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Renal vascular cytochrome P450-derived eicosanoids in androgen-induced hypertension

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Abstract:
Androgen has been linked to higher incidence of cardiovascular disease based on the simple observation that men have more cardiovascular and renal events than women at similar ages. The Cytochrome P450 (CYP)-derived eicosanoids, 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) have been implicated in the regulation of blood pressure *via* their vasoactive properties as vasoconstrictors and vasodilators, respectively, as well as *via* inhibition and activation of endothelial nitric oxide synthase. Since, 20-HETE and EETs have opposing vascular effects, their relative levels may determine vascular resistance and tone. We characterized the renal vascular production of 20-HETE and EETs in male and female rats before and after treatment with 5α-dihydrotestosterone (DHT). In renal interlobar arteries from male rats, the ratio between 20-HETE and EETs levels was 2-fold higher than that observed in arteries from female rats (1.86 ± 0.22 *vs*. 0.85 ± 0.13). Importantly, treatment with DHT significantly increased this ratio by 85 and 230% in arteries from male and female rats, respectively. Moreover, DHT treatment eliminated the difference in the ratio of 20-HETE to EETs between males and females. DHT treatment increased blood pressure in both male and female rats by 21.3 ± 4.0 and 15.3 ± 5.1 mmHg, respectively. The primary enzyme responsible for 20-HETE synthesis in the renal vasculature, CYP4A8, was significantly induced by treatment with DHT while the major epoxygenase in the kidney, CYP2C23, was down regulated by DHT. We conclude that increased vascular tone brought about by downregulation of CYP2C23 and decreased levels of vasodilatory EETs and by induction of CYP4A8 and enhanced production of 20-HETE may constitute important factors in androgen-induced hypertension.

Key words:
gender differences, arachidonic acid, blood pressure, kidney, 20-HETE, EET

Abbreviations: Ang-II – angiotensin II, CYP – cytochrome P450, DHT – 5α-dihydrotestosterone, EDHF – endothelial-derived hyperpolarizing factor, EET – epoxyeicosatrienoic acid, HETE – hydroxyeicosatetraenoic acid, RAS – renin-angiotensin system, sEH – soluble epoxide hydrolase, SHR – spontaneously hypertensive rats

Introduction

The cytochrome P450 (CYP)-derived arachidonic acid metabolites, including the four regioisomeric *cis*-epoxyeicosatrienoic acids (EETs; 5,6-, 8,9-, 11,12-, and 14,15-EETs) and the hydroxyeicosatetraenoic acids (18-, 19- and 20-HETE), have been increasingly recognized as important autocrine and paracrine mediators of cell functions. They have been implicated in the regulation of vascular tone, ion transport mechanisms, inflammation, cell proliferation and differentiation, renal hemodynamics and salt and water reabsorption and secretion (reviewed in [3, 11, 20, 21, 34]).

In the rat kidney, the CYP2C23 has been identified as the major EET producing enzyme [14], whereas synthesis of 19-HETE and 20-HETE is catalyzed primarily by enzymes of the CYP4A family, including

CYP4A1, 4A2, 4A3 and 4A8 [27]. Upon formation, EETs are subjected to rapid hydrolysis by a soluble epoxide hydrolase to their respective dihydroxyepoxytrienoic acids (DHETs) as well as to esterification primarily to glycerophospholipids [4]. The pathophysiological significance of EETs and DHETs stem from numerous studies describing potent stereospecific biological effects including vasodilation, stimulation of ion transport, inhibition of inflammatory response and stimulation of epithelial cell growth [17, 45]. Studies showing that induction of CYP2C23 in rats [23] or inhibition of the soluble epoxide hydrolase (sEH) [46] provides protection against angiotensin II (Ang II)-induced renal injury, and that mice lacking sEH have lower blood pressure than wild-type control mice [36] further enforced a role for EETs as important modulators in the cardiovascular system.

Androgen has been linked to higher incidence of cardiovascular disease based on the simple observation that men have more cardiovascular and renal events than women at similar ages. In fact, several population studies found statistically significant correlations between plasma levels of androgen and various cardiovascular risk factors [44]. Studies using 24 h ambulatory blood pressure monitoring have shown that blood pressure is higher in men than in women at similar ages [32]. Experimental evidence in humans indicates that renin activity is greater in men than in women and it is possible that the renin-angiotensin system (RAS) may play a role in mediating the gender difference in blood pressure regulation. These gender-associated differences observed in humans have also been documented in various animal models. Male SHR have higher blood pressure than do females of similar ages [5, 33]. Similar gender differences in the development of hypertension have also been found in Dahl salt-sensitive rats [8], DOCA-salt hypertensive rats [9], New Zealand genetically hypertensive rats [1] and rats fed high fat diet [47].

Holla et al. [13] were the first to uncover a possible link between CYP4A expression, 20-HETE synthesis and the development of androgen-induced hypertension. They reported that mice null for the *cyp4a14* (the murine homologue of the rat CYP4A2) displayed increased plasma androgen concentration and this was associated with increased renal expression of *cyp4a12* (the murine homologue of the rat CYP4A8) and formation of 20-HETE and the animals became hypertensive. Castration prevented the blood pressure increase and the enzyme induction, and androgen replacement restored the hypertension and *cyp4a12* expression and activity [13]. Additional studies in rats treated with 5α-dihydrotestosterone (DHT) substantiated this link [26] and provided additional evidence for a cause and effect relationship between vascular CYP4A expression and 20-HETE synthesis and the development of androgen-induced endothelial dysfunction and hypertension [37].

Materials and Methods

Animal experimentation

All experimental protocols were performed following an IACUC approved protocol in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Male and female Sprague-Dawley rats (200–250 g) were administered interperitoneal (*ip*) injections (50 µl) of either vehicle (20% benzyl alcohol in corn oil) or suspension of DHT in vehicle (56 mg/kg/day) and allowed free access to water and standard rat chow for 14 days. Systolic blood pressure was determined by calibrated tail cuff method (Natsume Tail Monometer Model # KN-210-1) at an ambient temperature, following the manufacturer's instructions. A minimum of 10 readings were obtained after discarding a few initial readings. Rats were preheated in a chamber at 40°C for 10 min, and then placed in plastic restrainers to become familiar with the environment. A cuff with a pneumatic pulse sensor was attached to the tail. Minimum 10 readings were obtained after discarding few initial readings. Values within ± 10% of their mean blood pressure measurements were obtained.

Isolation and microdissection of renal interlobar arteries

Rats were anesthetized with Phenobarbital (50 mg/kg). Kidneys were removed and freed of connective tissue, sliced into hemi sections and placed in Krebs bicarbonate buffer. Renal interlobar arteries (~80–100 µm diameter) were isolated as previously described [23]. The arteries were placed in Krebs bicarbonate buffer for measurement of HETEs and EETs synthesis or in Trizol for RNA isolation.

Measurement of HETE and EET synthesis

Levels of 20-HETE and EETs were measured by gas chromatography/mass spectrometry method as previously described [42]. Briefly, renal interlobar arteries (4 segments/tube) were placed in Kreb's bicarbonate buffer (pH 7.4) for 1 h at 37°C with gentle shaking. Reaction was stopped by using acetic acid (pH 4). The medium was collected and [$^2H_2$]-20-HETE (1 ng/ml) and [$^2H_8$]-8,9-EET, [$^2H_8$]-11,12-EET and [$^2H_8$]-14,15-EET (1 ng each) were added as internal standards. The mixture was then extracted thrice with 2 ml of ethyl acetate. The final extract was evaporated under nitrogen, resuspended in 30 µl of methanol, and purified by RP-HPLC on a C_{18} (Beckman Ultrasphere column (5 µm X 4.6 mm X 24 cm) using a linear gradient from acetonitrile : water : acetic acid (75:25: 0.05%) to acetonitrile (100%) over 20 min at a flow rate of 1 ml/min. Fractions containing CYP-HETEs and CYP EETs were collected based on the elution profile of standards monitored by UV absorbance (205 nm). The fractions were evaporated to dryness. Pentafluorobenzyl (PFB) esters were prepared using bromo-2,3,4,5,6-pentafluorotoluene and *N,N*-diisopropylethylamine and hydroxyl groups were converted to trimethylsilyl ether using N,O-bis(trimethylsilyl) trifluoroacetamide as described. To separate subterminal CYP-HETEs, samples were dissolved in isooctane, and 1 µl aliquots were injected into a GC (HP6890) column (HP-5 MS; 30 m; 0.25 mm, inner diameter: 0.25 µm film thickness, Agilent) by using a temperature program ranging from 150–280°C at 40°C/min. Methane was used as a reagent gas, and the MS (Hewlett-Packard 5973N) was operated in negative ionization mode. The endogenous CYP-HETEs were identified [ion mass-to-charge ratio (m/z) 391] by comparison of GC retention times with authentic CYP-HETE standards and quantitated by calculating the ratio of abundance with D_2 20-HETE (m/z 393) similarly endogenous CYP-EETs were identified [ion mass-to-charge ratio (m/z) 319] by comparison of GC retention times with authentic CYP-EETs standards and quantitated by calculating the ratio of abundance with D_8- EET (m/z 327). After extraction, the vessels were collected and suspended in 100–300 µl 1N NaOH in which they were left overnight to dissolve. Any intact vessels left were manually ground using a glass rod. Protein concentration was determined using BioRad Protein Assay (BioRad, Hercules, CA).

RT/PCR

To isolate mRNA, microdissected interlobar arteries were quickly frozen in liquid nitrogen with 1ml of Trizol solution. Microvessels were homogenized on ice and centrifuged at 12,000 g for 10 min at 4°C to remove insoluble materials. The supernatant was transferred to another tube and left for 5 min at room temperature. Chloroform was added to the sample and centrifuged again at 12,000 g for 10 min at 4°C; supernatant was transferred with 500 µl isopropranol to a new tube. The mixture was centrifuged and pellet was resuspended in Distilled DEPC-treated water. Total RNA was measured via spectrophotometry. A reverse transcription reaction was performed using Superscript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Briefly, mRNA (5 µg) from the microvessels was added to 10 µl reverse transcription reaction mixture containing 50 µM oligo dT, 10 mM dNTP mix and DEPC-treated water. After incubating the reaction at 65°C for 5 min, the final PCR was carried out in a final volume of 21 µl containing 25 mM $MgCl_2$, 100 mM DTT, 40 U RNaseOUT, 200 U superscript III reverse transcriptase, 10X RT Buffer, and 1 µl of RNase H. The samples were diluted to 25 µl with a final concentration of 200 ng/µl.

Real Time PCR

Quantitative Real-Time PCR was performed using Brilliant® SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and the Mx3000p™ Real-Time PCR System (Stratagene, La Jolla, CA). In this method, the double-stranded DNA specific dye (SYBR Green) is added to the PCR reaction where it binds the minor groove of double stranded DNA. Direct detection of PCR products by the LightCycler is monitored by measuring the increase in the fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. As PCR products accumulate, fluorescence increases. These measurements result in an amplification plot of the fluorescence signals Vs Cycle number. Because SYBR Green indiscriminately binds to double stranded DNA, other products in the PCR such as primer dimers may be detected along with the target gene. To verify that the SYBR Green dye only detected PCR products, the samples were subjected to a heat dissociation protocol after the final cycle characteristic melting temperature and produced

a single narrow dissociation peak, an indication that the assay detected only the target genes.

Specific primers were designed based on published sequences (GenBank). The CYP4A1, CYP4A2, CYP4A3, CYP4A8, CYP2C23 and 18S primers (GeneLink, USA) were designed to amplify 351, 317, 321, 404, 252 and 72 bp fragments respectively, from each of the corresponding cDNAs [37].

PCR was run according to the protocol for the Brilliant® SYBR Green QPCR Master Mix. Briefly, PCR was carried out using a final concentration of 2 μM of the primer pairs, 200 ng of cDNA template and 12.5 μl of Brilliant® SYBR Green QPCR Master Mix. The volume was adjusted to 25 μl by adding DEPC treated water. The thermocycling protocol began with a 15 min denaturation at 95°C, a 40 cycle amplification program consisting of 30 s denaturation at 95°C, 1 min annealing at 55°C and 30 s extension at 95°C. At the end, the temperature was brought up to 1 min at 95°C, 30 s 55°C and again 30 s at 95°C to melt any primer dimmers.

PCR efficiency for each primer pair was determined by quantitating amplification with the increasing concentration of template cDNA (0.001, 0.01, 0.1, 1 and 5 pg); specific amplification was verified by the subsequent analysis of melt curve profiles for each amplification. A non-template control served as negative control to exclude the formation of primer dimmers or any other non-specific PCR products possibly due to reagents. RNA expression of target genes was calculated based on real-time PCR efficiency (E) and the threshold crossing point (CP) and was normalized to the reference gene. The relative expression ratio of a given gene was calculated using the ΔCt method, as previously established [29].

Results

Androgen treatment induces blood pressure in both male and female rats

Blood pressure in age-matched male and female normotensive rats was not significantly different, viz, 126.6 ± 1.3 mmHg in male rats and 126.1 ± 1.5 mmHg in female rats. Daily treatment with DHT for 2 weeks significantly increased blood pressure in both females and males. As seen in Figure 1A, blood pressure in male rats increased by 21.3 ± 4.0 mmHg from 127.8 ± 2.5 mmHg before treatment to 148.1 ± 3.5 mmHg after DHT treatment. The increase in blood pressure in female rats was somewhat lower than the increase in male rats after DHT treatment. Blood pressure in female rats increased by 15.3 ± 5.1 mmHg from 128.1 ± 2.2 mmHg before treatment to 143.3 ± 3.5 mmHg after DHT treatment (Fig. 1B). Blood pressure in male or female rats treated with the vehicle control was unchanged (126.6 ± 1.3 and 126.1 ± 1.5 mmHg before *vs.* 126.8 ± 2.4 and 128 ± 0.5 mmHg 14 days after vehicle treatment in male and female rats, respectively).

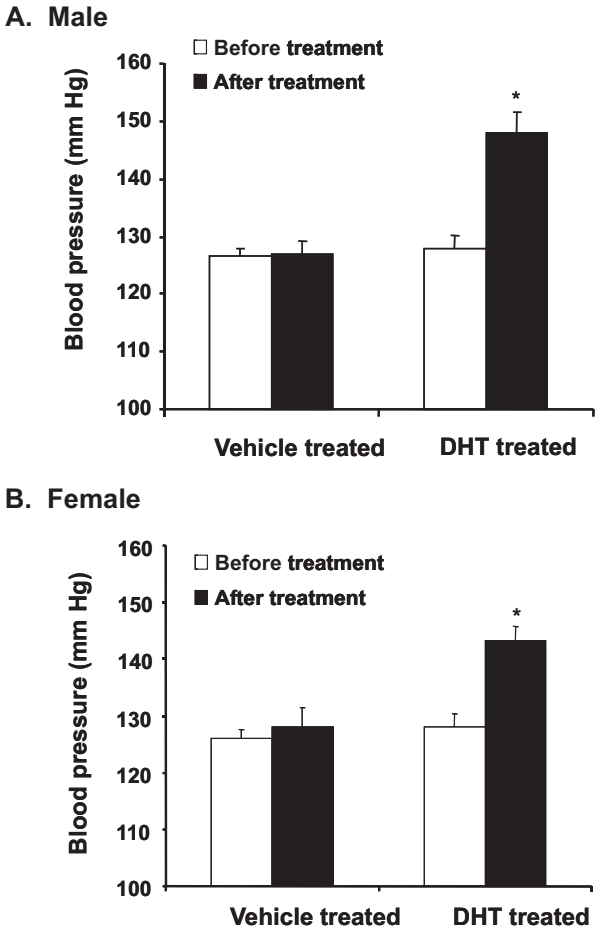

Fig. 1. Effect of DHT on systolic blood pressure in male (**A**) and female (**B**) rats. Results are mean ± SE; (n = 4); * $p < 0.05$ relative to before treatment in DHT treated group

Comparison of HETEs and EETs levels in female and male rats

Renal interlobar arteries are a rich source of CYP-derived HETEs and EETs as well as a target for their

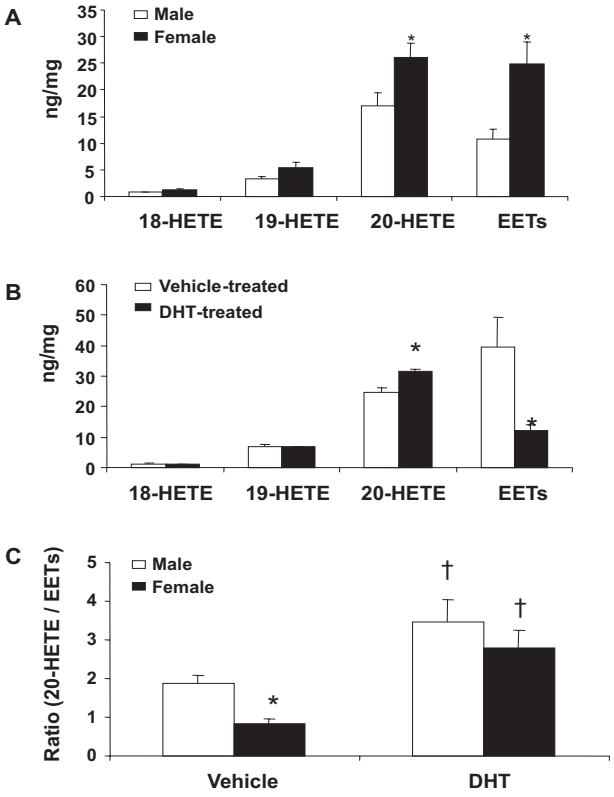

Fig. 2. Effect of DHT on the levels of renal vascular eicosanoids in male and female rats. **A**) Levels of 18-HETE (n = 18), 19-HETE (n = 18), 20-HETE (n = 18) and EETs (n = 11) in renal interlobar arteries from male and female rats; * $p < 0.05$ relative to male rats. **B**) Levels of 18-HETE (n = 16), 19-HETE (n = 16) 20-HETE (n = 16) and EETs (n = 6) in renal interlobar arteries from vehicle- and DHT-treated female rats; * $p < 0.05$ relative to vehicle-treated rats. **C**) Ratio of 20-HETE to EETs in vehicle and DHT-treated male and female rats; Results are mean ± SE (n = 6–11); * $p < 0.05$ relative to male rats; † $p < 0.05$ relative to vehicle-treated rats

bioactions as regulators of renal vascular tone [23]. Therefore, we compared and contrasted the levels of HETEs (18-, 19- and 20-HETE) and EETs, including 8,9-EET, 11,12-EET and 14,15-EET, in isolated arteries from male and female rats. As seen in Figure 2A, there was no significant difference between the levels of 18-HETE in male and female rats (0.76 ± 0.13 *vs*. 1.18 ± 0.25 ng/mg protein). The levels of 19-HETE were higher ($p = 0.05$) in females as compared to males (3.23 ± 0.45 *vs*. 5.43 ± 1.0 ng/mg protein). In contrast, 20-HETE levels were significantly greater by 52% in female (26.06 ± 2.7 ng/mg protein) when compared to male rats (17.06 ± 2.49 ng/mg protein). Likewise, EET levels in renal interlobar arteries were 2-fold higher in female (24.76 ± 4.27 ng/mg protein) as compared to male rats (10.85 ± 1.86 ng/mg protein) (Fig. 2A). In female rats, DHT treatment resulted in a significant 30% increase in the levels of 20-HETE (from 24.51 ± 1.51 to 31.7 ± 1.84 ng/mg protein) and a marked 2.5-fold decrease in the levels of EETs (from 39.4 ± 9.5 to 12.0 ± 2.3 ng/mg protein) (Fig. 2B). Noteworthy is the fact that the DHT-induced change in the levels of vascular HETE and EETs was different in male rats; while 20-HETE increased by about 50%, the levels of EETs were not significantly reduced as previously reported [37].

20-HETE and EETs have opposing effects in the renal vasculature; 20-HETE is a vasoconstrictor whereas EETs are vasodilators. Therefore, their relative levels may determine renal vascular resistance and tone. Interestingly, the ratio between 20-HETE and EETs levels in male was 2-fold higher than that observed in arteries from female rats (1.86 ± 0.22 *vs*. 0.85 ± 0.13) (Fig. 2C). Importantly, DHT treatment significantly increased this ratio by 85 and 230% in arteries from male and female rats, respectively. Moreover, DHT treatment eliminated the difference in the ratio of 20-HETE to EETs between male and female (Fig. 2C).

Vascular CYP mRNA expression in male and female rats

We measured the levels of expression of CYP proteins whose activity contributes to the production of 19-HETE, 20-HETE and EETs before and after treatment with DHT. As previously indicated, CYP4A proteins are the major arachidonic acid ω/ω-1 hydroxylases in the rat kidney, whereas CYP2C23 is the major arachidonic acid epoxygenase. As seen in Figure 3, treatment with DHT did not significantly increase the expression levels of CYP4A1 and CYP4A2 in arteries from either male or female rats. However, DHT treatment increased the relative expression levels of CYP4A8 by 1.9- and 4.1-fold over control levels in male and female rats, respectively (Fig. 3). Moreover, the levels of CYP2C23 were significantly decreased in male and female rats by 60% (Fig. 3).

Discussion

The current study points to significant gender differences in the renal vascular levels of the CYP-derived eicosanoids, namely 20-HETE and EETs. Given the distinct roles of these two groups of eicosanoids in the regulation of vascular tone and homeostasis and in the

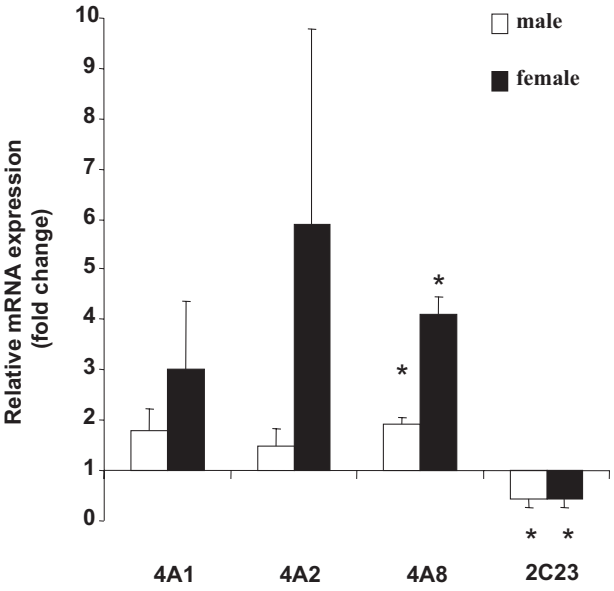

Fig. 3. Effect of DHT on CYP4A1, CYP4A2, CYP4A8 and CYP2C23 mRNA expression levels in renal interlobar arteries in male and female rats. Results are expressed as fold change to vehicle-treated rats, normalized to the levels of 18S (n = 3–5). Results are mean ± SE; * $p < 0.05$ relative to vehicle-treated rats

control of blood pressure, these differences may contributes to the gender differences in blood pressure and the risk for cardiovascular and renal disease.

Recent studies by Reckelhoff et al. [30, 31] have shown that blood pressure is significantly greater in men than in women at the same age. Similar observations have been made in animal models. For example, male SHR have higher blood pressure than age- and strain-matched female rats [5, 15]. Although, the specific mechanisms responsible for the gender-specific differences in blood pressure are not clear, androgen has been thought to play a role. Several studies have suggested that androgens increase arterial pressure by causing a hypertensive shift in the pressure-natriuresis relationship, either by having a direct effect to increase proximal tubular reabsorption or by activation of RAS [32, 33]. Others have postulated that androgens contribute to increased blood pressure, in part, by increasing oxidative stress *via* an NADPH oxidase-dependent mechanism in the SHR [15] and in normotensive rats [37]. Other mechanisms by which androgens have been thought to increase blood pressure include increased production of autocoids such as endothelins and Ang-II, which in turn promote oxidative stress, production of vasoconstrictor substances and reduction in nitric oxide. To this end, the renal vasoconstrictor action of Ang-II and endothelin-I has been shown to be mediated *via* increased production of the CYP-derived 20-HETE [7, 28].

The renal microcirculation is a major site for the synthesis of 20-HETE and EETs. The vascular wall expresses numerous CYP proteins including CYP2C epoxygenases and CYP4A ω-hydroxylases and has a substantial capacity to produce EETs and 20-HETE. The synthesis of EETs is primarily localized to the endothelium, whereas 20-HETE synthesis is localized to the smooth muscle cells. EETs and 20-HETE have prominent actions, mostly opposing effects, on the vasculature. EETs are primarily vasodilators and their dilatory action is believed to be related to their ability to open the Ca^{2+}-activated K (BK_{Ca}) channels thus evoking hyperpolarization of vascular smooth muscle cells. These properties together with their endothelial origin are the basis for their actions defining EETs as EDHFs [2]. EETs have also been shown to contribute to the maintenance of vascular homeostasis by up-regulating endothelial nitric oxide (eNOS) activity [41] and mediating the vasodilatory actions of autocoids such as bradykinin and adenosine [6]. On the other hand, 20-HETE is a potent vasoconstrictor in the microcirculation [24]. 20-HETE elicits vascular contraction *via* inhibition of the large conductance BK_{Ca} channel leading to vascular smooth muscle cell depolarization and elevation in cystolic Ca^{2+}; the latter is mostly related to activation of L-type Ca^{2+} cannels [24]. In addition, 20-HETE may contribute to increased vascular tone by mediating vascular responses to constrictor stimuli, modulating the myogenic response of arterioles to elevation in transmural pressure and reducing NO bioavailability *via* uncoupling eNOS [37, 42] and increasing superoxide production [12, 37]. Thus, in small resistance arteries, 20-HETE acts as a functional antagonist of the action of EETs. Therefore, the relative amounts of each of these bioactive eicosanoids may determine vascular tone and thereby blood pressure. Accordingly, a higher 20-HETE to EET ratio would be conducive to promoting pro-hypertensive mechanisms. The data in this study clearly shows that the 20-HETE to EET ratio is higher in male rats compared to age-matched female rats. It is possible that the relative increase in the vascular levels of 20-HETE contribute, at least in part, to the development of hypertension in males in animal models such as the SHR and rats fed high fat diet [40, 47].

This gender-difference in 20-HETE/EETs ratio may be the consequence of higher levels of androgens. Androgens have been shown to up-regulate the CYP4A proteins, specifically CYP4A2 and CYP4A8 [16, 26, 37]. These two enzymes are the main source of 20-HETE in the vasculature of the rat kidney [23]. The results of this study clearly show that androgen treatment increased the expression of primarily CYP4A8 in male and female rats. The results also indicate that androgens down-regulate the expression of CYP2C23, the major source of EETs in the rat kidney [14]. It is important to note that CYP4A8 and CYP2C23 have been implicated in the development of hypertension. Studies by Capdevila and colleagues have shown that in Cyp4a14 knockout mice, hypertension is more severe in the male and is associated with increased Cyp4a12 expression (the mouse homologue of the rat CYP4A8) and 20-HETE synthesis [13] and that androgen-induced hypertension is mediated, in part, *via* induction of CYP4A8 and production of 20-HETE [26]. The later finding was substantiated by Singh et al. [37] who demonstrated that inhibition of 20-HETE synthesis abolished androgen-induced hypertension. The CYP2C23 epoxygenase has been implicated in the regulation of salt induced hypertension: Increased dietary salt intake in the rat increased renal CYP2C23 epoxygenase activity and urinary EET levels while salt sensitive hypertension in animal models is associated with an inability to increase EET production [14, 22]. The dual action of androgens observed in the present study, i.e., upregulation of CYP4A8 and downregulation of CYP2C23, not only increased the absolute levels of 20-HETE but also reduced the levels of EETs in arteries from female rats and reversed the ratio of 20-HETE/EETs. Consequently, such changes may underlie the hypertensive actions of androgens.

In summary, androgen has been implicated as a contributing factor to gender-specific differences in blood pressure and susceptibility to cardiovascular morbidity. This study suggests that part of the mechanisms contributing to gender differences in blood pressure and to androgen-induced hypertension are alterations in the vascular levels of CYP-derived eicosanoids, which are important modulators of vascular tone. Androgens *via* induction of CYP4A8 and downregulation of CYP2C23 lead to an increase in the production of 20-HETE and a decrease in the production of EETs. Increased vascular tone brought about by the decreased levels of vasodilatory EETs and enhanced production of 20-HETE, a potent vasoconstrictor, may constitute important factors in androgen-induced hypertension.

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