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6β-HYDROXYTESTOSTERONE, A CYTOCHROME P450 1B1-TESTOSTERONE-METABOLITE, MEDIATES ANGIOTENSIN II-INDUCED RENAL DYSFUNCTION IN MALE MICE

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Abstract

6β-hydroxytestosterone, a cytochrome P450 1B1-derived metabolite of testosterone, contributes to the development of angiotensin II-induced hypertension and associated cardiovascular pathophysiology. In view of the critical role of angiotensin II in the maintenance of renal homeostasis, development of hypertension and end organ damage, this study was conducted to determine the contribution of 6β-hydroxytestosterone to angiotensin II actions on water consumption and renal function in male *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice. Castration of *Cyp1b1*$^{+/+}$ mice or *Cyp1b1*$^{-/-}$ gene disruption minimized the angiotensin II-induced increase in water consumption, urine output, proteinuria, and sodium excretion and decreases in urine osmolality. 6β-hydroxytestosterone did not alter angiotensin II-induced increases in water intake, urine output, proteinuria, and sodium excretion or decreases in osmolality in *Cyp1b1*$^{+/+}$ mice, but restored these effects of angiotensin II in *Cyp1b1*$^{-/-}$ or castrated mice *Cyp1b1*$^{+/+}$ mice. *Cyp1b1* gene disruption or castration prevented angiotensin II-induced renal fibrosis, oxidative stress, inflammation, urinary excretion of angiotensinogen, expression of angiotensin II type 1 receptor, and angiotensin converting enzyme. 6β-hydroxytestosterone did not alter angiotensin II-induced renal fibrosis, inflammation, oxidative stress, urinary excretion angiotensinogen, expression of angiotensin II type 1 receptor, or angiotensin converting enzyme in *Cyp1b1*$^{+/+}$ mice; however, in *Cyp1b1*$^{-/-}$ or castrated mice *Cyp1b1*$^{+/+}$ mice, it restored these effects of angiotensin II. These data indicate that 6β-hydroxytestosterone contributes to increased thirst, impairment of renal function, and end

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Conflicts of Interest/Disclosures

None.

organ injury associated with angiotensin II-induced hypertension in male mice and that cytochrome P450 1B1 could serve as a novel target for treating renal disease and hypertension in males.

Keywords

6β-hydroxytestosterone; CYP1B1; renal dysfunction; fibrosis; urinary angiotensinogen

Introduction

Men are more prone to develop hypertension and renal dysfunction compared to premenopausal women of the same age.[1–3] Sexual dimorphism in hypertension and renal dysfunction was demonstrated in various animal models of hypertension including angiotensin II (Ang II) and deoxycorticosterone acetate (DOCA)-salt-induced hypertension, Dahl salt-sensitive, and spontaneously hypertensive rats (SHR). In particular, males are more likely to develop hypertension and renal dysfunction compared to females in these models.[4–10] Testosterone stimulates the expression of renal angiotensinogen (AGT) or urinary excretion of AGT in SHRs and in Ang II-high salt (HS) diet-induced hypertension.[11–13] Moreover, castration decreases expression of renal renin and AGT and hepatic AGT in male SHRs.[11,12] Ang II infusion also increases Ang II type 1 receptor (AT1a) expression in male SHRs[14] or alters the Ang II type 2 receptor (AT2) to AT1a receptor balance in kidneys of male SHRs.[15] Furthermore, Ang II decreases the expression of Mas receptor and angiotensin converting enzyme 2 (ACE 2) mRNA in male SHR and Sprague-Dawley (SD) rats.[14,16] Dihydrotestosterone (DHT)-induced hypertension in rats expressing endothelial Cytochrome P-450 4A2 (CYP4A2) depends on the prohypertensive eicosanoid 20-hydroxyeicosatetraenoic acid (20-HETE),[17] and is associated with increased vascular expression of angiotensin converting enzyme (ACE).[18] Hypertension is also associated with inflammation.[19–21] In males, Ang II infusion increases vascular and renal infiltration of $CD4^+$ and $CD8^+$ T-lymphocytes.[21, 22] Adaptive transfer of T-cells from male mice into male *Rag1*$^{-/-}$ mice increases blood pressure (BP), but transfer of T-cells from female mice does not elicit a similar response.[23] These observations indicate that in males, testosterone downregulates the antihypertensive components and upregulates the hypertensive components of the renin-angiotensin and immune systems and thereby contributes to increased BP and renal dysfunction.

Cytochrome P-450 (CYP)1B1, which is highly expressed in the cardiovascular and renal systems and is capable of metabolizing fatty acids, retinoids, and sex steroids, contributes to the development of hypertension and renal dysfunction in male mice.[24–26] In female mice, where Ang II-induced increases in BP and renal dysfunction are minimized as compared to male mice, inhibition of CYP 1B1 activity with 2,3′,4,5′-tetramethoxystilbene (TMS) or *Cyp1b1* gene disruption (*Cyp1b1*$^{-/-}$) abrogated this protection against Ang II to increased BP and cause renal dysfunction.[27,28] These protective effects of CYP1B1 against Ang II in female mice are diminished by treatment with 2-methoxyestradiol, which is formed from a estradiol metabolite of *Cyp1b1*, 2-OH estradiol, by catechol-O-methyltransferase.[29,30] Testosterone is metabolized into dihydrotestosterone (DHT) by 5-alpha reductase, and 6β-

hydroxytestosterone (6β-OHT) and 16α-hydroxytestosterone (16α-OHT) by CYP1B1.[31,32] Ang II increases selectively the production of 6β-OHT but not 16α-OH or DHT, which is abolished by *Cyp1b1* gene disruption. Recently, we have shown and that 6β-OHT contributes to Ang II-induced increase in BP and associated cardiac pathophysiological changes in male mice.[33] These observations led us to hypothesize that 6β-OHT also contributes to Ang II-induced renal dysfunction and end organ damage in male mice. To test this hypothesis, we examined the effects of Ang II and 6β-OHT on Ang II-induced renal dysfunction and end organ damage in intact and castrated $Cyp1b1^{+/+}$ and $Cyp1b1^{-/-}$ male mice. The results show that Ang II-induced renal dysfunction and end organ damage are associated with increased renal oxidative stress, production of AGT, and inflammation; these effects are minimized in $Cyp1b1^{-/-}$ and castrated $Cyp1b1^{+/+}$ mice and restored by 6β-OHT.

Methods

All experiments were performed according to in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the protocols approved by our Institutional Animal Care and Use Committee. Experiments were performed in 8 week old, 20–25 g body weight, both intact and castrated $Cyp1b1^{+/+}$ and $Cyp1b1^{-/-}$ mice. These mice were infused with Ang II (700 ng/kg/min) or saline (vehicle) for 14 days with subcutaneously implanted micro osmotic pumps. In another series of experiments 6β-OHT (15 μg/g i.p. every third day) or its vehicle was given concurrently with Ang II infusion in both intact and castrated mice. Water intake, urine output, renal function, renal oxidative stress, end organ damage and T-cell infiltration were assessed in the same groups of mice that were used in the previous study[33] to reduce the number of animals used and to comply with the 3R guidelines set by the IACUC. Detailed experimental methods are available in the online-only Data Supplement.

Statistical Analysis

Data were analyzed by two-way analysis of variance followed by Tukey's multiple comparison post-hoc test or Student's *t*-test. The values of three to eight different experiments are expressed as the mean ± SEM. $P<0.05$ was considered statistically significant.

Results

Cyp1b1 Gene Disruption Minimized Ang II-Induced Increase in Water Intake and Renal Dysfunction

We have previously shown that 6β-OHT contributes to Ang II-induced increase in (BP) and associated cardiac pathophysiological changes in male mice.[33] In the present study, we examined in these animals Ang II-induced changes in renal function and associated pathogenesis. Infusion of Ang II for 2 weeks increased systolic blood pressure (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 (Table S1). Ang II also increased water intake and urine output in $Cyp1b1^{+/+}$ mice; this increase was minimized in $Cyp1b1^{-/-}$ mice (Table S1). Ang II infusion did not alter plasma levels of creatinine in $Cyp1b1^{+/+}$ or in

Cyp1b1$^{-/-}$ mice (Table S1). Ang II infusion decreased urine osmolality, increased urinary excretion of Na^+, and caused proteinuria in *Cyp1b1*$^{+/+}$ but not in *Cyp1b1*$^{-/-}$ mice (Table S1). The difference in the renal functional parameters observed between these two groups was consistent, reproducible, and similar to that reported previously.[24]

6β-OHT Treatment Restored Ang II-induced Increase in Water Intake and Renal Dysfunction in *Cyp1b1*$^{-/-}$ and Castrated *Cyp1b1*$^{+/+}$ Mice

As previously shown, the effect of Ang II to increase BP was minimized in intact *Cyp1b1*$^{-/-}$ and castrated *Cyp1b1*$^{+/+}$ mice. 6β-OHT, which did not alter basal BP, restored the effect of Ang II to increase SBP in intact *Cyp1b1*$^{-/-}$ and castrated *Cyp1b1*$^{+/+}$ mice (Table S1). *Cyp1b1* gene disruption mitigated the effects of Ang II to cause renal dysfunction; treatment with 6β-OHT in *Cyp1b1*$^{-/-}$ mice restored the ability of Ang II to cause an increase in water intake and renal dysfunction to the levels that were observed in *Cyp1b1*$^{+/+}$ mice (Table S1). Castration minimized the effect of Ang II to increase SBP, water intake or cause renal dysfunction in *Cyp1b1*$^{+/+}$ mice (Table S1). In castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice, administration of 6β-OHT restored the effects of Ang II to increase SBP, water intake and cause renal dysfunction. 6β-OHT did not alter any changes in water intake or renal function in animals infused with vehicle of Ang II. and treated only with 6β-OHT (Table S1).

Cyp1b1 Gene Disruption Attenuated Ang II Induced Renal Fibrosis but was Restored by 6β-OHT

Kidneys from Ang II-infused *Cyp1b1*$^{+/+}$ mice, but not from *Cyp1b1*$^{-/-}$ mice, displayed interstitial fibrosis as indicated by increased interstitial α-smooth muscle actin (α-SMA) staining and collagen deposition in the kidneys (Figure 1A–B, upper panel). Treatment with 6β-OHT restored the effects of Ang II in *Cyp1b1*$^{-/-}$ mice but did not alter these effects of Ang II in *Cyp1b1*$^{+/+}$ mice (Figure 1C–D, lower panel).

Castration prevents Ang II-Induced Renal Fibrosis, which is restored by 6β-OHT

Castration reduced Ang II-induced α-SMA and collagen deposition in the kidneys of Ang II infused *Cyp1b1*$^{+/+}$ mice, but castration did not have any further effects on *Cyp1b1*$^{-/-}$ mice infused with Ang II (Figure S1A–B). Concurrent treatment with 6β-OHT restored the ability of Ang II to cause renal fibrosis in castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice (Figure S2A–B). 6β-OHT alone did not cause renal fibrosis in castrated mice of these genotypes (Figure S1A–B and Figure S2A–B).

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

Ang II infusion increased renal NADPH oxidase activity, and ROS production as indicated by increased renal 2-hydroxyethidium fluorescence in *Cyp1b1*$^{+/+}$ mice, and this increase was minimized in *Cyp1b1*$^{-/-}$ mice (Figure 2A–C, upper panel). Treatment with 6β-OHT restored the loss of this action of Ang II in *Cyp1b1*$^{-/-}$ mice (Figure 2D–F, lower panel) but did not alter Ang II-induced increase in renal NADPH oxidase activity or ROS production in *Cyp1b1*$^{+/+}$ mice (Figure 2D–F, lower panel). 6β-OHT did not alter basal levels of NADPH

oxidase activity or ROS production in *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice (Figure 2D–F, lower panel).

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

Castration attenuated Ang II mediated increases in renal NADPH oxidase activity or ROS production, as indicated by decreases in 2-hydroxyethidium fluorescence in *Cyp1b1*$^{+/+}$ mice (Figure S3 A – C). In intact *Cyp1b1*$^{-/-}$ mice, Ang II did not increase renal NADPH oxidase activity or ROS production (Figure 2 upper panel A-C), and this was not altered by castration (Figure S3 A – C). Treatment with 6β-OHT restored the ability of Ang II to increase renal NADPH oxidase activity and ROS production in castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice (Figure S4). 6β-OHT did not alter the basal renal NADPH oxidase activity or ROS production in castrated *Cyp1b1*$^{+/+}$ *or Cyp1b1*$^{-/-}$ mice (Figure S4 A – C).

Cyp1b1 Gene Disruption or Castration Minimized Urinary Excretion of AGT, Which was Restored by 6β-OHT Treatment

Ang II infusion increases intrarenal accumulation and urinary excretion of AGT[34] and renal production of Ang II, which have been implicated in the development of hypertension.[34] Ang II increased urinary excretion of AGT in *Cyp1b1*$^{+/+}$ mice (Figure 3A). *Cyp1b1* gene disruption or castration minimized urinary AGT increase by Ang II without altering basal levels (Figure 3A,C). (Figure 3B,D).

Castration Decreased Renal Expression of ACE and AT1a but Not ACE2 or Androgen Receptor (AR), Which Were Restored by 6β-OHT

Castration decreased mRNA expression of AT1a and ACE in both *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice; this effect reversed and increased by treatment with 6β-OHT in both phenotypes. (Figures S5A and S6B). Castration or 6β-OHT did not alter the expression of ACE2 and AR in either *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice (Figures S6A and S6B).

Ang II Infusion Decreased Renal mRNA Expression of CYP4A12A but Not CYP1B1 in Mice

Infusion of Ang II decreased renal mRNA encoding *CYP4A12A* in both *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ male mice (Figure S7A). Ang II infusion did not alter *Cyp1b1* mRNA expression in *Cyp1b1*$^{+/+}$ mice, and, as expected, no *Cyp1b1* mRNA was detected in *Cyp1b1*$^{-/-}$ mice (Figure S7B).

Cyp1b1 Gene Disruption or Castration Minimized Renal Accumulation of CD4^{+} T-Lymphocytes, Which was Restored by 6β-OHT Treatment in *Cyp1b1*$^{-/-}$ but Not in Castrated *CYP1b1*$^{+/+}$ Mice

Ang II infusion increased renal accumulation of CD4^{+} T-lymphocytes in *Cyp1b1*$^{+/+}$ mice, which was not altered by treatment with 6β-OHT (Figure 4). *Cyp1b1* gene disruption or castration of *Cyp1b1*$^{+/+}$ mice attenuated the renal accumulation of CD4^{+} T-cells (Figures 4 and S8). Treatment with 6β-OHT restored the effect of Ang II to increase renal accumulation of CD4^{+} T-lymphocytes in intact *Cyp1b1*$^{-/-}$ mice but not in castrated *Cyp1b1*$^{+/+}$ mice (Figures 4 and S8).

Cyp1b1 Gene Disruption or Castration Decreased IL-6 and IFN-γ mRNA Expression, Which was Restored by 6β-OHT

As shown above, *Cyp1b1* gene disruption or castration decreased renal accumulation of $CD4^+$ T-cells, which was restored by 6β-OHT. We examined the effects of *Cyp1b1* gene disruption and castration on cytokine IL-6 and IFN-γ mRNA expression and their restoration by 6β-OHT. *Cyp1b1* disruption or castration decreased IL-6 and IFN-γ expression (Figure S9A and S9B), and treatment with 6β-OHT restored expression of mRNA encoding IL-6 and IFN-γ in castrated $Cyp1b1^{+/+}$ and $Cyp1b1^{-/-}$ mice (Figure S9A, B).

Restoration by 6β-OHT of the Effect of Ang II on Renal Fibrosis is Dependent on Systolic BP in $Cyp1b1^{-/-}$ Mice

To determine if the effect of 6β-OHT to restore the effect of Ang II on renal fibrosis in $Cyp1b1^{-/-}$ mice is dependent on systolic BP, we examined the effect of a direct vasodilator hydralazine (250 mg/L in drinking water) on systolic BP and renal fibrosis. Hydralazine reduced SBP (Figure S10A) and renal fibrosis as indicated by decreased absence of interstitial α-smooth muscle actin (α-SMA) staining and collagen deposition in the kidneys of $Cyp1b1^{-/-}$ mice infused with Ang II and concurrently treated with 6β-OHT (Figure S10B).

Renal Fibrosis is independent of Systolic BP Blood Pressure in $Cyp1b1^{+/+}$ mice

Infusion of Norepinephrine (10 mg/kg/day) increased systolic BP blood pressure in $Cyp1b1^{+/+}$ mice (Figure S10A) but did not cause and norepinephrine infusion did not increase renal fibrosis as indicated by decreased absence of interstitial α-smooth muscle actin (α-SMA) staining and collagen deposition in the kidneys (Figure S10 B).

Discussion

The main findings of this study are that 6β-OHT, a CYP1B1-generated metabolite of testosterone, contributes to Ang II-induced: a) increase in water intake, b) renal dysfunction, and c) end organ damage by increasing BP and oxidative stress, promoting intrarenal production of AGT, and activating T-cells in male mice. Previously, we reported that *Cyp1b1* gene disruption or inhibition of CYP1B1 activity minimized Ang II-induced renal dysfunction and end organ damage in male mice.[25] Moreover, we showed that Ang II stimulates the production of 6β-OHT, a testosterone-derived metabolite of CYP1B1, in $Cyp1b1^{+/+}$, but not in $Cyp1b1^{-/-}$ mice, and contributes to Ang II-induced hypertension and associated cardiac pathophysiology by acting as a permissive factor.[33] The current study that Assessment of assessed the renal function and associated pathophysiological changes in these mice[33] has revealed that Ang II infusion for 14 days increased water intake and urine output in $Cyp1b1^{+/+}$ mice, and these effects of Ang II were minimized in $Cyp1b1^{-/-}$ mice thus confirming our previous results.[25] Administration of 6β-OHT to $Cyp1b1^{-/-}$ mice that do not generate this testosterone metabolite[33] restored the effects of Ang II to increase thirst and urine output, suggesting that 6β-OHT mediates contributes to the this effect of Ang II in male mice , most likely by acting in the brain.

Ang II increased urinary Na^+ excretion in *Cyp1b1*$^{+/+}$ mice, and *Cyp1b1* gene disruption minimized Na^+ excretion in male mice.[25] In the present study, we found that administering 6β-OHT to *Cyp1b1*$^{-/-}$ mice infused with Ang II markedly increased Na^+ excretion. The increase in urinary Na^+ excretion by Ang II in the *Cyp1b1*$^{+/+}$ mice, and its restoration by 6β-OHT in *Cyp1b1*$^{-/-}$ mice is most likely the consequence of increase in BP. Further studies are needed to determine the fractional Na^+ excretion and its time-course and total body Na^+ to address the role of CYP1B1 and 6β-OHT in Ang II actions to alter Na^+ balance in male mice. 6β-OHT also contributed to the effect of Ang II to decrease osmolality, cause proteinuria, and renal fibrosis as indicated by interstitial α-SMA and collagen accumulation, because these effects of Ang II that were abrogated in *Cyp1b1* gene disrupted mice, were significantly restored by 6β-OHT. Further support for the role of 6β-OHT in Ang II-induced renal dysfunction and end organ damage was obtained in castrated mice. Castration attenuated Ang II-induced renal dysfunction and fibrosis in *Cyp1b1*$^{+/+}$ mice, but, as expected, castration did not have any effect in *Cyp1b1*$^{-/-}$ mice infused with Ang II. However, concurrent administration of 6β-OHT restored the effects of Ang II to cause renal dysfunction and fibrosis in both castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice. The mechanism by which 6β-OHT mediates the effect of Ang II to cause end organ damage in intact *Cyp1b1*$^{-/-}$ and castrated *Cyp1b1*$^{+/+}$ mice could be due to restoration of the effect of Ang II on BP by 6β-OHT.[33] Supporting this view was our observation that hydralazine that abolished the increase in systolic BP prevented renal fibrosis caused by Ang II in *Cyp1b1*$^{-/-}$ mice treated with 6β-OHT. However, we cannot exclude any possible action of hydralazine on one or more signaling mechanisms involved in the action of Ang II, because Ang II has also been reported to cause fibrosis independent of an increase in BP.[35–38]

Ang II increases production of ROS, which contributes to the development of hypertension and end organ damage in male SHR rats[39–41]. Moreover, *Cyp1b1* gene disruption and/or inhibition of its activity decreases vascular and renal oxidative stress in rats and mice.[23–25] Our finding that administration 6β-OHT to *Cyp1b1*$^{-/-}$ mice or castrated *Cyp1b1*$^{+/+}$ mice infused with Ang II increased renal NADPH oxidase activity and ROS production suggests that 6β-OHT, by restoring the effect of Ang II to increase oxidative stress, contributes to the development of renal dysfunction and end organ damage in male mice.

Treatment with DHT has been shown to increase the renal vascular expression of CYP4A8 and its orthologue CYP4A12 in male mice, thereby stimulating prohypertensive eicosanoid 20-HETE production and increasing the activity of the renin-angiotensin system and BP.[18,42,43] DHT-induced hypertension was minimized in AGT-deficient mice.[44] *Cyp1b1* gene disruption in male mice did not alter renal expression of CYP4A10/12/14 (mouse orthologues of rat CYP4A1/A2/A3).[25] In the present study, expression of CYP4A12A mRNA was decreased in the kidneys of both *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ male mice infused with Ang II, whereas Ang II did not alter renal *Cyp1b1* mRNA in *Cyp1b1*$^{+/+}$ mice. However, we cannot exclude the possible contribution of CYP4F2 that also metabolizes arachidonic acid (AA) into 20-HETE[45] to the actions of Ang II and 6β-OHT. Also, in view of the demonstration that 20-HETE mediates the renal vasoconstrictor effect of Ang II[46] but exert natriuretic effect[47] further studies are required to assess contribution of 20-HETE to Ang II induced renal dysfunction and end organ damage. Therefore, DHT-generated 20-HETE does not appear to contribute to Ang II-induced renal dysfunction and end organ

damage in at least in this genotype of mice. However, in view of the demonstration that 20-HETE mediates the renal vasoconstrictor effect of Ang II[45] but exert natriuretic effect[46] further studies are required to assess contribution of 20-HETE to Ang II induced renal dysfunction and end organ damage. Moreover, whether alterations in expression and activities of other CYP isoforms including CYP2C and 2J, that metabolize AA into epoxyeicosatrienoic acids, that exert anti-hypertensive, anti-inflammatory and pro-fibrinolytic effects[48] are involved in the modulation by 6β-OHT of Ang II actions on BP, renal dysfunction and end organ damage remains to be determined.

Male SHR kidneys have higher AT1a receptor expression in the kidneys as compared to female SHR kidneys, whereas female SHRs have higher AT2 receptor expression in the kidneys.[49] Infusion of Ang II increased AT1a receptor expression in male SHRs but not SD rats.[14, 16] Renal mRNA expression of ACE2, AT2, and plasma levels of Ang 1–7 are increased in female SHRs compared with male SHRs, and this increase in AT2 mRNA and Ang 1–7 has been proposed to be responsible for the lower BP in female SHRs than in male SHRs.[14–16] In the present study, Ang II infusion did not alter renal AT1a or ACE expression in either *Cyp1b1*$^{+/+}$ or *Cyp1b1*$^{-/-}$ mice. However, castration attenuated both AT1a and ACE mRNA expression in both genotypes, and treatment with 6β-OHT increased AT1a and ACE mRNA expression in both mouse genotypes. Castration or 6β-OHT had no effect on expression of ACE2 expression in either group of mice infused with Ang II or its vehicle, suggesting that castration downregulated the prohypertensive arm of the renin-angiotensin system (RAS), and treatment with 6β-OHT upregulated the prohypertensive arm of the RAS.

An increase in urinary excretion of AGT is a marker for increased intrarenal RAS activity.[34,50] Ang II infusion increased renal expression and urinary excretion of AGT in male rats, suggesting that there is an increased AGT availability for conversion to Ang II in kidneys.[13,34,51–54] In Dahl-salt sensitive male rats, a high salt diet increased intrarenal AGT expression, BP, proteinuria and glomerulosclerosis, which were prevented by castration and restored by administering testosterone, suggesting that Ang II-induced intrarenal AGT production depends on androgens.[55] Our previous study showing that Ang II stimulated production of testosterone metabolite 6β-OHT via CYP1B1[33] together with the demonstration that the Ang II-induced increase in urinary excretion of AGT was inhibited by *Cyp1b1* gene disruption or castration in *Cyp1b1*$^{+/+}$ mice, and that treatment with 6β-OHT restored the increase in urinary excretion of AGT in these mice suggest that 6β-OHT contributes to Ang II- induced intrarenal AGT production. Increased availability of intrarenal AGT coupled with increased renal ACE expression and AT1a receptor expression may augment the effects of Ang II to cause renal dysfunction and end organ damage. The mechanism by which 6β-OHT mediates increased urinary excretion of AGT that could be due to enhanced transcription of AGT by Ang II remains to be determined.

Ang II also stimulates the immune system and causes infiltration of macrophages and T-cells in blood vessels, kidneys, and central nervous system resulting in increased BP and end organ damage.[56] This increase in BP is attenuated in *Rag1*$^{-/-}$ deficient male mice and restored by adoptive transfer of male wild type (WT) T-cells.[57] Recent studies also showed that adoptive transfer of male WT T-cells to *Rag1*$^{-/-}$ female mice did not increase BP in female mice infused with Ang II[58], whereas adoptive transfer of WT female T-cells to male

Rag1$^{-/-}$ mice minimized the increase in BP[23], indicating that females are protected against inflammation mediated by Ang II. In a recent study, gonadectomy of male and female SHRs increased proinflammatory cells in both sexes.[59] However, we found that *Cyp1b1* gene disruption or castration of *Cyp1b1*$^{+/+}$ mice attenuated Ang II-induced infiltration of CD4$^+$ T-lymphocytes and treatment with 6β-OHT restored renal accumulation of CD4$^+$ T-cells in *Cyp1b1*$^{-/-}$ mice but failed to do so in castrated mice. These results suggest that 6β-OHT is insufficient to restore Ang II-induced infiltration of CD4$^+$ T-cells, and testosterone and/or its metabolite DHT is required for accumulation of these cells in castrated mice. However, further in-depth studies are required to assess the role of 6β-OHT in the action of Ang II on other subtypes of T-lymphocytes and dendritic cells and production of cytokines in renal dysfunction and end organ damage. Moreover, in view of the recent evidence that the dendritic-cell isoketal-modified proteins activate T-cells and promote hypertension, the possibility that testosterone and/or its metabolite 6β-OHT-dependent activation of T-cells by Ang II might be mediated via formation of dendritic-cell isoketal-modified proteins[60] remains to be explored.

The mechanism by which 6β-OHT contributes to the effects of Ang II to increase thirst, renal dysfunction, oxidative stress, inflammation, and end organ damage is unclear. Since 6β-OHT restored these effects of Ang II in both intact *Cyp1b1*$^{-/-}$ and castrated *Cyp1b1*$^{+/+}$ mice without producing these effects alone, it appears that it acts as a permissive factor to increase sensitivity to the action of Ang II, most likely by interacting with AR, which is located on mesangial cells, glomeruli, proximal tubules, and cortical collecting ducts.[61,62] Therefore, whether 6β-OHT could restores the renal actions of Ang II through genomic and/or nongenomic AR or independent of AR remains to be determined.[63,64] Since expression of AR mRNA in the kidney was not altered either in *Cyp1b1* or castrated mice treated with 6β-OHT or its vehicle, the effect of 6β-OHT-mediated Ang II-induced renal pathophysiological changes seems to be independent of AR expression. This observation together with our finding that 6β-OHT did not alter either basal BP or renal function, or Ang II-induced increase in BP or renal dysfunction and end organ damage in *Cyp1b1*$^{+/+}$ mice, suggest that its effects are unlikely to be due to competition with testosterone or DHT at the AR.

In conclusion, this study provides evidence that the testosterone metabolite of *Cyp1b1*, 6β-OHT stimulated by Ang II, contributes to its actions to increase thirst, renal dysfunction, fibrosis, oxidative stress, AGT production, and inflammation in male mice. In contrast, we have shown that, in female mice, CYP1B1 protects against Ang II-induced hypertension and renal dysfunction via metabolism of estradiol to 2-hydroxyestradiol and subsequently its metabolism by catechol-O-methyltransferase to 2-methoxyestradiol[29,30] that acts as a permissive factor to suppress Ang II actions.[26,27] Therefore, it appears that 17β-estradiol and testosterone metabolites of CYP1B1 contribute to sex differences in Ang II-induced renal dysfunction and end organ damage associated with hypertension. Finally, CYP1B1 could serve as a novel target for developing agents that inhibit CYP1B1 for treating renal dysfunction in males, but inhibitors of CYP1B1 could be detrimental in treating renal dysfunction and hypertension in females. Since CYP1B1 is known to cause congenital glaucoma[65] due to a developmental abnormality in the eye [65] and thus acute or chronic inhibition of CYP1B1 in adults may not cause increased ocular pressure. However, this

possibility needs to be assessed in the development of CYP1B1 inhibitors in the treatment of renal dysfunction and hypertension in males.

Perspectives

Sex differences in renal dysfunction in various models of experimental hypertension have been attributed to sex chromosome complement and sex hormones. The present study provides evidence that the CYP1B1 metabolite of testosterone, 6β-OHT, in male mice contributes to Ang II-induced increases in thirst, renal dysfunction, and end organ damage most likely by increased oxidative stress and intrarenal RAS activity and activation of T-cells. The levels of testosterone decreases with age in men, and many individuals including females are prescribed testosterone for sexual dysfunction and to increase muscle mass and boost energy. However, the use of testosterone as regards the cardiovascular effects, benefits vs. risk are controversial. Since Ang II increases the production of 6β-OHT that contributes to its hypertensive and pathophysiological effects, it would be important to determine the levels of this testosterone metabolite in individuals of different ages with increased activity of renin-angiotensin system. Moreover, in view of polymorphism in human CYP1B1gene[65] and differences in the catalytic properties of polymorphic CYP1B1 variants for steroid hormones including testosterone[66] it would be important to determine association of CYP1B1 gene polymorphism to the levels of testosterone and its metabolites, BP and renal function.

Future studies should characterize interactions of 6β-OHT with genomic and nongenomic AR and the mechanism by which it permits Ang II to express its renal pathophysiological effects including possible activation of T-cells via dendritic isoketal-modified proteins.[58] Moreover, development of selective inhibitors of the effects of 6β-OHT and CYP1B1 activity could be useful for treating renal dysfunction and end organ damage and hypertension in males.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What is New?

- Demonstrates that 6â-hydroxytestosterone (6β-OHT), a testosterone-derived metabolite of CYP1B1, contributes to Ang II-induced increases in thirst and renal dysfunction in male mice.
- Provides the evidence that 6β-OHT mediates the effect of Ang II to produce renal fibrosis and oxidative stress in male mice.
- Demonstrates that *Cyp1b1* gene disruption or castration decreases Ang II-induced urinary excretion of angiotensinogen (AGT), which is restored by 6β-OHT.
- Demonstrates that 6β-OHT mediates the effect of Ang II to increase renal infiltration of $CD4^+$ T-cells.

What is Relevant?

- This study advances our knowledge of the mechanism by which CYP1B1-testosterone-derived metabolite 6β-OHT contributes to Ang II-induced renal dysfunction, fibrosis, oxidative stress, and inflammation in males.
- Our demonstration that 6β-OHT mediates the action of Ang II to stimulate intrarenal AGT production coupled with increased mRNA expression of mRNA encoding AT1a and ACE furthers our understanding of the mechanism of testosterone-dependent activation of intrarenal RAS by Ang II and has important pathophysiological significance in the development of renal disease and hypertension.
- Finally, our study has translational significance in developing selective CYP1B1 inhibitors for treating testosterone-dependent Ang II-induced renal dysfunction, end organ damage, and hypertension in men.

Summary

Ang II increased renal dysfunction, fibrosis, oxidative stress, inflammation, and urinary excretion of AGT in male mice. *Cyp1b1* gene disruption or castration in $Cyp1b1^{+/+}$ mice minimized these effects of Ang II. Administering the testosterone metabolite formed by CYP1B1, 6β-OHT to $Cyp1b1^{-/-}$ or castrated $Cyp1b1^{+/+}$ mice restored the ability of Ang II to increase thirst and cause renal dysfunction, fibrosis, oxidative stress, inflammation, and urinary excretion of AGT in male mice. Since 6β-OHT increased intrarenal AGT and mRNA expression of AT1a receptor and ACE in *Cyp1b1* gene-disrupted or castrated mice infused with Ang II, it appears that 6β-OHT promotes increased formation of Ang II in the kidneys, thereby contributing to renal dysfunction and associated pathogenesis in male mice. Therefore, developing selective inhibitors of CYP1B1 could be useful for treating renin-angiotensin and testosterone-dependent renal disease and hypertension in males.

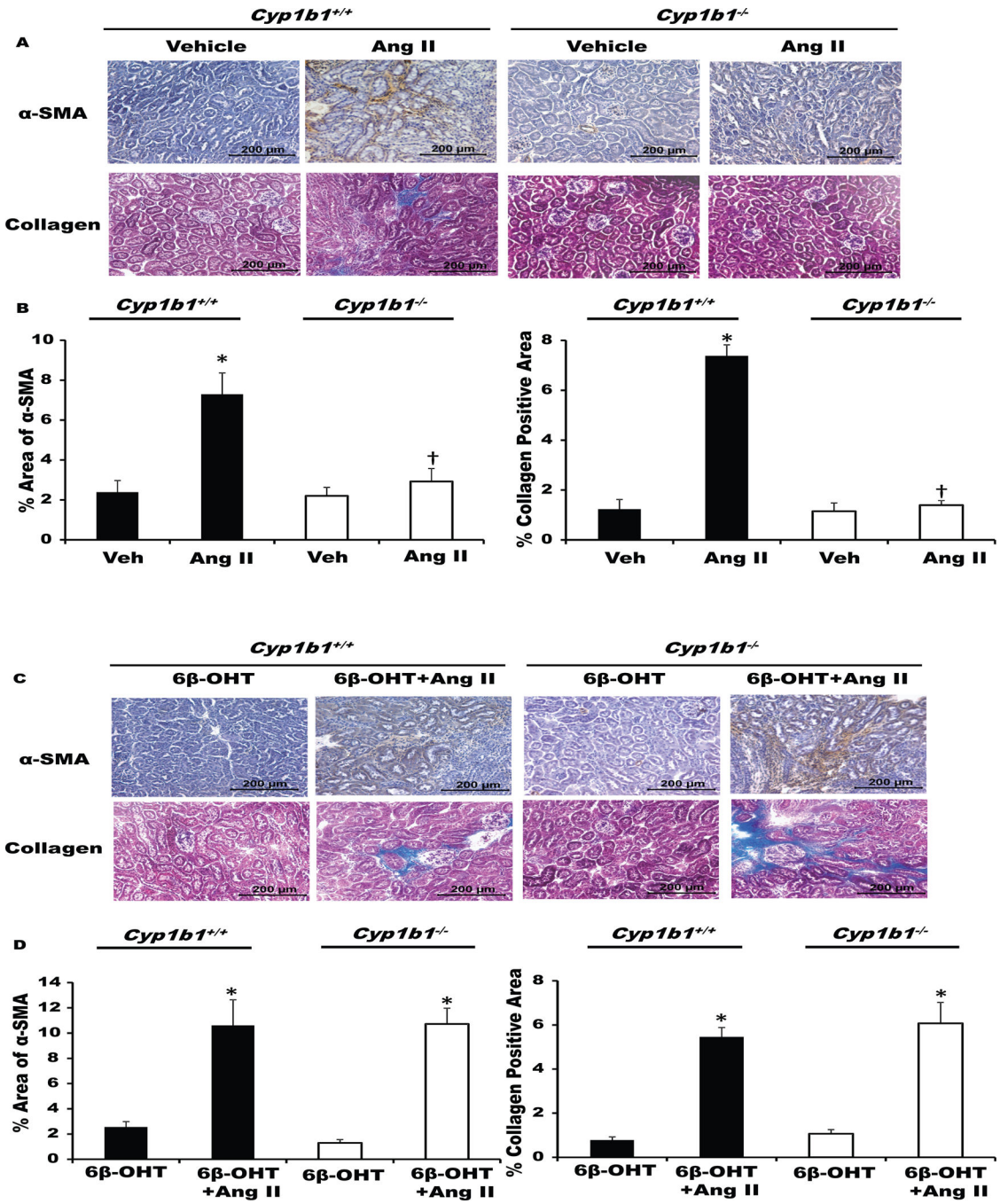

Figure 1. ***Cyp1b1*** **gene disruption minimizes angiotensin II (Ang II)-induced renal fibrosis that is restored by 6β-hydroxytestosterone (6β-OHT)**

Cyp1b1$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were infused with vehicle or Ang II (upper panel) and treated with 6β-OHT (15 μg/g i.p.) or 6β-OHT+Ang II (lower panel) for 2 weeks. After the end of Ang II infusion, kidneys were removed and processed. (A&C) Ang II infusion increased interstitial accumulation of α-smooth muscle actin (α-SMA) and renal collagen deposition in *Cyp1b1*$^{+/+}$ mice, which were minimized in *Cyp1b1*$^{-/-}$ mice, restored by concurrent treatment with 6β-OHT, but not in mice treated with 6β-OHT alone. (B&D) Bar graphs showing quantified data. Data are expressed as means ± SE; $n = 3$ for each treatment

group. *P< 0.05, Ang II vs. vehicle and 6β-OHT vs. 6β-OHT+Ang II; †P< 0.05, Ang II-infused *Cyp1b1*$^{-/-}$ mice vs. Ang II-infused *Cyp1b1*$^{+/+}$ mice.

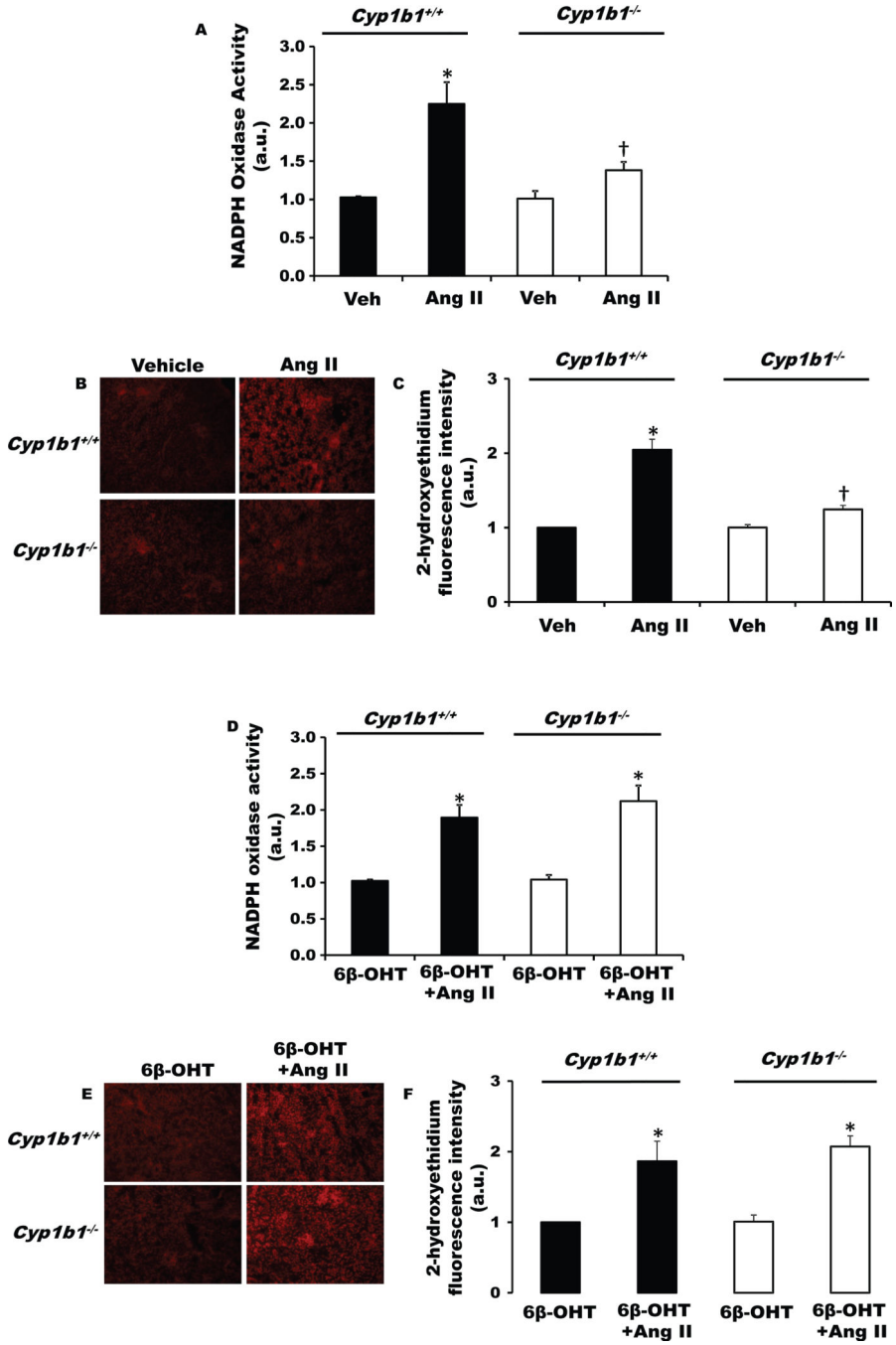

Figure 2. *Cyp1b1* gene disruption minimizes angiotensin II (Ang II)-induced renal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and superoxide production in *Cyp1b1*$^{-/-}$ mice, which are restored by concurrent treatment with 6β-hydroxytestosterone (6β-OHT)

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 kidney homogenates. (B) Renal superoxide production was determined by the fluorescence intensity of 2-hydoxyethidium. Photomicrographs are representative of kidneys 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=4–5 for each treatment group and data are expressed as mean±SEM).

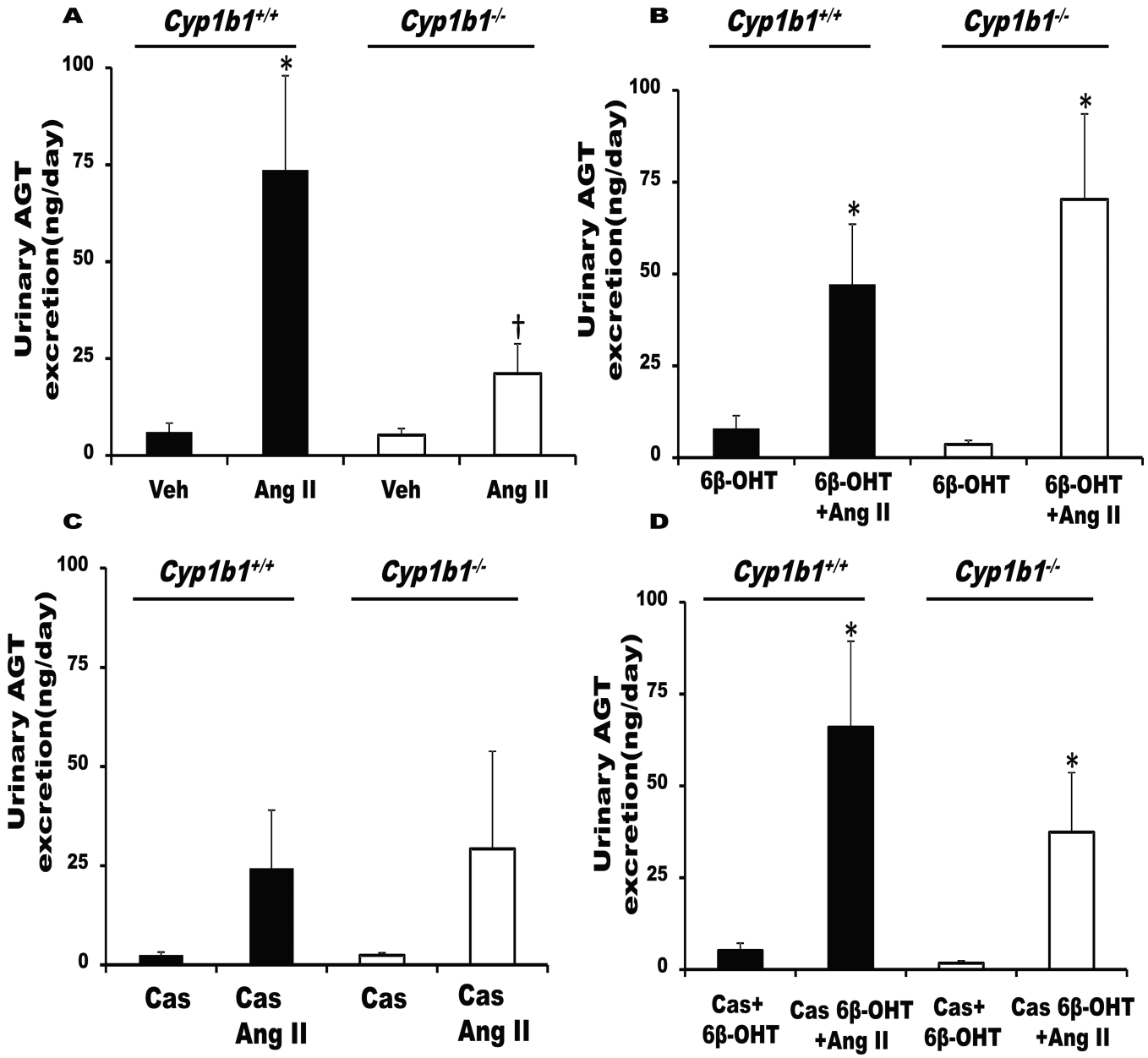

Figure 3. *Cyp1b1* gene disruption or castration in *Cyp1b1*$^{+/+}$ mice attenuates urinary angiotensinogen (AGT) excretion, which is restored by 6β-hydroxytestosterone (6β-OHT)

Intact or castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were infused with vehicle (Veh) or Ang II and given intraperitoneal injections of testosterone metabolite, 6β-OHT, every third day for 2 weeks. At the end of Ang II infusion, urine was collected, and urinary AGT excretion was measured. (A) Intact *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice infused with vehicle or Ang II. (B) Intact *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice infused with vehicle or Ang II and treated with 6β-OHT for 2 weeks. (C) Castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice infused with vehicle or Ang II. (D) Castrated *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ *mice* infused with vehicle or Ang II and treated with 6β-OHT. *P<0.05 Ang II vs. vehicle, Ang II+6β-OHT vs. vehicle+6β-OHT, and

Cas+6β-OHT+Ang II vs. Cas+6β-OHT; †P<0.05, *Cyp1b1*$^{+/+}$ Ang II vs. *Cyp1b1*$^{-/-}$ Ang II (n=4–8 for each group of experiments; data are expressed as mean±SEM).

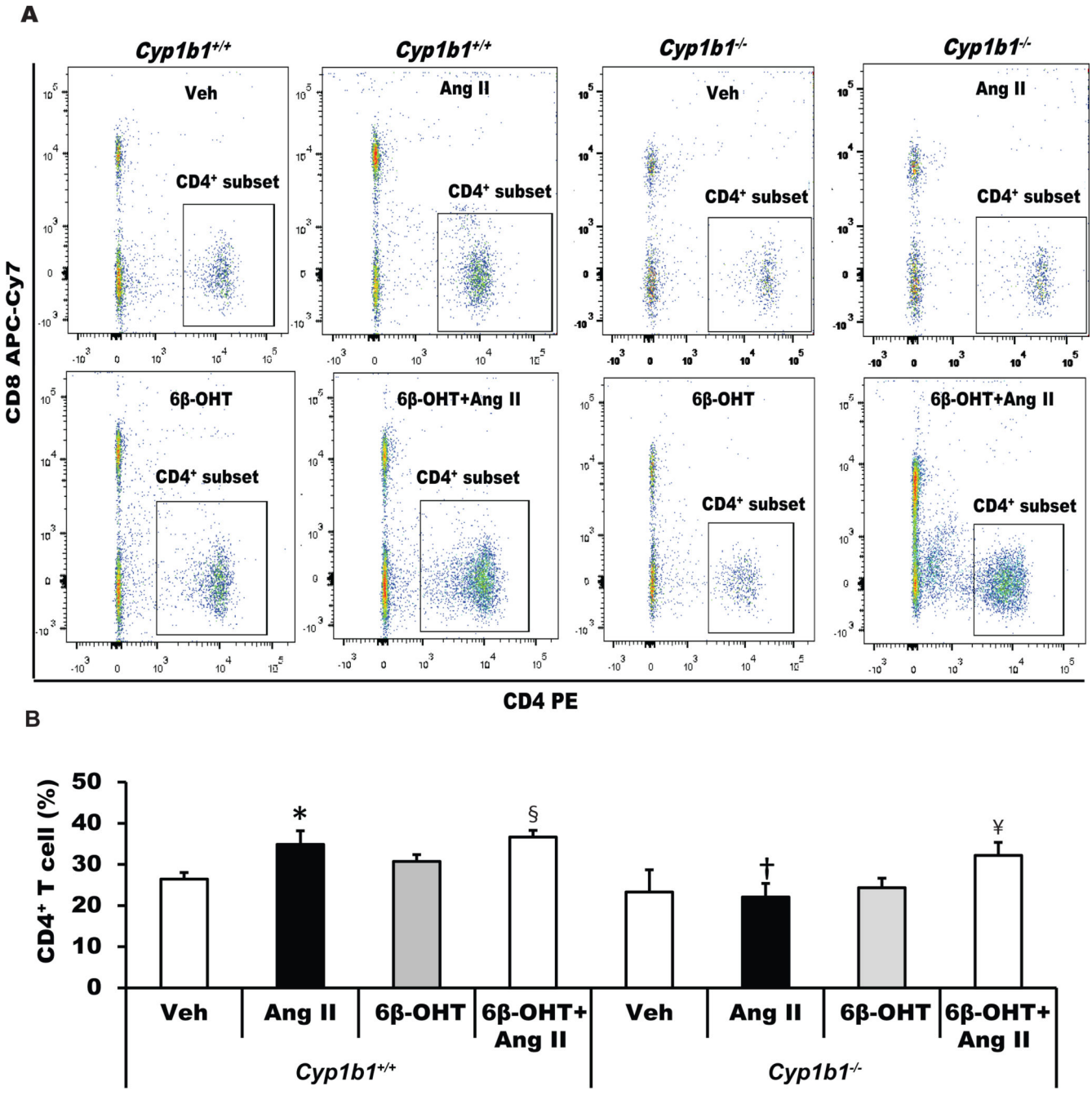

Figure 4. *Cyp1b1* gene disruption minimizes angiotensin II (Ang II)-induced renal accumulation of renal CD4$^+$ T-cells in *Cyp1b1*$^{-/-}$ mice, which is restored by 6β-hydroxytestosterone (6β-OHT)

Cyp1b1$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice were infused with vehicle or Ang II and treated with 6β-OHT or 6β-OHT+Ang II for 2 weeks. At the end of Ang II infusion, kidneys were removed and processed, and cells were isolated, counted, and used for flow cytometry. (A) Dot plot images of intact *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice infused with vehicle or Ang II and treated with 6β-OHT for 2 weeks. (B) the average percentage of CD4$^+$ T cell subset is represented by a histogram. *P<0.05 Ang II vs. vehicle; †P<0.05 *Cyp1b1*$^{-/-}$ Ang II vs. *Cyp1b1*$^{+/+}$ Ang II; § *Cyp1b1*$^{+/+}$ 6β-OHT Ang II vs *Cyp1b1*$^{+/+}$ 6β-OHT; ¥ P<0.05 *Cyp1b1*$^{-/-}$ 6β-OHT Ang II

vs. *Cyp1b1*$^{-/-}$ 6β-OHT (n=3 for each treatment group and data are expressed as mean ±SEM).