Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2015 ; 0: 124–130. doi:10.1016/j.prostaglandins.2014.12.001.

ANDROGEN-INDUCED HYPERTENSION IN ANGIOTENSINOGEN DEFICIENT MICE: ROLE OF 20-HETE AND EETS

Victor Garcia¹, Jennifer Cheng¹, Adam Weidenhammer¹, Yan Ding¹, Cheng-Chia Wu¹, Fan Zhang¹, Katherine Gotlinger¹, John R. Falck², and Michal L. Schwartzman¹

¹Department of Pharmacology, New York Medical College, Valhalla, NY

²Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX

Abstract

20-HETE is a potent inducer of endothelial ACE in vitro and administration of lisinopril or losartan attenuates blood pressure in models of 20-HETE-dependent hypertension. The present study was undertaken to further define the relationship between 20-HETE and the renin-angiotensin system in hypertension using an angiotensinogen-deficient mouse (Agt^{+/-}). Treatment of male AGT^{+/-} with 5 α -dihydrotestosterone (DHT) increased systolic BP from 102 $\pm$ 2 to 125 $\pm$ 3 mmHg; in comparison, the same treatment raised BP in wild type (WT) from 110 $\pm$ 2 to 138 $\pm$ 2 mmHg. DHT increased vascular 20-HETE levels in AGT^{+/-} and WT from 1.5 $\pm$ 0.7 and 2.1 $\pm$ 0.6 to 13.0 $\pm$ 2.0 and 15.8 $\pm$ 4.0 ng/mg, respectively. Concurrent treatment with the 20-HETE antagonist, 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (20-HEDE) prevented the increases in BP in both AGT^{+/-} and WT mice. Administration of 20-HEDE at the peak of the DHT-induced BP increase (12 days) reduced BP to basal levels after 48 hours. Interestingly, basal levels of renal microvascular EETs were higher in AGT^{+/-} compared to WT (55.2 $\pm$ 9.7 vs 20.0 $\pm$ 4.1 ng/mg) and treatment of AGT^{+/-} with DHT decreased the levels of EETs (28.4 $\pm$ 5.1 ng/mg). DHT-mediated changes in vascular EET level were not observed in WT mice. Vascular Cyp4a12 and ACE protein levels were increased in both AGT^{+/-} and WT by 30–40% and decreased with concomitant administration of 20-HEDE. Lisinopril was as effective as 20-HEDE in preventing DHT-mediated increases in BP in both AGT^{+/-} and WT mice. This study substantiates our previous findings that the RAS plays an important role in 20-HETE-mediated hypertension. It also proposes a novel interaction between 20-HETE and EETs.

Keywords

20-HETE; Angiotensinogen; Androgen; ACE; Hypertension

© 2014 Elsevier Inc. All rights reserved.

Corresponding Author: Victor Garcia, New York Medical College, 15 Dana Road, BSB Room 519, Valhalla, NY 10595, TEL (914) 594-415; FAX (914) 347-4956; Victor_Garcia@nyc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DISCLOSURES

No conflicts of interest.

INTRODUCTION

The cytochrome P450-derived eicosanoids, including 20-HETE and EETs, have been increasingly acknowledged 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. Some of these properties contribute significantly to the control of blood pressure. The contribution of these eicosanoids to the development of hypertension and its complication has been documented in numerous animal models. In general, EETs are considered anti-hypertensive whereas 20-HETE effects on tubular transport and vascular tone render it anti- and pro-hypertensive, respectively [1, 2].

The renin-angiotensin system (RAS) has been long recognized as a critical regulator of blood pressure and fluid homeostasis. Components of the RAS, including renin, angiotensin-converting enzyme (ACE), and angiotensin type 1 receptors (AT1R), are generally expressed in tissues (e.g., kidney, brain, arterial vessels, adrenals) that impact on BP control. Angiotensin II (Ang II), the product of sequential degradation of angiotensinogen by renin and ACE, increases BP by mechanisms that include (i) vasoconstriction via AT1R in the vasculature and via increasing sympathetic tone and the release of arginine vasopressin, (ii) modulation of renal sodium and water reabsorption by stimulating renal AT1R, the production and release of aldosterone from the adrenal glands, or the sensation of thirst in the central nervous system. Blocking the synthesis or actions of Ang II lowers BP in hypertensive patients. Mice null for angiotensinogen, renin, ACE and AT_{1A} (the closest murine homologue to the human AT1R gene) exhibit marked reduction in BP, indicating the role of RAS in normal BP homeostasis [3, 4].

Studies have documented interactions between the RAS, EETs and 20-HETE in hypertension. Angiotensin II has been shown to transcriptionally activate soluble epoxide hydrolase (sEH), which hydrolyzes EETs to their corresponding diols (DHETs), in vitro and in vivo [5]. Administration of sEH inhibitors lowers blood pressure in angiotensin-induced hypertension, presumably through EET-dependent suppression of the RAS [6–8]. Indeed, a recent study clearly demonstrated that administration of an EET analog attenuates angiotensin II-dependent hypertension and renal injury in SD rats [9]. On the other hand, Ang II has been shown to stimulate the release of 20-HETE in isolated preglomerular vessels [10] and the renal synthesis of 20-HETE [11]. Increased 20-HETE in the peripheral vasculature contributes to the acute vasoconstrictor response to Ang II [12] and inhibition of 20-HETE synthesis attenuates the renal pressor response to Ang II [11] and the development of Ang II-dependent hypertension [13]. In cultured aortic VSM cells, 20-HETE mediates Ang II-induced mitogenic effects and contributes to the vascular injury, hypertrophy and hypertension caused by Ang II in rats [14–16]. Experimental models of hypertension that show increased vascular 20-HETE production such as the SHR [17, 18] and the androgen-induced hypertension [19–22] are also RAS-mediated. Interestingly, treatment with ACE inhibitors altered renal CYP-mediated eicosanoids [23] and reversed the suppression of hepatic CYP epoxygenase activity and induction of renal CYP ω -hydroxylase activity in mice fed a high fat diet [24]. Recent studies in our lab identified 20-HETE as a potent inducer of endothelial ACE [25] and inhibition of ACE or blockade of AT1R [26] abrogate

blood pressure increase in a rat model of 20-HETE-dependent hypertension [27], suggesting that the pro-hypertensive effect of 20-HETE are mediated and/or amplified by activation of the RAS.

The present study was undertaken to further define the relationship between 20-HETE and RAS in hypertension using the angiotensinogen-deficient mice (Agt^{+/-}). We used the model of androgen-induced hypertension in which the increase in blood pressure is mediated by 20-HETE [19, 28, 29]. Here we show that androgen-induced blood pressure increase in Agt^{+/-} mice is tempered compared to WT mice despite the fact that expression and activity of the Cyp4a12-20-HETE synthase, the sole 20-HETE synthesizing enzyme and the driving force of androgen-induced hypertension in mice [29], were not different between the two strains of mice. This tempered hypertensive response was associated with a moderated increase in vascular reactivity and a lack of impaired relaxation to acetylcholine as seen in WT mice treated with DHT. It is possible that the increased capacity of microvessels to produce EETs along with the limited RAS expression contributed to the tempered hypertensive response to androgen in the Agt^{+/-} mice.

METHODS AND MATERIALS

Animal studies

All experimental protocols were approved by the Institutional Animal Care and Use Committee. The Angiotensinogen-deficient mice (AGT^{+/-}, 4–6-week-old) were direct descendants of angiotensinogen-mutants developed by Kim et al [30]. AGT^{+/-} mice were obtained from Jackson Laboratory (B6.129P2-Agtm1Unc/J; Bar Harbor, ME), bred and offspring genotyped using methods and gene primer sequences available from Jackson Laboratories (<http://jaxmice.jax.org/protocolsdb/f?p=116:1:0>). Wild type offspring served as controls. Placebo or 5 α -dihydrotestosterone (DHT) pellets (5 mg/day; Innovative Research Group of America, Sarasota, FL) were subcutaneously implanted in the mice on day 0. Mice were treated with 20-HETE daily (10 mg/kg/day, ip) from either day 0 or day 12. Some mice were given lisinopril in drinking water (10 mg/kg/day) at day 0.

Blood pressure measurements

Systolic blood pressure measurements were taken via the CODA tail-cuff system (Kent Scientific), which utilizes volume pressure recording sensor technology. Mice were acclimated to the machine for one week prior to day 0 and blood pressure was monitored throughout the length of the experiment, from day 0 to day 18. Values within $\pm 10\%$ of their mean blood pressure measurements were obtained. On day 18, mice were anesthetized with ketamine (70 mg/kg) and xylazine (70 mg/kg) and laparotomy was performed. Renal interlobar arteries, aortas and kidneys were collected for western blot analysis, real-time PCR analysis, lipid extraction and functional studies.

Measurement of levels of vascular 20-HETE, EETs and DHETs

Renal interlobar arteries (3–5) were incubated in 1 ml oxygenated Krebs buffer with 1 mM NADPH for 1 hr in a 37°C shaking water bath. Reactions were terminated by acidification with acetic acid to pH 4. Internal standard mix (500 pg containing d6–20-HETE, d8–11,12-

EET and d11-11,12-DHET) was added to each sample, followed by extraction with ethyl acetate. Identification and quantification of 20-HETE, EETs (5,6-,8,9-, 11,12- and 14,15-EET) and DHETs were performed with a Q-trap 3200 linear ion trap quadrupole liquid chromatography/tandem mass spectrometer equipped with a Turbo V ion source operated in negative electrospray mode (Applied Biosystems, Grand Island, NY).

Measurements of vascular function

Renal interlobar arteries were mounted on wires in the chambers of a multivessel myograph (JP Trading, Aarhus, Denmark) filled with Krebs buffer (37°C) gassed with 95% O₂ / 5% CO₂. After mounting and 30–60 min of equilibration, the vessels were set to an internal circumference equivalent to 90% of that which they would have in vitro when placed under a transmural pressure of 100 mmHg. Isometric tension was monitored continuously before and after the experimental interventions. A cumulative concentration-response curve to phenylephrine (1×10^{-9} – 1×10^{-4} M) was constructed. Additional experiments were conducted to determine concentration-response curves for acetylcholine-induced relaxation after maximal contraction. A cumulative relaxation-response curve to acetylcholine (1×10^{-8} – 1×10^{-4} M) was constructed and the maximal relaxation recorded.

Western blot analysis—Aortas were homogenized in tissue protein extraction reagent containing with protease and phosphatase inhibitors (TPER; Roche, Indianapolis, IN). Western blot analysis was performed using primary antibodies against ACE (Abcam, Cambridge, MA), Cyp4a12 (donated by Dr. Jorge Capdevila) and β -actin (Sigma, Saint Louis, MI).

RNA extraction and real-time PCR—Kidneys (30 mg) were homogenized in Buffer RLT containing beta-mercaptoethanol (Qiagen, Germantown, MD). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD), according to the manufacturer's instructions, and quantified with NanoDrop (ThermoScientific, Waltham, MA). RT reaction of total RNA (100–500 ng) was performed using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). Real-time PCR analysis was performed using the PerfeCTa SYBR Green FastMix Low ROX Kit (Quanta Biosciences, Gaithersburg, MD) and the Mx3000p Real-Time PCR System (Stratagene, Santa Clara, CA). Primers used are as follows: angiotensinogen sense: 5'- GCA CCC TGG TCT CTT TCT ACC -3', angiotensinogen antisense: 5'-TGT GTC CAT CTA GTC GGG AGG -3', 18S rRNA sense: 5'- GAT GGG CGG CGG AAA ATA G -3'; 18S rRNA antisense: 5'- GCG TGG ATT CTG CAT AAT GGT - 3'.

Statistical analysis—Data are expressed as means $\pm$ SEM. Significance of difference in mean values was determined using t-test and one-way analysis of variance followed by the Newman-Keul's post hoc test (Graphpad Prism). $P < 0.05$ was considered to be significant.

RESULTS

DHT increases blood pressure in male and female Agt+/- mice

Real time PCR analysis indicated that renal preglomerular vessels from Agt+/- mice express about half of the levels of Agt mRNA in the WT mice. Moreover, DHT did not significantly alter Agt levels in the Agt+/- mice (Figure 1A). As seen in Figure 1B, administration of DHT increased blood pressure in both male and female Agt+/- mice. Systolic blood pressure reached 125 ± 3 mmHg in male and 119 ± 2 mmHg in female Agt+/- mice after 18 days as compared to placebo-treated mice (102 ± 2 mmHg and 101 ± 2 mmHg male and female Agt+/- mice, respectively). There was no significant difference between male and female in their response to DHT.

20-HETE antagonist normalizes DHT-induced hypertension in Agt+/- mice

DHT induced hypertension in both WT and Agt+/- mice (Figure 1C). However, the increase in blood pressure in Agt+/- mice was significantly lower than in WT mice (125 ± 3 vs 138 ± 2 mmHg in Agt+/- and WT mice, respectively, at day 18 of DHT treatment). The blood pressure increase was 20-HETE-dependent in both strains. As seen in Figure 1C, administration of the 20-HETE antagonist, 20-HEDE, at the peak of the DHT-induced hypertension (day 12), reduced blood pressure after 48 hours. 20-HEDE reduced blood pressure from 138 ± 1 mmHg to 101 ± 2 mmHg and 120 ± 1 mmHg to 100 ± 2 mmHg in DHT-treated WT and Agt+/- mice, respectively. 20-HEDE alone had no effect on placebo-treated mice. In another set of experiments, daily administration of 20-HEDE was initiated upon implantation of the DHT pellet. As seen in Figure 2, 20-HEDE prevented the DHT-induced blood pressure increases.

The RAS has been implicated as a mediator of 20-HETE-dependent hypertension presumably through 20-HETE-induced activation of ACE [25, 26]. Here, we showed that administration of either lisinopril or losartan abrogated DHT-induced increases in blood pressure in both WT (not shown) and Agt+/- mice (Figure 2), suggesting that the RAS as well as 20-HETE participate in DHT-induced hypertension.

DHT increases expression of Cyp4a12-20-HETE synthase and ACE in Agt+/- mice

We have previously implicated Cyp4a12 as the main source for 20-HETE in mice and as the leading force of androgen-driven 20-HETE-dependent hypertension [29]. Here, we show that DHT treatment increases Cyp4a12 protein levels by 55% in renal preglomerular arterioles of both WT and Agt+/- (Figure 3A). Co-administration of the 20-HETE antagonist, while lowering blood pressure, does not alter DHT-mediated increases in Cyp4a12 protein levels.

As indicated above, 20-HETE is a potent inducer of endothelial ACE and this action of 20-HETE comprises part of the mechanism by which 20-HETE increases blood pressure. Treatment of either WT or Agt+/- mice with DHT increased ACE protein levels in preglomerular microvessels by 30–40% (Figure 3B). Importantly, co-treatment with 20-HEDE in both WT and Agt+/- mice prevented the DHT-mediated increases in ACE

expression indicating that induction of ACE in response to DHT is 20-HETE-dependent (Figure 3B).

20-HETE and EET production in preglomerular microvessels

In agreement with previous studies [29, 31], the increased protein level of Cyp4a12-20-HETE synthase was associated with several-fold increases in 20-HETE production in preglomerular microvessels from WT and Agt+/- mice (from 2.1 ± 0.6 and 3.5 ± 1.8 to 15.8 ± 4.0 and 13.0 ± 1.2 ng/mg in vessels from WT and Agt+/- mice, respectively). Administration of 20-HEDE to DHT-treated WT or Agt+/- mice did not significantly alter the androgen-induced elevation in 20-HETE levels (Figure 4).

DHT administration did not alter the levels of EETs in microvessels of WT mice; however, it significantly reduced EET levels in Agt+/- microvessels to levels not different than that of DHT-treated WT (28.4 ± 5.1 vs 26.1 ± 7.7 ng/mg in Agt+/- and WT mice, respectively) (Figure 5A). Co-administration of 20-HEDE did not alter the effect of DHT on EET levels in either WT or Agt+/- microvessels. Further analysis of DHETs and evaluation of epoxygenase activity (the sum of EETs and DHETs, Figure 5B) indicated that total epoxygenase activity followed the same pattern as observed for levels of EETs (Figure 5B). Moreover, measurements of DHETs (not shown) and analysis of EETs to DHETs ratio (Figure 5C), as an index of epoxygenase against soluble epoxide hydrolase (sEH) activity suggested that Agt+/- mice display higher microvascular epoxygenase activity as compared to WT microvessels (3.4 ± 0.7 , 4.4 ± 1.0 and 9.6 ± 2.9 in placebo, DHT and DHT+20-HEDE treated WT mice vs 12.1 ± 3.1 , 13.82 ± 3.2 and 16.03 ± 7.9 in placebo, DHT and DHT+20-HEDE treated Agt+/- mice, respectively) (Figure 5C). The 14,15-EET to its DHET ratio was elevated in Agt+/- mice compared to WT (3.2 ± 0.8 and 1.1 ± 0.2 , in Agt+/- and WT mice, respectively) (Figure 5D) suggesting that increased epoxygenase activity and/or suppression of soluble epoxide hydrolase activity contributed to increased in EETs in mice deficient for angiotensinogen.

Effect of DHT on vascular reactivity in Agt+/- mice

We have shown that DHT-induced hypertension is associated with increased vascular reactivity and endothelial dysfunction as measured by relaxation to acetylcholine [19, 28]. We measured constrictor activity to phenylephrine and relaxation to acetylcholine in interlobar arteries from WT and Agt+/- mice that were treated with placebo or DHT. There were no observed differences between WT and Agt+/- placebo-treated mice in EC₅₀ to phenylephrine (0.50 ± 0.06 vs 0.56 ± 0.16 μ M, n=4) or relaxation at 5×10^{-6} M acetylcholine (90 ± 2 % vs 87 ± 2 % μ M, n=4) (Table 1). The EC₅₀ to phenylephrine in vessels of DHT-treated mice was greater ($p < 0.05$) in Agt+/- than WT, indicating attenuated vascular reactivity in AGT-deficient mice (0.22 ± 0.06 vs 0.12 ± 0.03 μ M, n=8). The constrictor activity in both WT and Agt+/- vessels was greatly decreased ($p < 0.05$) by addition of 20-HEDE to the myograph bath as indicated by increased EC₅₀ to phenylephrine (0.60 ± 0.25 and 0.53 ± 0.14 μ M in WT and Agt+/- mice, respectively), suggesting the contribution of 20-HETE as previously demonstrated. Treatment with lisinopril also resulted in attenuation of vascular reactivity albeit to lesser extent than that of 20-HEDE as indicated by a modest

increase in the EC₅₀ to phenylephrine, which was significant in WT but not Agt+/- mice (0.37±0.09 and 0.28±0.04 μM in WT and Agt+/- mice, respectively).

Relaxations to acetylcholine in vessels from DHT-treated WT mice were impaired amounting to only 26±6% (n=4) at 5×10⁻⁶ M acetylcholine; this attenuated response was reversed in vessels from mice co-treated with either 20-HEDE or lisinopril (74±9 and 70±9% relaxation at 5×10⁻⁶ M acetylcholine, respectively). The impaired relaxation to acetylcholine in DHT-treated WT mice was not seen in Agt+/- mice treated with DHT. Interlobar arteries from Agt+/- mice displayed 77±4% relaxation to 5×10⁻⁶ M acetylcholine and was not significantly altered by either 20-HEDE or lisinopril co-treatment (71±8 and 79±9%, respectively, n=4). However, addition of 20-HETE to the myograph bath significantly decreased (p<0.05) relaxations of microvessels from either WT or Agt+/- mice co-treated with DHT and 20-HEDE by approximately 50% (from 74±9 to 34±13 % in WT and from 71±8 to 48±7 % in Agt+/- microvessels, n=5).

DISCUSSION

The current study demonstrates that in mice heterozygous for angiotensinogen, the hypertensive response to androgen administration is tempered. This tempered response was associated with attenuated increases in vascular reactivity, despite the fact that induction of Cyp4a12-20-HETE synthase and production of 20-HETE were not different than in WT mice, suggesting that deficiency in angiotensinogen moderates androgen-induced and 20-HETE driven hypertension possibly through mechanisms that include impaired RAS activation and potential contribution of EETs to attenuated hypertensive response.

Previous studies from our laboratory and others demonstrated that androgen-induced hypertension is mediated in large part by increased production of 20-HETE via transcriptional activation of the expression of CYP4A-20-HETE synthases in rats and mice [19–21, 28, 29, 32]. In other studies, we showed that the hypertensive response to androgen could be prevented or reversed by administration of a 20-HETE biosynthesis inhibitor or a 20-HETE antagonist. On the other hand, we also showed that administration of ACE inhibitors or ATR1 blockers prevents and reverses blood pressure elevation in rats made hypertensive by transduction of CYP4A2-20-HETE-synthase [26]. Androgen has been shown to induce angiotensinogen [33, 34] and interfering with the RAS mitigates androgen-stimulated hypertension [35]. Mice homozygous for targeted disruption of the ATG gene have a low survival rate and display major pathological changes in kidneys and blood vessels [30, 36, 37]; however, the mice with one copy of the ATG gene (Agt+/-) are normal and do display lower levels of plasma AGT and blood pressure than the WT [30]. We used these mice to assess the impact of AGT deficiency on 20-HETE and EETs in androgen-induced hypertension.

In this study, Agt+/- mice displayed a significantly lower blood pressure than their corresponding WT mice. Administration of DHT increased blood pressure in both WT and Agt+/- mice with no significant gender differences. However, the increase in blood pressure in Agt+/- mice was largely attenuated; blood pressure increased by 30 mmHg in WT and by 20 mmHg in Agt+/- mice. The blood pressure increases in response to DHT treatment was

abrogated by either administration of the 20-HETE antagonist 20-HEDE, indicating that both 20-HETE and the RAS contribute to androgen-induced hypertension in these strains. Notably, basal and androgen-stimulated levels of 20-HETE in renal microvessels did not differ between the two strains, suggesting that the low level of angiotensinogen, which was not altered by DHT, is the main contributor to the attenuated blood pressure increase in Agt +/- mice [30]. This notion is supported by the observation that in both strains DHT induced Cyp4a12 expression to the same levels, further indicating that there are no significant differences between WT and Agt +/- mice in the ability of DHT to induce and activate the Cyp4a12-20-HETE pathway.

We have previously shown that ACE is activated in response to 20-HETE in vitro [25] and in vivo [26]. In this study, we showed that DHT-induced hypertension is associated with increased expression of ACE in the renal vasculature and that this increase is mediated by 20-HETE since administration of the 20-HETE antagonist, 20-HEDE, negated DHT effect on ACE expression. Importantly, the effect of DHT on ACE expression as well as the effect of the 20-HETE antagonist was not different between WT and Agt +/- mice. This finding suggests that ACE activation may not play a significant role in determining the differences between WT and Agt +/- mice in the hypertensive response to DHT. It is possible that the low levels of AGT in the Agt +/- mice [38] resulted in lower levels of the pro-hypertensive Ang II which has been shown to work in concert with 20-HETE in increasing vascular resistance and blood pressure [12, 26]. However, additional studies are needed to assess the relative contribution of Ang II and 20-HETE to the hypertension in this animal model.

The catalytic ability of ACE is not exclusively tied to conversion of angiotensin I to angiotensin II as ACE has been shown to degrade bradykinin [39] as well as other substances. Bradykinin has been documented as a stimulator of vascular EET production in porcine coronary arteries [40, 41], thus promoting dilation. The bradykinin/EET interactions into our current working model of 20-HETE-dependent hypertension and ACE activation may account for several of the observations seen in the angiotensinogen-deficient mice. Increased ACE expression in DHT-treated Agt +/- mice and subsequent increases in bradykinin degradation could result in reduced EET production as seen when comparing placebo and DHT-treated Agt +/- mice. In addition, it is possible that the administration of DHT along with RAS deficiency observed in Agt +/- mice influence a host of CYP enzymes such as *Cyp4a* (*4a10/4a14/4a12*), *Cyp2c44* and *Cyp2J2* and consequently alter EET levels.

Increased vascular reactivity and endothelial dysfunction have been shown to underlie the hypertensive effect of 20-HETE [42]. As previously documented, the administration of androgen to WT mice increased vascular reactivity as measured by increased constrictor responsiveness of renal microvessels to phenylephrine. This increase was attenuated in vessels from Agt +/- mice. Administration of 20-HEDE decreased vascular reactivity by 5- and 2.5-fold in WT and Agt +/- mice, respectively, while administration of lisinopril was less effective. It increased the EC₅₀ to phenylephrine by 3-fold in WT and did not significantly alter the EC₅₀ in Agt +/- mice, suggesting that the influence of the RAS/AngII on vascular reactivity and on 20-HETE-mediated increase in constrictor responsiveness in AGT-deficient mice is marginal. Measurements of relaxation to acetylcholine as an index of endothelial function in renal microvessels from WT indicated again that DHT-induced

hypertension is associated with endothelial dysfunction that is primarily mediated by increased production of 20-HETE in the vasculature [19, 28]. Interestingly, relaxation to acetylcholine in microvessels of Agt+/- mice was not altered by DHT nor was it changed by co-administration of either 20-HEDE or lisinopril. This finding suggests that despite the increase in the production of 20-HETE in response to DHT, endothelial function was unaffected. These results can be explained by our finding that vascular epoxygenase activity is significantly higher in Agt+/- mice than WT mice providing higher levels of the functional 20-HETE antagonists, EETs, to promote vasodilation.

The increased epoxygenase activity in the Agt+/- is likely the consequence of RAS deficiency. Angiotensin II has been shown to affect the levels of EETs and 20-HETE in vitro and in vivo. Hence, renal and vascular CYP2C expression and EET production are suppressed in rats administered angiotensin II and rats overexpressing the human renin and angiotensinogen genes [6, 43, 44]. Other studies showed increases in soluble epoxide hydrolase activity in angiotensin II-mediated hypertension and documented a direct stimulatory effect of angiotensin II on soluble epoxide hydrolase expression through AT1 receptor at the transcriptional level [5, 45], thus reducing the levels of the biologically active EETs. Consequently, numerous studies showed that administration of soluble epoxide hydrolase inhibitors lower blood pressure in numerous animal models of hypertension [6–8]. In the current study, analysis of EET and DHET levels in renal blood vessels suggested that AGT deficiency resulted in increasing epoxygenase activity. Hence, EET:DHET ratio, which presents an index of soluble epoxide hydrolase activity, in blood vessels from Agt+/- mice was significantly greater when compared to corresponding WT mice and was unaffected by DHT. DHT treatment, which increases RAS activity [42], lowered vascular EET levels in both WT and Agt+/- mice. However, the fact that DHT reduced EET levels but did not alter EET:DHET ratio in Agt+/- mice implies suppression of epoxygenase rather than soluble epoxide hydrolase activity [43]. Interestingly, EET:DHET ratio in blood vessels from WT mice was significantly increased in mice treated with DHT and 20-HEDE inferring the possibility that 20-HETE has an inhibitory influence on epoxygenase activity. The fact that 20-HETE levels are not affected by 20-HEDE indicates that the observed increase in EET:DHET ratio is not a consequence of substrate shunting. Furthermore, the EET:DHET and 14,15-EET:14,15-DHET data obtained in vessels from Agt+/- mice suggests the possibility that mice deficient in angiotensinogen exhibit modified epoxygenase or soluble epoxide hydrolase function. Interactions between 20-HETE and EETs on biochemical or molecular levels are yet to be explored, although it is possible that under conditions in which 20-HETE actions are antagonized, excess 20-HETE is metabolized by epoxygenases to 20-hydroxy-EETs that induce CYP-epoxygenases through activation of the PPAR α receptors [46, 47].

In summary, the current study shows that androgen-induced blood pressure increase in Agt +/- mice is tempered compared to WT mice, despite the fact that expression and activity of the Cyp4a12-20-HETE synthase, the sole 20-HETE synthesizing enzyme and the driving force of androgen-induced hypertension in mice [29] and expression of ACE, were not different between the two strains of mice. This tempered hypertensive response was associated with a moderated increase in vascular reactivity and a lack of impaired relaxation to acetylcholine as seen in WT mice treated with DHT. It is possible that the increased

capacity of microvessels to produce EETs along with the limited RAS expression contributed to the tempered hypertensive response to androgen in the Agt+/- mice.

Acknowledgments

GRANTS

This study was supported by NIH grants HL034300 (MLS) and GM31278 (JRF) and Diversity Supplement Award HL34300-26A1S1 (VG).

REFERENCES

1. Imig JD. Epoxides and soluble epoxide hydrolase in cardiovascular physiology. *Physiol Rev.* 2012; 92(1):101–130. [PubMed: 22298653]
2. Williams JM, et al. 20-hydroxyeicosatetraenoic acid: a new target for the treatment of hypertension. *J Cardiovasc Pharmacol.* 2010; 56(4):336–344. [PubMed: 20930591]
3. Le TH, Coffman TM. Targeting genes in the renin-angiotensin system. *Curr Opin Nephrol Hypertens.* 2008; 17(1):57–63. [PubMed: 18090671]
4. Cvetkovic B, Sigmund CD. Understanding hypertension through genetic manipulation in mice. *Kidney Int.* 2000; 57(3):863–874. [PubMed: 10720939]
5. Ai D, et al. Angiotensin II up-regulates soluble epoxide hydrolase in vascular endothelium in vitro and in vivo. *Proc Natl Acad Sci U S A.* 2007; 104(21):9018–9023. [PubMed: 17495027]
6. Imig JD, et al. Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension.* 2002; 39:690–694. [PubMed: 11882632]
7. Jung O, et al. Soluble epoxide hydrolase is a main effector of angiotensin II-induced hypertension. *Hypertension.* 2005; 45(4):759–765. [PubMed: 15699457]
8. Varcabova S, et al. Antihypertensive action of soluble epoxide hydrolase inhibition in Ren-2 transgenic rats is mediated by suppression of the intrarenal renin-angiotensin system. *Clin Exp Pharmacol Physiol.* 2013; 40(4):273–281. [PubMed: 23039246]
9. Khan AH, et al. Epoxyeicosatrienoic acid analog attenuates angiotensin II hypertension and kidney injury. *Front Pharmacol.* 2014; 5:216. [PubMed: 25295006]
10. Croft KD, et al. Angiotensin II releases 20-HETE from rat renal microvessels. *Am J Physiol Renal Physiol.* 2000; 279:F544–F551. [PubMed: 10966934]
11. Alonso-Galicia M, et al. Role of 20-hydroxyeicosatetraenoic acid in the renal and vasoconstrictor actions of angiotensin II. *Am J Physiol Regul Integr Comp Physiol.* 2002; 283:R60–R68. [PubMed: 12069931]
12. Joly E, et al. Increased renal vascular reactivity to ANG II after unilateral nephrectomy in the rat involves 20-HETE. *Am J Physiol Regul Integr Comp Physiol.* 2006; 291(4):R977–R986. [PubMed: 16675634]
13. Chabova VC, et al. Effects of chronic cytochrome P-450 inhibition on the course of hypertension and end-organ damage in Ren-2 transgenic rats. *Vascul Pharmacol.* 2007; 47(2–3):145–159. [PubMed: 17604232]
14. Muthalif MM, et al. Angiotensin II-induced hypertension: contribution of Ras GTPase/Mitogen-activated protein kinase and cytochrome P450 metabolites. *Hypertension.* 2000; 36:604–609. [PubMed: 11040243]
15. Parmentier JH, et al. 20-Hydroxyeicosatetraenoic acid mediates angiotensin ii-induced phospholipase d activation in vascular smooth muscle cells. *Hypertension.* 2001; 37:623–629. [PubMed: 11230346]
16. Yaghini FA, et al. Contribution of arachidonic acid metabolites derived via cytochrome P450A to angiotensin II-induced neointimal growth. *Hypertension.* 2005; 45(6):1182–1187. [PubMed: 15897359]
17. Dunn KM, et al. Elevated production of 20-HETE in the cerebral vasculature contributes to severity of ischemic stroke and oxidative stress in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol.* 2008; 295(6):H2455–H2465. [PubMed: 18952718]

18. Zhang F, et al. Modulation by 20-HETE of phenylephrine-induced mesenteric artery contraction in spontaneously hypertensive and Wistar-Kyoto rats. *Hypertension*. 2001; 38:1311–1315. [PubMed: 11751709]
19. Singh H, et al. Vascular Cytochrome P450 4A Expression and 20-Hydroxyeicosatetraenoic Acid Synthesis Contribute to Endothelial Dysfunction in Androgen-Induced Hypertension. *Hypertension*. 2007; 50:123–129. [PubMed: 17548721]
20. Nakagawa K, et al. Androgen-mediated induction of the kidney arachidonate hydroxylases is associated with the development of hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2003; 284:R1055–R1062. [PubMed: 12531784]
21. Holla VR, et al. Alterations in the regulation of androgen-sensitive Cyp 4a monooxygenases cause hypertension. *Proc Natl Acad Sci U S A*. 2001; 98:5211–5216. [PubMed: 11320253]
22. Muller DN, et al. Mouse Cyp4a isoforms: enzymatic properties, gender- and strain-specific expression, and role in renal 20-hydroxyeicosatetraenoic acid formation. *Biochem J*. 2007; 403(1): 109–118. [PubMed: 17112342]
23. Ito O, et al. Effects of converting enzyme inhibitors on renal P-450 metabolism of arachidonic acid. *Am J Physiol Regul Integr Comp Physiol*. 2001; 280:R822–R830. [PubMed: 11171663]
24. Theken KN, et al. Enalapril reverses high-fat diet-induced alterations in cytochrome P450-mediated eicosanoid metabolism. *Am J Physiol Endocrinol Metab*. 2012; 302(5):E500–E509. [PubMed: 22185841]
25. Cheng J, et al. Induction of Angiotensin-Converting Enzyme and Activation of the Renin-Angiotensin System Contribute to 20-Hydroxyeicosatetraenoic Acid-Mediated Endothelial Dysfunction. *Arterioscler Thromb Vasc Biol*. 2012; 32(8):1917–1924. [PubMed: 22723444]
26. Sodhi K, et al. CYP4A2-Induced Hypertension Is 20-Hydroxyeicosatetraenoic Acid-and Angiotensin II-Dependent. *Hypertension*. 2010; 56:871–878. [PubMed: 20837888]
27. Inoue K, et al. Endothelial-specific CYP4A2 overexpression leads to renal injury and hypertension via increased production of 20-HETE. *Am J Physiol Renal Physiol*. 2009
28. Wu CC, et al. Androgen-Dependent Hypertension Is Mediated by 20-Hydroxy-5,8,11,14-Eicosatetraenoic Acid-Induced Vascular Dysfunction: Role of Inhibitor of κ B Kinase. *Hypertension*. 2011; 57(4):788–794. [PubMed: 21321301]
29. Wu CC, et al. Androgen-Sensitive Hypertension Associates with Upregulated Vascular CYP4A12-20-HETE Synthase. *J Am Soc Nephrol*. 2013
30. Kim HS, et al. Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci U S A*. 1995; 92(7):2735–2739. [PubMed: 7708716]
31. Ding Y, et al. 20-HETE INDUCES REMODELING OF RENAL RESISTANCE ARTERIES INDEPENDENT OF BLOOD PRESSURE ELEVATION IN HYPERTENSION. *Am J Physiol Renal Physiol*. 2013; 305:10.
32. Vasudevan H, Yuen VG, McNeill JH. Testosterone-dependent increase in blood pressure is mediated by elevated Cyp4A expression in fructose-fed rats. *Mol Cell Biochem*. 2012; 359(1–2): 409–418. [PubMed: 21894443]
33. Ellison KE, et al. Androgen regulation of rat renal angiotensinogen messenger RNA expression. *J Clin Invest*. 1989; 83(6):1941–1945. [PubMed: 2723066]
34. Chen YF, Naftilan AJ, Oparil S. Androgen-dependent angiotensinogen and renin messenger RNA expression in hypertensive rats. *Hypertension*. 1992; 19(5):456–463. [PubMed: 1568764]
35. Lima R, et al. Roles played by 20-HETE, angiotensin II and endothelin in mediating the hypertension in aging female spontaneously hypertensive rats. *Am J Physiol Regul Integr Comp Physiol*. 2013; 304(3):R248–R251. [PubMed: 23220478]
36. Davisson RL, et al. Complementation of reduced survival, hypotension, and renal abnormalities in angiotensinogen-deficient mice by the human renin and human angiotensinogen genes. *J Clin Invest*. 1997; 99(6):1258–1264. [PubMed: 9077534]
37. Ding Y, Stec DE, Sigmund CD. Genetic evidence that lethality in angiotensinogen-deficient mice is due to loss of systemic but not renal angiotensinogen. *J Biol Chem*. 2001; 276(10):7431–7436. [PubMed: 11096065]
38. Tanimoto K, et al. Angiotensinogen-deficient mice with hypotension. *J Biol Chem*. 1994; 269(50): 31334–31337. [PubMed: 7989296]

39. Skidgel RA, Erdos EG. The broad substrate specificity of human angiotensin I converting enzyme. *Clin Exp Hypertens A*. 1987; 9(2–3):243–259. [PubMed: 2440624]
40. Weston AH, et al. Bradykinin-induced, endothelium-dependent responses in porcine coronary arteries: involvement of potassium channel activation and epoxyeicosatrienoic acids. *Br J Pharmacol*. 2005; 145(6):775–784. [PubMed: 15895105]
41. Gauthier KM, et al. 14,15-epoxyeicosa-5(Z)-enoic-mSI: a 14,15- and 5,6-EET antagonist in bovine coronary arteries. *Hypertension*. 2003; 42(4):555–561. [PubMed: 12953017]
42. Wu CC, Schwartzman ML. The role of 20-HETE in androgen-mediated hypertension. *Prostaglandins Other Lipid Mediat*. 2011; 96(1–4):45–53. [PubMed: 21722750]
43. Zhao X, et al. Salt-sensitive hypertension after exposure to angiotensin is associated with inability to upregulate renal epoxygenases. *Hypertension*. 2003; 42(4):775–780. [PubMed: 12900436]
44. Kaergel E, et al. P450-dependent arachidonic acid metabolism and angiotensin II-induced renal damage. *Hypertension*. 2002; 40(3):273–279. [PubMed: 12215466]
45. Ai D, Shyy JY, Zhu Y. Linking an insect enzyme to hypertension: angiotensin II-epoxide hydrolase interactions. *Kidney Int*. 2010; 77(2):88–92. [PubMed: 19847158]
46. Muller DN, et al. A peroxisome proliferator-activated receptor- α activator induces renal CYP2C23 activity and protects from angiotensin II-induced renal injury. *Am J Pathol*. 2004; 164:521–532. [PubMed: 14742258]
47. Cowart LA, et al. The CYP4A isoforms hydroxylate epoxyeicosatrienoic acids to form high affinity peroxisome proliferator-activated receptor ligands. *J Biol Chem*. 2002; 277:35105–35112. [PubMed: 12124379]

HIGHLIGHTS

- This study further explores the relationship between 20-HETE and RAS in hypertension using the angiotensinogen-deficient mice (Agt+/-).
- Angiotensinogen deficiency lessen androgen-mediated blood pressure increase despite the fact that androgen-induced expression of Cyp4a12, the sole 20-HETE synthesizing enzyme and the driving force of androgen-induced hypertension in mice, was not altered.
- The diminished hypertensive response to androgen in Agt+/- mice was associated with a moderated increase in vascular reactivity and a lack of impaired relaxation to acetylcholine.
- The increased capacity of microvessels to produce EETs in Agt+/- mice along with the limited RAS expression contributed to the diminished hypertensive response to androgen in the Agt+/- mice.

Figure 1. Androgen induced hypertension in WT and Agt^{+/-} mice

Agt mRNA (A). Systolic blood pressure of male or female DHT-treated WT or Agt^{+/-} mice (B). Systolic blood pressure of DHT-treated mice co-administered 20-6,15-HEDE (20-HEDE) after blood pressure plateaus at day 12 (C). Results are mean $\pm$ SE (n=3-4), * p<0.05 vs Placebo-treated mice, # p<0.05 vs DHT-treated mice.

Figure 2. The 20-HETE antagonist, 20-HEDE, or angiotensin converting enzyme inhibitor, lisinopril and AT1R blocker, losartan prevent androgen-induced hypertension in Agt+/- mice. Systolic blood pressure of WT (A) or Agt+/- (B) mice treated with DHT $\pm$ concurrent 20-HEDE, lisinopril or losartan treatment. Results are mean $\pm$ SE (n=3–4), * p<0.05 vs DHT+-treated mice, # p<0.05 vs DHT+20-HEDE-treated mice.

Figure 3. DHT treatment leads to increased Cyp4a12 and ACE expression

Representative Western blots and densitometry analysis of Cyp4a12 (A) and ACE (B) in renal interlobar arteries from WT and Agt^{+/-} mice treated for 18 days with placebo, DHT and DHT+20-HEDE (HEDE). Results are mean $\pm$ SE (n=3-4), * p<0.05 vs WT Placebo (P), # p<0.05 vs Agt^{+/-} Placebo (P), † p<0.05 vs WT DHT, ** p<0.05 vs AGT^{+/-} DHT.

Figure 4. Preglomerular microvessel 20-HETE production

20-HETE levels of renal interlobar arteries from WT and Agt +/- mice were measured at the end of the experiment as described. Results are mean $\pm$ SE (n=3–4), * p<0.05 vs Placebo-treated mice.

Figure 5. Preglomerular microvessel EET and DHET production

Respective EET (5,6-,8,9-, 11,12- and 14,15-EET) (A), EET+DHET (B), EET/DHET (C) and 14,15-EET/DHET (D) levels of renal interlobar arteries from WT and Agt +/- mice measured at the end of the experiment as described. Results are mean $\pm$ SE (n=3–4), * p<0.05 vs WT, # p<0.05 vs placebo-treated Agt +/- mice, § p<0.05 vs placebo-treated WT mice.

Table 1

Vascular Reactivity

Effect of DHT on WT and Agt+/- mice vascular reactivity in response to phenylephrine-induced constriction (EC50 (μM)) (A) and % relaxation to acetylcholine at 5 × 10⁻⁶ M Acetylcholine (B).

A				
EC50 (μM) to Phenylephrine	Placebo	DHT	DHT + 20-HEDE	DHT+ Lisinopril
WT Mice	0.50 ± 0.06	0.12 ± 0.03 *	0.60 ± 0.25 #	0.37 ± 0.09 **
Agt+/- Mice	0.56 ± 0.16	0.22 ± 0.06 * †	0.53 ± 0.14 #	0.28 ± 0.04 *

B				
% Relaxation (5 × 10 ⁻⁶ M Acetylcholine)	Placebo	DHT	DHT + 20-HEDE	DHT + 20-HEDE + 20-HEDE (ex vivo)
WT Mice	90 ± 2	26 ± 6 *	74 ± 9 #	34 ± 13 **
Agt+/- Mice	87 ± 2	77 ± 4	71 ± 8	48 ± 7 **

Results are mean ±SE (n=3-4).

* p<0.05 vs Placebo-treated mice,

p<0.05 vs DHT-treated mice,

† p<0.05 vs DHT-treated WT mice,

** p<0.05 vs DHT+20-HEDE-treated mice.