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6β-HYDROXYTESTOSTERONE, A CYTOCHROME P450 1B1 METABOLITE OF TESTOSTERONE, CONTRIBUTES TO ANGIOTENSIN II-INDUCED HYPERTENSION AND ITS PATHOGENESIS IN MALE MICE

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Abstract

Previously, we showed that *Cyp1b1* gene disruption minimizes angiotensin II-induced hypertension and associated pathophysiological changes in male mice. This study was conducted to test the hypothesis that cytochrome P450 1B1-generated metabolites of testosterone, 6β-hydroxytestoterone and 16α-hydroxytestosterone, contribute to angiotensin II-induced hypertension and its pathogenesis. Angiotensin II infusion for 2 weeks increased cardiac cytochrome P450 1B1 activity and plasma levels of 6β-hydroxytestosterone, but not 16α-hydroxytestosterone, in *Cyp1b1*$^{+/+}$ mice without altering *Cyp1b1* gene expression; these effects of angiotensin II were not observed in *Cyp1b1*$^{-/-}$ mice. Angiotensin II-induced increase in systolic blood pressure and associated cardiac hypertrophy, and fibrosis, measured by intracardiac accumulation of α-smooth muscle actin, collagen and transforming growth factor-β, and increased nicotinamide adenine dinucleotide phosphate oxidase activity and production of reactive oxygen species; these changes were minimized in *Cyp1b1*$^{-/-}$ or castrated *Cyp1b1*$^{+/+}$ mice, and restored by treatment with 6β-hydroxytestoterone. In *Cyp1b1*$^{+/+}$ mice, 6β-hydroxytestosterone did not alter the angiotensin II-induced increase in systolic blood pressure; the basal systolic blood pressure was also not affected by this agent in either genotype. Angiotensin II or castration did not alter cardiac, angiotensin II type 1 receptor, angiotensin converting enzyme, Mas receptor, or androgen receptor mRNA levels in *Cyp1b1*$^{+/+}$ or in *Cyp1b1*$^{-/-}$ mice. These data suggest that the testosterone metabolite, 6β-hydroxytestosterone, contributes to angiotensin II-induced hypertension and associated cardiac pathogenesis in male mice, most likely by acting as a permissive factor. Moreover, cytochrome P450 1B1 could serve as a novel target for developing

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Conflicts of Interest/Disclosures

None.

agents for treating renin-angiotensin and testosterone-dependent hypertension and associated pathogenesis in males.

Keywords

6β-hydroxytestosterone; CYP1B1; hypertension; castration; oxidative stress

Introduction

Hypertension is a major cause of cardiovascular disease, and studies in several animal models of hypertension, and epidemiological and clinical studies, have demonstrated a sexual dimorphism in the development of hypertension and cardiovascular disease.[1–4] Sex differences that appear at puberty are maintained throughout adulthood.[5] Men have a higher risk of developing hypertension than do premenopausal women of the same age.[6] These sex differences in blood pressure (BP) control have been attributed to sex chromosomes and gonadal hormones.[7] Angiotensin (Ang) II also increases BP to a greater degree in males than in females, and castration protects males, whereas ovariectomy prevents protection in female mice against Ang II-induced hypertension.[8] Castration or testosterone receptor antagonists also reduce blood pressure in spontaneously hypertensive rats (SHR) or Dahl-salt sensitive rats.[9–11] However, the mechanism by which testosterone contributes to Ang II-induced hypertension is not known. In male SHR or Dahl-salt sensitive rats, the rise in BP has been attributed to the effect of testosterone to increase plasma renin levels and hepatic and/or renal angiotensinogen mRNA expression[12] and oxidative stress.[13–15] Sex-specific differences in BP have also been observed in *Cyp4a14*$^{-/-}$ mice (an increase in males but not females).[16] Hypertension in male *Cyp4a14*$^{-/-}$ mice that was attributed to increased expression of CYP4A12A and associated increase in testosterone and its metabolite dihydrotestosterone (DHT), and production of 20-hydroxyeicosatetraenoic acid (20-HETE), a prohypertensive eicosanoid, was abolished by castration.[16] Administration of DHT to rats increases renal expression of CYP4A2, a mouse homologue of CYP4A14, and conversion of arachidonic acid to 20-HETE that increases vascular tone, response to vasoconstrictor agents, and inhibition of nitric oxide synthesis, oxidative stress, endothelial dysfunction, and BP.[17]

Previous studies revealed that CYP1B1, which is highly expressed in various extra-hepatic tissues including the cardiovascular system,[18] contributes to Ang II and deoxycorticosterone acetate salt-induced hypertension in male mice or rats[19–22] and in SHR[23] and associated cardiovascular and renal pathogenesis, most likely via increased oxidative stress. However, in female mice where Ang II-induced increase in BP and associated pathophysiological changes are minimized as compared to male mice, inhibition of CYP1B1 activity with 2,3′, 4,5′-tetramethoxystilbene (TMS) or *Cyp1b1* gene disruption (*Cyp1b1*$^{-/-}$) produced opposite effects and increased BP and associated pathophysiological changes that were not different from those observed in male mice.[24] The protective effect of CYP1B1 against Ang II-induced hypertension and its pathogenesis in female mice was minimized by 2-methoxyestradiol that is generated from an estradiol metabolite of *Cyp1b1*, 2-OH estradiol, by catechol-O-methyltransferase.[25,26] Since testosterone can also be metabolized by

CYP1B1 into 6β-hydroxytestosterone (6β-OHT) and 16α-hydroxytestosterone (16α-OHT),[27,28] it led us to hypothesize that these testosterone metabolites contribute to Ang II-induced hypertension and associated cardiovascular pathophysiological changes in male mice. To test this hypothesis, we examined the effect of Ang II on plasma levels of testosterone and its metabolites and effect of the testosterone metabolites of CYP1B1, 6β-OHT and 16α-OHT, on Ang II-induced hypertension and associated cardiovascular pathological changes in intact and castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ male mice. The results show that Ang II selectively increased plasma levels of 6β-OHT in *Cyp1b1*$^{+/+}$ mice but not in *Cyp1b1*$^{-/-}$ or castrated mice. Moreover, Ang II-induced increase in systolic blood pressure (SBP) and associated cardiac pathophysiological changes, including fibrosis and oxidative stress that were minimized in *Cyp1b1*$^{-/-}$ and castrated *Cyp1b1*$^{+/+}$ mice, were reversed by 6β-OHT.

Methods

For detailed methods, see the online-only data supplement http://hyper.ahajournals.org.

Results

Ang II Infusion Increased Cardiac CYP1B1 Activity and Plasma Levels of its Testosterone Metabolite, 6β-OHT, in *Cyp1b1*$^{+/+}$ but Not *Cyp1b1*$^{-/-}$ Mice

Ang II infusion increased cardiac CYP1B1 activity without altering *Cyp1b1* mRNA expression in *Cyp1b1*$^{+/+}$ mice (Figure 1A, 1B). Ang II infusion also increased plasma levels of 6β-OHT but did not alter that of testosterone, DHT, or 16α-OHT in *Cyp1b1*$^{+/+}$ mice as measured by ultra-performance liquid chromatography-coupled with-quadrupole time of flight-mass spectrometer (UPLC-qTOFMS) (Table 1). The coefficient correlation of standard curves prepared from different concentrations of testosterone and its metabolites, and the representative ion chromatograms of authentic testosterone and its metabolites and their plasma levels from *Cyp1b1*$^{+/+}$ mice infused with Ang II, analyzed by UPLC-qTOFMS are shown in Table S1 and Figure S1, respectively. In *Cyp1b1*$^{-/-}$ mice, the basal plasma levels of testosterone, DHT, and 16α-OHT were not significantly different compared to the corresponding levels of these steroids observed in *Cyp1b1*$^{+/+}$ mice; 6β-OHT was not detected. During infusion of Ang II in *Cyp1b1*$^{-/-}$ mice, the plasma levels of testosterone, but not DHT or 16α-OHT, was reduced compared to corresponding values obtained in *Cyp1b1*$^{+/+}$ mice; 6β-OHT was not detected.

Cyp1b1 Gene Disruption Minimized Ang II-Induced Increase in Systolic Blood Pressure (SBP)

Infusion of Ang II (700 ng/kg/min) for 2 weeks increased SBP, measured by tail cuff every 3rd day, in *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice, but the increase was significantly less in *Cyp1b1*$^{-/-}$ than in *Cyp1b1*$^{+/+}$ mice (Figure 2A). The difference in SBP increase observed between these two groups was consistent, reproducible, and similar to that reported previously.[19]

6β-OHT Treatment Restored Ang II-Induced Hypertension in *Cyp1b1*$^{-/-}$ and Castrated *Cyp1b1*$^{+/+}$ Mice

To investigate whether testosterone metabolites generated by CYP1B1 contribute to Ang II-induced increase in SBP in male mice, we examined the effect of 6β-OHT and 16α-OHT on the actions of Ang II on SBP in intact and castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice. The ability of Ang II to increase SBP is significantly reduced in *Cyp1b1*$^{-/-}$ mice compared to that observed in *Cyp1b1*$^{+/+}$ mice,[19] which we confirmed in this study. Administration of 6β-OHT (Figure 2B), but not 16α-OHT (Figure S2), in *Cyp1b1*$^{-/-}$ mice restored the ability of Ang II to increase SBP to levels not different from those obtained in *Cyp1b1*$^{+/+}$ mice. It is well established that the ability of Ang II to increase SBP in male mice is diminished by castration.[8] Ang II infusion in castrated *Cyp1b1*$^{+/+}$ mice produced a small increase in SBP on 3rd and 6th day which was diminished by the 9th and 12th day of infusion to levels not different from those observed in intact or castrated non-Ang II-infused *Cyp1b1*$^{+/+}$ mice. The Ang II-induced increase in SBP that was reduced in *Cyp1b1*$^{-/-}$ mice (Figure 3A), was further diminished and reached levels at the 12th day not different from those of intact or castrated *Cyp1b1*$^{-/-}$ mice (Figure 3B). Castration did not alter basal BP in either *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice. In castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice, administration of 6β-OHT (Figure 3C and 3D) restored the hypertensive effect of Ang II to levels similar to those observed in intact *Cyp1b1*$^{+/+}$ mice without any change in SBP in animals treated with only 6β-OHT. Since Ang II did not alter plasma levels of 16α-OHT in *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice, and 16α-OHT did not restore the effect of Ang II on SBP in *Cyp1b1*$^{-/-}$ mice (Figure S2), we did not investigate its effect on cardiac remodeling or in castrated mice.

Cyp1b1 Gene Disruption Attenuated Cardiac Hypertrophy and Fibrosis Associated with Ang II-Induced Hypertension, which was Reversed by 6β-OHT

Infusion of Ang II increased heart:body weight ratio (HW/BW), an indicator of cardiac hypertrophy, in *Cyp1b1*$^{+/+}$, which was diminished in *Cyp1b1*$^{-/-}$ mice (Table S2). Hearts from Ang II-infused *Cyp1b1*$^{+/+}$ mice, but not from *Cyp1b1*$^{-/-}$ mice, also displayed fibrosis as indicated by α-smooth muscle actin (α-SMA) positive myofibroblasts and collagen deposition in the myocardium and increased transforming growth factor (TGF)-β staining cells (Figure 4A). Treatment with 6β-OHT did not alter these effects of Ang II in *Cyp1b1*$^{+/+}$ mice but restored them in *Cyp1b1*$^{-/-}$ mice (Table S2 and Figure 4B).

Castration Reduced Cardiac Hypertrophy and Fibrosis Associated with Ang II-Induced Hypertension, which was Prevented by 6β-OHT Treatment

Eight week old mice were castrated, and after a 7-day washout period for the depletion of residual testosterone, the mice were divided into two groups and infused with vehicle or Ang II as described in methods. Castration reduced Ang II-induced increase in HW/BW ratio (Table S2), α-SMA, collagen deposition and TGF-β staining in *Cyp1b1*$^{+/+}$ mice; these effects of Ang II that were minimized in *Cyp1b1*$^{-/-}$ mice were abolished by castration (Figure S3). Concurrent treatment with 6β-OHT restored the ability of Ang II to cause cardiac hypertrophy (Table S2) and fibrosis in castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice (Figure S4). 6β-OHT alone did not cause cardiac hypertrophy or fibrosis in either intact or castrated mice of these genotypes (Figure 4B and Figure S4).

6β-OHT Restored the Ability of Ang II to Increase Cardiac Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NADPH) Activity and Production of Reactive Oxygen Species (ROS), which was Diminished in Male *Cyp1b1*$^{-/-}$ Mice

ROS have been implicated in hypertension and cardiovascular dysfunction in male models of experimental hypertension.[14] Ang II infusion increased cardiac NADPH oxidase activity, and ROS production as indicated by increased cardiac 2-hydroxyethidium fluorescence, in *Cyp1b1*$^{+/+}$, but not *Cyp1b1*$^{-/-}$ mice (Figure 5 upper panel A–C). Treatment with 6β-OHT did not alter Ang II-induced increase in cardiac NADPH oxidase activity or ROS production in *Cyp1b1*$^{+/+}$ mice, but restored the loss of this action of Ang II in *Cyp1b1*$^{-/-}$ mice (Figure 5 lower panel A–C). 6β-OHT did not alter basal levels of NADPH oxidase activity or ROS production in *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice (Figure 5 lower panel A–C).

6β-OHT Restored Loss of Effect of Ang II-Induced Increase in Cardiac NADPH Oxidase Activity and ROS Production in Castrated Male *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ Mice

Castration is known to reduce oxidative stress in SHR rats. [14] The effect of Ang II to increase cardiac NADPH oxidase activity or ROS production was diminished in, as indicated by decrease in 2-hydroxyethidium fluorescence in *Cyp1b1*$^{+/+}$ mice (Figure S5 A–C). In intact *Cyp1b1*$^{-/-}$ mice where Ang II did not increase cardiac NADPH oxidase activity or ROS production (Figure 5 upper panel A–C), were not altered by castration (Figure S5 A–C). Treatment with 6β-OHT restored the ability of Ang II to increase cardiac NADPH oxidase activity and ROS production in castrated *Cyp1b1*$^{+/+}$ *and Cyp1b1*$^{-/-}$ mice (Figure S6 A–C). 6β-OHT did not alter the basal NADPH oxidase activity or ROS production in castrated *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice (Figure S6 A–C).

Cyp1b1 Gene Disruption or Castration Did Not Alter Cardiac Angiotensin-Converting Enzyme (ACE), Ang II Type 1 Receptor (AT1a), and Mas Receptors and Androgen Receptor (AR) or CYP4A12A mRNA Expression in Ang II-Infused Male Mice

To determine whether alterations in Ang II actions in *Cyp1b1*$^{-/-}$ or castrated *Cyp1b1*$^{+/+}$ mice are due to changes in *At1a* receptor, *Ace*, *Mas* receptor, *Ar*, or *Cyp4a12a* mRNA expression, we measured their respective mRNA expression in cardiac tissue. Cardiac *At1a* receptor, *Ace*, *Mas* and *Ar* receptor mRNA expression were not altered in intact (Figure S7 A–D) or castrated (Figure S8 A–D) *Cyp1b1*$^{-/-}$ and *Cyp1b1*$^{+/+}$ mice during infusion of Ang II or its vehicle. *Cyp4a12a* expression was not detected in cardiac tissues of *Cyp1b1*$^{-/-}$ or *Cyp1b1*$^{+/+}$ mice (data not shown).

Discussion

This study demonstrates for the first time that CYP1B1 maintains testosterone levels during Ang II infusion, and Ang II selectively stimulates production of 6β-OHT, which contributes to its hypertensive effect and associated cardiac hypertrophy, fibrosis, and oxidative stress in male mice. Previously, we showed that *Cyp1b1* gene disruption or inhibition of CYP1B1 activity with TMS minimized Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress.[19] However, the mechanism by which CYP1B1 contributes to Ang II-induced hypertension was not known. Since CYP1B1 is constitutively active, it needs a substrate to produce a product that mediates and/or modulates the effect of

Ang II. CYP1B1 can metabolize several substrates including retinoids, fatty acids, and steroids.[29–31] Therefore, more than one product generated from these substrates could contribute to the CYP1B1-dependent hypertensive effect of Ang II and associated pathogenesis. Testosterone is known to promote cardiac hypertrophy and fibrosis[32] and to contribute to Ang II-induced hypertension.[8] In hypertrophic human and SHR left ventricles, the expression of AR and the metabolism of testosterone into more than one metabolite including 6β-OHT, but not 16α-OHT, is increased, and is diminished in humans with a left ventricle-assisted device.[33] Testosterone is also metabolized into 6β-OHT in adult rat cultured myocytes.[34] In the present study in male *Cyp1b1*$^{+/+}$ mice, Ang II infusion selectively increased plasma levels of testosterone metabolite of CYP1B1 6β-OHT, but not testosterone, DHT, or 16α-OHT. The lack of reduction in plasma levels of testosterone despite an increase in 6β-OHT levels in *Cyp1b1*$^{+/+}$ mice, suggests that testosterone levels are maintained most likely by its increased production by Ang II. Supporting this view was our observation that in *Cyp1b1*$^{-/-}$ mice, the plasma level of testosterone during Ang II infusion was markedly diminished and 6β-OHT was undetectable. The plasma levels of DHT were also reduced by Ang II but it did not reach statistical significance; the basal plasma levels of testosterone, DHT, and 16α-OHT levels were not altered, and 6β-OHT was undetectable in *Cyp1b1*$^{-/-}$ mice. The mechanism by which CYP1B1 contributes to testosterone production by Ang II that could involve hydroxylation of one or more of its precursors remains to be determined. Moreover, further studies of the measurement of protein bound and tissue levels of testosterone are required to address an insignificant effect of Ang II on plasma levels of DHT despite significant decrease in testosterone levels in *Cyp1b1*$^{-/-}$ mice. Our demonstration that the ability of Ang II to selectively stimulate production of 6β-OHT, but not 16α-OHT, in *Cyp1b1*$^{+/+}$ mice that was abolished by *Cyp1b1* gene disruption raises the possibility that it might contribute to Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress. Therefore, we examined the effect of 6β-OHT on Ang II-induced hypertension and associated cardiac effects. Administration of 6β-OHT did not alter the effect of Ang II to increase SBP and associated cardiac hypertrophy as determined by increased HW/BW ratio in male *Cyp1b1*$^{+/+}$ mice. It also failed to alter the effect of Ang II to cause cardiac fibrosis as assessed by increased intracardiac accumulation of α-SMA, TGF-β, or collagen and oxidative stress as determined by increased cardiac NADPH oxidase activity and ROS production in *Cyp1b1*$^{+/+}$ mice. However, these effects of Ang II that were minimized in male *Cyp1b1*$^{-/-}$ mice were restored by concurrent treatment with 6β-OHT, indicating that this CYP1B1 testosterone metabolite contributes to Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress in male *Cyp1b1*$^{+/+}$ mice. The effect of 6β-OHT in restoring the effects of Ang II to increase SBP and associated cardiac effects in *Cyp1b1*$^{-/-}$ mice were selective, because the CYP1B1-generated testosterone metabolite 16α-OHT failed to restore these effects of Ang II in male *Cyp1b1*$^{-/-}$ mice. Although recombinant CYP1B1 *in vitro* produces 16α-OHT,[28] *Cyp1b1* gene disruption did not alter plasma levels of 16α-OHT during infusion of Ang II or its vehicle, suggesting that it is most likely produced by ≥ 1 CYPs other than CYP1B1 in male mice.

Supporting evidence for the role of the testosterone metabolite, 6β-OHT, in Ang II-induced hypertension and its associated cardiac effects was obtained in castrated mice. In castrated

Cyp1b1$^{+/+}$ mice, Ang II-induced increase in SBP was minimized at days 3 and 6 and thereafter abolished at 12th day of Ang II infusion. In *Cyp1b1*$^{-/-}$ mice, a component of Ang II-induced increase in SBP that was independent of CYP1B1 was also abolished by castration at 12th day of Ang II infusion. However, concurrent administration of 6β-OHT restored Ang II-induced increase in SBP and associated cardiac hypertrophy, fibrosis, and oxidative stress in both castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice. Since testosterone can be also metabolized into 6β-OHT by CYP3A4,[35] the *Cyp1b1*-independent, but gonad-dependent, component of Ang II-induced increase in SBP in mice could be due to formation of 6β-OHT by *Cyp3a4*. However, it is unlikely, because Ang II-induced production of 6β-OHT was abolished in *Cyp1b1*$^{-/-}$ mice. Therefore, the CYP1B1-independent component of Ang II-induced increase in SBP could be due to the direct effect of the fraction of testosterone not metabolized by CYP1B1 and of DHT on AR to modulate the effect of Ang II. The effect of testosterone is unlikely to be due to its conversion to DHT, because Ang II did not stimulate production of DHT in *Cyp1b1*$^{+/+}$ mice or decrease its levels in *Cyp1b1*$^{-/-}$ mice. Also, AR antagonist, flutamide, but not finasteride, an inhibitor of 5α reductase that converts testosterone into DHT, lowers BP in SHR.[10] Exogenous DHT that increases renal vascular expression in male rats of CYP4A8, and its orthologue CYP4A12 in male mice, stimulates 20-HETE production and increases the activity of the renin-angiotensin system and BP.[36–38] Moreover, DHT-induced hypertension is minimized in angiotensinogen deficient mice.[39] However, *Cyp1b1* gene disruption in male mice does not alter renal expression of CYP4A1/A2/A3 (rat orthologues of mouse CYP4A10/12/14).[20] In the present study, expression of *Cyp4a12a* mRNA was also not detected in the heart after infusion of Ang II or its vehicle in male *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice. Therefore, DHT does not appear to contribute to Ang II-induced hypertension in male mice.

The ability of *Cyp1b1* gene disruption and castration to inhibit Ang II-induced hypertension and associated cardiac effects and their restoration by 6β-OHT could be due to alterations in expression of the pressor (AT1a receptor, ACE) or depressor (Mas receptor) components of the renin-angiotensin system. However, it is unlikely, because neither *Cyp1b1* gene disruption nor castration altered cardiac expression of mRNA of AT1a receptor and ACE or Mas receptor expression in mice.

The restoration by 6β-OHT of cardiac remodeling by Ang II in *Cyp1b1*$^{-/-}$ mice and castrated *Cyp1b1*$^{+/+}$ mice could be due to restoration of the effect of Ang II on BP. Although we cannot exclude this possibility, Ang II also produces cardiac remodeling independent of an increase in BP.[40] Although in our study 6β-OHT restored the hypertensive effects of Ang II cardiac hypertrophy, fibrosis, and oxidative stress in *Cyp1b1*$^{-/-}$ mice and in castrated *Cyp1b1*$^{+/+}$ mice, it did not alter the basal BP, suggesting that 6β-OHT acts as a permissive factor in the development of Ang II-induced hypertension and associated cardiac effects. The mechanism by which 6β-OHT modulates the effects of Ang II is not known. Whether the effect of 6β-OHT to restore Ang II actions is mediated via genomic or non-genomic AR[41,42] remains to be determined. Restoration by 6β-OHT of Ang II actions in *Cyp1b1* gene-disrupted or castrated mice was not due to alterations in expression of AR, because mRNA expression of AR was not altered in these mice. Testosterone-dependent enhanced pressor response to Ang II in growth-restricted[43] and

increased uterine arterial contraction to Ang II in testosterone-treated pregnant rats has also been observed.[44] Ang II produces vascular contraction and hypertension by increasing RhoA activity via Jak2-induced phosphorylation of Rho exchange factor Arhgef1 in mice.[45] Moreover, testosterone-dependent renal vascular response to Ang II in New Zealand genetic hypertensive male rats has been attributed to upregulation of Rho kinase signaling pathway.[46] Therefore, further studies are required to determine a) if 6β-OHT contributes to the actions of elevated testosterone levels on vascular effects of Ang II, b) role of 6β-OHT in the actions of Ang II including endothelial and renal dysfunction and end-organ damage and the possible role of Rho kinase signaling pathway in male mice, c) contribution of 6β-OHT to the vascular actions of other vasoconstrictor agents, norepinephrine, endothelin-1 and vasopressin, and to the other models of experimental hypertension and SHR. An androgen-dependent increased sensitivity to Ang II-induced renal damage and hypertension has also been demonstrated by early life stress caused by maternal-pup separation in male rats.[47] This androgen-dependent increased sensitivity to Ang II-induced hypertension by early life stress that could be mediated by 6β-OHT remains to be determined. Moreover, the ability of peripherally administered Ang II to increase SBP is mediated by its action in subfornical organ via generation of superoxides,[48] in the kidney,[49] and activation of immune cells.[50] Therefore, it is possible that the effect of 6β-OHT to restore Ang II-induced hypertension in *Cyp1b1*$^{-/-}$ and castrated male mice could be mediated by its action to modulate the effect of Ang II in the brain, immune cells, and the kidney. Since CYP1B1[51] and AR are present in the brain,[52] 6β-OHT could also be generated locally in the brain and modulate the central actions of peripheral Ang II.

In conclusion, this study provides evidence that the testosterone metabolite of *Cyp1b1* 6β-OHT formed by Ang II acts as a permissive factor and contributes to the hypertensive effect of this peptide and associated cardiac hypertrophy, fibrosis, increased NADPH oxidase activity, and oxidative stress in male mice. In contrast, we have shown that, in female mice, CYP1B1 protects against Ang II-induced hypertension and its pathogenesis via metabolism of estradiol into 2-hydroxyestradiol and subsequently its metabolism by catechol-O-methyltransferase into 2-methoxyestradiol that acts as a permissive factor to suppress Ang II actions.[24] However, recently the effect of sex chromosome complement has been implicated in Ang II-induced hypertension,[7] Therefore, it appears that, in addition to sex chromosomes, 17β-estradiol and testosterone metabolites of CYP1B1 contribute to sex differences in Ang II-induced hypertension and associated pathogenesis in male and female mice, respectively. Finally, CYP1B1 could serve as a novel target for developing agents that inhibit CYP1B1 for treating hypertension and associated pathogenesis in males, but inhibitors of CYP1B1 could be detrimental in treating hypertension in females.

Perspectives

Sex differences in BP in various models of experimental hypertension have been attributed to sex chromosome complement and female and male sex hormones. The present study provides the first evidence that the CYP1B1 metabolite of testosterone, 6β-OHT, in male mice contributes to Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress, most likely by acting as a permissive factor. Therefore, characterization of the interaction of 6β-OHT with testosterone receptor and underlying

mechanism of its action to promote Ang II-induced hypertension and development of selective inhibitors of the effects of 6β-OHT and CYP1B1 activity could be useful for treating hypertension and associated pathogenesis in males.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What is New?

- Demonstration for the first time that CYP1B1 participates in maintaining testosterone plasma levels during Ang II infusion in male mice.
- Provides first evidence that Ang II selectively stimulates production of CYP1B1-generated metabolite of testosterone, 6β-OHT, which contributes to hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress caused by Ang II in male mice.
- 6β-OHT contributes to Ang II-induced hypertension by acting as a permissive factor in male mice.

What is Relevant?

- The results of this study advance our knowledge of the mechanism by which CYP1B1, through metabolism of testosterone to 6β-OHT, contributes to Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress in males.
- Furthermore, it has an important pathophysiological significance in hypertension and cardiac disease such as heart failure associated with increased activity of the renin-angiotensin system and increased use of testosterone, whereby production of 6β-OHT would promote cardiac hypertrophy, fibrosis, and oxidative stress in males.
- Finally, our study also has translational significance in developing selective CYP1B1 inhibitors for treating renin-angiotensin and testosterone-dependent hypertension in men.

Summary

Cyp1b1 gene disruption or castration in *Cyp1b1*$^{+/+}$mice minimized Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress. Ang II increased cardiac CYP1B1 activity and stimulated production of CYP1B1-generated testosterone metabolite, 6β-OHT, and not 16α-OHT. Administration of 6β-OHT to *Cyp1b1*$^{-/-}$ or castrated *Cyp1b1*$^{+/+}$mice restored Ang II-induced hypertension and associated cardiac hypertrophy, fibrosis, and oxidative stress. Since 6β-OHT did not alter basal BP in *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice, it appears that 6β-OHT acts as a permissive factor in Ang II actions to cause hypertension and associated cardiac pathogenesis in male mice. Therefore, developing selective inhibitors of CYP1B1 could be useful for treating renin-angiotensin and testosterone-dependent hypertension in males.

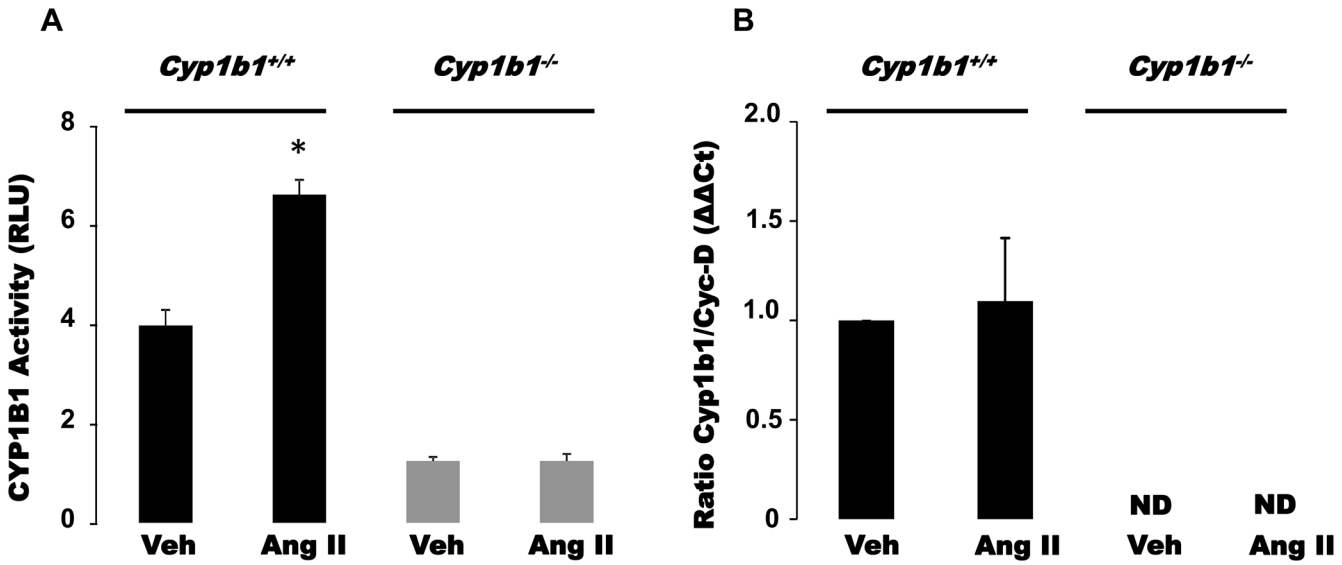

Figure 1. Angiotensin II (Ang II) increased cardiac cytochrome P450 (CYP) 1B1 activity in *Cyp1b1*$^{+/+}$ mice without altering *Cyp1b1* gene expression

Cyp1b1$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were infused with vehicle or Ang II for 2 weeks. At the end of Ang II or its vehicle (Veh) infusion, cardiac tissue was collected to measure CYP1B1 activity using the P450-Glo assay kit and expressed as relative luminescence units (RLU) (A). *Cyp1b1* mRNA expression was determined by real time PCR. *Cyp1b1* mRNA expression was normalized against cyclophilin-D (Cyc-D) (B). **p*<0.05 vehicle vs. corresponding value from Ang II-infused animals (n=3–5 for all experiments; data are expressed as mean±SEM).

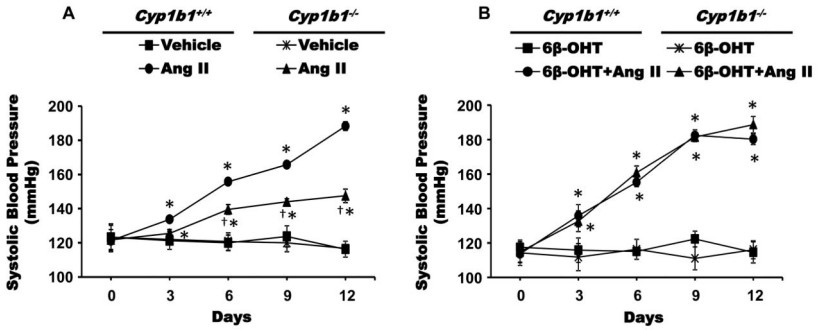

Figure 2. *Cyp1b1* gene disruption reduced hypertensive effect of angiotensin (Ang II), which was restored by 6β-hydroxytestosterone (6β-OHT)

Cyp1b1$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were infused with Ang II or vehicle for 2 weeks and given intraperitoneal injections of the *Cyp1b1*-derived metabolite of testosterone, 6β-OHT (15 μg/g, i.p.) every 3rd day for 2 weeks. (A) and (B) systolic blood pressure was measured by tail cuff method twice weekly. *p<0.05 Ang II vs vehicle, Ang II+ 6β-OHT vs. vehicle+ 6β-OHT; †p<0.05, *Cyp1b1*$^{+/+}$ Ang II vs. *Cyp1b1*$^{-/-}$ Ang II (n=4–5 for all experiments; data are expressed as mean±SEM).

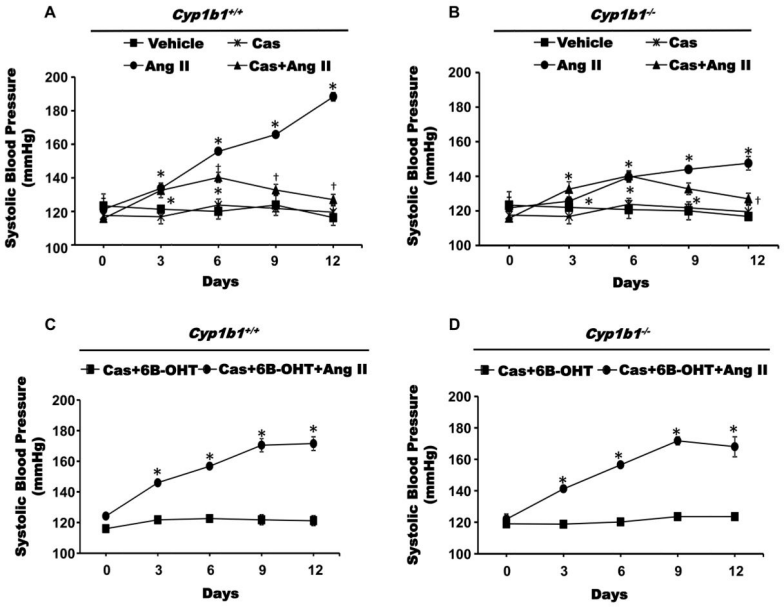

Figure 3. Castration (Cas) reduced the hypertensive effect of angiotensin II (Ang II) in *Cyp1b1*$^{+/+}$, which was restored by 6β-hydroxytestosterone (6β-OHT)

Cyp1b1$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were sham operated or castrated and subsequently infused with Ang II or vehicle for 2 weeks and given 6β-OHT, as described in Figure 2. (A) and (B) systolic blood pressure was measured by tail cuff method twice weekly. *p<0.05 Ang II vs. vehicle, Cas+Ang II vs. Cas, and Cas+6β-OHT+Ang II vs. Cas+6β-OHT; †p<0.05, Cas +Ang II vs. Ang II (n=4–5 for all experiments; data are expressed as mean±SEM).

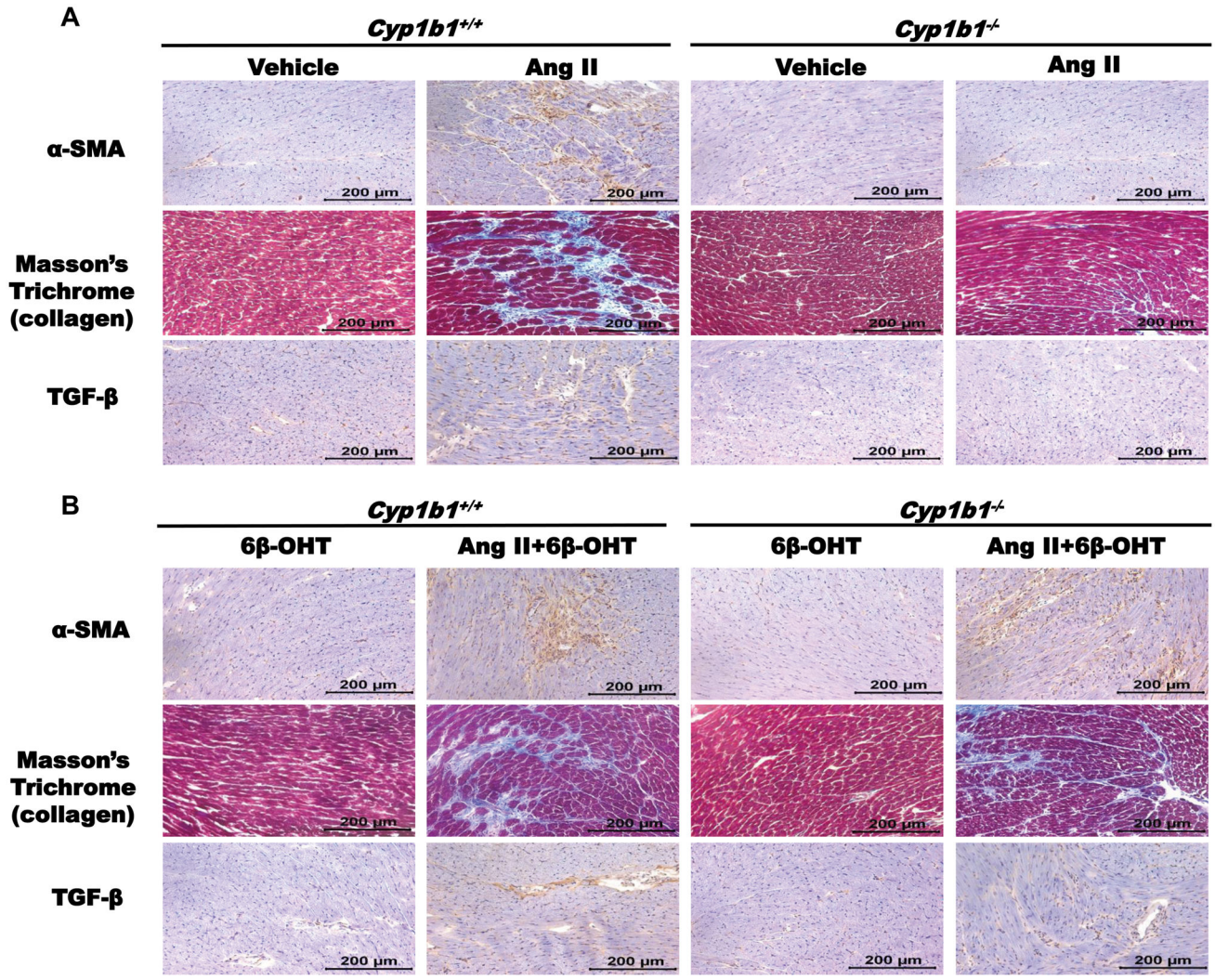

Figure 4. *Cyp1b1* gene disruption minimizes angiotensin II (Ang II)-induced cardiac fibrosis which is restored by 6β-hydroxytestosterone (6β-OHT)

Mice infused with vehicle or Ang II for 2 weeks and injected with 6β-OHT as described in methods. After the end of Ang II infusion, heart was removed and processed for immunohistochemistry. (A) α-smooth muscle actin (α-SMA) positive myofibroblasts, Masson's trichrome staining revealed intracardiac collagen deposition (intense blue staining) and intracardiac transforming growth factor-β (TGF-β). Ang II infusion in *Cyp1b1*$^{-/-}$ mice treated with 6β-OHT but not in mice treated with 6β-OHT alone, produced cardiac fibrosis in *Cyp1b1*$^{-/-}$ mice as evident by (B) α-smooth muscle actin (α-SMA) positive myofibroblasts, collagen deposition and intracardiac transforming growth factor-β (TGF-β). Photomicrographs are representative images of at least three animals from each experimental group.

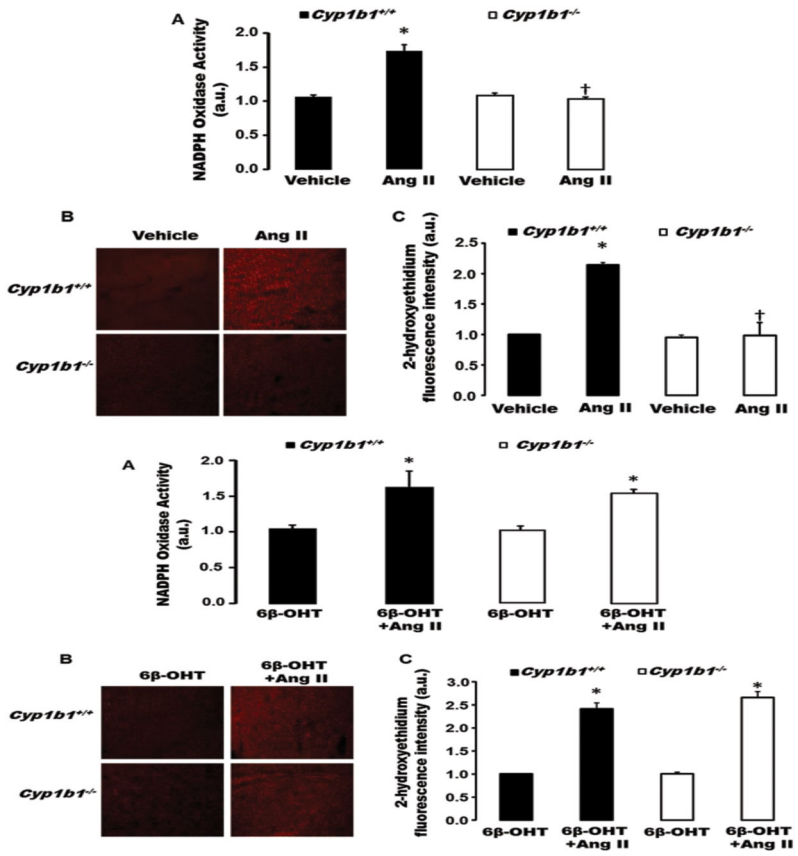

Figure 5. *Cyp1b1* gene disruption minimizes angiotensin II (Ang II)-induced cardiac nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and superoxide production in *Cyp1b1*$^{-/-}$ mice which is reversed by 6β-hydroxytestosterone (6β-OHT)

Cyp1b1$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were infused with vehicle or Ang II (upper panel) and treated with 6β-OHT or 6β-OHT+Ang II (lower panel) for 2 weeks. (A) NADPH oxidase activity was measured in cardiac homogenates. (B) Cardiac superoxide production was determined by the fluorescence intensity of 2-hydoxyethidium. Photomicrographs are representative of hearts from mice in each of the different treatment groups following incubation with dihydroethidium. (C) Graph depicts quantified data. *p<0.05 Ang II vs. vehicle; †p<0.05 *Cyp1b1*$^{-/-}$ Ang II vs. *Cyp1b1*$^{+/+}$ Ang II (n=4 for all experiments and data are expressed as mean±SEM).

Table 1

Plasma levels (ng/ml) of testosterone and its metabolites in *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice.

	***Cyp1b1*$^{+/+}$**		***Cyp1b1*$^{-/-}$**	
Compound	**Vehicle**	**Ang II**	**Vehicle**	**Ang II**
Testosterone	1.45±0.27	1.43±0.19	0.75±0.06	0.01±0.00†
Dihydrotestosterone	0.72±0.23	1.05±0.27	0.56±0.14	0.33±0.05†
6β-hydroxytestosterone	Undetected	0.50±0.0*	Undetected	Undetected
16α-hydroxytestosterone	0.20±0.14	0.24±0.12	0.10±0.05	0.12±0.08

At the termination of Ang II infusion, plasma was collected and analyzed for levels of testosterone and its metabolites by ultra-performance liquid chromatography-coupled with quadrupole time of flight-mass spectrometer (UPLC-QTOFMS) (Table 1).

*P<0.05 vehicle vs. corresponding value from Ang II-infused animals;

†p < 0.05, *Cyp1b1*$^{+/+}$ Ang II vs. *Cyp1b1*$^{-/-}$ Ang II (n=3–5 for all experiments; data are expressed as mean±SEM).