OPEN

Brain Testosterone-CYP1B1 (Cytochrome P450 1B1) Generated Metabolite 6β-Hydroxytestosterone Promotes Neurogenic Hypertension and Inflammation

Purnima Singh, Shubha Ranjan Dutta, Chi Young Song, SaeRam Oh, Frank J. Gonzalez, Kafait U. Malik

Abstract—Previously, we showed that peripheral administration of 6β-hydroxytestosterone, a CYP1B1 (cytochrome P450 1B1)-generated metabolite of testosterone, promotes angiotensin II-induced hypertension in male mice. However, the site of action and the underlying mechanism by which 6β-hydroxytestosterone contributes to angiotensin II-induced hypertension is not known. Angiotensin II increases blood pressure by its central action, and CYP1B1 is expressed in the brain. This study was conducted to determine whether testosterone-CYP1B1 generated metabolite 6β-hydroxytestosterone locally in the brain promotes the effect of systemic angiotensin II to produce hypertension in male mice. Central CYP1B1 knockdown in wild-type (*Cyp1b1*$^{+/+}$) mice by intracerebroventricular-adenovirus-GFP (green fluorescence protein)-CYP1B1-short hairpin (sh)RNA attenuated, whereas reconstitution of CYP1B1 by adenovirus-GFP-CYP1B1-DNA in the paraventricular nucleus but not in subfornical organ in *Cyp1b1*$^{-/-}$ mice restored angiotensin II-induced increase in systolic blood pressure measured by tail-cuff. Intracerebroventricular-testosterone in orchidectomized (Orchi)-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$, and intracerebroventricular-6β-hydroxytestosterone in the Orchi-*Cyp1b1*$^{-/-}$ mice restored the angiotensin II-induced: (1) increase in mean arterial pressure measured by radiotelemetry, and autonomic imbalance; (2) reactive oxygen species production in the subfornical organ and paraventricular nucleus; (3) activation of microglia and astrocyte, and neuroinflammation in the paraventricular nucleus. The effect of intracerebroventricular-6β-hydroxytestosterone to restore the angiotensin II-induced increase in mean arterial pressure and autonomic imbalance in Orchi-*Cyp1b1*$^{-/-}$ mice was inhibited by intracerebroventricular-small interfering (si)RNA-androgen receptor (AR) and GPRC6A (G protein-coupled receptor C6A). These data suggest that testosterone-CYP1B1-generated metabolite 6β-hydroxytestosterone, most likely in the paraventricular nucleus via AR and GPRC6A, contributes to angiotensin II-induced hypertension and neuroinflammation in male mice.

Graphic Abstract—An online graphic abstract is available for this article. **(*Hypertension*. 2020;76:1006-1018. DOI: 10.1161/HYPERTENSIONAHA.120.15567.)** ● **Data Supplement**

Key Words: blood pressure ■ hypertension ■ mice ■ microglia ■ testosterone

Men display higher blood pressure (BP) than do women of similar reproductive age.[1] This protection against high BP in females has been attributed to 17β-estradiol, the predominant estrogen. Recently, we showed that a 17β-estradiol-CYPIB1 (cytochrome P450 1B1)-catechol-*O*-methyltransferase-generated metabolite, 2-methoxyestradiol in the brain protects against neurogenic hypertension induced by Ang II (angiotensin II), and DOCA (deoxycorticosterone acetate) salt in female mice.[2] However, this protection against hypertension was absent in the males. Studies in humans and experimental animal models suggest that the male sex hormone testosterone is a prohypertensive hormone.[3] Gonadectomy minimized the increase in BP in male spontaneously hypertensive rats and hypertension induced by DOCA salt in male rats, and Ang II in the male mice and rats,[4–8] and peripheral testosterone replacement restored DOCA salt and Ang II-induced increase in BP in the rats.[5,8] These observations suggest that testosterone, unlike estrogen, promotes the effect of Ang II to increase BP in males.

A chronic increase in circulating Ang II produces its hypertensive effects (neurogenic hypertension), primarily through sympathoexcitation via its action in the circumventricular organs, including the subfornical organ (SFO).[9] This, in turn, leads to increased neuronal activity in the paraventricular

Received May 19, 2020; first decision June 12, 2020; revision accepted July 7, 2020.

From the Department of Pharmacology, Addiction Science, and Toxicology, College of Medicine, University of Tennessee Health Science Center, Memphis (P.S., S.R.D., C.Y.S.); and Laboratory of Metabolism, National Cancer Institute, Bethesda, MD (F.J.G.).

The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.120.15567.

Correspondence to Kafait U. Malik, Department of Pharmacology, Addiction Science, and Toxicology, College of Medicine, University of Tennessee Health Science Center, 71 S. Manassas St, Memphis, TN 38103. Email kmalik@uthsc.edu

nucleus (PVN) and rostral ventrolateral medulla, and subsequently, the preganglionic sympathetic nerves.[10] Plasma renin activity in ovariectomized female rats was increased with testosterone treatment, whereas it was reduced by castration in male rats.[11] Therefore, testosterone by itself or through the generation of a metabolite might act in the brain to promote the hypertensive effects of Ang II. Supporting this view is the observation that intracerebroventricular (ICV) injection of the androgen receptor (AR) antagonist flutamide reduced the Ang II-induced increase in BP in male mice.[12] However, the mechanism by which testosterone contributes to the effect of Ang II to increase BP and associated neuroinflammation in the brain is not known.

Nongenomic GPRC6A (G protein-coupled receptor C6A) is present in both human and mouse brain,[13,14] and testosterone-mediated metabolic functions such as stimulation of insulin secretion in pancreatic islets were shown to be dependent on testosterone-GPRC6A rapid signaling.[15] However, the role of GPRC6A in the brain in the action of testosterone on Ang II-induced hypertension has not been explored. Testosterone is metabolized by CYP1B1 into 6β-OHT (6β-hydroxytestosterone),[16,17] and CYP1B1 is widely expressed in the male mouse brains[18] and in human neurons and astrocytes.[19] These observations together with our previous findings[7] that systemic administration of 6β-OHT contributes to Ang II-induced hypertension and its pathogenesis led us to the hypothesis that the testosterone-CYP1B1 generated metabolite 6β-OHT, produced in the brain contributes to the effect of Ang II on autonomic function, neuroinflammation, and hypertension, via genomic (AR) and nongenomic (GPRC6A) receptors in male mice.

Materials and Methods

The authors declare that a detailed Methods section and all supporting data are available within the article and in the Data Supplement. Other details regarding analytic methods, study materials, and the data will be made available from the corresponding author upon reasonable request.

Animals

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under protocols approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Experiments were conducted on 8- to 10-week-old, 20- to 25-g body weight, intact and orchidectomized (Orchi) *Cyp1b1*$^{+/+}$ (Wild-type, C57BL/6J background) and *Cyp1b1*$^{-/-}$ (C57BL/6J background) male mice. Mice were maintained on a 12:12-h light-dark cycle in temperature (22°C±0.2°C)-controlled rooms with access to mouse chow and water ad libitum. The mice were randomly divided into different groups for the experiments. The effects of Ang II (infused for 14 days, 700 ng/kg per minute, subcutaneous, osmotic minipump) was investigated in mice injected ICV with (1) adenovirus (Ad)-GFP (green fluorescence protein)-scrambled (Scr)-short hairpin (sh)RNA (a single injection of 2 µL, 6.4×10^{11} pfu/mL) and Ad-GFP-CYP1B1-shRNA (a single injection of 2 µL, 1.3×10^{13} pfu/mL); (2) Ad-GFP-DNA (a single injection of 2 µL, 1.0×10^{12} pfu/mL) and Ad-GFP-CYP1B1-DNA (a single injection of 2 µL, 1.0×10^{12} pfu/mL); (3) testosterone (3 µg/2 µL per alternate day, starting 6 days before implantation of osmotic minipump till the end of the experiment), 6β-OHT (1.5 µg/2 µL per alternate day), and their vehicle (Veh, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid, 2 µL per alternate day); and (4) after selective injection of Ad-GFP-DNA/Ad-GFP-CYP1B1-DNA in SFO (200 nL, single injection) and PVN (200 nL, bilaterally), respectively. Saline was infused as a vehicle for Ang II.

Statistical Analysis

The data were expressed as the mean±SEM with $P<0.05$ considered statistically significant. For the BP data, a comparison between the groups was performed using 2-way ANOVA with repeated measures. Unpaired *t*-test was used for comparisons between 2 groups with normally distributed data. Multiple groups with normally distributed variables were compared by 1-way ANOVA. In most of the experiments, the primary outcomes and main comparisons exceeded a power of 0.8 with the number of animals used (Data Supplement).

Results

Central CYP1B1 Knockdown in *Cyp1b1*$^{+/+}$ Attenuates, and Its Reconstitution in *Cyp1b1*$^{-/-}$, but Not in Orchi-*Cyp1b1*$^{-/-}$ Mice, Restores Ang II-Induced Hypertension

Ang II increased systolic BP (SBP) in *Cyp1b1*$^{+/+}$ mice transduced with ICV-Ad-GFP-Scr-shRNA (Figure 1A). However, ICV-Ad-GFP-CYP1B1-shRNA that reduced the *Cyp1b1* messenger (m)RNA expression in both PVN and SFO (Figure S1A and S1B in the Data Supplement) minimized the effect of Ang II to increase SBP in *Cyp1b1*$^{+/+}$ mice (Figure 1A).

Ang II caused a small increase in SBP in *Cyp1b1*$^{-/-}$ mice transduced with ICV-Ad-GFP-DNA (Figure 1B). However, transduction with ICV-Ad-GFP-CYP1B1-DNA restored the expression of *Cyp1b1* mRNA in both the PVN and SFO (Figure S1C and S1D), and the Ang II-induced increased SBP in intact *Cyp1b1*$^{-/-}$ (Figure 1B) but not in Orchi-*Cyp1b1*$^{-/-}$ mice (Figure S1E).

GFP expression in the PVN and SFO (Figure 1C and 1D; Figure S1F) confirmed the transduction with adenoviral vectors given by ICV. The CYP1B1 adenoviral vectors selectively downregulated expression of *Cyp1b1* mRNA, as indicated by a lack of effect on the expression of *Cyp4a10* and *Cyp1a1* mRNA in the PVN (Figure S1A and S1C).

CYP1B1 Contributes to Ang II-Induced Reactive Oxygen Species Production in the Brain

Ang II-induced hypertension is mediated via reactive oxygen species (ROS) production in the SFO and PVN.[20] Moreover, the central infusion of tempol, a superoxide dismutase mimetic, prevents Ang II-induced hypertension in male mice.[12] Therefore, we investigated the contribution of CYP1B1 in the brain to ROS production, as indicated by enhanced 2-hydroxyethidium fluorescence (yellow color) generated after staining with dihydroethidium and visualized using a dual-wavelength filter, excitation at 375 nm and emission at 585 nm.[21] Ang II increased the ROS production in the PVN and SFO in *Cyp1b1*$^{+/+}$ mice with ICV-Ad-GFP-Scr-shRNA (Figure 1E; Figure S2A). However, this effect of Ang II to increase ROS production was lower in the PVN than in the SFO in *Cyp1b1*$^{+/+}$ mice with ICV-Ad-GFP-CYP1B1-shRNA (Figure 1E; Figure S2A). Transduction with ICV-Ad-GFP-CYP1B1-DNA but not its control Ad-GFP-DNA restored 2-hydroxyedithium fluorescence in the PVN completely and only partially in the SFO in *Cyp1b1*$^{-/-}$ mice (Figure 1F; Figure S2B). Ang II-induced increase in 2-hydroxyedithium fluorescence in PVN or SFO was

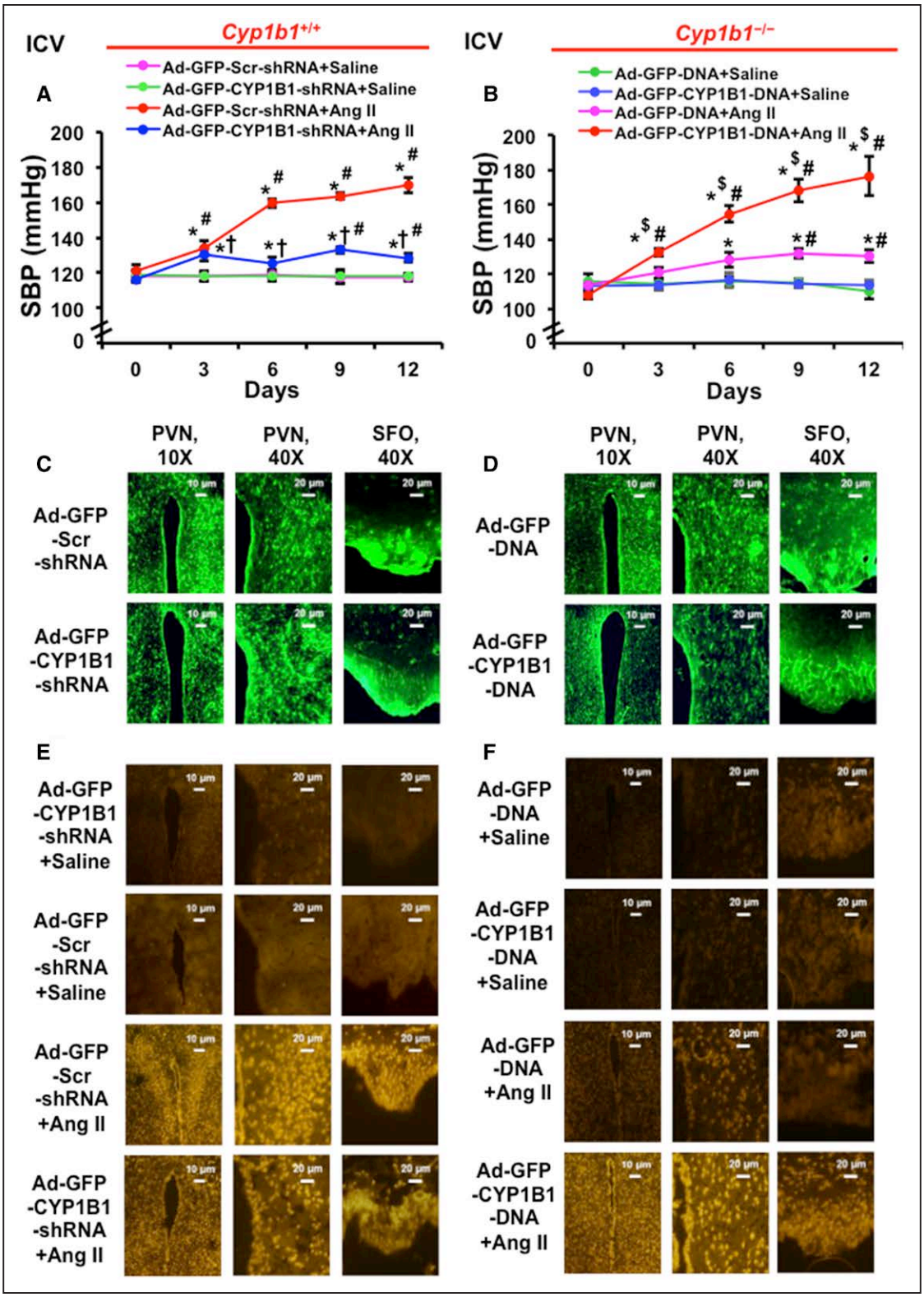

Figure 1. Central CYP1B1 (cytochrome P450 1B1) knockdown with intracerebroventricular (ICV)-injection of adenovirus (Ad)-GFP (green fluorescence protein)-CYP1B1 short hairpin (sh)RNA (Ad-GFP-CYP1B1-shRNA) but not Ad-scrambled (Scr)-shRNA in *Cyp1b1*$^{+/+}$ mice attenuated (**A**) and reconstitution with ICV-injection of Ad-GFP-CYP1B1-DNA but not Ad-GFP-DNA in *Cyp1b1*$^{-/-}$ mice restored (**B**) the Ang II (angiotensin II)-induced increase in systolic blood pressure (SBP) as measured by tail-cuff method. Transduction by ICV-Ad-GFP-CYP1B1-shRNA/Ad-GFP-Scr-shRNA (**C**) and Ad-GFP-DNA/Ad-GFP-CYP1B1-DNA (**D**) was confirmed by their fluorescence in the subfornical organ (SFO) and paraventricular nucleus (PVN). Ang II in *Cyp1b1*$^{+/+}$ mice transduced with ICV-Ad-GFP-Scr-shRNA increased the reactive oxygen species production in PVN and SFO as indicated by 2-hydroxyethidium (2-HE) fluorescence (**E**). However, Ang II-induced increase in 2-HE fluorescence was lower in PVN than in the SFO in *Cyp1b1*$^{+/+}$ mice transduced with ICV-Ad-GFP-CYP1B1-shRNA (**E**). Transduction with ICV-Ad-GFP-CYP1B1-DNA but not its control Ad-GFP-DNA restored 2-HE fluorescence in PVN and partially in SFO in *Cyp1b1*$^{-/-}$ mice (**F**). Saline was used as a vehicle for Ang II. **P*<0.05 vs Day 0 values (before the start of Ang II infusion) within the group, #*P*<0.05 vs corresponding saline-treated groups; †*P*<0.05 vs Ad-GFP-Scr-shRNA+Ang II; $*P*<0.05 vs Ad-GFP-DNA+Ang II, n=5–8/group.

not observed in orchi-*Cyp1b1*$^{-/-}$ mice transduced with ICV-Ad-GFP-CYP1B1-DNA (Figure S2C and S2D).

Reconstitution of CYP1B1 Expression in the PVN, but Not in SFO Exacerbates Ang II-Induced Hypertension in *Cyp1b1*$^{-/-}$ Mice

We recently reported that in female mice, *Cyp1b1* mRNA is expressed in both the SFO and PVN and reconstitution of *Cyp1b1* mRNA in PVN but not SFO in the *Cyp1b1*$^{-/-}$ mice protected against Ang II-induced hypertension.[2] This observation raised the possibility that Ang II-induced hypertension might also depend on CYP1B1 in the PVN in male mice. Therefore, we examined the expression of *Cyp1b1* mRNA in PVN and SFO and the effect of selective transduction of the SFO and PVN with Ad-GFP-CYP1B1-DNA on the Ang II-induced increase in SBP in male *Cyp1b1*$^{-/-}$ mice. Like in females, *Cyp1b1* mRNA was expressed in both SFO and PVN (Figure S3A). However, in contrast to females,[2] transduction with Ad-GFP-CYP1B1-DNA in PVN but not in SFO restored the Ang II-induced increase in SBP in *Cyp1b1*$^{-/-}$ male mice (Figure 2A and 2B). The selective transduction of the adenoviral vectors in PVN and SFO was confirmed by GFP fluorescence (Figure 2C and 2D) and the restoration of the expression of *Cyp1b1* mRNA in the PVN or SFO but not in the adjoining areas—ventromedial hypothalamic nucleus and ventral hippocampal commissure, respectively (Figure S3B and S3C).

Testosterone via Its CYP1BI-Generated Metabolite 6β-OHT in the Brain Contributes to Ang II-Induced Hypertension

Since transduction with Ad-GFP-CYP1B1-DNA by ICV or selectively injecting into PVN restored the Ang II-induced increase in SBP in *Cyp1b1*$^{-/-}$ mice, we investigated whether testosterone in the brain contributes to Ang II-induced hypertension via its metabolism to 6β-OHT. Ang II failed to increase mean arterial pressure (MAP), SBP, and diastolic BP in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice (Figure 3A and 3B; Figure S4A through S4D). ICV-injection of testosterone restored the effect of Ang II to increase MAP (Figure 3A and 3B), SBP (Figure S4A and S4B), and diastolic BP (Figure S4C and S4D) to a higher degree in Orchi-*Cyp1b1*$^{+/+}$ than in Orchi-*Cyp1b1*$^{-/-}$ mice. However, ICV-injection of the testosterone-CYP1B1 metabolite 6β-OHT, but not testosterone, restored the Ang II-induced increase in MAP, SBP, and diastolic BP (Figure 3B; Figure S4B and S4D) in Orchi-*Cyp1b1*$^{-/-}$ mice. Ang II did not alter heart rate and locomotor activity in any of the above groups (Figure S4E through S4H).

Central 6β-OHT Mediates the Effect of Testosterone to Promote Ang II-Induced Impairment of Autonomic Function

Systemic Ang II in mice is associated with impairment of autonomic function.[22,23] Therefore, we assessed whether testosterone via its CYP1B1-generated metabolite 6β-OHT in the brain promotes this effect of Ang II. The autonomic function was identical in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice infused with saline (Figure 3C through 3F). Power spectral analysis of heart rate variability showed no increase in the ratio of low frequency to high frequency oscillations or LF/HF ratio (Figure 3C) during Ang II infusion in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice, indicating intact sympathoparasympathetic balance. Also, in these mice, Ang II did not alter either the vascular (decreased MAP to ganglionic blocker hexamethonium, 30 mg/kg, intraperitoneal, Figure 3D) or cardiac (bradycardia to β-blocker propranolol, 4 mg/kg, intraperitoneal, Figure 3E) sympathetic activity, or vagal tone (tachycardia to muscarinic antagonist atropine, 1 mg/kg, intraperitoneal, Figure 3F). However, systemic administration of Ang II resulted in sympathoparasympathetic imbalance (Figure 3C), increased both vascular (Figure 3D), and cardiac (Figure 3E) sympathetic activity, and reduced vagal tone (Figure 3F) in ICV-testosterone injected Orchi-*Cyp1b1*$^{+/+}$ mice, and ICV-6β-OHT but not ICV-testosterone injected Orchi-*Cyp1b1*$^{-/-}$ mice.

During Ang II infusion, ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ and ICV-6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice, increased urinary levels of norepinephrine (Figure S5A), an index of sympathetic outflow, and the vasopressin prosegment, copeptin (Figure S5B), a biomarker for vasopressin release.[24]

ICV-testosterone injection over the duration of the experiment did not result in an increase in plasma testosterone levels (Figure S5C) in Orchi-*Cyp1b1*$^{+/+}$ or Orchi-*Cyp1b1*$^{-/-}$ mice, suggesting that there was no leakage of testosterone from the brain.

Testosterone-CYP1B1-Generated Metabolite 6β-OHT Promotes Ang II-Induced ROS Production in the Brain

We also investigated the CYP1B1-dependent action of testosterone on ROS production. Ang II failed to stimulate the production of ROS as indicated by 2-hydroxyethidium fluorescence in SFO and PVN in Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice (Figure 4A; Figure S6A). ICV-testosterone increased Ang II-induced ROS production in both the SFO and PVN in Orchi-*Cyp1b1*$^{+/+}$ (Figure 4A; Figure S6A) but not in Orchi-*Cyp1b1*$^{-/-}$ (Figure 4A; Figure S6B) mice. ICV-6β-OHT caused a larger increase in ROS production in the PVN than in the SFO in the Orchi-*Cyp1b1*$^{-/-}$ mice (Figure 4A; Figure S6B).

Since transduction with Ad-GFP-CYP1B1-DNA in PVN but not in SFO in *Cyp1b1*$^{-/-}$ mice restored the Ang II-induced increase in SBP, further studies of the action of testosterone and 6β-OHT were performed only in the PVN, which are described below.

Testosterone Increases *Adam17* but Not *nNos* mRNA in PVN in Orchi-*Cyp1b1*$^{+/+}$ and 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ Mice

The ADAM17 (a disintegrin and metalloprotease 17)-mediated impairment of ACE2 (angiotensin-converting enzyme 2) compensatory function in the mouse hypothalamus has been shown to contribute to the development of neurogenic hypertension.[25] Hence, we investigated the CYP1B1-dependent action of 6β-OHT on *Adam17* mRNA expression in PVN in response to Ang II. Orchidectomy reduced, and Ang II infusion increased PVN *Adam17* mRNA expression in *Cyp1b1*$^{+/+}$, but not in *Cyp1b1*$^{-/-}$ mice (Figure 4B). Testosterone in

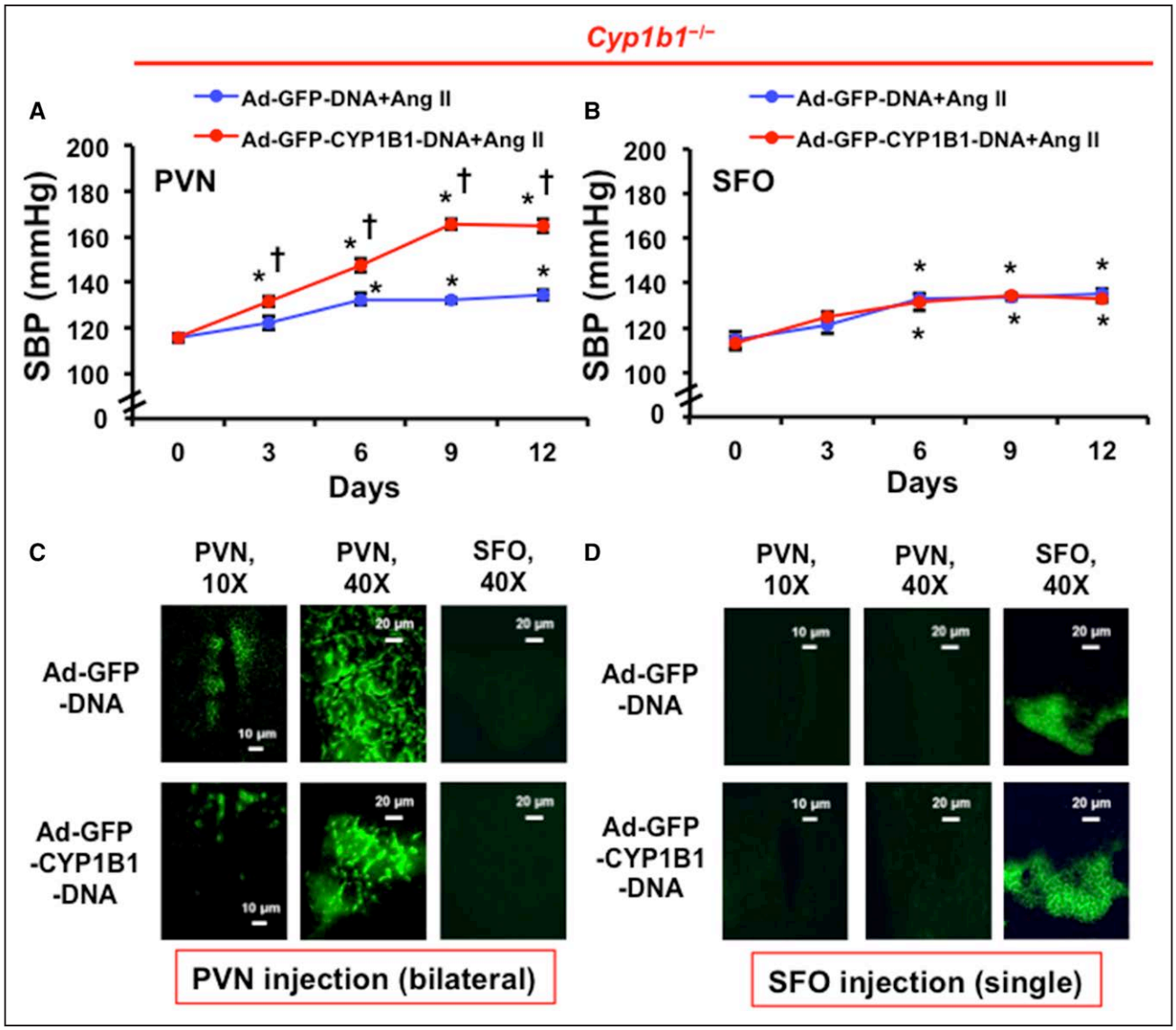

Figure 2. Selective reconstitution of CYP1B1 (cytochrome P450 1B1) in the paraventricular nucleus (PVN) bilaterally (**A**), but not in the subfornical organ (SFO; **B**) of *Cyp1b1*$^{-/-}$ mice with adenovirus (Ad)-GFP (green fluorescence protein)-CYP1B1-DNA but not Ad-GFP-DNA restored Ang II (angiotensin II)-induced increase in systolic blood pressure (SBP) measured by tail-cuff method. The selectivity of Ad-GFP-DNA/Ad-GFP-CYP1B1-DNA injection in PVN (**C**) and SFO (**D**) was confirmed by its GFP fluorescence. Saline was used as a vehicle for Ang II. **P*<0.05 vs Day 0 values (before the start of Ang II infusion) within the group, †*P*<0.05 vs Ad-GFP-DNA+Ang II; n=12–13/group.

Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$, and 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice increased the *Adam17* mRNA expression in PVN in response to Ang II infusion (Figure 4B).

Central inhibition of NOS (nitric oxide synthase) has been shown to contribute to Ang II-induced increase in the discharge of renal sympathetic nerves and elevated arterial BP and heart rate in male rats.[26] However, in the present study, orchidectomy or *Cyp1b1* gene disruption and Ang II infusion did not affect PVN nNos-mRNA expression (Figure 4C). Neither testosterone in Orchi-*Cyp1b1*$^{+/+}$ nor 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice altered the *nNos* mRNA expression in the PVN in response to Ang II infusion (Figure 4C).

6β-OHT Promotes Ang II-Induced Increase in PVN c-Fos+ Cell Population

The effect of testosterone and 6β-OHT on PVN neuronal activity was also assessed by examining c-Fos immunoreactivity in response to Ang II. As expected, Ang II failed to increase the total number of c-Fos+ cells in both Orchi-*Cyp1b1*$^{+/+}$ or Orchi-*Cyp1b1*$^{-/-}$ mice (Figure S7A and S7B). However, ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$, and 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$, but not ICV-testosterone in Orchi-*Cyp1b1*$^{-/-}$ mice increased the number of c-Fos+ cells in PVN (Figure S7).

6β-OHT Promotes Ang II-induced PVN Neuroinflammation

Systemic Ang II increases activation of microglia and pro-inflammatory cytokines including IL (interleukin)-1β), IL-6, TNF-α (tumor necrosis α), and decreases anti-inflammatory cytokine IL-10 mRNA levels in the PVN.[27] We examined the contribution of CYP1B1-dependent effects of testosterone on the neuroinflammatory markers in the PVN in the male mice in response to Ang II. Ang II failed to increase the number of activated microglia (ionized calcium-binding

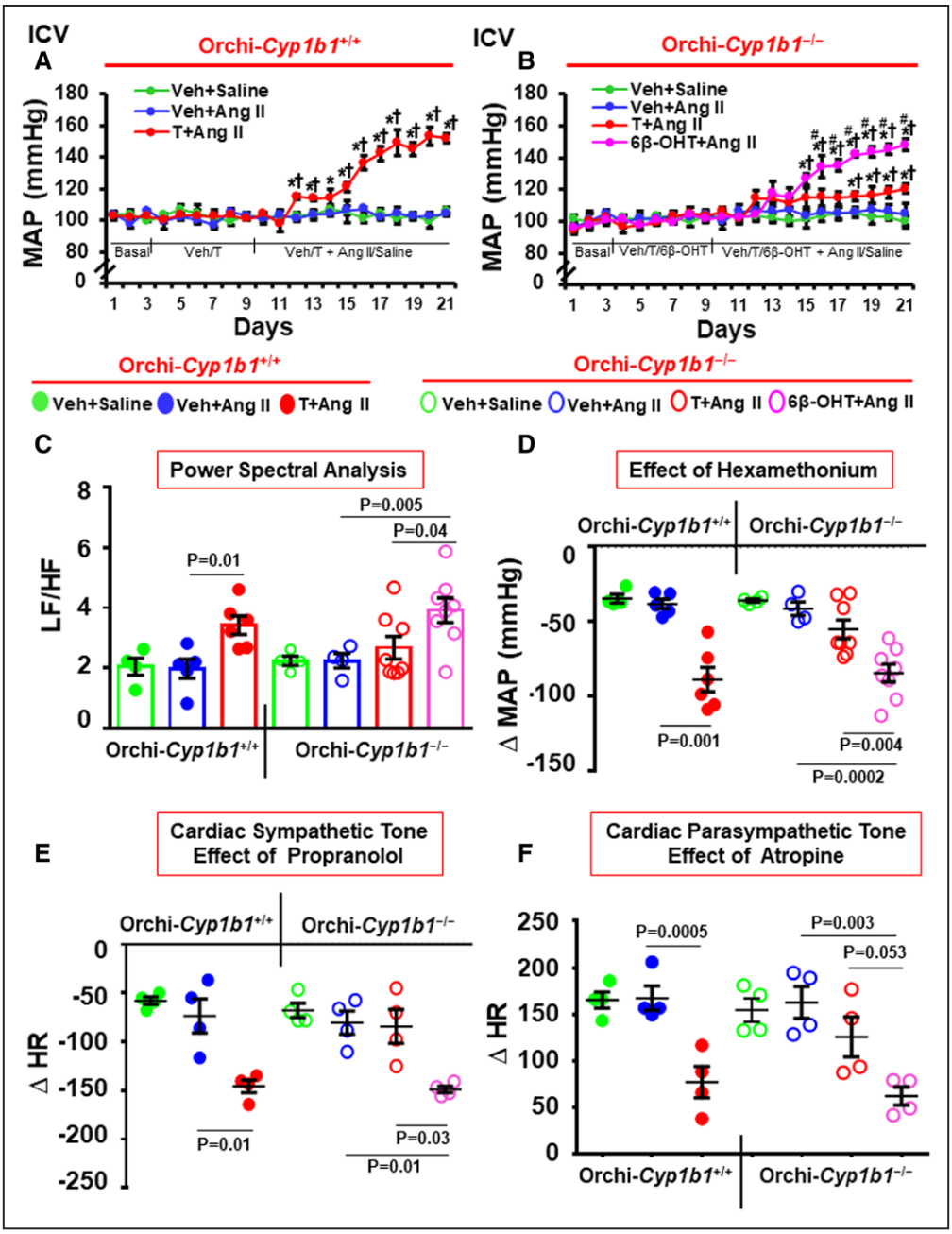

Figure 3. Ang II (angiotensin II) increased the mean arterial pressure (MAP) in Orchidectomized (Orchi)-*Cyp1b1*$^{+/+}$ mice with intracerebroventricular (ICV)-administration of T (testosterone) but not its vehicle (Veh, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid; **A**) as measured by radiotelemetry. However, ICV-6β-OHT (6β-hydroxytestosterone) but not T, restored the Ang II-induced increase in MAP (**B**) in Orchi-*Cyp1b1*$^{-/-}$ mice. T in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ mice restored the Ang II-induced increased low frequency (LF)/high frequency (HF) ratio of heart rate (HR) variability, **C**), decreased MAP in response to ganglionic blocker hexamethonium (**D**), bradycardia in response to β-blocker propranolol (**E**) and tachycardia in response to muscarinic antagonist atropine (**F**). In Orchi-*Cyp1b1*$^{-/-}$ mice, ICV-6β-OHT restored these effects of Ang II (**C** through **F**). Saline was used as a vehicle for Ang II. **P*<0.05 vs Day 9 value (the day before implantation of the osmotic minipump) within the group, †*P*<0.05 vs Vehicle (Veh) Ang II in the corresponding Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ groups, #*P*<0.05 vs T+Ang II in Orchi-*Cyp1b1*$^{-/-}$ group; n=4–8/group. Δ indicates change.

adaptor molecule 1 positive or IBA+ cells) in the PVN in Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice (Figure 5A and 5B; Figure S7C). ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$, and 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$, but not testosterone in Orchi-*Cyp1b1*$^{-/-}$ mice, increased the number of activated microglia in the PVN (Figure 5A and 5B; Figure S7C). Most of these microglia appeared to have globular cell bodies with shorter and thicker cell processes (Figure 5A, lower panel). The remaining microglia appeared to have thinner and longer processes and reduced cell bodies as observed in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ or in ICV-testosterone injected Orchi-*Cyp1b1*$^{-/-}$ mice with or without Ang II (Figure 5A, lower panel).

Astrocytes contribute to Ang II-mediated sympathoexcitatory effects in the PVN.[28] In our study, Ang II failed to increase the number of activated astrocytes (glial fibrillary acidic protein, GFAP+ cells) in the PVN in Orchi-*Cyp1b1*$^{+/+}$ or Orchi-*Cyp1b1*$^{-/-}$ mice with ICV-Veh (Figure 5A and 5B;

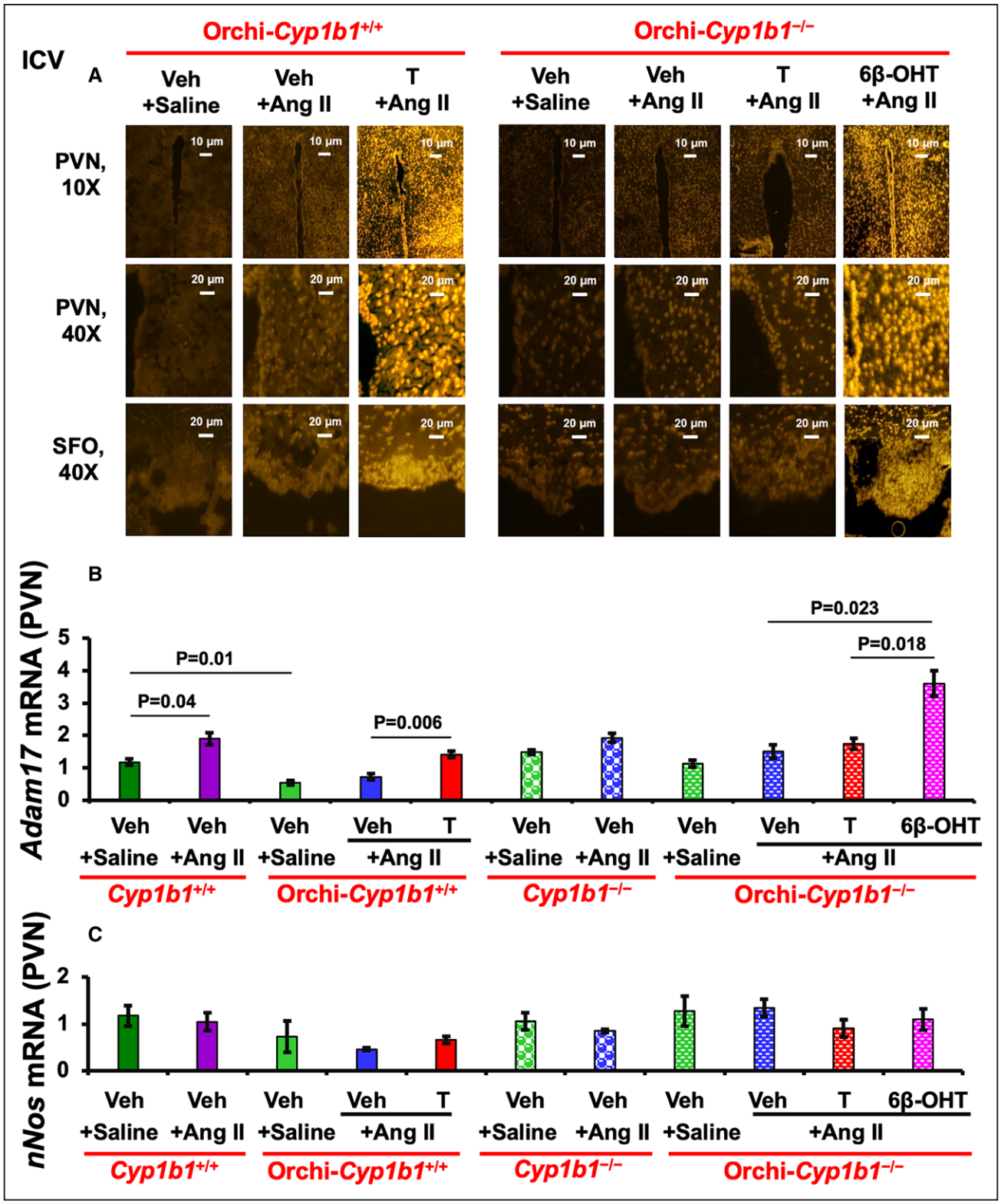

Figure 4. Ang II (angiotensin II) increased 2-hydroxyethidium fluorescence to a greater degree in the paraventricular nucleus (PVN) and minimally in the subfornical organ (SFO) in orchidectomized (Orchi)-*Cyp1b1*$^{+/+}$ with intracerebroventricular (ICV)-T (testosterone) but not in Orchi-*Cyp1b1*$^{-/-}$ mice (**A**). ICV-6β-OHT (6β-hydroxytestosterone) caused a greater increase in the reactive oxygen species production in the PVN than in the SFO in the Orchi-*Cyp1b1*$^{-/-}$ mice (**A**). Ang II increased *Adam17* messenger (m)RNA expression in the PVN in the *Cyp1b1*$^{+/+}$ but not in Orchi- *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice, and this effect was restored by ICV-T in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ mice (**B**). ICV-6β-OHT increased the *Adam17* mRNA expression in PVN in Ang II-infused Orchi-*Cyp1b1*$^{-/-}$ mice (**B**). Ang II did not alter *nNos* mRNA in intact or Orchi- *Cyp1b1*$^{+/+}$ and *Cyp1b1*$^{-/-}$ mice with or without ICV-T or 6β-OHT (**C**). Saline was used as a vehicle for Ang II. Vehicle (Veh) for T and 6β-OHT, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid. n=3 samples/group, each sample consist of bilateral PVN punches pooled from 3 to 4 mice.

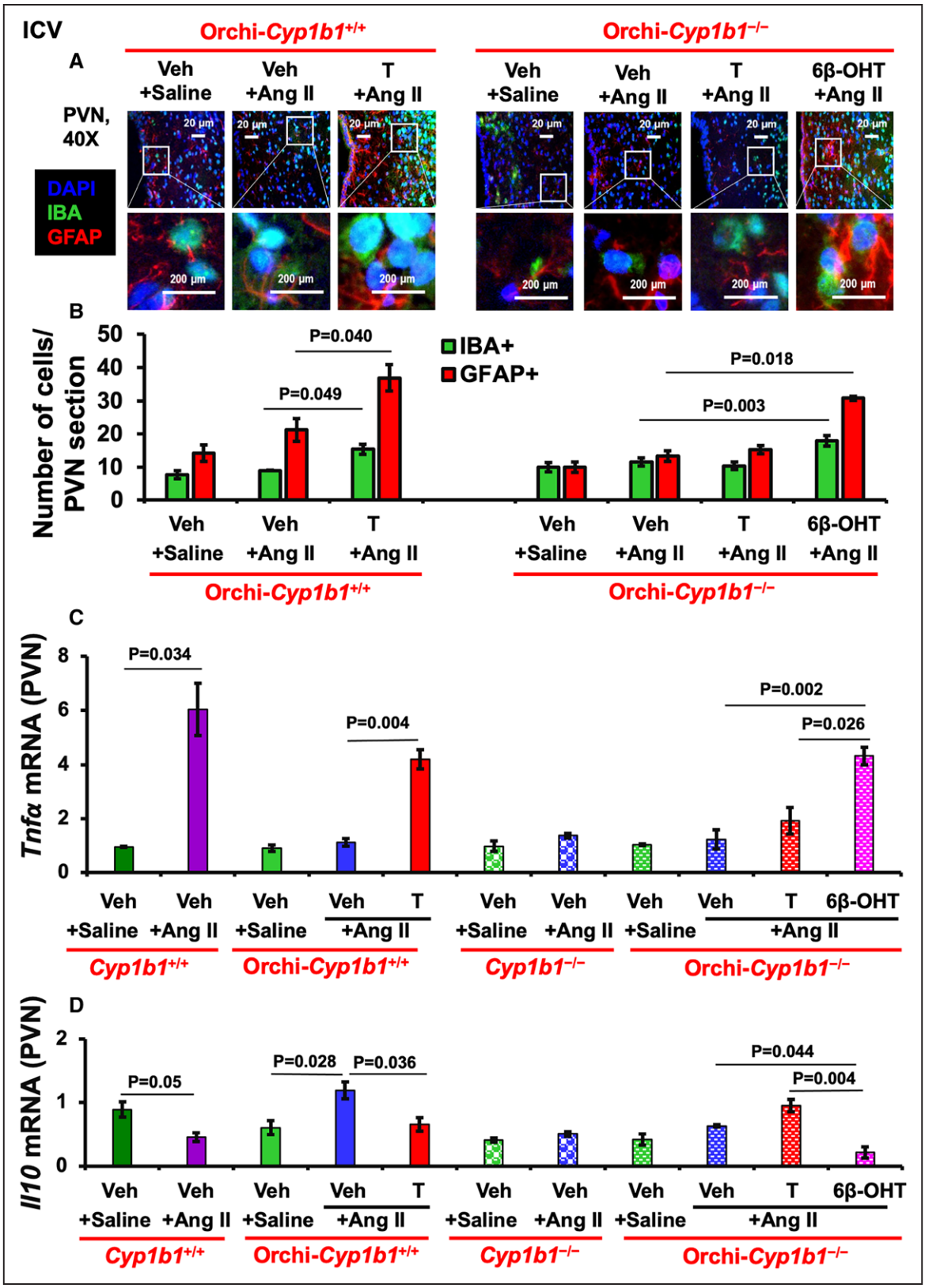

Figure 5. Intracerebroventricular (ICV)-T (testosterone) in orchidectomized (Orchi)-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$, and ICV-6β-OHT (6β-hydroxytestosterone) in Orchi-*Cyp1b1*$^{-/-}$ mice restored the Ang II (angiotensin II)-induced increase in the number of activated microglia (ionized calcium-binding adaptor molecule 1 [IBA], **A** and **B**) and astrocytes (glial fibrillary acidic protein [GFAP], **A** and **B**) in the paraventricular nucleus (PVN). The enlarged images in the lower panel (**A**) show the colocalization of microglia and astrocyte and their morphology. Ang II failed to increase *Tnfα*-mRNA (**C**) and reduce *Il10*-mRNA (**D**) in PVN, which were restored by ICV-T in Orchi-*Cyp1b1*$^{+/+}$ (**C** and **D**) or 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ (**C** and **D**) mice. Saline was used a vehicle for Ang II. Vehicle (Veh) for T and 6β-OHT, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid. n=3/group for histological analysis; for mRNA expression: n=3 samples/group, each sample consist of bilateral PVN punches pooled from 3 to 4 mice.

Figure S7C). Ang II infusion in ICV-testosterone injected Orchi-*Cyp1b1*$^{+/+}$, and ICV-6β-OHT injected Orchi-*Cyp1b1*$^{-/-}$, but not ICV-testosterone injected Orchi-*Cyp1b1*$^{-/-}$ mice increased the PVN activated astrocytes (Figure 5A and 5B; Figure S7C), mostly having shorter and thicker cell processes (Figure 5A, lowe;r panel). The remaining astrocytes showed longer and thinner processes resembling those observed in the ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ or ICV-testosterone injected Orchi-*Cyp1b1*$^{-/-}$ mice with or without Ang II (Figure 5A, lower panel).

Ang II increased *Tnf*α mRNA (Figure 5C) and reduced *Il10* mRNA (Figure 5D) in the PVN of *Cyp1b1*$^{+/+}$ mice, but these effects were reversed by orchidectomy or CYP1B1 gene disruption. ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ or 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice increased *Tnf*α-mRNA (Figure 5C) and reduced *Il10*-mRNA (Figure 5D) in PVN in response to Ang II.

ICV-Small Interfering RNA of AR and GPRC6A Blunts the Effect of 6β-OHT to Restore Ang II-Induced Hypertension in Orchi-*Cyp1b1*$^{-/-}$ Mice

Testosterone produces its actions via both genomic AR[29,30] and nongenomic GPRC6A receptors.[15] ICV-6β-OHT failed to restore the effect of Ang II to increase MAP (Figure 6A), SBP, diastolic BP (Figure S4B and S4D), and LF/HF ratio (Figure 6B) in mice injected with ICV-small interfering (si)RNA-AR or siRNA-GPRC6A. In addition, these siRNAs attenuated the effect of ICV-6β-OHT on the restoration of the decrease in Ang II-induced increased MAP in response to hexamethonium (Figure 6C), a decrease in heart rate in response to propranolol (Figure 6D), or to prevent an increase in heart rate in response to atropine (Figure 6E) in Orchi-*Cyp1b1*$^{-/-}$ mice. Interestingly, Ang II increased the *Gprc6a*- but not *Ar* mRNA in the PVN in *Cyp1b1*$^{+/+}$mice (Figure S8). ICV-siRNA-AR and siRNA-GPRC6A (0.4 nmol/2 µL, single injection) selectively reduced the expression of *Ar*- and *Gprc6a* mRNA, respectively, in PVN (Figure S8).

Central CYP1B1 Contributes to the Development of DOCA Salt-Induced Hypertension in Male Mice

We also examined the contribution of brain CYP1B1 in uninephectromized-DOCA (50 mg/kg, subcutaneous) salt (1% NaCl)-induced hypertension for 4 weeks. DOCA salt increased SBP in *Cyp1b1*$^{+/+}$ but not in *Cyp1b1*$^{-/-}$ mice (Figure S9A). ICV-Ad-GFP-CYP1B1-shRNA but not Ad-GFP-Scr-shRNA minimized the SBP in response to DOCA salt treatment in *Cyp1b1*$^{+/+}$ mice (Figure S9B). Transduction in the brain by ICV-Ad-GFP-CYP1B1-DNA but not Ad-GFP-DNA restored the DOCA salt-induced increase in SBP in the *Cyp1b1*$^{-/-}$ mice (Figure S9C).

Discussion

The present study is the first to show the contribution of central testosterone in Ang II-induced neurogenic hypertension via its CYP1B1-generated metabolite 6β-OHT, most likely by its action in the PVN by increasing (1) sympathetic outflow, (2) vasopressin release, (3) ROS production, (4) *Adam17* mRNA, (5) activation of microglia and astrocytes, and (6) proinflammatory cytokine *Tnf*α mRNA, while reducing (7) anti-inflammatory cytokine *Il10* mRNA expression. Also, we show for the first time that 6β-OHT in the brain promotes Ang II-induced hypertension through both the AR and GPRC6A receptors in Orchi-*Cyp1b1*$^{-/-}$ mice.

CYP1B1 is present in human and mouse brain,[18,19] and the central administration of the AR antagonist, flutamide in male mice attenuated Ang II-induced hypertension.[12] Our findings that (1) ICV-Ad-GFP-CYP1B1-shRNA in the *Cyp1b1*$^{+/+}$ mice attenuated, and ICV-Ad-GFP-CYP1B1-DNA in *Cyp1b1*$^{-/-}$ mice restored Ang II-induced increase in SBP, and (2) ICV-Ad-GFP-CYP1B1-DNA in Orchi-*Cyp1b1*$^{-/-}$ mice that lack endogenous testosterone, failed to restore the Ang II-induced increase in SBP suggests that testosterone contributes to Ang II-induced hypertension via a CYP1B1-generated metabolite, most likely 6β-OHT locally in the brain. Ang II is known to produce neurogenic hypertension via its action in brain circumventricular organs, including SFO,[9] through its communication with the PVN of the hypothalamus.[10] The central effect of Ang II-induced hypertension is dependent on ROS production in these areas.[20] In the present study, transduction with ICV-Ad-GFP-CYP1B1-shRNA showed a trend of greater reduction in ROS production in *Cyp1b1*$^{+/+}$ mice, whereas the ICV-Ad-GFP-CYP1B1-DNA caused a greater increase in ROS production in *Cyp1b1*$^{-/-}$ mice in response to Ang II in PVN than in SFO. These observations suggest that CYP1B1 in the PVN contributes to the Ang II-induced ROS production via the metabolism of testosterone into 6β-OHT. Supporting this view was our observation that transduction with Ad-GFP-CYP1B1-DNA in PVN, but not SFO, restored the Ang II-induced increase in BP in *Cyp1b1*$^{-/-}$ mice. Further evidence that testosterone contributes to Ang II-induced hypertension via its metabolism by CYP1B1 locally in the brain was that Ang II failed to increase BP or impair autonomic function in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice. The effects of Ang II to increase BP and impair autonomic function were restored by ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ and by 6β-OHT in *Cyp1b1*$^{-/-}$ mice. Moreover, urinary levels of norepinephrine were also increased by ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ mice, and ICV-6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice. In as much as ICV-6β-OHT alone did not affect BP, it seems to act as a permissive factor (ie, it is required) for Ang II-induced hypertension. The effect of ICV-testosterone appeared to be limited to the brain since plasma levels of testosterone that were attenuated in Orchi-*Cyp1b1*$^{+/+}$, and Orchi-*Cyp1b1*$^{-/-}$ mice were not increased by ICV-injected testosterone. From these observations, it follows that the testosterone-CYP1B1-generated metabolite 6β-OHT locally in the brain promotes Ang II-induced hypertension primarily by its central action of increased sympathetic activity independent of its direct vascular and renal effects.

Ang II in the brain increases vasopressin release that contributes to its pressor effect.[31] In our study, Ang II in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$, and Orchi-*Cyp1b1*$^{-/-}$ mice increased urinary concentrations of the vasopressin prosegment, copeptin which was further enhanced by ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ mice, and 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice. These observations indicate that 6β-OHT contributes to the Ang II-induced increase in vasopressin release that could complement the hypertensive effect of Ang II. The mechanism by which 6β-OHT promotes

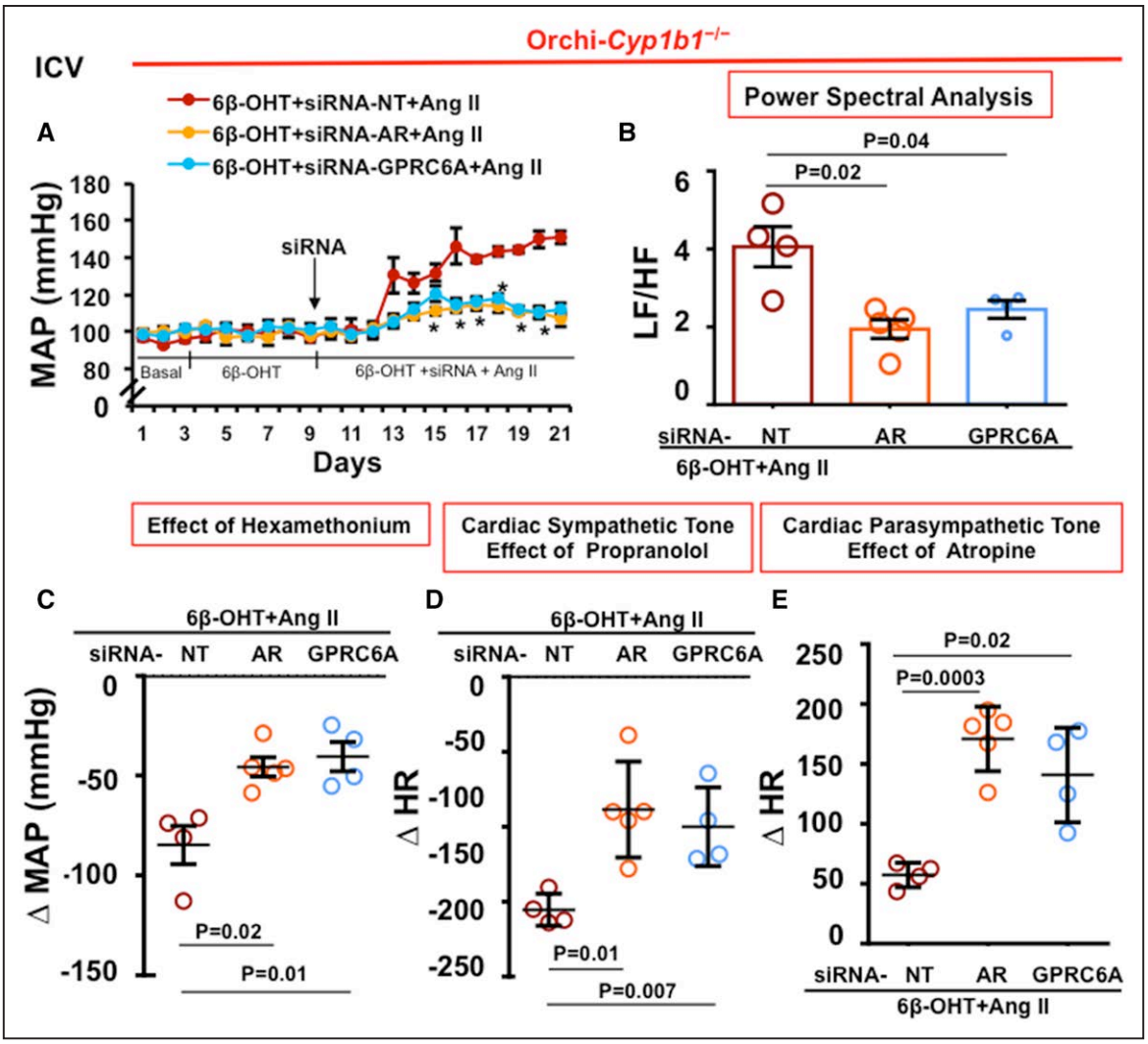

Figure 6. Intracerebroventricular (ICV)-6β-OHT (6β-hydroxytestosterone) failed to restore Ang II (angiotensin II)-induced increase in mean arterial pressure (MAP; **A**), low frequency (LF)/high frequency (HF) ratio (power spectral analysis; **B**), decreased MAP in response to ganglionic blocker hexamethonium (**C**), bradycardia in response to β-blocker propranolol (**D**), and tachycardia in response to muscarinic antagonist atropine (**E**) in orchidectomized (Orchi)-*Cyp1b1*$^{-/-}$ mice with ICV-small interfering (si)RNA-AR (androgen receptor) or siRNA-GPRC6A (G-protein coupled receptor C6A). Saline was used as a vehicle for Ang II. *P<0.05 vs Day 9 value (the day before implantation of the osmotic minipump) within the group, #P<0.05, vs 6β-OHT +siRNA-NT+Ang II; n=4–5/group. Δ indicates change; and NT, nontargeted control.

the Ang II-induced increase in vasopressin prosegment release remains to be determined.

Recently, we have shown that the effect of 17β-estradiol to inhibit ROS production and increase *nNos* mRNA expression in the PVN, and to protect against Ang II-induced hypertension, is mediated by its CYP1B1-generated metabolite 2-methoxyestradiol in the PVN of female mice.[2] In the present study, ICV-testosterone caused a greater increase in Ang II-induced ROS production as determined by 2-hydroxyethidium fluorescence in the PVN than in SFO of the male Orchi-*Cyp1b1*$^{+/+}$ mice. However, testosterone failed to increase Ang II-induced ROS production in the SFO and PVN in Orchi-*Cyp1b1*$^{-/-}$ mice, most likely due to lack of its CYP1B1-generated metabolite 6β-OHT. Supporting this conclusion was our demonstration that ICV-6β-OHT in the Orchi-*Cyp1b1*$^{-/-}$ mice caused a greater increase in Ang II-induced ROS production in the PVN than in SFO. These observations further support our contention that 6β-OHT acts primarily in the PVN. Ang II-induced ROS production leads to increased calcium (Ca^{2+}) signaling and neuronal firing.[32] Our finding that the Ang II-induced increase in the number of c-Fos+ cells in the PVN by ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$, and by ICV-6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice, suggests that 6β-OHT increases neuronal activity most likely by increasing Ca^{2+} signaling. It has been reported that castration rescued mice from heatstroke-induced apoptosis and neuronal damage in the hypothalamus, which was reversed by the restoration of circulating testosterone levels.[33] Whether or not this effect of testosterone is mediated by its metabolite 6β-OHT is not known.

6β-OHT in the PVN could mediate the effect of testosterone to enhance the effect of Ang II by increasing ADAM17-glutamatergic or by inhibiting NO-GABA signaling.[25,26,34–37] In the present study, Ang II increased *Adam17*- but not the *nNos*-mRNA expression in the PVN of ICV-testosterone injected Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$, and

ICV-6β-OHT injected Orchi-*Cyp1b1*$^{-/-}$ mice. These observations suggest that ADAM17 and not NO most likely contribute to the action of 6β-OHT to promote the effect of Ang II in the PVN to increase sympathetic activity and BP in male mice. The mechanism by which 6β-OHT contributes to ADAM17-mediated effects of Ang II on glutamatergic neurons remains to be determined. Our observation that Ang II did not alter PVN *nNOS* mRNA is consistent with previous reports that the Ang II-induced increases in BP and sympathetic activity is independent of nNOS (neuronal NOS) in the PVN or SFO in male mice.[37] Therefore, it appears that in male mice, unlike in rats,[35] GABAergic inhibitory input to presympathetic neurons in the PVN is independent of NO. Further studies are required to assess the interaction of the ACE2-GABAergic system[35] in 6β-OHT-mediated effects of Ang II on the activity of presympathetic neurons in the PVN and BP.

The present study demonstrates, for the first time, the critical role of testosterone in neuroinflammation associated with Ang II-induced hypertension that is mediated by the CYP1B1-generated metabolite 6β-OHT. Supporting this view was our demonstration that ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not *Cyp1b1*$^{-/-}$, and the testosterone metabolite 6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice increased *Tnf*α mRNA, and reduced *Il10* mRNA levels in PVN. Stimulation with TNF-α in the PVN increases sympathetic outflow and enhances cardiac sympathetic afferent reflex with subsequent BP elevation.[38]

Neurogenic hypertension involves activation of microglia in the PVN, and inhibition of PVN microglia activation prevents Ang-II-induced hypertension.[2,27] Our findings that Ang II increased PVN-activated microglia IBA+ cells in ICV-testosterone injected Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$ and ICV-6β-OHT injected Orchi-*Cyp1b1*$^{-/-}$ mice indicates that the testosterone metabolite 6β-OHT promotes this effect of Ang II. Ang II also stimulates hypothalamic astrocytes and decreases PVN glutamate currents by decreasing extracellular glutamate uptake, thereby resulting in an increase in extracellular glutamate and presympathetic nerve activity, and hypertension in male rats.[28] In a recent study, we showed that 17β-estradiol via CYP1B1-generated metabolite 2-methoxyestradiol inhibited activation of microglia and astrocytes in the PVN in response to Ang II in female mice.[2] However, in the present study, Ang II produced opposite effects on PVN astrocytes in male mice. Ang II failed to induce an increase in astrocyte activation in the PVN in ICV-Veh injected Orchi-*Cyp1b1*$^{+/+}$ and Orchi-*Cyp1b1*$^{-/-}$ mice; these effects of Ang II were reversed by ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ but not in Orchi-*Cyp1b1*$^{-/-}$, and by ICV-6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ mice, as indicated by the increased number of GFAP+ cells in the PVN. From these observations, it follows that the testosterone metabolite 6β-OHT also promotes the effect of Ang II to cause activation of astrocytes that contribute to neuroinflammation, sympathetic outflow, and hypertension in male mice.[28] Importantly, both human neurons and astrocytes contain CYP1B1 protein.[19] The mechanism by which 6β-OHT promotes these effects of Ang II that could be due to increased extracellular glutamate as a result of decreased uptake by astrocytes[28] remains to be determined.

Testosterone produces its actions via both genomic AR[29,30] and nongenomic GPRC6A receptors.[15] While the role of GPRC6A in the brain is unknown, the role of AR in the brain has been explored traditionally due to its relationship with behavior involving the administration of a nonaromatizable AR agonist (usually dihydrotestosterone) or antagonist (eg, flutamide). The effects of dihydrotestosterone may not be AR specific, as dihydrotestosterone can be metabolized into 3α-androstanediol (3α-diol), which has a low affinity for ARs but a high affinity for GABA receptors, and 3βdiol, an estrogenic compound which binds estrogen receptors.[39] The observations that AR is expressed in the PVN and other hypothalamic regions in male mice[40] and central administration of the AR antagonist flutamide in male mice attenuated the Ang II-induced hypertension[12] suggest that testosterone contributes to Ang II-induced hypertension via its action on AR in the brain, most likely in the PVN. In this study, we found that ICV-6β-OHT in Orchi-*Cyp1b1*$^{-/-}$ produced a similar increase in MAP and autonomic imbalance as ICV-testosterone in Orchi-*Cyp1b1*$^{+/+}$ in response to Ang II but failed to do so in mice injected with ICV-siRNA-AR or siRNA-GPRC6A. These observations suggest that 6β-OHT contributes to the effect of testosterone-dependent Ang II-induced autonomic imbalance and hypertension via both the receptors AR and GPRC6A in the brain, most likely by acting in the PVN. 6β-OHT could act via AR and GPRC6A receptors through independent pathways or by crosstalk acting on membrane GPRC6A that in turn leads to activation of nuclear receptor AR.[41] Our observation that Ang II increased the *Gprc6a-* but not *Ar* mRNA in the PVN in *Cyp1b1*$^{+/+}$ mice support the possibility of the latter mechanism. However, further studies are required to elucidate the cellular signaling pathways and the mechanism of interaction between AR and GPRC6A in mediating the effects of 6β-OHT in the brain on Ang II-induced hypertension, autonomic imbalance, and neuroinflammation.

There are epidemiological studies showing that high levels of androgens increased the incidence of cerebrovascular disease in young adult men[42] and high doses of testosterone produced deleterious effects in a mouse model of cerebral ischemia.[43] Therefore, based upon the findings of our study, it is possible that the CYP1B1-generated metabolite 6β-OHT might contribute to the deleterious effect of high levels of testosterone on the pathogenesis of the cardiovascular, renal, and cerebrovascular diseases associated with increased activity of the renin-angiotensin system.

There are some limitations in our study that need to be acknowledged. First, we did not measure levels of testosterone or its metabolite 6β-OHT in the PVN. This would have required many mice for analysis of these steroids by LC/MS, and currently, there is no ELISA assay available for the measurement of 6β-OHT. Therefore, the doses of the drugs used were based on preliminary experiments. Second, Ang II can also alter autonomic function and BP by its action in other areas in the brain, including the nucleus of the solitary tract and ventrolateral medulla. Therefore, the testosterone-CYP1B1 generated metabolite 6β-OHT that could also contribute to the action of Ang II by acting in these sites cannot be excluded and needs to be explored. Third, whether the observed effects in our study were independent or the consequence of hypertension per se remains to be addressed.

In conclusion, our study provides novel and significant information, and it demonstrates that CYP1B1-generated testosterone metabolite 6β-OHT contributes to Ang II-induced autonomic imbalance, hypertension, and neuroinflammation, most likely by its action in the PVN in male mice. Moreover, 6β-OHT promotes Ang II-induced autonomic imbalance and hypertension via both AR and GPRC6A (Graphical abstract, Figure S10). Furthermore, our preliminary experiments show that in the male mouse brain, CYP1B1 also contributes to DOCA salt-induced hypertension, which is known to be mediated by central Ang II.[25] Whether it is mediated by the testosterone-CYP1B1-generated metabolite 6β-OHT remains to be investigated. Moreover, the potential role of 6β-OHT in other models of neurogenic hypertension and neuroinflammatory diseases needs further exploration.

Perspectives

Our study is the first to demonstrate the mechanism by which testosterone via its central CYP1B1-generated metabolite 6β-OHT in the hypothalamic PVN contributes to Ang II-induced hypertension, autonomic imbalance, and increases neuroinflammation. Therefore, inhibitors of CYP1B1 activity would be useful for protection against the deleterious effects of Ang II in the development of neurogenic hypertension and neuroinflammatory diseases, which could be exacerbated using testosterone in males. However, in view of our recent study[2] that the 17β-estradiol-CYP1B1-catechol-O-methyltransferase generated metabolite 2-methoxyestradiol via its action most likely in the PVN protects against Ang II-induced hypertension and neuroinflammation, CYP1B1 inhibitors would be detrimental in females but could be treated with 2-methoxyestradiol, which can also be used in males.

Our study could have significant implications in the pathogenesis of COVID-19 infection, which is associated with cardiovascular dyshomeostasis, inflammation, and higher mortality in men than in women, and in patients with hypertension, which are known to have exacerbated activities of renin-angiotensin and sympathetic nervous system. A recent study shows that estradiol and AR antagonist enzalutamide downregulate the SARS-CoV-2 host factor transmembrane serine protease TMPRSS2 that is required for viral entry into cells.[44] Therefore, the exploration of the contribution of CYP1B1 to the sex differences in the pathogenesis of COVID-19 infection is warranted and could provide information on potential clinical significance and hence the use of CYP1B1 inhibitors in males and 2-methoxyestradiol in both females and males to protect cardiovascular damage and inflammation associated with COVID-19.

Acknowledgments

We thank Dr Amanda S. Clarke, Director, Office of Scientific Writing, University of Tennessee Health Science Center for her editorial assistance.

Sources of Funding

This work was supported by the National Institutes of Health National Heart, Lung, and Blood Institute grants R01HL-19134–44, R01HL-079109-09, and UTHSC CORNET Award (K.U. Malik); and UTHSC Neuroscience Institute Postdoctoral Partial Support to P. Singh. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Heart, Lung, and Blood Institute.

Disclosures

None.

References

1. Messerli FH, Garavaglia GE, Schmieder RE, Sundgaard-Riise K, Nunez BD, Amodeo C. Disparate cardiovascular findings in men and women with essential hypertension. *Ann Intern Med*. 1987;107:158–161. doi: 10.7326/0003-4819-107-2-158
2. Singh P, Song CY, Dutta SR, Gonzalez FJ, Malik KU. Central CYP1B1 (Cytochrome P450 1B1)-estradiol metabolite 2-methoxyestradiol protects from hypertension and neuroinflammation in female mice. *Hypertension*. 2020;75:1054–1062. doi: 10.1161/HYPERTENSIONAHA.119.14548
3. Reckelhoff JF. Gender differences in the regulation of blood pressure. *Hypertension*. 2001;37:1199–1208. doi: 10.1161/01.hyp.37.5.1199
4. Reckelhoff JF, Zhang H, Srivastava K. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system. *Hypertension*. 2000;35(1 Pt 2):480–483. doi: 10.1161/01.hyp.35.1.480
5. Crofton JT, Share L. Gonadal hormones modulate deoxycorticosterone-salt hypertension in male and female rats. *Hypertension*. 1997;29(1 Pt 2):494–499. doi: 10.1161/01.hyp.29.1.494
6. Xue B, Pamidimukkala J, Hay M. Sex differences in the development of angiotensin II-induced hypertension in conscious mice. *Am J Physiol Heart Circ Physiol*. 2005;288:H2177–H2184. doi: 10.1152/ajpheart.00969.2004
7. Pingili AK, Kara M, Khan NS, Estes AM, Lin Z, Li W, Gonzalez FJ, Malik KU. 6β-hydroxytestosterone, a cytochrome P450 1B1 metabolite of testosterone, contributes to angiotensin II-induced hypertension and its pathogenesis in male mice. *Hypertension*. 2015;65:1279–1287. doi: 10.1161/HYPERTENSIONAHA.115.05396
8. Mishra JS, More AS, Gopalakrishnan K, Kumar S. Testosterone plays a permissive role in angiotensin II-induced hypertension and cardiac hypertrophy in male rats. *Biol Reprod*. 2019;100:139–148. doi: 10.1093/biolre/ioy179
9. Johnson AK, Gross PM. Sensory circumventricular organs and brain homeostatic pathways. *FASEB J*. 1993;7:678–686. doi: 10.1096/fasebj.7.8.8500693
10. Koba S, Hanai E, Kumada N, Kataoka N, Nakamura K, Watanabe T. Sympathoexcitation by hypothalamic paraventricular neurons projecting to the rostral ventrolateral medulla. *J Physiol*. 2018;596:4581–4595. doi: 10.1113/JP276223
11. Ellison KE, Ingelfinger JR, Pivor M, Dzau VJ. Androgen regulation of rat renal angiotensinogen messenger RNA expression. *J Clin Invest*. 1989;83:1941–1945. doi: 10.1172/JCI114102
12. Xue B, Johnson AK, Hay M. Sex differences in angiotensin II-induced hypertension. *Braz J Med Biol Res*. 2007;40:727–734. doi: 10.1590/s0100-879x2007000500018
13. Wellendorph P, Bräuner-Osborne H. Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. *Gene*. 2004;335:37–46. doi: 10.1016/j.gene.2004.03.003
14. Kuang D, Yao Y, Lam J, Tsushima RG, Hampson DR. Cloning and characterization of a family C orphan G-protein coupled receptor. *J Neurochem*. 2005;93:383–391. doi: 10.1111/j.1471-4159.2005.03025.x
15. Pi M, Kapoor K, Wu Y, Ye R, Senogles SE, Nishimoto SK, Hwang DJ, Miller DD, Narayanan R, Smith JC, et al. Structural and functional evidence for testosterone activation of GPRC6A in peripheral tissues. *Mol Endocrinol*. 2015;29:1759–1773. doi: 10.1210/me.2015-1161
16. Crespi CL, Penman BW, Steimel DT, Smith T, Yang CS, Sutter TR. Development of a human lymphoblastoid cell line constitutively expressing human CYP1B1 cDNA: substrate specificity with model substrates and promutagens. *Mutagenesis*. 1997;12:83–89. doi: 10.1093/mutage/12.2.83
17. Song J, Wadhwa L, Bejjani BA, O'Brien WE. Determination of 3-keto-4-ene steroids and their hydroxylated metabolites catalyzed by recombinant human cytochrome P450 1B1 enzyme using gas chromatography-mass spectrometry with trimethylsilyl derivatization. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2003;791:127–135. doi: 10.1016/s1570-0232(03)00228-9
18. Yamaori S, Jiang R, Maeda C, Ogawa R, Okazaki H, Aramaki H, Watanabe K. Expression levels of 39 Cyp mRNAs in the mouse brain and neuroblastoma cell lines, C-1300N18 and NB2a–strong expression of Cyp1b1. *Fundamental Toxicological Sci*. 2017;4:195–200.
19. Muskhelishvili L, Thompson PA, Kusewitt DF, Wang C, Kadlubar FF. In situ hybridization and immunohistochemical analysis of cytochrome P450 1B1 expression in human normal tissues. *J Histochem Cytochem*. 2001;49:229–236. doi: 10.1177/002215540104900210

20. Davisson RL, Zimmerman MC. Angiotensin II, oxidant signaling, and hypertension: down to a T? *Hypertension*. 2010;55:228–230. doi: 10.1161/HYPERTENSIONAHA.109.144477
21. Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS. Selective fluorescent imaging of superoxide *in vivo* using ethidium-based probes. *Proc Natl Acad Sci U S A*. 2006;103:15038–15043. doi: 10.1073/pnas.0601945103
22. Bruner CA, Fink GD. Neurohumoral contributions to chronic angiotensin-induced hypertension. *Am J Physiol*. 1986;250(1 Pt 2):H52–H61. doi: 10.1152/ajpheart.1986.250.1.H52
23. Merrill DC, Thompson MW, Carney CL, Granwehr BP, Schlager G, Robillard JE, Sigmund CD. Chronic hypertension and altered baroreflex responses in transgenic mice containing the human renin and human angiotensinogen genes. *J Clin Invest*. 1996;97:1047–1055. doi: 10.1172/JCI118497
24. Littlejohn NK, Siel RB Jr, Ketsawatsomkron P, Pelham CJ, Pearson NA, Hilzendeger AM, Buehrer BA, Weidemann BJ, Li H, Davis DR, et al. Hypertension in mice with transgenic activation of the brain renin-angiotensin system is vasopressin dependent. *Am J Physiol Regul Integr Comp Physiol*. 2013;304:R818–R828. doi: 10.1152/ajpregu.00082.2013
25. Xia H, Sriramula S, Chhabra KH, Lazartigues E. Brain angiotensin-converting enzyme type 2 shedding contributes to the development of neurogenic hypertension. *Circ Res*. 2013;113:1087–1096. doi: 10.1161/CIRCRESAHA.113.301811
26. Li YF, Wang W, Mayhan WG, Patel KP. Angiotensin-mediated increase in renal sympathetic nerve discharge within the PVN: role of nitric oxide. *Am J Physiol Regul Integr Comp Physiol*. 2006;290:R1035–R1043. doi: 10.1152/ajpregu.00338.2004
27. Shi P, Diez-Freire C, Jun JY, Qi Y, Katovich MJ, Li Q, Sriramula S, Francis J, Sumners C, Raizada MK. Brain microglial cytokines in neurogenic hypertension. *Hypertension*. 2010;56:297–303. doi: 10.1161/HYPERTENSIONAHA.110.150409
28. Stern JE, Son S, Biancardi VC, Zheng H, Sharma N, Patel KP. Astrocytes contribute to angiotensin II stimulation of hypothalamic neuronal activity and sympathetic outflow. *Hypertension*. 2016;68:1483–1493. doi: 10.1161/HYPERTENSIONAHA.116.07747
29. Davey RA, Grossmann M. Androgen receptor structure, function and biology: from bench to bedside. *Clin Biochem Rev*. 2016;37:3–15.
30. Heinlein CA, Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Mol Endocrinol*. 2002;16:2181–2187. doi: 10.1210/me.2002-0070
31. Khanmoradi M, Nasimi A. Angiotensin II in the paraventricular nucleus stimulates sympathetic outflow to the cardiovascular system and make vasopressin release in rat. *Neurosci Lett*. 2016;632:98–103. doi: 10.1016/j.neulet.2016.08.040
32. Sumners C, Raizada MK, Kang J, Lu D, Posner P. Receptor-mediated effects of angiotensin II on neurons. *Front Neuroendocrinol*. 1994;15:203–230. doi: 10.1006/frne.1994.1009
33. Lin CY, Lin MT, Cheng RT, Chen SH. Testosterone depletion by castration may protect mice from heat-induced multiple organ damage and lethality. *J Biomed Biotechnol*. 2010;485306:1–9. doi: 10.1155/2010/485306
34. Xu J, Molinas AJR, Mukerjee S, Morgan DA, Rahmouni K, Zsombok A, Lazartigues E. Activation of ADAM17 (A disintegrin and metalloprotease 17) on glutamatergic neurons selectively promotes sympathoexcitation. *Hypertension*. 2019;73:1266–1274. doi: 10.1161/HYPERTENSIONAHA.119.12832
35. Mukerjee S, Gao H, Xu J, Sato R, Zsombok A, Lazartigues E. ACE2 and ADAM17 interaction regulates the activity of presympathetic neurons. *Hypertension*. 2019;74:1181–1191. doi: 10.1161/HYPERTENSIONAHA.119.13133
36. Latchford KJ, Ferguson AV. Angiotensin II activates a nitric-oxide-driven inhibitory feedback in the rat paraventricular nucleus. *J Neurophysiol*. 2003;89:1238–1244. doi: 10.1152/jn.00914.2002
37. Xue B, Singh M, Guo F, Hay M, Johnson AK. Protective actions of estrogen on angiotensin II-induced hypertension: role of central nitric oxide. *Am J Physiol Heart Circ Physiol*. 2009;297:H1638–H1646. doi: 10.1152/ajpheart.00502.2009
38. Shi Z, Gan XB, Fan ZD, Zhang F, Zhou YB, Gao XY, De W, Zhu GQ. Inflammatory cytokines in paraventricular nucleus modulate sympathetic activity and cardiac sympathetic afferent reflex in rats. *Acta Physiol (Oxf)*. 2011;203:289–297. doi: 10.1111/j.1748-1716.2011.02313.x
39. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*. 1998;139:4252–4263. doi: 10.1210/endo.139.10.6216
40. Fan W, Yanase T, Nishi Y, Chiba S, Okabe T, Nomura M, Yoshimatsu H, Kato S, Takayanagi R, Nawata H. Functional potentiation of leptin-signal transducer and activator of transcription 3 signaling by the androgen receptor. *Endocrinology*. 2008;149:6028–6036. doi: 10.1210/en.2008-0431
41. Pi M, Parrill AL, Quarles LD. GPRC6A mediates the non-genomic effects of steroids. *J Biol Chem*. 2010;285:39953–39964. doi: 10.1074/jbc.M110.158063
42. Wang Y, Rudd AG, Wolfe CD. Age and ethnic disparities in incidence of stroke over time: the South London stroke register. *Stroke*. 2013;44:3298–3304. doi: 10.1161/STROKEAHA.113.002604
43. Uchida M, Palmateer JM, Herson PS, DeVries AC, Cheng J, Hurn PD. Dose-dependent effects of androgens on outcome after focal cerebral ischemia in adult male mice. *J Cereb Blood Flow Metab*. 2009;29:1454–1462. doi: 10.1038/jcbfm.2009.60
44. Wang X, Dhindsa RS, Povysil G, Zoghbi A, Motelow JE, Hostyk JA, Nickols N, Rettig M, Goldstein D. TMPRSS2 transcriptional inhibition as a therapeutic strategy for COVID-19. *Preprints*. 2020:2020030360. doi: 10.20944/preprints202003.0360.v2

Novelty and Significance

What Is New?

- This provides the first evidence that central testosterone contributes to the development of angiotensin II-induced increases in blood pressure and sympathetic outflow, most likely via the CYP1B1 (cytochrome P450 1B1)-generated metabolite 6β-hydroxytestosterone locally in the paraventricular nucleus of the hypothalamus.
- Testosterone increases reactive oxygen species production and neuroinflammation associated with angiotensin II-induced hypertension via the CYP1B1-generated metabolite 6β-hydroxytestosterone in the paraventricular nucleus.
- 6β-Hydroxytestosterone in the brain contributes to the development of angiotensin II-induced neurogenic hypertension via genomic AR (androgen receptor) and nongenomic GPRC6A (G protein-coupled receptor C6A) receptors.
- Central CYP1B1 also contributes to the development of deoxycorticosterone acetate salt–induced hypertension in male mice.

What Is Relevant?

- This study is the first to demonstrate that testosterone-CYP1B1-generated metabolite 6β-hydroxytestosterone locally in the brain, most likely in the paraventricular nucleus, via its action on AR and GPRC6A promotes angiotensin II-induced hypertension, autonomic imbalance, and associated neuroinflammation in male mice.
- Interfering with 6β-hydroxytestosterone generation from testosterone by inhibiting CYP1B1 in the brain could protect hypertestosteronemic males or hypogonadal males on testosterone replacement therapy against the development of neurogenic hypertension induced by angiotensin II and deoxycorticosterone acetate salt.

Summary

The testosterone-CYP1B1-generated metabolite 6β-hydroxytestosterone contributes to the development of angiotensin II-induced hypertension, reactive oxygen species production, and neuroinflammation in male mice, most likely in the paraventricular nucleus via AR and GPRC6A. Central CYP1B1 also contributes to the development of neurogenic hypertension induced by deoxycorticosterone acetate salt in male mice, probably through a similar mechanism.