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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 5α-androstane-3β, 17β-diol (3β-diol), which has recently been shown to be a selective estrogen receptor (ERβ) agonist. Therefore, we hypothesized that DHT's anti-inflammatory properties following cytokine stimulation are mediated through ERβ. Using primary human brain vascular smooth muscle cells (HBVSMC), we tested whether DHT's effect on IL-1β induced COX-2 expression was mediated via AR or ERβ. The metabolism of DHT to 3β-diol is a viable pathway in HBVSMC since mRNA for enzymes necessary for the synthesis and metabolism of 3β-diol [3alpha-hydroxysteroid dehydrogenase (HSD), 3β-HSD, 17β-HSD, CYP7B1] was detected. In addition, the expression of AR, ERα, and ERβ mRNA was detected. When applied to HBVSMC, DHT (10 nM; 18 h) attenuated IL-1β-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 ERβ antagonist PHTPP (1 μM) inhibited the effect of DHT, suggesting that DHT actions are ERβ-mediated. In HBVSMC and in rat mesenteric arteries, 3β-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 3β-diol and activation of ERβ.

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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 (NFκB) 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 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

Abbreviations: 3α-HSD, 3 alpha hydroxysteroid dehydrogenase; 3β-diol, 5α-androstane-3β,17β-diol; 3β-HSD, 3 beta hydroxysteroid dehydrogenase; 17β-HSD, 17 beta hydroxysteroid dehydrogenase; Adiol, 5α-androstene-3β,17β-diol; AR, androgen receptor; COX-2, cyclooxygenase-2; DHT, dihydrotestosterone; E2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; HBVSMC, human brain vascular smooth muscle cells; ICI, ICI 182,780; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; NFκB, 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.

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androgen, DHT [12,13]. Alternatively, testosterone can be metabolized to 17β-estradiol (E2) by the aromatase enzyme [14] and subsequently activate estrogen receptor alpha (ERα) or beta (ERβ) [15,16]. A third and less well explored pathway for androgen action is through the conversion of DHT to 5α-androstane-3β, 17β-diol (3β-diol), a known selective ERβ agonist [16–18]. This metabolic pathway occurs by the action of the enzymes 3beta-hydroxysteroid dehydrogenase (3β-HSD), 3alpha-hydroxysteroid dehydrogenase (3α-HSD) or 17beta-hydroxysteroid dehydrogenase (17β-HSD) [18–22]. 3β-Diol is further converted to inactive metabolites by the enzyme CYP7B1 [18,23]. Since blood vessels contain AR, ERα, ERβ, 5α reductase, aromatase, and 3β-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, 3β-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 3β-diol and subsequent activation of ERβ in human brain vascular smooth muscle cells (HBVSMC). Additional experiments were performed in rodent peripheral arteries to determine if 3β-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 α-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-1β (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 α-actin and smoothelin (data not shown). Cells were treated with DHT (10 nM), 3β-diol (10 nM) or vehicle (0.001% ethanol) for 18 h followed by interleukin-1beta (IL-1β, 1 ng/ml or 5 ng/ml) for an additional 6 h. In a separate set of experiments, cells were pre-treated for 1 h with vehicle (0.001% ethanol) or the selective ERβ antagonist 4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP, 1 μM; Tocris Bioscience; dissolved in ethanol) followed by 18 h of co-treatment with either vehicle (0.001% ethanol), DHT (10 nM), or 3β-diol (10 nM), then 6 h of IL-1β (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-1β (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 17β-HSD and 3α-HSD primer sets and a 55 °C annealing temperature was used for the ERβ primer set. Samples were assayed alongside a cDNA standard curve for each primer to determine the absolute cDNA concentration present. For standard curve generation, cDNA was diluted to a stock concentration of 10 pg/ml then serially diluted from 1 pg/ml to 0.0001 fg/ml in PCR grade sterile water to generate eight working standards for each primer pair. The size of the amplified cDNA was confirmed by 2% agarose gel electrophoresis. 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.