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*Hypertension* 2007;50:123-129; originally published online Jun 4, 2007;

DOI: 10.1161/HYPERTENSIONAHA.107.089599

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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## Vascular Cytochrome P450 4A Expression and 20-Hydroxyeicosatetraenoic Acid Synthesis Contribute to Endothelial Dysfunction in Androgen-Induced Hypertension

Harpreet Singh, Jennifer Cheng, Huan Deng, Rowena Kemp, Tsuneo Ishizuka, Alberto Nasjletti, Michal Laniado Schwartzman

**Abstract**—Epidemiological evidence suggests a role for sex-dependent mechanisms in the pathophysiology of hypertension. It has been shown that 5 $\alpha$ -dihydrotestosterone (DHT) administration (56 mg/kg of body weight per day IP for 14 days) increases blood pressure, cytochrome P450 4A expression, and 20-hydroxyeicosatetraenoic acid synthesis in rats. We examined whether increased vascular 20-hydroxyeicosatetraenoic acid synthesis underlies endothelial dysfunction and hypertension in DHT-treated male Sprague–Dawley rats by using HET0016, a selective cytochrome P450 4A inhibitor. Coadministration of HET0016 (10 mg/kg per day IP for 14 days) to DHT-treated rats markedly reduced DHT-induced interlobar arterial production of 20-hydroxyeicosatetraenoic acid ( $14.3 \pm 1.5$  versus  $1.5 \pm 0.5$  ng/mg of protein per hour;  $P < 0.05$ ), superoxide anion ( $246 \pm 47$  versus  $31 \pm 8$  cpm/ $\mu$ g of protein), and the levels of gp91-phox, p47-phox, and 3-nitrosylated proteins. Moreover, the maximal relaxing response to acetylcholine in phenylephrine-precontracted renal interlobar arteries from DHT-treated rats ( $42.8 \pm 4.8\%$ ) significantly ( $P < 0.05$ ) increased in the presence of HET0016 ( $81.5 \pm 10.8\%$ ). Importantly, the administration of HET0016 negated DHT-induced hypertension; systolic blood pressure was reduced from  $146 \pm 2$  mm Hg in DHT-treated rats to  $130 \pm 1$  mm Hg ( $P < 0.05$ ). The results strongly implicate vascular cytochrome P450 4A–derived 20-hydroxyeicosatetraenoic acid in the development of androgen-induced endothelial dysfunction and hypertension. (*Hypertension*. 2007;50:123-129.)

**Key Words:** hypertension ■ endothelial dysfunction ■ cytochrome P450 ■ NO ■ superoxide anion ■ 20-HETE

20-hydroxyeicosatetraenoic acid (20-HETE) is a primary eicosanoid in the microcirculation, where it participates in the regulation of vascular tone by sensitizing the smooth muscle cells to constrictor stimuli<sup>1</sup> and contributes to myogenic, mitogenic, and angiogenic responses.<sup>2–5</sup> The synthesis of 20-HETE is catalyzed primarily by enzymes of the cytochrome P450 (CYP) 4A family.<sup>6,7</sup> CYP4A proteins are present in vascular tissues and show distinct distribution along the vascular tree.<sup>8</sup> Suppression and overexpression of CYP4A proteins in small arteries and arterioles decreases and increases, respectively, vascular reactivity and myogenic tone.<sup>7,9,10</sup>; these effects can be reversed by the addition of 20-HETE or inhibition of its synthesis.

CYP4A and 20-HETE synthesis have been linked to hypertension in numerous experimental models. In the spontaneously hypertensive rat, depletion or inhibition of CYP4A activity lowers blood pressure (BP).<sup>11,12</sup> Inhibition of vascular 20-HETE synthesis by intravenous administration of CYP4A1 or CYP4A2 antisense oligonucleotides decreases BP in normotensive and hypertensive rats,<sup>6,7</sup> whereas transduction with adenoviruses expressing the CYP4A2 protein increases vascular CYP4A expression and 20-HETE levels and augments BP.<sup>13</sup>

A role for androgens in promoting elevation of BP is well recognized<sup>14</sup> and, according to recent studies, such a role may rely on increased synthesis of vascular 20-HETE. Hence, mice deficient in *cyp4a14* (the mouse homologue of CYP4A2) displayed androgen-sensitive hypertension, which was reversed by castration.<sup>15</sup> In these mice, *cyp4a12* expression (the mouse homologue of CYP4A8) is elevated and so is renal microsomal 20-HETE synthesis. Similarly, androgen-induced hypertension in rats treated with 5 $\alpha$ -dihydrotestosterone (DHT) has been associated with increased CYP4A8 expression and renal vascular 20-HETE synthesis.<sup>16</sup>

The mechanisms by which 20-HETE promotes hypertension are primarily linked to its ability to sensitize constrictor responsiveness and increase vascular resistance.<sup>17–19</sup> However, recent studies<sup>13,20–22</sup> have raised the possibility that the CYP4A–20-HETE pathway is an important determinant of endothelial function and suggested that endothelial dysfunction may constitute part of the mechanisms by which this pathway promotes hypertension. The current study was undertaken to support a cause-and-effect relationship among the CYP4A–20-HETE pathway, endothelial dysfunction, and

Received February 19, 2007; first decision March 5, 2007; revision accepted May 3, 2007.

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*Hypertension* is available at <http://www.hypertensionaha.org>

DOI: 10.1161/HYPERTENSIONAHA.107.089599

hypertension in a model of androgen-sensitive hypertension. We showed that administration of a CYP4A inhibitor decreases the androgen-induced increase in BP and prevents the associated endothelial dysfunction while inhibiting 20-HETE synthesis.

## Methods

### Animal Experimentation

All of the experimental protocols were performed following an Institutional Animal Care and Use Committee–approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sprague–Dawley male rats (8 to 9 weeks old) were administered (50  $\mu$ L, IP) DHT (56 mg/kg of body weight per day) or its vehicle (20% benzyl alcohol in corn oil) for 14 days. In some experiments, rats were concomitantly given (50  $\mu$ L, IP) the CYP4A selective inhibitor *N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)-formamidin<sup>23</sup> (HET0016; 10 mg/kg of body weight per day) or its vehicle (10% weight per volume of lecithin in saline). Systolic BP was determined before and at days 5, 9, and 14 of treatment by the tail cuff method. At day 14, rats were anesthetized with phenobarbital (50 mg/kg of body weight); kidneys were perfused in situ, and renal interlobar arteries were microdissected as described.<sup>13</sup>

### Agonist-Induced Vasorelaxation

Relaxation responses of phenylephrine-constricted arteries to acetylcholine ( $10^{-9}$  to  $10^{-4}$   $\mu$ mol/L) were studied in the presence of indomethacin (10  $\mu$ mol/L) with and without *N*<sup>G</sup>-nitro-L-arginine methyl ester (1 mmol/L) or *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS; 30  $\mu$ mol/L), a selective CYP4A inhibitor,<sup>24</sup> as described.<sup>13</sup>

### Western Blot Analysis

Western blot analysis of arterial segments was performed as described previously<sup>13</sup> using the following primary antibodies: CYP 4A1 polyclonal antibody (Daiichi Chemical Co); nitrotyrosine polyclonal antibody (Cayman Chemicals), gp91<sup>phox</sup> and p47<sup>phox</sup> polyclonal antibodies (Upstate Co), and endothelial NO synthase (eNOS) polyclonal antibody (Santa Cruz Biotechnology).

### Real-Time PCR

Quantitative real-time PCR was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene) and the Mx3000p Real-Time PCR System (Stratagene) and analyzed as described.<sup>25,26</sup> For details please see the data supplement available at <http://hyper.ahajournals.org>.

### Measurement of HETEs, 11,12-Epoxyeicosatrienoic Acids, and Superoxide

HETE and 11,12-epoxyeicosatrienoic acid (EET) production levels in renal interlobar arteries were quantified by selected ion monitoring gas chromatography/mass spectrometry analysis as described.<sup>13</sup> Superoxide anion levels were measured in isolated renal interlobar arteries by lucigenin chemiluminescence as described.<sup>13,27</sup>

### Statistical Analysis

Concentration–response data derived from each vessel were fitted separately to a logistic function by nonlinear regression and analyzed by a 2-way ANOVA followed by a Duncan multiple-range test. Other data were analyzed by a Student's *t* test for paired or unpaired observations as appropriate. Data are expressed as mean  $\pm$  SE. *N* represents the number of rats for each group. The null hypothesis was rejected at  $P < 0.05$ .

## Results

### DHT Treatment Induces Vascular CYP4A Expression and 20-HETE Synthesis

Treatment with DHT resulted in a 2-fold increase in vascular CYP4A protein expression compared with treatment with the vehicle control (Figure 1A). This increase was likely accounted for by increased levels of mRNA for CYP4A8 and, possibly, CYP4A1 and CYP4A2 (Figure 1B). DHT treatment increased ( $P < 0.05$ ) the relative expression of CYP4A8 mRNA by 2.2-fold, whereas the 1.9- and 1.6-fold increases in CYP4A1 and CYP4A2 mRNA levels, respectively, were not statistically significant. It should be noted the level of CYP4A2 mRNA expression (copy number) in untreated rats was the highest, namely,  $66 \pm 47 \times 10^6$ ,  $1.5 \pm 0.8 \times 10^6$  and  $1.1 \pm 0.5 \times 10^6$  for CYP4A2, CYP4A1, and CYP4A8, respectively. CYP4A3 mRNA levels were undetectable (data not shown). The mRNA expression levels of CYP4F proteins, which have also been shown to metabolize arachidonic acid to 20-HETE,<sup>28</sup> were not affected by DHT treatment (Figure 1C). The increased expression of CYP4A proteins after DHT treatment was associated with a 31% increase in 20-HETE production by renal interlobar arteries (Figure 1D). The levels of other HETEs, including 18- and 19-HETEs, were unchanged. Likewise, the levels of EETs in arteries from control and DHT-treated rats were not significantly different (Figure 1D).

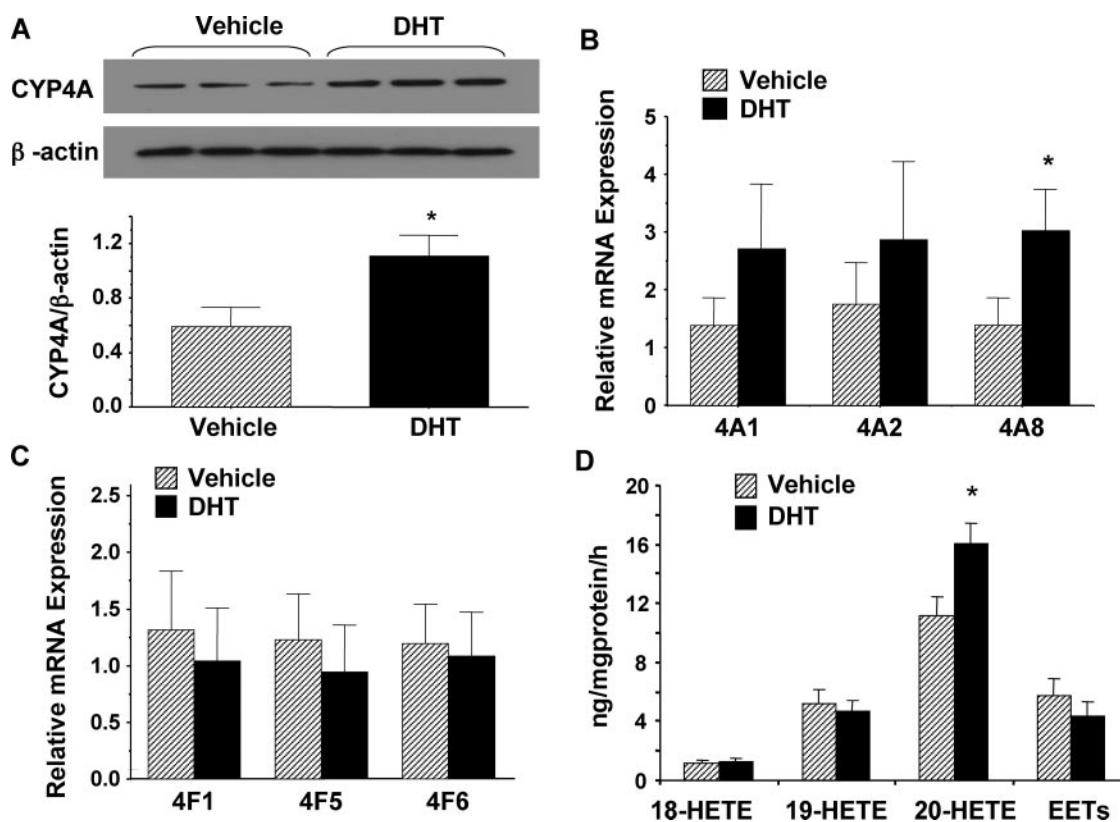
### DHT-Induced Increase in BP Is Prevented by HET0016 Treatment

Treatment with DHT daily for 14 days resulted in a time-dependent increase in BP that was not seen in rats treated with the vehicle control (Figure 2A). BP increase was evident as early as 5 days after treatment. At day 14 of treatment, BP in DHT-treated rats was increased by 16%, namely,  $126 \pm 1$  mm Hg before and  $146 \pm 2$  mm Hg 14 days after DHT treatment ( $P < 0.002$ ). BP in rats treated with the vehicle control was largely unchanged ( $126 \pm 1$  mm Hg before and  $129 \pm 1$  mm Hg 14 days after vehicle treatment). Importantly, concurrent administration of the CYP4A inhibitor HET0016 abolished the DHT-driven BP increase (Figure 2A).

That HET0016 treatment associated with inhibition of 20-HETE production was confirmed by measuring 20-HETE levels in interlobar arteries from treated rats. As seen in Figure 2B, 20-HETE levels in arteries from rats treated with both DHT and HET0016 ( $1.53 \pm 0.57$  ng/mg of protein per hour) were 10% of values in arteries from rats treated with DHT alone ( $14.36 \pm 1.53$  ng/mg of protein per hour). Treatment with HET0016 alone decreased basal 20-HETE levels by 82% ( $9.70 \pm 1.25$  versus  $1.68 \pm 0.48$  ng/mg of protein per hour in vessels from vehicle- and HET0016-treated rats, respectively). At the dose used, HET0016 had no significant effect on arterial levels of EETs (Figure 2B).

### DHT-Treated Rats Display Endothelial Dysfunction, Which Is Corrected by CYP4A Inhibition

Relaxing responses to acetylcholine were examined in renal interlobar arteries precontracted with phenylephrine. Arteries were relaxed in a concentration-dependent manner. At the



**Figure 1.** Effect of DHT on CYP4A protein and mRNA expression and 20-HETE levels in renal interlobar arteries. A, Representative immunoblot of CYP4A and densitometry analysis. Results are relative density normalized to  $\beta$ -actin levels (N=7). B and C, Real-time PCR analysis of CYP 4A1, 4A2, 4A8, 4F1, 4F5, and 4F6 mRNA levels. Results are depicted as relative expression normalized to the levels of 18S (N=5). D, Levels of 18-HETE (N=13), 19-HETE (N=13), 20-HETE (N=13), and EETs (N=7). \* $P < 0.05$  vs vehicle-treated rats.

maximally tested concentration, the response to acetylcholine in arteries from DHT-treated rats was significantly lower ( $42 \pm 4\%$  relaxation) than in arteries from vehicle-treated rats ( $84 \pm 3\%$  relaxation; Figure 3A). After the addition of  $N^G$ -nitro-L-arginine methyl ester, the residual (NO-independent) relaxing effect of acetylcholine, at the maximal concentration, was similar in arteries from rats treated with DHT ( $37 \pm 5\%$  relaxation) or vehicle ( $31 \pm 4\%$  relaxation; Figure 3B). The addition of DDMS, a selective CYP4A inhibitor,<sup>24</sup> to the bath significantly enhanced acetylcholine-induced relaxation in arteries from DHT-treated rats (from  $42 \pm 4\%$  to  $70 \pm 7\%$  relaxation) but not in arteries from rats treated with the vehicle only ( $80 \pm 6\%$  relaxation; Figure 3B). Importantly, addition of 20-HETE ( $10 \mu\text{mol/L}$ ) to the organ bath reversed the DDMS effect in arteries from DHT-treated rats from  $70 \pm 7\%$  to  $41 \pm 4\%$  relaxation ( $n=3$ ;  $P < 0.05$ ; data not shown).

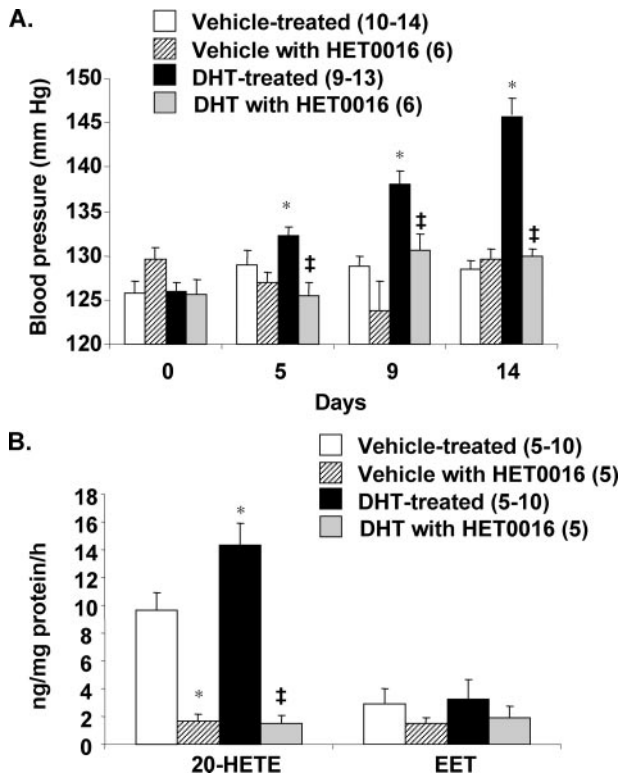
To further link endothelial dysfunction to the increased expression of the CYP4A-20-HETE pathway, acetylcholine-induced relaxation was examined in arteries from rats receiving HET0016 along with DHT. As seen in Figure 4, HET0016 administration corrected the diminished relaxing effect of acetylcholine in the arteries of DHT-treated rats from  $43 \pm 4\%$  to  $83 \pm 5\%$  relaxation at  $10^{-5}$  mol/L acetylcholine. In fact, arteries from rats treated with both DHT and HET0016 exhibited a similar relaxing response to acetylcholine, as did arteries from vehicle-treated rats ( $83 \pm 5\%$  and

$84 \pm 3\%$  relaxation at  $10^{-5}$  mol/L acetylcholine, respectively). Moreover, HET0016 had no effect on acetylcholine-induced relaxation in arteries from control rats treated with the vehicle only.

### Oxidative Stress in DHT Treated Rats Is Diminished With CYP4A Inhibition

Oxidative stress is believed to be one of the underlying mechanisms contributing to endothelial dysfunction and hypertension.<sup>29</sup> We measured oxidative indices in renal interlobar arteries from rats treated with DHT with and without HET0016. As seen in Figure 5, DHT treatment increased the expression levels of  $p47^{\text{phox}}$  and  $gp91^{\text{phox}}$ , components of the vascular superoxide-generating reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase system,<sup>30</sup> by 3- and 2-fold, respectively. Importantly, HET0016 markedly inhibited DHT-induced  $p47^{\text{phox}}$  and  $gp91^{\text{phox}}$  protein levels by 65% and 61%, respectively, while having no significant effect on basal (vehicle-treated) levels of either  $p47^{\text{phox}}$  or  $gp91^{\text{phox}}$  (Figure 5).

We also measured levels of nitrotyrosine, a marker for peroxynitrite, and superoxide anion in renal interlobar arteries. As seen in Figure 6A, the levels of 3-nitrosylated proteins increased by 3-fold after DHT treatment. Likewise, DHT treatment increased vascular superoxide anion levels by 3-fold (Figure 6B). That the increase in these oxidants is linked to increased CYP4A activity is further supported by



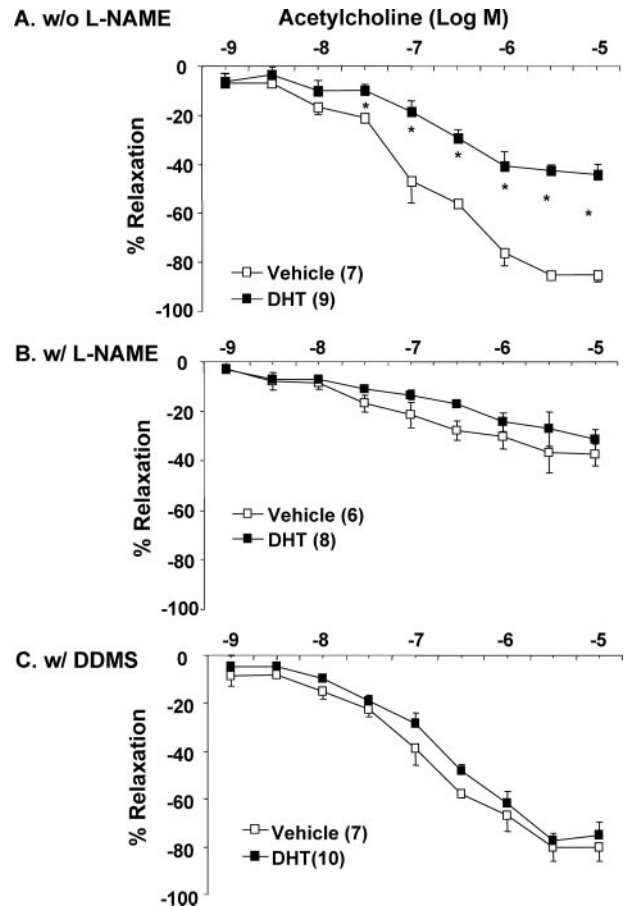
**Figure 2.** A, Effect of DHT and HET0016 treatment on systolic BP and (B) levels of 20-HETE and EETs in renal interlobar arteries. N is given in parentheses; \* $P < 0.05$  vs vehicle-treated rats; ‡ $P < 0.005$  vs DHT-treated rats.

the fact that HET0016 inhibited the DHT-induced increases in both 3-nitrosylated proteins and superoxide anion levels (Figure 6).

### Discussion

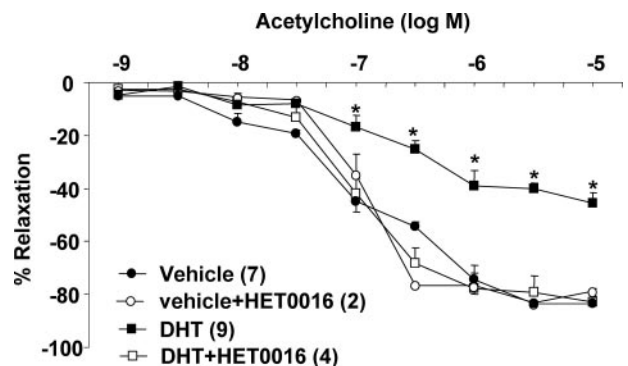
Gender-specific differences in BP and susceptibility to cardiovascular morbidity have led to a search for the possible effects of sex hormones on cardiovascular function. Epidemiological and clinical studies demonstrate that men have higher BP than women and point to a significant correlation between androgen levels and various cardiovascular diseases.<sup>31</sup> These gender-associated differences were also documented in experimental animal models<sup>14,32</sup> and further suggested that androgen contributes, at least in part, to these differences.

The androgen-regulated CYP4A proteins<sup>16,33</sup> have been linked to the pathogenesis of hypertension through their catalytic activity as arachidonate  $\omega$ -hydroxylases. This link was originally derived from studies indicating that depletion of CYP4A and inhibition of arachidonic acid  $\omega$ -hydroxylation reduced BP in SHR.<sup>11,12</sup> It was substantiated in other experimental models of hypertension<sup>34-37</sup> and further supported by numerous studies showing that the arachidonic acid  $\omega$ -hydroxylase metabolite 20-HETE promotes vasoconstriction and increases vascular resistance.<sup>38</sup> Recent studies<sup>15,16,39</sup> provided additional support for an association among androgen, CYP4A expression, 20-HETE synthesis, and hypertension. However, a cause-and-effect relationship has not been established. The current study is the first to

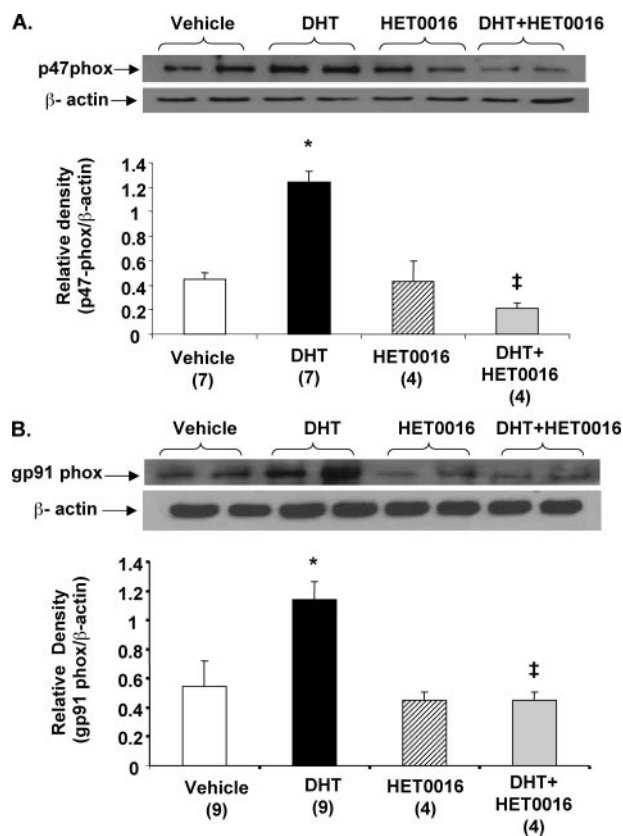


**Figure 3.** Acetylcholine-induced relaxation of phenylephrine-precontracted renal interlobar arteries from vehicle- and DHT-treated rats in the absence (A) and presence (B) of  $N^G$ -nitro-L-arginine methyl ester and (C) DDMS. N is given in parentheses; \* $P < 0.05$  vs vehicle-treated rats.

demonstrate that inhibition of CYP4A activity abrogates androgen-induced hypertension. It also substantiates previous observations that 20-HETE prohypertensive mechanisms may include, among others, activation of endothelial dysfunction through inhibition of NO-dependent vasorelaxation possibly via increased oxidative stress and diminished NO bioavailability.<sup>13</sup>



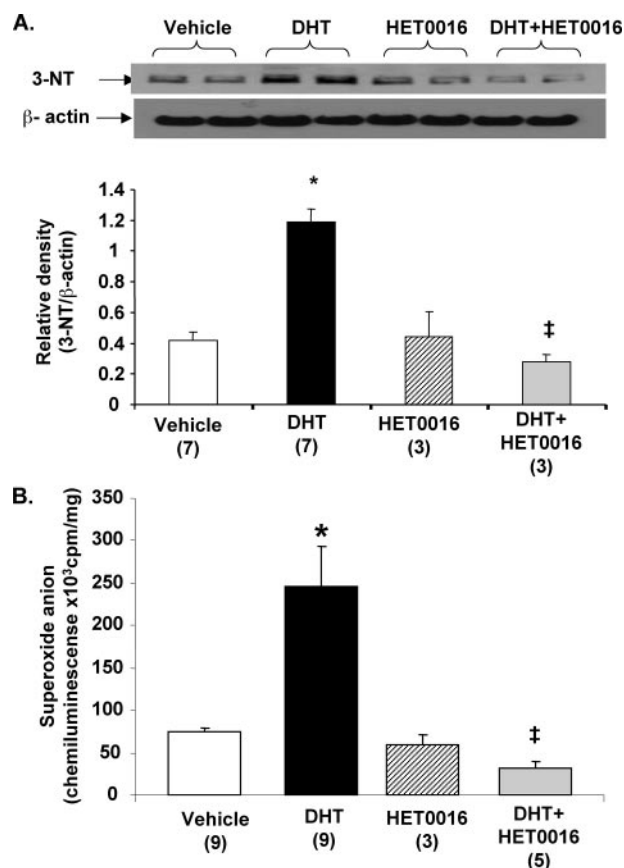
**Figure 4.** Acetylcholine-induced relaxation of phenylephrine-precontracted renal interlobar arteries from vehicle- and DHT-treated rats with and without concurrent HET0016 treatment. N is given in parentheses; \* $P < 0.05$  vs vehicle-treated rats.



**Figure 5.** p47<sup>phox</sup> (A) and gp91<sup>phox</sup> (B) expression levels in renal interlobar arteries. N is given in parentheses; \* $P < 0.005$  vs vehicle-treated rats; ‡ $P < 0.005$  vs DHT-treated rats.

In this study, normotensive rats were treated with DHT for 14 days at a dose shown previously to increase plasma testosterone.<sup>16</sup> The results show that BP increased within 5 days of treatment and continued to increase, reaching levels of  $\approx 20$  mm Hg higher than those observed in rats treated with the vehicle. The DHT-induced increase in BP was associated with increased vascular CYP4A protein expression which, based on real-time PCR analysis, was derived from increased mRNA levels of primarily CYP4A8 and possibly CYP4A1 and CYP4A2.

That DHT administration brought about endothelial dysfunction concurs with numerous studies showing that chronic, but not acute, treatment with androgens diminished endothelial-dependent relaxation to acetylcholine<sup>40</sup>; the current study suggests that such an effect may be mediated by 20-HETE. 20-HETE has been identified as a major eicosanoid in the microcirculation, which acts by sensitizing the vasculature to constrictor stimuli<sup>1</sup> and interfering with NO-dependent vasodilation.<sup>13,21</sup> This notion is supported by our results showing that treatment with the CYP4A-20-HETE inhibitor HET0016 prevented DHT-induced endothelial dysfunction. Given the fact that HET0016 abolished vascular 20-HETE synthesis without significantly changing the levels of other CYP-derived eicosanoids, it is reasonable to assume that 20-HETE plays a causative role in DHT-induced endothelial dysfunction. Similarly, the fact that treatment with HET0016 abolished the DHT-induced increase in BP implicates this pathway in androgen-induced hypertension. That ex vivo



**Figure 6.** 3-Nitrotyrosine (3-NT) expression levels (A) and superoxide anion production (B) in renal interlobar arteries. N is given in parentheses; \* $P < 0.005$  vs vehicle-treated rats; ‡ $P < 0.005$  vs DHT-treated rats.

treatment with DDMS of vessels from rats receiving DHT corrected the impairment in acetylcholine-induced relaxation implies that the endothelial dysfunction in these animals is primarily the result of a local action of 20-HETE rather than the consequence of DHT-induced hypertension. This conclusion is in line with the observation that exogenous 20-HETE prevents DDMS from correcting endothelial dysfunction in DHT-treated rats.

The mechanism(s) by which increased expression and activity of the CYP4A-20-HETE contributes to endothelial dysfunction are yet to be identified. The fact that the DHT-induced endothelial dysfunction was readily reversed by CYP4A inhibitors and reinstated by adding back 20-HETE points to the possibility that 20-HETE interferes with NO bioavailability, as was suggested previously.<sup>13</sup> Mechanisms that may account for such results include an effect on the phosphorylation state of eNOS<sup>41</sup> and/or the uncoupling of eNOS activity via interference with heat shock protein 90 association<sup>42</sup> and/or rapid activation of reactive oxygen species generation, which, in turn, scavenge NO and reduce its bioavailability.<sup>43</sup> A recent report<sup>44</sup> demonstrated that 20-HETE increases superoxide anion levels in cultured endothelial cells. The other possibility is an effect on eNOS itself. Western blot analysis of eNOS in blood vessels showed no effect on total eNOS expression (data not shown); however, this does not exclude the possibility that 20-HETE interferes

with the process of eNOS activation (heat shock protein 90 coupling and phosphorylation/dephosphorylation), as was suggested in recent reports.<sup>13,45</sup>

The underlying mechanism(s) by which the CYP4A-20-HETE pathway affects BP is yet to be elucidated. Both endothelial dysfunction and oxidative stress have been suggested as causatives in androgen-dependent hypertension. A study in male spontaneously hypertensive rats<sup>46</sup> demonstrated that administration of apocynin resulted in the lowering of BP and suggested that increased oxidative stress via an NADPH oxidase-dependent mechanism precedes androgen-mediated hypertension. It is possible that CYP4A-20-HETE mediates DHT-induced oxidative stress by upregulating the NADPH oxidase system, thereby increasing BP; this notion is substantiated by the demonstration that suppression of the CYP4A-20-HETE pathway by HET0016 diminished the DHT-induced expression of the NADPH oxidase system together with decreasing the DHT-induced increase in BP. Accordingly, the effect on endothelial function may be a consequence of increased NADPH oxidase expression mediated via increased production of 20-HETE. However, the action of DDMS *ex vivo* to readily correct DHT-induced endothelial dysfunction suggests that acute deactivation of the CYP4A-20-HETE pathway ameliorates DHT-induced endothelial dysfunction by mechanisms other than downregulation of NADPH oxidase expression. Hence, CYP4A-20-HETE may exert a short-term effect on NO bioavailability, possibly via increased superoxide generation as suggested by Guo *et al*<sup>44</sup> and a long-term effect via increased expression/protein levels of the NADPH oxidase system. The question of which of these effects contribute to DHT-induced hypertension need to be further investigated.

### Perspectives

Androgen has been implicated as a contributing factor to gender-specific differences in BP and susceptibility to cardiovascular morbidity. In recent years, the CYP4A enzymes have been implicated in the development and maintenance of hypertension. Their prohypertensive role has been based on the vasoconstrictor properties of 20-HETE, the CYP4A-derived arachidonic acid  $\omega$ -hydroxylation metabolite. The CYP4A enzymes are readily induced by androgen; the current study provides evidence that androgen-induced endothelial dysfunction and hypertension are mediated by increased vascular CYP4A expression and enhanced production of 20-HETE, because inhibition of 20-HETE synthesis by a selective CYP4A inhibitor abrogated BP increase and ameliorated acetylcholine-induced relaxations. 20-HETE is a prominent eicosanoid in the microcirculation, and its bioactions in this milieu are primarily a consequence of its ability to sensitize smooth muscle to constrictor stimuli via inhibition of the large conductance calcium-activated potassium channels. However, recent studies, including the current one, suggest that 20-HETE has other actions that may promote both endothelial dysfunction and hypertension. These include interference with eNOS-NO production and NO bioavailability and stimulation of the production of reactive oxygen species. Of interest are 2 reports of association among urinary excretion of 20-HETE, oxidative stress, and endothelial

dysfunction in hypertensive subjects,<sup>21,47</sup> which lend support to the relevance of 20-HETE in the regulation of vascular function.

### Source of Funding

This work was supported by National Heart, Lung, and Blood Institute grant HL34300.

### Disclosures

None.

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