containing 2% 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, Carlsbad, CA). A 6-h time point for COX-2 induction by an inflammatory stimulus (hypoxia or HGD) 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 our 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 (34). Depending on the experiment, cells were pretreated with DHT (10 nM) or vehicle (0.001% ethanol) for 18 h followed by hypoxia (1% $O_2$; 6 h) or HGD (1% $O_2$; 6 h) using a plexi-glass hypoxic chamber (BioSpherix). For HGD experiments, normal growth media were replaced with DMEM without glucose (Invitrogen) and then immediately placed in the hypoxic chamber. In a separate set of experiments, the involvement of AR stimulation was tested by pretreating (1 h) cells with the AR-antagonist bicalutamide (1 μM; dissolved in DMSO) followed by 18 h of cotreatment with either vehicle (0.001% ethanol + 0.01% DMSO) or DHT and then 6 h of HGD. A bicalutamide dose of 1 μM is effective for AR antagonism in our in vitro model as previously shown in our earlier studies (34).

*Immunocytochemistry.* Cells were plated on sterilized glass coverslips and grown in a 5% $CO_2$ incubator/room air at 37°C in smooth muscle growth medium with smooth muscle growth supplement containing 2% FBS (ScienCell). When cells reached ~60% confluency, they were divided into groups and culture medium was replaced with IL-1β (5 ng/ml), saline, or $CoCl_2$ (100 μM). Some cells did not receive treatment and were used to verify the presence of smooth

muscle actin and smoothelin. Following incubation or treatment, cells were fixed (4% formaldehyde), washed in filtered PBS (pH 7.4), and permeabilized in methanol (−20°C). Next, cells were incubated in 2% BSA/PBS to block nonspecific binding and then incubated with COX-2 (1:200) monoclonal antibody (Cayman Chemical, Ann Arbor, MI), HIF-1α (1:200) polyclonal antibody (Thermo Fisher Scientific), α-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 overnight at 4°C. Next cells were washed in PBS (4 × 5 min) and incubated with Alexa Fluor 555 and Alexa Fluor 488 (1:6,000; Invitrogen) for 1 h in PBS containing 2% BSA and washed in PBS (4 × 5 min). Coverslips 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). 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).

*Western blot.* Levels of HIF-1α and COX-2 protein were examined using standard immunoblotting methods, as previously described (34). 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-PAGE. Separated proteins were transferred to nitrocellulose membranes, and nonspecific 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 (1:1,000) mouse monoclonal antibody (Cayman Chemical, Ann Arbor, MI), HIF-1α (1:1,000) rabbit polyclonal antibody for human cell experiments (Thermo Fisher Scientific), HIF-1α (1:500) mouse monoclonal for rat artery experiments (Stressgen), 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 (see Fig. 6*B*). Following additional TPBS washes, proteins were visualized using an Odyssey Infrared Imager and data were analyzed using Odyssey V3.0 software (LI-COR).

*Reagents.* All reagents were purchased from Sigma-Aldrich Chemical 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 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 allows pooling of multiple blots from each experiment. Each figure represents data obtained from four to eight different membranes. For HIF-1α DNA binding assay data, values are reported as optical density at a 450-nm wavelength. All values are reported as means ± SE. Data were compared using 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

*DHT 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 with values measured in intact male rats (941 ± 32 pg/ml). In contrast, gonadectomized rats receiving replacement DHT pellets had comparable DHT serum levels (1,089 ± 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 (45-mg pellet, 18 days) or placebo followed by 6 h of normoxia (21% $O_2$) or hypoxia (1% $O_2$) ex vivo in continued presence of hormone (Fig. 1). Figure 1*A* illustrates a representative Western blot of COX-2 levels from each group tested, and Fig. 1*B* illustrates the data analysis. Under simulated normoxic conditions, COX-2 protein levels were increased in pial arteries following DHT administration in vivo compared with arteries isolated from the placebo-treated rats. In addition, hypoxic exposure also resulted in an increase in COX-2 protein levels in rat pial arteries. DHT administration in vivo attenuated 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 (Fig. 1*B*).

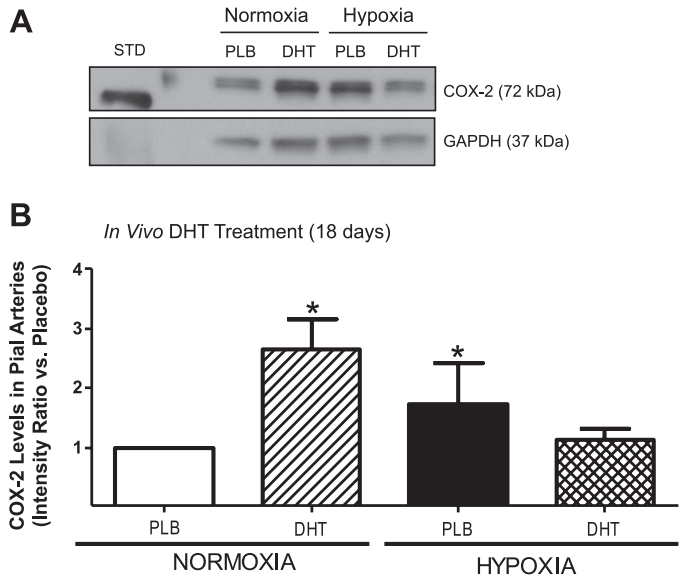



Fig. 1. Chronic in vivo dihydrotestosterone (DHT) treatment increases cyclooxygenase-2 (COX-2) under normoxic conditions and inhibits hypoxia-induced COX-2 levels following hypoxic exposure in rat pial arteries. COX-2 levels following normoxic and hypoxic exposure ex vivo (1% $O_2$; 6 h) in pial arteries isolated from castrated male rats treated in vivo with placebo (PLB) or DHT (45-mg pellet; 18 days). *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* side of blot. *B*: data analysis of mean intensity ratios of DHT treated groups compared with placebo following normoxic or hypoxic exposure. Each bar represents the means ± SE of $n \geq 5$ per group. *$P < 0.05$ vs. vehicle (Veh).

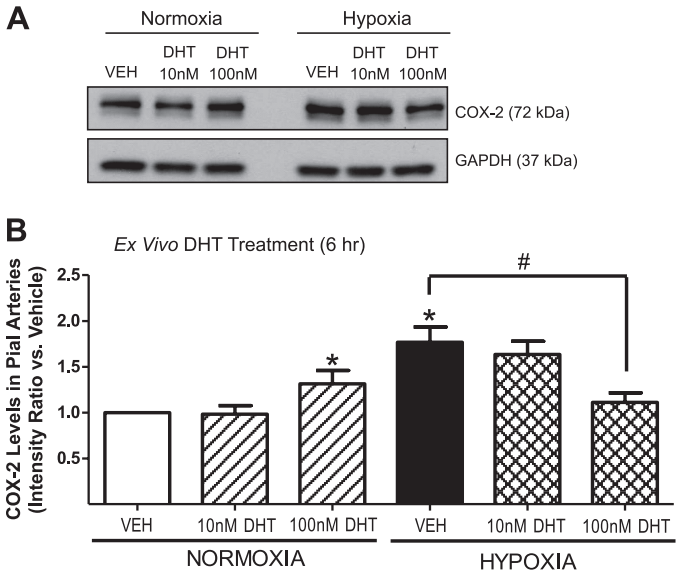



Fig. 2. Ex vivo DHT treatment increases COX-2 levels under normoxic conditions and decreases COX-2 following hypoxia in rat pial arteries. COX-2 levels in pial arteries, dissected from castrated male rats, treated with DHT (10 or 100 nM; 6 h) or vehicle ex vivo followed by exposure to normoxia or hypoxia 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 with vehicle. Each bar represents means ± SE of $n \geq 5$ per group. *$P < 0.05$ vs. normoxia vehicle; #$P < 0.05$ vs. hypoxia vehicle.

*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; 6 h) or vehicle followed by 6 h of normoxia (21% $O_2$) or hypoxia (1% $O_2$) ex vivo in continued presence of hormone. Figure 2*A* illustrates a representative Western blot of COX-2 levels from each group tested, and Fig. 2*B* illustrates the data analysis. The higher dose of DHT (100 nM) administered ex vivo increased COX-2 levels under normoxic conditions compared with vehicle treated pial arteries. During hypoxia, COX-2 was significantly increased compared with the normoxic vehicle control. This increase in hypoxic-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 (45-mg pellet; 18 days) and then exposed to ex vivo to normoxia (21% $O_2$, 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 (Fig. 3, *A* 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 with hypoxia alone.

*HIF-1α protein stabilization following hypoxia in rat pial arteries.* In addition to determining activation of HIF-1α, protein levels using anti-HIF-1α were assessed in rat pial arteries (Fig. 4). Similar to the COX-2 studies, artery segments were isolated from gonadectomized male rats treated in vivo with DHT or placebo followed by normoxia (21% $O_2$) or hypoxia (1% $O_2$) ex vivo in continued presence of hormone (Fig. 4*A*). In separate experiments, arteries were isolated from gonadectomized male rats treated ex vivo with DHT (100 nM; 6 h) or vehicle followed by 6 h of normoxia (21% $O_2$) or hypoxia (1% $O_2$) ex vivo in continued presence of DHT or vehicle (Fig. 4*B*). Bands for HIF-1α protein were faint under normoxic conditions. However, following hypoxia levels of HIF-1α were increased compared with normoxic controls, suggesting enhanced protein stability under hypoxic conditions. Similar to the HIF-1α activation studies, in the presence of DHT administered ex vivo or in vivo HIF-1α levels were attenuated following hypoxic exposure.

*Increased COX-2 expression in HBVSMC following cytokine stimulation.* Before we assessed the effects of DHT and hypoxia on COX-2 levels in our primary HBVSMC model, we first assessed the regulation of COX-2 expression using a known stimulus of the proinflammatory mediator via immunocytochemistry. We confirmed the induction of COX-2 (red) in response to IL-1β (5 ng/ml; 3 h) compared with saline treatment (Fig. 5*A*). Also shown in Fig. 5*A* are panels of nuclei labeling with DAPI (blue) to demonstrate nuclear borders. In separate experiments, markers for vascular smooth muscle

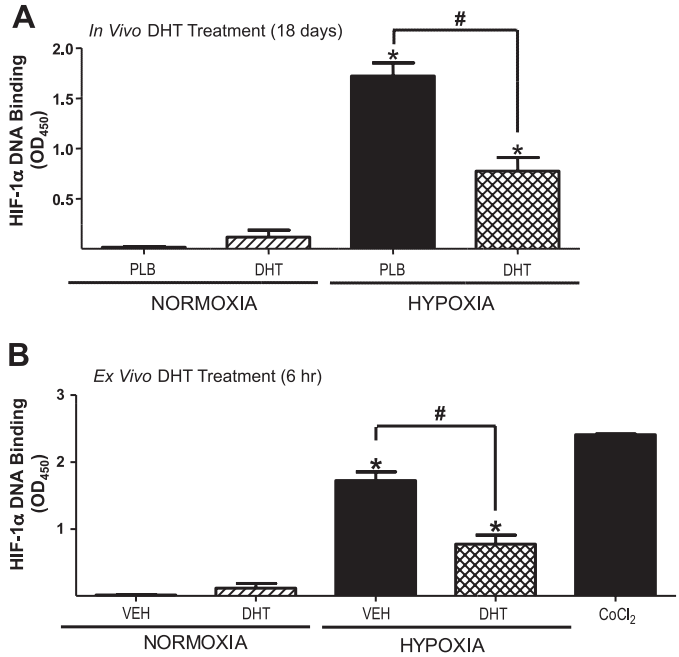



Fig. 3. DHT blunted hypoxia-induced nuclear hypoxia inducible factor-1α (HIF-1α) activation in rat pial arteries. HIF-1α DNA binding was assessed in pial artery nuclear isolates. *A*: HIF-1α activation in nuclear isolates from castrated rat pial arteries pretreated ex vivo with vehicle or DHT (100 nM; 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. *B*: assessment of HIF-1α activation in nuclear isolates from pial arteries isolated from castrated male rats treated in vivo with vehicle or DHT (45-mg pellet, 18 days) followed by exposure to hypoxia ex vivo (1% $O_2$; 1 h). $OD_{450}$, optical density at 450-nm wavelength. Each bar represents means ± SE of $n \geq 4$ per group. *$P < 0.001$ vs. normoxia vehicle; #$P < 0.001$ vs. hypoxia vehicle.

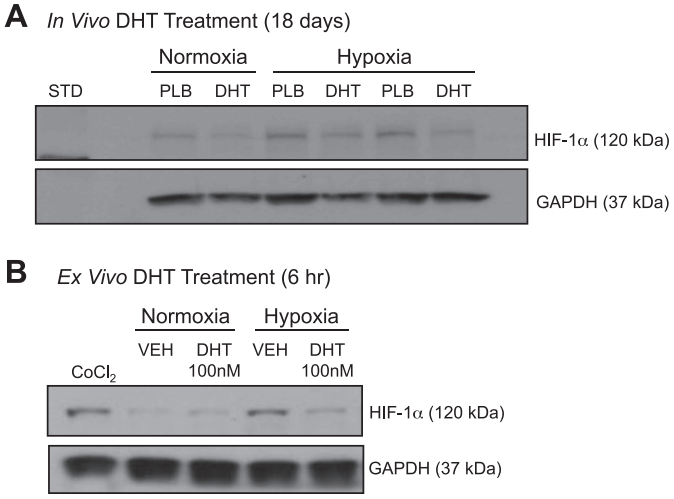



Fig. 4. HIF-1α protein levels following in vivo or ex vivo DHT treatment in the presence of normoxia or hypoxia in rat pial arteries. *A*: representative blot for HIF-1α and GAPDH (loading control) protein levels in pial arteries dissected from castrated male rats treated with DHT in vivo (18 days) or placebo exposed to hypoxia (1% $O_2$) or normoxia for 6 h. *B*: representative blot for HIF-1α and GAPDH (loading control) protein levels following *ex* vivo treatment of DHT (100 nM) or vehicle exposed to hypoxia (1% $O_2$) or normoxia for 6 h.

were also assessed (Fig. 5*B*). 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 (Fig. 6). A representative Western blot for COX-2 in all the treatment groups is illustrated in Fig. 6*A*. Figure 6*B* 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).

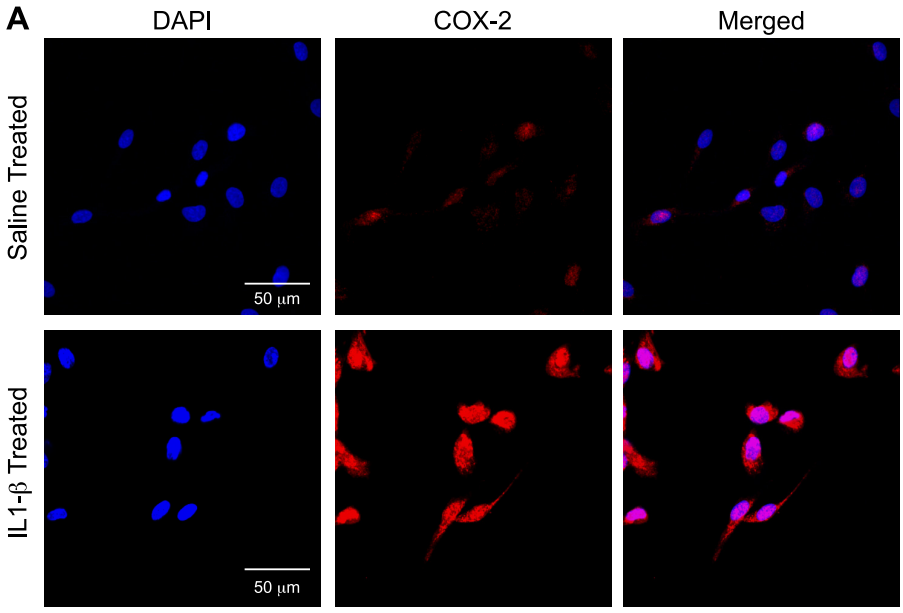
*DHT blunted hypoxia-induced increases in HIF-1α protein levels in HBVSMC.* HIF-1α localization was confirmed using an anti-HIF-1α antibody in HBVSMC (Fig. 7*A*) following $CoCl_2$ induction in vitro. To determine the effect of DHT treatment on hypoxia-induced HIF-1α in HBVSMC, cells were treated with 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 HIF-1α protein levels were measured via Western blot (Fig. 7*B*). Under normoxic conditions, DHT treatment did not alter HIF-1α levels compared with vehicle. 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 was 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.* 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, HGD was used to determine the effects of DHT on COX-2 (Fig. 8*A*) and HIF-1α (Fig. 8*B*) 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 bicalutamide (1 μM; 1-h pretreatment + 18-h cotreatment 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 with 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 mimic early vascular ischemic injury using a hypoxic ex vivo cerebral artery model and a hypoxic glucose deprivation vascular cell culture model to investigate the mechanisms associated with the influence of DHT on vascular inflammatory responses to severe pathophysiological conditions. Using COX-2 as marker for vascular inflammation, we determined the effects of the potent androgen DHT on COX-2 levels during hypoxia in rat pial arteries and primary HBVSMC. In separate experiments, human cells were exposed to HGD to more closely model some of the cellular insults that occur during ischemia. We found that DHT increased COX-2 levels under normoxic conditions but decreased COX-2 levels during hypoxic conditions or following HGD. Furthermore, since HIF-1α is a transcription factor controlling COX-2 levels and it is stabilized/activated during hypoxia or ischemia, we tested the hypothesis that changes in HIF-1α activation or levels would correlate with changes in COX-2 levels. Our 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, we also demonstrated that the effects of DHT 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 our knowledge, this is the first report of the effects of DHT on vascular inflammation during hypoxia or HGD in the cerebral vasculature. Previously, we have shown that under normal physiological conditions DHT increases COX-2 levels in both rat pial arteries (14) and human coronary artery smooth muscle cells via an AR-dependent mechanism (34). The current data also support a proinflammatory 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, we (34) have also previously shown that during cytokine-induced inflammation DHT de-



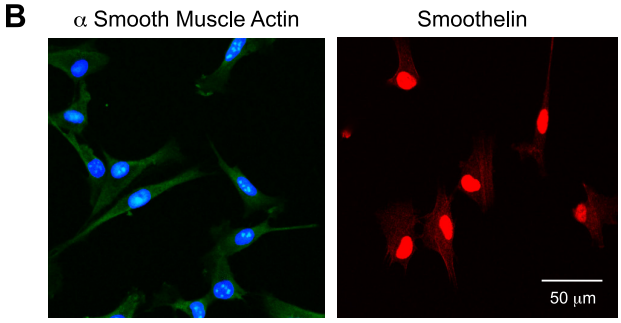




Fig. 5. IL-1β-induced COX-2 expression in human brain vascular smooth muscle cells (HBVSMC). *A*: HBVSMC were treated with saline or IL-1β (5 ng/ml; 3 h) and immunocytochemistry verified that COX-2 (red) levels were upregulated following cytokine treatment. DAPI (blue) was used as a nuclear marker. *B*: HBVSMC labeled with either α-smooth muscle actin (green) or smoothelin (red) antibodies to verify levels of vascular smooth muscle cell markers.

creases COX-2 protein levels via an AR-independent mechanism. Our in vitro data point to vascular smooth muscle cells as important mediators of this response to DHT. However, we would like to point out that it is likely that the endothelium also plays an important role in the response we observed in the rat pial arteries. For instance, Norata et. al. (32) has shown that DHT also attenuates proinflammatory markers during cytokine-induced inflammation in human endothelial cells. The current data show similar patterns during the pathophysiological conditions of hypoxic stress in both rodent pial arteries and HBVSMC. Interestingly, the dose of DHT needed to attenuate COX-2 or HIF-1α levels was higher in the rat pial vessels (100 nM) compared with 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 our current findings with our previous results, it appears that DHT may be proinflammatory 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 with pathophysiological conditions. We 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 HGD, not under normoxia. Therefore, it is possible that DHT has differing actions during normoxia and hypoxia due to interactions with the HIF-1α pathway. HIF-1α stimulates transcription of COX-2 mRNA by binding to the hypoxia response element on the COX-2 promoter (4, 5, 20). Aside from being a transcription factor in the pathway leading to increased 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 (18). During hypoxia, HIF-1α binds to the Toll-like receptor 4 promoter causing upregulation of Toll-like receptor 4. This enhances the response of macrophages to the endotoxin LPS, resulting in increased COX-2 (20a). 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 (2). Therefore, it appears that hypoxia may enhance susceptibility to subsequent inflammatory signals and vice versa. Whether the NF-κB 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 NF-κB inhibition (25). However, this response was shown to be NF-κB-dependent in human vascular endothelial cells (37). A detailed examination of human pulmonary artery smooth muscle cells showed that hypoxia increased NF-κB activity and that NF-κB inhibition could reduce HIF-1α. Furthermore, mutation of the NF-κB binding site in the HIF-1α promoter prevented hypoxia-induced

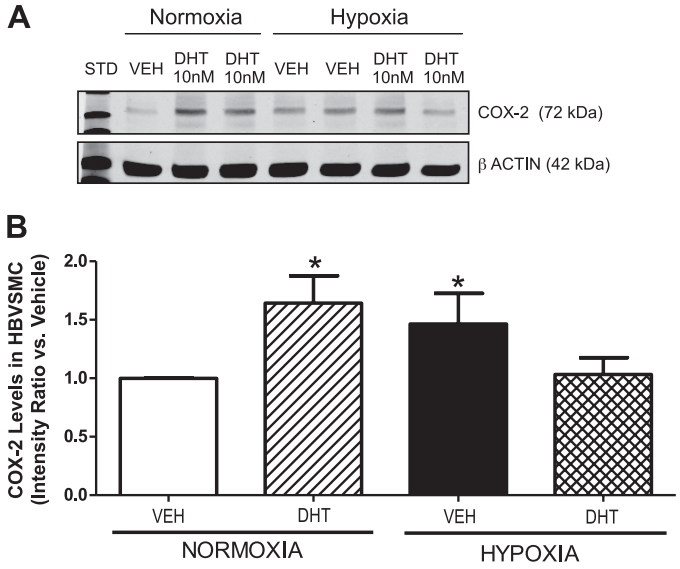



Fig. 6. DHT differentially modulates COX-2 during normoxia and hypoxia in human HBVSMC. COX-2 protein levels was assessed in cells pretreated in vitro with vehicle or DHT (10 nM; 18 h) then exposed in vitro to normoxia or hypoxia (1% $O_2$; 6 h). *A*: representative blot for COX-2 protein levels in all groups. β-Actin served as a loading control. *B*: data analysis of COX-2 mean intensity ratio compared with vehicle. Each bar represents the means ± SE of $n \geq 9$ per group. *$P < 0.05$ vs. vehicle.

HIF-1α transcription (1). 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 NF-κB activity. Previous studies (14) have shown that DHT

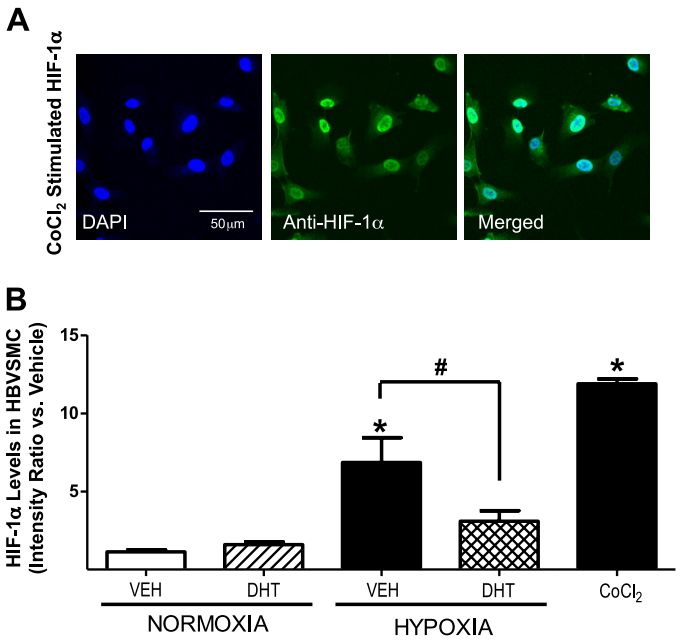



Fig. 7. DHT blunted hypoxia-induced increases in HIF-1α protein in HBVSMC. *A*: HIF-1α localization via immunocytochemistry in HBVSMC stimulated with cobalt chloride ($CoCl_2$; 3 h). *B*: HIF-1α protein levels were assessed via western blot in HBVSMC pretreated in vitro with vehicle or DHT (10 nM; 18 h) and 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 means ± SE of $n \geq 6$ per group. *$P < 0.01$ vs. normoxia vehicle; #$P < 0.01$ vs. hypoxia vehicle.

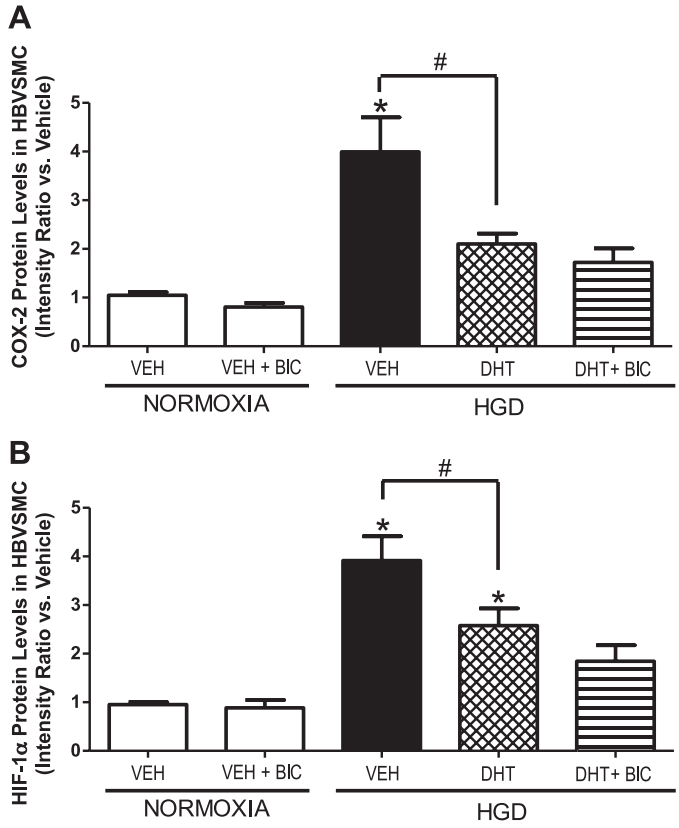



Fig. 8. DHT blunted hypoxia with glucose deprivation (HGD)-induced COX-2 and HIF-1α levels HBVSMC via an androgen receptor-independent mechanism. COX-2 and HIF-1α protein levels were assessed in HBVSMC pretreated in vitro with vehicle, DHT (10 nM), or DHT + bicalutamide (androgen receptor antagonist, 1 μM) for 18 h and then exposed to normoxia or HGD (1% $O_2$; 6 h). *A*: COX-2 Western analysis results; each bar represents means ± SE of $n \geq 9$ per group. *$P < 0.001$ vs. vehicle; #$P < 0.001$ vs. HGD vehicle. *B*: HIF-1α Western analysis results; each bar represents means ± SE of $n \geq 6$ per group. *$P < 0.01$ vs. vehicle; #$P < 0.01$ vs. HGD vehicle.

can alter NF-κB activity in rat pial arteries under normoxia, 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 the paradoxical actions of DHT is AR involvement. Our previous and current results support such a hypothesis since, during normal conditions the effects of DHT 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 HGD, the effects of DHT 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 AR directly or following its conversion by 5α-reductase to the more potent androgen DHT (15, 24). Alternatively, testosterone can be metabolized to estradiol by the aromatase enzyme (36) and subsequently activate ERα

or ERβ (21, 23). A third and less explored pathway for androgen action is through the conversion of DHT to 3β-diol, an ERβ agonist (21, 22, 47), by the enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD), 3α-hydroxysteroid dehydrogenase (3α-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (12, 19, 41, 44, 47). Blood vessels contain AR, ERα, ERβ, 5α-reductase, aromatase, and 3β-HSD (13, 30, 38, 39); therefore, the presence of these enzymes and receptors allows for potential androgenic and estrogenic effects that can influence vascular inflammation through a variety of pathways. Our current data point to ERβ as a possible alternative receptor pathway for the action of DHT 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 (31).

In summary, we have shown that during hypoxia or HGD 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 (10, 50). Physiologically, circulating androgen levels decrease as men age (11); 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, we investigated the actions of androgens on cerebrovascular inflammation during pathophysiological conditions of hypoxia or HGD. 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.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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