Renin-Angiotensin System

Central CYP1B1 (Cytochrome P450 1B1)-Estradiol Metabolite 2-Methoxyestradiol Protects From Hypertension and Neuroinflammation in Female Mice

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Abstract—Previously, we showed that peripheral administration of 2-ME (2-methoxyestradiol), a CYP1B1 (cytochrome P450 1B1)-catechol-*O*-methyltransferase (COMT) generated metabolite of E2 (17β-Estradiol), protects against angiotensin II-induced hypertension in female mice. The demonstration that central E2 inhibits angiotensin II-induced hypertension, together with the expression of CYP1B1 in the brain, led us to hypothesize that E2-CYP1B1 generated metabolite 2-ME in the brain mediates its protective action against angiotensin II-induced hypertension in female mice. To test this hypothesis, we examined the effect of intracerebroventricularly (ICV) administered E2 in ovariectomized (OVX)-wild-type (*Cyp1b1$^{+/+}$*) and OVX-*Cyp1b1$^{-/-}$* mice on the action of systemic angiotensin II. ICV-E2 attenuated the angiotensin II-induced increase in mean arterial blood pressure, impairment of baroreflex sensitivity, and sympathetic activity in OVX-*Cyp1b1$^{+/+}$* but not in ICV-injected short interfering (si)RNA-COMT or OVX-*Cyp1b1$^{-/-}$* mice. ICV-2-ME attenuated the angiotensin II-induced increase in blood pressure in OVX-*Cyp1b1$^{-/-}$* mice; this effect was inhibited by ICV-siRNA estrogen receptor-α (ERα) and G protein-coupled estrogen receptor 1 (GPER1). ICV-E2 in OVX-*Cyp1b1$^{+/+}$* but not in OVX-*Cyp1b1$^{-/-}$* mice and 2-ME in the OVX-*Cyp1b1$^{-/-}$* inhibited angiotensin II-induced increase in reactive oxygen species production in the subfornical organ and paraventricular nucleus, activation of microglia and astrocyte, and neuroinflammation in paraventricular nucleus. Furthermore, central CYP1B1 gene disruption in *Cyp1b1$^{+/+}$* mice by ICV-adenovirus-GFP (green fluorescence protein)-CYP1B1-short hairpin (sh)RNA elevated, while reconstitution by adenovirus-GFP-CYP1B1-DNA in the paraventricular nucleus but not in subfornical organ in *Cyp1b1$^{-/-}$* mice attenuated the angiotensin II-induced increase in systolic blood pressure. These data suggest that E2-CYP1B1-COMT generated metabolite 2-ME, most likely in the paraventricular nucleus via estrogen receptor-α and GPER1, protects against angiotensin II-induced hypertension and neuroinflammation in female mice. (*Hypertension*. 2020;75:1054-1062. DOI: 10.1161/HYPERTENSIONAHA.119.14548.) ● Online Data Supplement

Key Words: angiotensin II ■ brain ■ estradiol ■ female ■ hypertension

Sex differences in blood pressure (BP) in both humans and experimental animal models are well documented.[1–3] Both gonadal hormones and sex chromosomes have been implicated in sexual dimorphism in BP.[2] Chronic infusion of Ang II (angiotensin II) elevates BP by increasing sympathetic activity and blunts baroreflex function by its central action on circumventricular organs including the subfornical organ (SFO).[4] It conveys this information through the paraventricular nucleus (PVN) to the rostral ventrolateral medulla and then to preganglionic sympathetic neurons.[5] However, circulating Ang II can also reach the PVN via disruption of the blood-brain barrier.[6] Peripherally infused Ang II produces a greater increase in BP in intact males than in female mice.[3] E2 (17β-Estradiol), the primary female estrogen protects against Ang II-induced hypertension by its action in the brain via ERα (estrogen receptor-α) in the SFO.[7,8] Estrogen also protects against aldosterone-induced hypertension via its effect on ERβ in the PVN and in the rostral ventrolateral medulla.[9] However, estrogen in the brain exerts behavioral effects via nongenomic GPER1 (G protein-coupled estrogen receptor 1).[10] Whether GPER1 is involved in the protective effect of E2 in Ang II-induced hypertension in the brain has not been explored.

Recently, we reported that the protective effect of peripheral E2 against Ang II-induced hypertension is mediated via its metabolism by CYP1B1 (cytochrome P450 1B1) to 2-hydroxyestradiol, followed by its conversion to 2-ME (2-methoxyestradiol) by COMT (catechol-*O*-methyltransferase).[11,12] However, the site and mechanism of action of 2-ME that protects against Ang II-induced hypertension are unknown. Both CYP1B1 and COMT are present in the brain, and microglia (BV2 cell line) has been reported to metabolize

E2 to 2-hydroxyestradiol that is further converted into 2-ME.[13] These observations led us to the hypothesis that the central E2-CYP1B1-COMT generated metabolite, 2-ME, mediates the protective effect of E2 on Ang II-induced hypertension.

Materials and Methods

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

Animal Experiments

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 (UTHSC) Institutional Animal Care and Use Committee. Experiments were conducted on 8- to 10-week-old, 20- to 25-g body weight, intact, and ovariectomized (OVX) *Cyp1b1*⁺/⁺ (Wild-type, C57BL/6J background) and *Cyp1b1*⁻/⁻ (C57BL/6J background) female mice.

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*-tests were used for the comparison between the 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 (online-only Data Supplement).

Results

Central E2 Minimizes Ang II-Induced Hypertension via Its CYP1B1-COMT-Generated Metabolite 2-ME

E2 minimizes Ang II-induced hypertension by its action in the brain.[7] In the present study, we investigated whether E2 inhibits Ang II-induced hypertension via its metabolism in the brain to 2-ME formed by COMT from 2-hydroxyestradiol, which is generated from E2 by CYP1B1. Ang II produced greater increase in mean arterial BP (MAP), systolic BP (SBP), and diastolic BP in intracerebroventricularly (ICV)-injected vehicle (Veh for E2 and 2-ME, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid) in OVX-*Cyp1b1*⁺/⁺, and intact or OVX-*Cyp1b1*⁻/⁻ as compared with *Cyp1b1*⁺/⁺ mice (Figure 1A and 1C; Figures S1 and S2 in the online-only Data Supplement). ICV injection of E2 minimized Ang II-induced increase in MAP (Figure 1A and 1C), SBP, and diastolic BP (Figure S2) in OVX-*Cyp1b1*⁺/⁺ but not in OVX-*Cyp1b1*⁻/⁻ mice.

ICV-short interfering (si)RNA-COMT but not siRNA-NT (nontargeted) that selectively reduced *Comt*-messenger (m)RNA examined in PVN (Figure S3) prevented the effect of E2 to minimize the Ang II-induced increase in MAP, SBP, and diastolic BP (Figure 1B; Figure S2) in OVX-*Cyp1b1*⁺/⁺ mice. In the OVX-*Cyp1b1*⁻/⁻ mice, ICV injection of the E2-COMT generated metabolite 2-ME, but not E2, attenuated Ang II-induced increase in MAP, SBP, and diastolic BP (Figure 1C; Figure S2). Ang II did not alter heart rate in any of the above groups (Figures S1 and S2).

Central 2-ME Mediates the Protective Effect of E2 Against Ang II-Induced Impairment of Baroreflex Sensitivity and Autonomic Function

Next, we assessed whether central 2-ME also mediates the protective action of E2 against Ang II-induced impairment of baroreceptor reflex sensitivity,[14] by power spectral analysis of BP and heart rate variability, which is expressed by the ratio of low frequency (LF) to high frequency (HF) oscillations in the above-described groups. The LF/HF ratio was increased similarly in the intact and OVX-*Cyp1b1*⁺/⁺ and OVX-*Cyp1b1*⁻/⁻ mice with ICV-injected Veh following Ang II infusion (Figure 1D; Figure S1E), although there was a trend toward a higher increase in the intact and OVX-*Cyp1b1*⁻/⁻ mice. ICV-E2 in OVX-*Cyp1b1*⁺/⁺, and 2-ME in OVX-*Cyp1b1*⁻/⁻, but not E2 in OVX-*Cyp1b1*⁻/⁻ mice or OVX-*Cyp1b1*⁺/⁺ with ICV-injected siRNA-COMT, reduced the LF/HF ratio in response to Ang II (Figure 1D). The contribution of autonomic nervous system activation to the effect of Ang II on BP was also assessed by using the ganglion blocker hexamethonium. Hexamethonium caused a greater reduction of MAP in Ang II-induced hypertension in OVX-*Cyp1b1*⁺/⁺, intact or OVX-*Cyp1b1*⁻/⁻ compared with *Cyp1b1*⁺/⁺ mice with ICV-Veh. ICV-E2 in OVX-*Cyp1b1*⁺/⁺ or 2-ME in OVX-*Cyp1b1*⁻/⁻, but not E2 in OVX-*Cyp1b1*⁻/⁻ mice minimized the reduction by hexamethonium in Ang II-induced increase in MAP (Figure 1E; Figure S1F). ICV-E2 showed a trend of not reducing the effect of hexamethonium in OVX-*Cyp1b1*⁺/⁺ mice with ICV-injected siRNA-COMT ($P=0.054$; Figure 1E).

During Ang II infusion, ICV-E2 in OVX-*Cyp1b1*⁺/⁺, and 2-ME in OVX-*Cyp1b1*⁻/⁻ mice, reduced the urinary levels of norepinephrine, an index of sympathetic activity. However, E2 in OVX-*Cyp1b1*⁻/⁻ or OVX-*Cyp1b1*⁺/⁺ mice with ICV-siRNA-COMT failed to reduce the urinary levels of norepinephrine (Figure S4).

Central CYP1B1 Gene Disruption Accentuates in the Intact *Cyp1b1*⁺/⁺, and Reconstitution Attenuates in the Intact but Not in OVX-*Cyp1b1*⁻/⁻ Mice Ang II-Induced Hypertension

Ang II caused a small increase in SBP in *Cyp1b1*⁺/⁺ mice transduced with ICV-adenovirus (Ad)-GFP (green fluorescence protein)-scrambled (Scr)-short hairpin (sh)RNA (Figure 2A). However, ICV-Ad-GFP-CYP1B1-shRNA that selectively reduced *Cyp1b1*, but not *Cyp4a10*-mRNA expression determined in PVN (Figure S5A), enhanced this effect of Ang II to increase SBP in *Cyp1b1*⁺/⁺ mice (Figure 2A).

Ang II increased SBP in *Cyp1b1*⁻/⁻ mice transduced with ICV-Ad-GFP-DNA (Figure 2B). However, transduction with ICV-Ad-GFP-CYP1B1-DNA that restored the expression of *Cyp1b1*-mRNA determined in PVN (Figure S5B) attenuated the Ang II-induced increased SBP in intact *Cyp1b1*⁻/⁻ mice but not in OVX-*Cyp1b1*⁻/⁻ mice (Figure 2B; Figure S6).

GFP expression in the SFO and PVN (Figure 5A and 5B) confirmed the transduction with adenoviral probes given ICV.

ICV-siRNA-ERα and siRNA-GPER1 in OVX-*Cyp1b1*⁻/⁻ Mice Blunts the Protective Effect of 2-ME Against Ang II-Induced Hypertension

E2 produces its actions via both genomic and nongenomic receptors.[7–10] ICV-2-ME failed to minimize the Ang II-induced
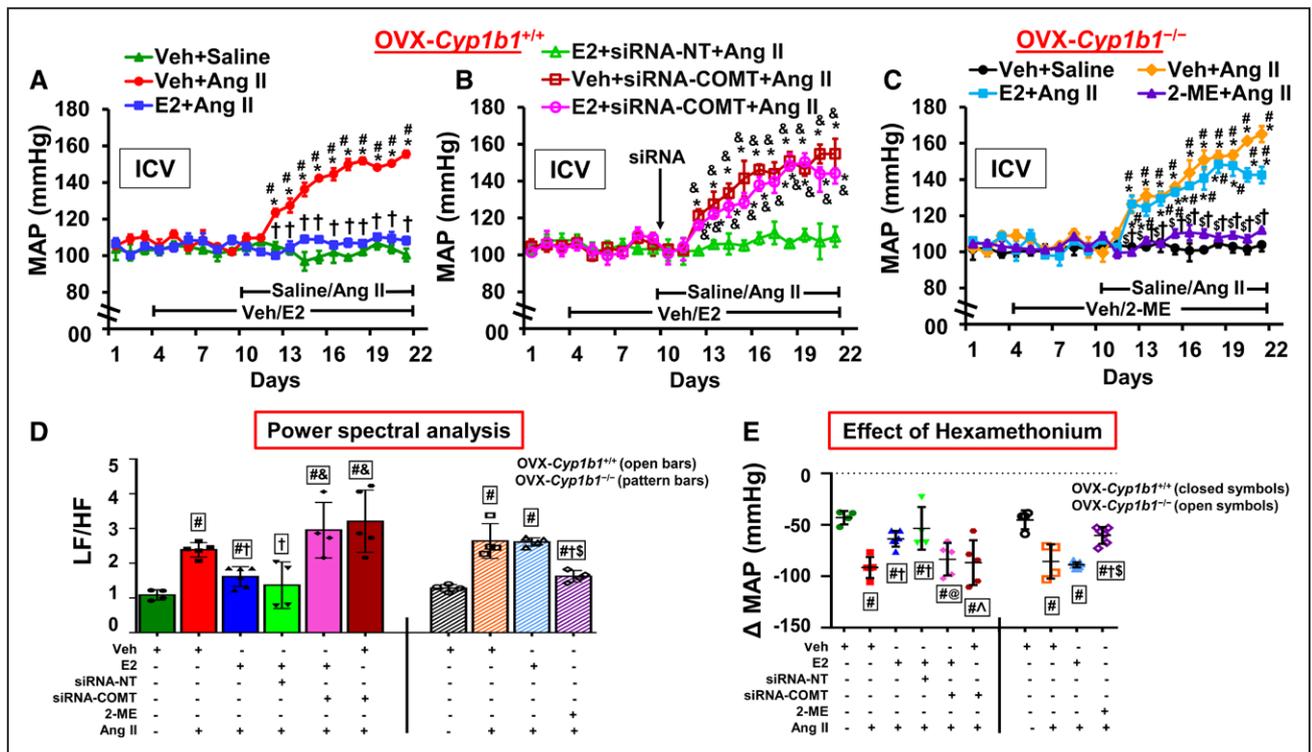
Figure 1. Ang II (angiotensin II)-induced increase in mean arterial blood pressure (MAP) was attenuated in ovariectomized (OVX)-*Cyp1b1*+/+ mice by intracerebroventricular (ICV) administration of E2 (17β-estradiol, 1.5 µg/2 µL per alternate day) but not its vehicle (Veh, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid, 2 µL/alternate day; **A**) or with ICV-injected short interfering (si)RNA-COMT (catechol-*O*-methyltransferase, 0.4 nmol/2 µL, single injection; **B**) as measured by telemetry. However, ICV-2-ME (2-methoxyestradiol, 1.5 µg/2 µL per alternate day) but not E2 attenuated the Ang II-induced increase in MAP (**C**) in OVX-*Cyp1b1*−/− mice. E2 in OVX-*Cyp1b1*+/+ but not in OVX-*Cyp1b1*−/− mice blunted the Ang II-induced increased low frequency (LF)/high frequency (HF) ratio (power spectral analysis, **D**), and reduced MAP in response to hexamethonium (30 mg/kg, intraperitoneal, **E**). ICV-E2 failed to blunt the Ang II-induced increased LF/HF ratio and showed a similar trend of not minimizing the effect of hexamethonium in OVX-*Cyp1b1*+/+ mice with ICV-injected siRNA-COMT. In OVX-*Cyp1b1*−/− mice, ICV-2-ME reduced these effects of Ang II (**D** and **E**). Ang II (700 ng/kg per minute) or its vehicle saline was systemically administered for 14 d with subcutaneously implanted micro-osmotic pumps. *P<0.05 vs Day 9 value (day before implantation of osmotic pump) within the group, #P<0.05 vs Veh+Saline in the corresponding OVX-*Cyp1b1*+/+ and OVX-*Cyp1b1*−/− groups, †P<0.05 vs Veh+Ang II in the corresponding OVX-*Cyp1b1*+/+ and OVX-*Cyp1b1*−/− groups, &P<0.05, @P=0.054, ^P=0.052 vs E2+siRNA-NT+Ang II in OVX-*Cyp1b1*+/+ group, $P<0.05 vs E2+Ang II in OVX-*Cyp1b1*−/− group; n=4–6/group. Δ indicates change; and NT, nontargeted control.

increase in MAP (Figure 3A), SBP, diastolic BP (Figure S2D and S2E), LF/HF ratio (Figure 3B), and exhibited a trend of not minimizing the effect of hexamethonium (*P*=0.058 and 0.052; Figure 3C) in OVX-*Cyp1b1*−/− mice injected with ICV-siRNA-ERα or siRNA-GPER1. ICV-siRNA-ERα and siRNA-GPER1 selectively reduced the expression of *Erα*- and *Gper1*-mRNAs, respectively, in PVN (Figure S7).

Reconstitution of CYP1B1 Expression in the PVN but Not in SFO Minimizes Ang II-Induced Hypertension in *Cyp1b1*−/− Mice

ERs and AT1R (Ang II receptor type I) are present in both SFO and PVN,[15,16] and E2 can act in both these sites to reduce Ang II-induced hypertension.[7,17,18] Therefore, this raised the possibility that 2-ME generated from E2 in both areas might be responsible for protecting against Ang II-induced hypertension. *Cyp1b1*-mRNA was found to be expressed in both these areas in *Cyp1b1*+/+ but not in *Cyp1b1*−/− mice (Figure S8). Therefore, we examined the effect of selective transduction of SFO and PVN with Ad-GFP-CYP1B1-DNA on the Ang II-induced increase in SBP in the *Cyp1b1*−/− mice. Transduction with Ad-GFP-CYP1B1-DNA in PVN but not in SFO reduced Ang II-induced increase in SBP in these mice (Figure 4A and 4B) even though the *Cyp1b1*-mRNA expression was higher in SFO than in PVN (Figure S8).

The selective transduction of the adenoviral probes in PVN and SFO was confirmed by GFP fluorescence (Figure 5C).

E2-CYP1B1-COMT-Generated Metabolite 2-ME Attenuates Ang II-Induced Reactive Oxygen Species Production in the Brain

Ang II-induced hypertension is mediated via reactive oxygen species (ROS) production in SFO and PVN.[19,20] Therefore, we investigated the CYP1B1-dependent action of E2 on ROS production. Ang II stimulated the production of ROS as indicated by enhanced 2-hydroxyethidium (2-HE) fluorescence in the SFO, and PVN generated after staining with dihydroethidium in OVX-*Cyp1b1*+/+ and OVX-*Cyp1b1*−/− mice (Figure S9). ICV-E2 minimized ROS production in both SFO and PVN in OVX-*Cyp1b1*+/+ (Figure S9A) but not in OVX-*Cyp1b1*−/− (Figure S9B) mice. In the OVX-*Cyp1b1*−/− mice, ICV-2-ME caused a greater reduction in the ROS production in the PVN than in the SFO (Figure S9B).

Ang II in *Cyp1b1*+/+ mice with ICV-Ad-GFP-Scr-shRNA increased the 2-HE fluorescence in the SFO and insignificantly in the PVN (Figure S10A). However, Ang II-induced increase in 2-HE fluorescence in *Cyp1b1*+/+ mice with ICV-Ad-GFP-CYP1B1-shRNA was much higher in the PVN than in the SFO (Figure S10A). Transduction with ICV-Ad-GFP-CYP1B1-DNA but not its control Ad-GFP-DNA abolished 2-HE fluorescence
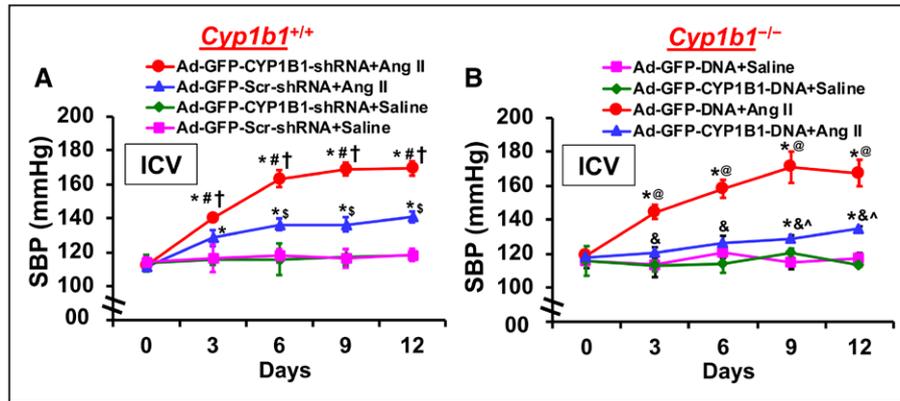
Figure 2. Central CYP1B1 (cytochrome P450 1B1) gene disruption with intracerebroventricular (ICV)-injection of adenovirus (Ad)-GFP (green fluorescence protein)-CYP1B1 short hairpin (sh)RNA (Ad-GFP-CYP1B1-shRNA; single injection of 2 μL, 1.3×10^13 pfu/mL) but not Ad-scrambled (Scr)-shRNA (single injection of 2 μL, 6.4×10^11 pfu/mL) in *Cyp1b1*^+/+ mice accentuated (**A**) and reconstitution with ICV-injection of Ad-GFP-CYP1B1-DNA (single injection of 2 μL, 1.0×10^12 pfu/mL) but not Ad-GFP-DNA (single injection of 2 μL, 1.0×10^12 pfu/mL) in *Cyp1b1*^−/− abrogated (**B**) the Ang II (angiotensin II)-induced increase in systolic blood pressure (SBP) as measured by tail-cuff method. Saline was used as a vehicle for Ang II. *P<0.05 vs Day 0 values (before start of Ang II infusion) within the group, $P<0.05 vs Ad-GFP-Scr-shRNA+Saline; #P<0.05 vs Ad-GFP-Scr-shRNA+Ang II, †P<0.05 vs Ad-GFP-CYP1B1-shRNA+Saline, @P<0.05 vs Ad-GFP-DNA+Saline, &P<0.05 vs Ad-GFP-DNA+Ang II, ^P<0.05 vs Ad-GFP-CYP1B1-DNA+Saline; n=4–9/group.

in PVN and minimized it in SFO in *Cyp1b1*^−/− mice (Figure S10B). Since transduction with Ad-GFP-CYP1B1-DNA in PVN but not in SFO in *Cyp1b1*^−/− mice also reduced the Ang II-induced increase in SBP, further studies on the action of E2 and 2-ME described below were performed only in PVN.

2-ME Increases Neuronal Nitric Oxide Synthase Expression in PVN

E2 attenuates PVN neuronal activity by enhancing NO production.[18] Hence, we investigated the CYP1B1-dependent action of E2 on *nNos*-mRNA expression in the PVN. Ovariectomy or *Cyp1b1* gene disruption reduced PVN *nNos*-mRNA expression (Figure 6A). Ang II increased *nNos*-mRNA expression only in the *Cyp1b1*^+/+ mice. ICV-E2 in OVX-*Cyp1b1*^+/+ or 2-ME in OVX-*Cyp1b1*^−/− but not E2 in OVX-*Cyp1b1*^−/− mice infused with Ang II restored PVN *nNos*-mRNA levels (Figure 6A).

2-ME Reduces Ang II-Induced Increase in PVN c-Fos+ Cell Population

The effect of E2 and 2-ME on PVN neuronal activity was also assessed by examining c-Fos immunoreactivity in response to Ang II. As expected, Ang II increased the total number of c-Fos+

cells in both OVX-*Cyp1b1*^+/+ (Figure S11A) or OVX-*Cyp1b1*^−/− (Figure S11B) mice. However, ICV-E2 in OVX-*Cyp1b1*^+/+, and 2-ME in OVX-*Cyp1b1*^−/−, but E2 not in OVX-*Cyp1b1*^−/− or with ICV-siRNA-COMT in OVX-*Cyp1b1*^+/+ mice reduced the number of c-Fos+ cells in PVN (Figure S11).

2-ME Protects Against Ang II-Induced PVN Gliosis and Neuroinflammation

Ang II-induced hypertension involves microglial activation, increases in proinflammatory cytokine including IL-6, and decreases in anti-inflammatory cytokine IL-10 levels in the PVN.[21] Thus, we examined CYP1B1-COMT dependent effects of E2 on these neuroinflammatory markers in the PVN. Ang II increased the number of microglia (ionized calcium-binding adaptor molecule 1 positive or IBA+ cells) in PVN in OVX-*Cyp1b1*^+/+ and OVX-*Cyp1b1*^−/− mice (Figure 5D and 5F; Figure S12), most of which had globular cell bodies with shorter and thicker cell processes (Figure S12), a feature similar to activated microglia.[21] ICV-E2 in OVX-*Cyp1b1*^+/+, and 2-ME in OVX-*Cyp1b1*^−/−, but not E2 in OVX-*Cyp1b1*^−/− or with ICV-siRNA-COMT in OVX-*Cyp1b1*^+/+ mice reduced the number of microglia in PVN. Moreover, the remaining microglia had thinner and
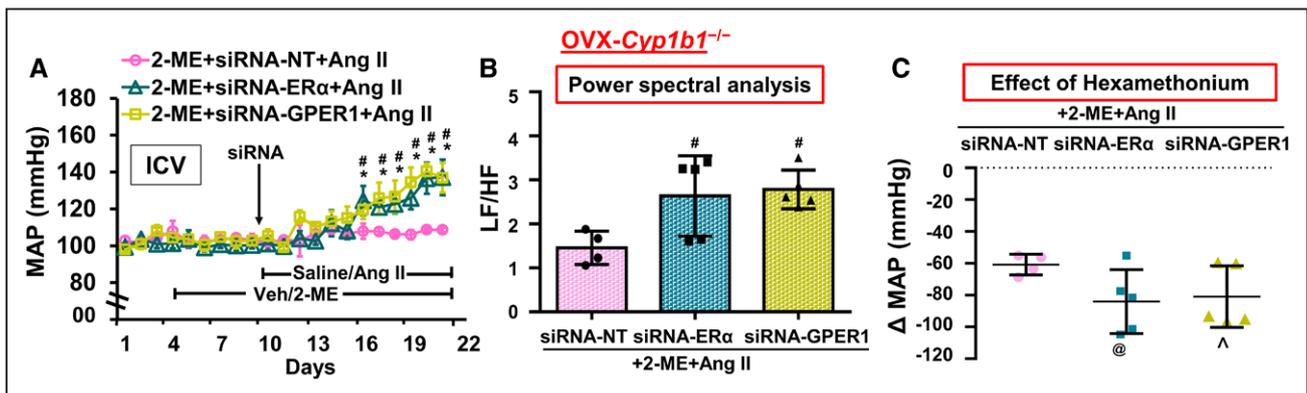

Figure 3. Intracerebroventricular (ICV)-2-ME (2-methoxyestradiol) failed to attenuate Ang II (angiotensin II)-induced increase in mean arterial blood pressure (MAP; **A**), and low frequency (LF)/high frequency (HF) ratio (**B**), and exhibited a trend of not reducing the effect of hexamethonium on Ang II-induced increase in MAP (**C**) in ovariectomized (OVX)-*Cyp1b1*^−/− mice with ICV-short interfering (si)RNA-ERα (estrogen receptor α) or siRNA-GPER1 (G-protein coupled estrogen receptor 1; 0.4 nmol/2 μL). Saline was used as a vehicle for Ang II. *P<0.05 vs Day 9 value (day before implantation of osmotic pump) within the group, #P<0.05, @P=0.058, ^P=0.052, vs 2-ME+siRNA-NT+Ang II; n=4–6/group. Δ indicates change; and NT, nontargeted control.
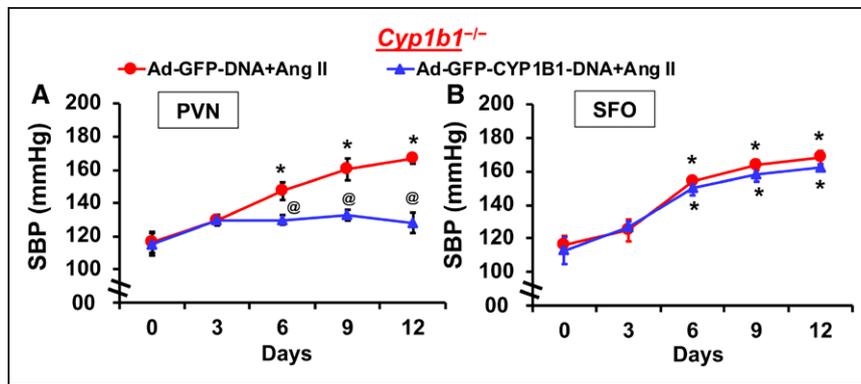
Figure 4. Selective reconstitution of CYP1B1 (cytochrome P450 1B1) gene in the paraventricular nucleus (PVN) bilaterally (single injection, **A**), but not in the subfornical organ (SFO; single injection, **B**) of *Cyp1b1*^{-/-} mice with adenovirus (Ad)-GFP (green fluorescence protein)-CYP1B1-DNA (1.0×10^{12} pfu/mL, 0.5 μL/injection) but not Ad-GFP-DNA (1.0×10^{12} pfu/mL, 0.5 μL/injection) abrogated Ang II (angiotensin II)-induced increase in systolic BP (SBP) measured by tail-cuff method. *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=4–5/group.

longer processes and reduced cell bodies as similar to those observed in the saline-infused mice (Figure S12).

Astrocytes contribute to Ang II-mediated sympathoexcitatory effects in PVN.[22] In our study, Ang II increased the number of astrocytes (glial fibrillary acidic protein, GFAP+ cells) in the PVN, and most of these had shorter and thicker cell processes in OVX-*Cyp1b1*^{+/+} or OVX-*Cyp1b1*^{-/-} mice with ICV-Veh (Figure 5E and 5F; Figure S13). ICV-E2 in OVX-*Cyp1b1*^{+/+} and 2-ME in OVX-*Cyp1b1*^{-/-}, but not E2 in OVX-*Cyp1b1*^{-/-} or with siRNA-COMT in OVX-*Cyp1b1*^{+/+} mice infused with Ang II reduced the PVN astrocyte number (Figure 5E and 5F), and the remaining astrocytes showed longer and thinner processes similar to those observed in the saline-infused mice (Figure S13).

E2 protects against traumatic brain injury by both reducing brain proinflammatory cytokine and enhancing anti-inflammatory cytokine production.[23] In our study, Ang II increased *Il6*-mRNA (Figure 6B) and reduced *Il10*-mRNA (Figure 6C) in PVN, but these effects were reversed by ICV-E2 in OVX-*Cyp1b1*^{+/+} or 2-ME in OVX-*Cyp1b1*^{-/-} (Figure 6B and 6C). However, ICV-injected E2 failed to reduce *Il6*-mRNA expression but caused a minimal increase in *Il10*-mRNA expression in OVX-*Cyp1b1*^{-/-} mice (Figure 6C).

Central CYP1B1 Protects Against Deoxycorticosterone Acetate-Salt-Induced Hypertension in Female Mice

We also examined the contribution of brain CYP1B1 in uninephectromized-DOCA (deoxycorticosterone acetate; 50 mg/kg, subcutaneous)-salt (1% NaCl)-induced hypertension. DOCA-salt-induced hypertension was attenuated in *Cyp1b1*^{+/+} but not in *Cyp1b1*^{-/-} mice (Figure S14A). ICV-Ad-GFP-CYP1B1-shRNA but not Ad-GFP-Scr-shRNA increased the SBP in response to DOCA-salt treatment in *Cyp1b1*^{+/+} mice (Figure S14B). Reconstitution of CYP1B1-mRNA in the brain by ICV-Ad-GFP-CYP1B1-DNA but not Ad-GFP-DNA minimized the DOCA-salt-induced increase in SBP in the *Cyp1b1*^{-/-} mice (Figure S14C).

Discussion

The novel findings of this study are that E2 via its central CYP1B1 and COMT generated metabolite, 2-ME, mitigated Ang II-induced increase in BP by its action in the PVN by reducing (1) sympathetic outflow, (2) ROS production, (3) activation of microglia and astrocytes, and (4) proinflammatory cytokine *Il6*-mRNA while increasing *nNos*- and anti-inflammatory cytokine

Il10-mRNA expression. Also, we show for the first time that 2-ME in the brain minimized Ang II-induced hypertension through both the receptors ERα and GPER1 in OVX-*Cyp1b1*^{-/-} mice. This conclusion is supported by the findings that CYP1B1 is present in human and mouse brain,[24,25] and ICV administration of exogenous E2 in the OVX-*Cyp1b1*^{+/+} mice ameliorated the Ang II-induced increases in BP and sympathetic outflow.[7] Moreover, in the present study, ICV-E2 in OVX-*Cyp1b1*^{+/+} mice minimized the Ang II-induced impairment of baroreflex sensitivity. Like ovariectomy, CYP1B1 gene disruption increases sensitivity to the hypertensive effect of Ang II, indicating the critical role of central CYP1B1 in the action of E2. The demonstration that COMT is widely distributed in the brain[26] and our findings that ICV-E2 failed to protect against Ang II-induced increase in BP and sympathetic activity and impaired baroreflex sensitivity in OVX-*Cyp1b1*^{+/+} mice injected with ICV-siRNA-COMT, suggest that these effects are most likely due to reduced metabolism of E2 to 2-ME. The systemic COMT inhibitor or siRNA also caused hypersensitivity of the pressor response to Ang II, which was prevented by 2-ME in male mice.[27] The effect of ICV-E2 appeared to be limited to the brain since plasma levels of E2 that were attenuated in OVX-*Cyp1b1*^{+/+}, and OVX-*Cyp1b1*^{-/-} mice were not altered by ICV-injected E2 (Figure S15). Further evidence that the effect of E2 in the brain is mediated via its metabolism to 2-ME locally by the central CYP1B1 was our finding that ICV-E2 failed to minimize the above effects of Ang II in OVX-*Cyp1b1*^{-/-} mice. Supporting this view was our demonstration that ICV-2-ME in the OVX-*Cyp1b1*^{-/-} mice reduced Ang II-induced hypertension, sympathetic activity, and impairment of baroreflex sensitivity. In as much as ICV-2-ME alone did not affect BP, it would appear that it acts as a permissive factor for the protective action of E2 against Ang II-induced hypertension.

The effect of ICV-E2 in OVX-*Cyp1b1*^{+/+} but not in OVX-*Cyp1b1*^{-/-} mice, and 2-ME in OVX-*Cyp1b1*^{-/-} mice to reduce the Ang II-induced increase in sympathetic outflow was also indicated by reduced urinary levels of norepinephrine. Since COMT can metabolize catecholamines,[26] one might argue that increased norepinephrine metabolism by COMT in the PVN could contribute to the effect of 2-ME to reduce Ang II-induced increased sympathetic activity and BP. However, this is unlikely because siRNA-COMT did not enhance the Ang II-induced increase in urinary norepinephrine levels. Therefore, it seems that CYP1B1-COMT-E2 generated 2-ME locally in the brain lowers Ang II-induced hypertension primarily by inhibiting the central action of Ang II from increasing sympathetic activity independent of its direct vascular and renal effects.
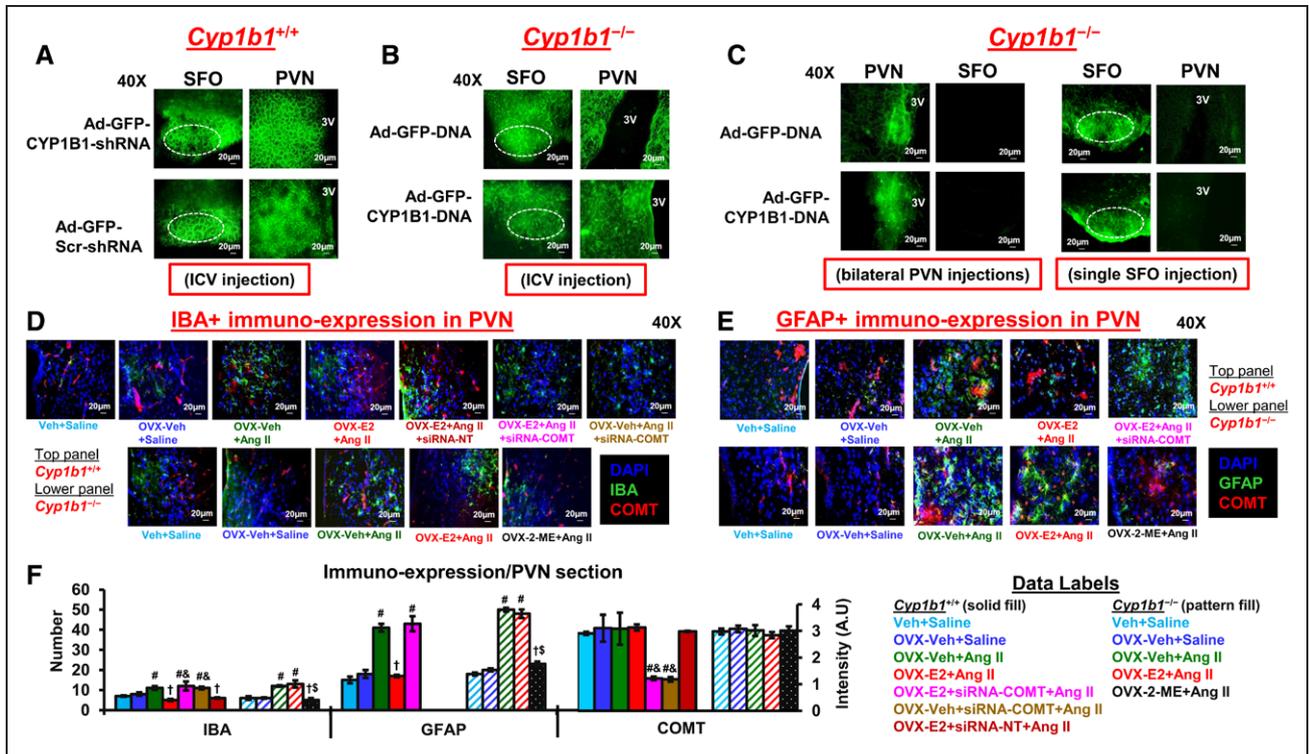
Figure 5. Transduction in the brain by intracerebroventricular (ICV) injections of adenovirus (Ad)-GFP (green fluorescence protein)-CYP1B1 (cytochrome P450 1B1) -short hairpin (sh)RNA/Ad-GFP-scrambled (Scr)-shRNA (**A**) and Ad-GFP-DNA/Ad-GFP-CYP1B1-DNA (**B**) was confirmed by their fluorescence in the subfornical organ (SFO) and paraventricular nucleus (PVN). The selectivity of Ad-GFP-DNA/Ad-GFP-CYP1B1-DNA injection in PVN and SFO was confirmed by its GFP fluorescence (**C**). ICV-E2 in OVX-*Cyp1b1*^{+/+} but not in those with ICV-short interfering (si)RNA-COMT (catechol-*O*-methyltransferase) or OVX-*Cyp1b1*^{−/−} reduced the Ang II (angiotensin II)-induced increased number of microglia (ionized calcium-binding adaptor molecule 1., **D** and **F**) and astrocytes (glial fibrillary acidic protein, **E** and **F**) in PVN. ICV-2-ME (2-methoxyestradiol) reduced the microglia and astrocyte numbers (**D**–**F**) in PVN in the OVX-*Cyp1b1*^{−/−} mice. siRNA-COMT reduced the COMT expression in PVN (**D**–**F**). Saline was used a vehicle for Ang II. Veh: Vehicle for E2 and 2-ME, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid. #$P<0.05$ vs Veh+Saline in the corresponding OVX-*Cyp1b1*^{+/+} and OVX-*Cyp1b1*^{−/−} groups, †$P<0.05$ vs Veh+Ang II in the corresponding OVX-*Cyp1b1*^{+/+} and OVX-*Cyp1b1*^{−/−} groups, &$P<0.05$ vs E2+siRNA-NT+Ang II in OVX-*Cyp1b1*^{+/+} group, \$$P<0.05$ vs E2+Ang II in OVX-*Cyp1b1*^{−/−} group; n=3/group. A.U indicates arbitrary unit; and NT, nontargeted control.

Further evidence that E2 minimizes Ang II-induced hypertension via its metabolism by CYP1B1 locally in the brain were our findings that (1) ICV-Ad-GFP-CYP1B1-shRNA in the *Cyp1b1*^{+/+} mice accentuated, and ICV-Ad-GFP-CYP1B1-DNA in *Cyp1b1*^{−/−} mice minimized the Ang II-induced increase in SBP and (2) ICV-Ad-GFP-CYP1B1-DNA in OVX-*Cyp1b1*^{−/−} mice that lack endogenous E2, failed to decrease the Ang II-induced increase in SBP.

E2 produces its actions via both genomic and nongenomic receptors.[7–10] In the brain, E2 protects against Ang II-induced hypertension via both its genomic receptors ERα and ERβ.[7,17,18] ER stimulation inhibits neuronal activity in PVN.[28] 2-ME exerts its effects via ER-dependent and -independent pathways.[13,29–34] For example, 2-ME inhibits microglia (BV2 cells)[13] and vascular smooth muscle cell proliferation independent of ERs.[29] However, 2-ME by binding to GPR30 (GPER1) downregulates AT1 receptor in rat liver membranes[30] and via MMP9/EGF/ERK1/2-dependent pathway in rat aortic smooth muscle cells.[31] On the other hand, 2-ME exerts bone sparing effect in male mice via ERα receptor.[32] Also, *Gper1* deletion exacerbates Ang II-induced increase in pulse pressure without altering its pressor response.[35] However, in the present study, the effects of ICV-2-ME to reduce Ang II-induced increase in BP and impaired baroreflex sensitivity in OVX-*Cyp1b1*^{−/−} mice were inhibited by ICV-siRNA-ERα or siRNA-GPER1, suggesting that 2-ME

exerts these effects via both these receptors. 2-ME could act via ERα and GPER1 receptors through independent pathways or by crosstalk by acting on membrane GPER1 that in turn leads to activation of nuclear ERα.[33] However, further studies are required to elucidate the cellular signaling pathways and the interaction between ERα and GPER1 in the protective action of 2-ME in PVN against Ang II-induced hypertension.

ERs and AT1R are present in both SFO and PVN,[15,16] and E2 can act in both these sites to reduce Ang II-induced hypertension.[7,17,18] Therefore, the E2-CYP1B1-COMT-generated metabolite 2-ME in both PVN and SFO could protect against Ang II-induced hypertension. However, we observed that even though the expression of *Cyp1b1*-mRNA was much higher in SFO than in PVN, transduction with Ad-GFP-CYP1B1-DNA in the PVN, but not SFO, abrogated the Ang II-induced increase in BP in *Cyp1b1*^{−/−} mice. Therefore, it seems that CYP1B1 and COMT in the PVN are responsible for the protective effect of E2, most likely through the production of 2-ME. The transduction of PVN with the adenoviral probes, as indicated by GFP expression, did not spread to the SFO and vice versa. However, we cannot exclude the possible participation of other areas adjoining to these structures as a large injection volume (0.5 μL) was used in these experiments. Moreover, the significance of CYP1B1 in SFO is not known and remains to be investigated.
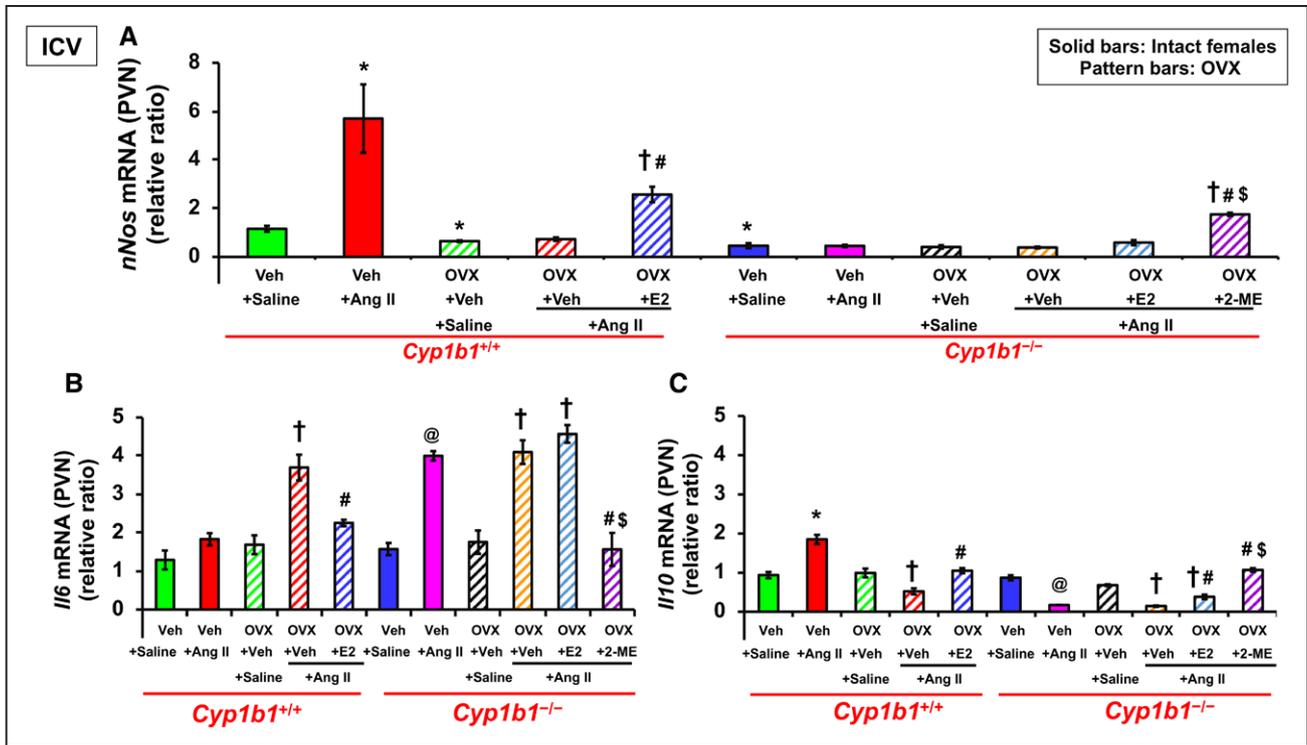
Figure 6. Ang II (angiotensin II) produced a greater increase in *nNos* messenger (m)RNA expression in paraventricular nucleus (PVN) in the *Cyp1b1*^{+/+} mice than in ovariectomized (OVX)-*Cyp1b1*^{+/+}, an effect, which was absent, in *Cyp1b1*^{−/−} mice (**A**). Intracerebroventricular (ICV)-E2 (17β-Estradiol) increased the *nNos* mRNA expression in PVN in Ang II (angiotensin II) infused OVX-*Cyp1b1*^{+/+} but not in OVX-*Cyp1b1*^{−/−} mice, which was restored by ICV-2-ME (2-methoxyestradiol) in OVX-*Cyp1b1*^{−/−} mice (**A**). Ang II increased *Il6* mRNA (**B**) and reduced *Il10* mRNA (**C**) in PVN, which were reversed by ICV-E2 in OVX-*Cyp1b1*^{+/+} (**B**) or 2-ME in OVX-*Cyp1b1*^{−/−} (**C**) mice. However, ICV-E2 in OVX-*Cyp1b1*^{−/−} mice failed to reduce *Il6* mRNA expression (**B**) but caused a minimal increase in *Il10* mRNA expression (**C**). Saline was used as a vehicle for Ang II. Veh: vehicle for E2 and 2-ME, 20% w/v 2-hydroxypropyl-β-cyclodextrin in artificial cerebrospinal fluid. *$P<0.05$ vs Veh+Saline in *Cyp1b1*^{+/+} mice, @$P<0.05$ vs Veh+Saline in *Cyp1b1*^{−/−} mice, †$P<0.05$ vs Veh+Saline in the corresponding OVX-*Cyp1b1*^{+/+} or OVX-*Cyp1b1*^{−/−} groups, #$P<0.05$ vs Veh+Ang II in the corresponding OVX-*Cyp1b1*^{+/+} or OVX-*Cyp1b1*^{−/−} groups, $$P<0.05$ vs E2+Ang II in OVX-*Cyp1b1*^{−/−} mice; n=3 samples/group, each sample containing bilateral PVN punches pooled from 3 mice.

E2 protects against Ang II-induced increases in sympathetic activity and hypertension by stimulating nNOS (neuronal nitric oxide synthase) and reducing ROS production in the SFO and PVN.[3,7,18] In the present study, ICV-E2 caused a greater reduction in Ang II-induced increase in ROS production as determined by 2-HE fluorescence, in the PVN than in SFO in OVX-*Cyp1b1*^{+/+}. However, E2 failed to minimize Ang II-induced increase in ROS production in the SFO and PVN of the OVX-*Cyp1b1*^{−/−} mice, most likely due to lack of its CYP1B1-COMT-generated metabolite 2-ME. Supporting this conclusion was our demonstration that ICV-2-ME in the OVX-*Cyp1b1*^{−/−} mice caused a greater reduction in Ang II-induced ROS production in PVN than in SFO. Moreover, our observation that ICV-Ad-GFP-CYP1B1-shRNA in *Cyp1b1*^{+/+} produced a larger increase, while the ICV-Ad-GFP-CYP1B1-DNA in *Cyp1b1*^{−/−} mice caused a greater decrease in ROS production in response to Ang II in PVN than in SFO, support our contention that the E2-CYP1B1-COMT-generated metabolite 2-ME acts primarily in the PVN. Ang II-induced ROS production leads to increased calcium (Ca2+) signaling and neuronal firing.[36] Our finding that the observed Ang II-induced increase in the number of c-Fos+ cells in the PVN was reduced by E2 in OVX-*Cyp1b1*^{+/+} but not in OVX-*Cyp1b1*^{−/−}, and by 2-ME in OVX-*Cyp1b1*^{−/−} mice, suggests that 2-ME inhibits neuronal activity most likely by reducing Ca2+ signaling. Since (1) E2 in OVX-*Cyp1b1*^{+/+} but not in OVX-*Cyp1b1*^{−/−}, and 2-ME in OVX-*Cyp1b1*^{−/−} mice increased *nNos*-mRNA levels in the PVN

in response to Ang II and (2) nNOS in PVN co-localizes with GPER1,[37] it is possible that 2-ME acts via ERα and GPER1 by inhibiting the effect of Ang II on intracellular Ca2+. 2-ME could also produce its protective effect against Ang II-induced hypertension by (1) downregulating AT1 receptor,[30,34] (2) stimulating NO-GABA pathways,[38] and/or (3) by reducing ADAM17-glutamate signaling[39] in the PVN. However, further studies are required to assess the contribution of these pathways to the action of 2-ME in the PVN.

E2 abrogates the release of proinflammatory molecules from the activated microglia via ERs.[40] Moreover, in BV2 cultured microglia cells, 2-ME generated from E2 via CYP1B1 and COMT inhibits the proliferation and activation of these cells.[13] Furthermore, COMT colocalizes with microglia in the brain,[41] and the present study in the PVN. Moreover, Ang II-induced increased microglia (IBA+ cells) number, and proinflammatory cytokine *Il6*-mRNA, and decreased anti-inflammatory cytokine *Il10*-mRNA expressions in PVN was minimized by ICV-E2 in OVX-*Cyp1b1*^{+/+} mice but not those injected ICV-siRNA-COMT (that reduced COMT immunoexpression in PVN) or in OVX-*Cyp1b1*^{−/−} mice. These observations, together with our finding that 2-ME blunted these effects of Ang II in OVX-*Cyp1b1*^{−/−} mice, suggests that 2-ME generated locally in microglia via CYP1B1-COMT in PVN mediates the protective effect of E2 on Ang II-induced hypertension. This is supported by a previous report showing that 2-ME exerts anti-inflammatory effects in animal

models and inhibits LPS-induced IL-6 expression.[42] Whether these effects of 2-ME are mediated by reduced intracellular Ca^{2+} and activity of one or more signaling molecules or reduced tubulin polymerization[29] in the PVN remains to be determined.

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.[22] E2 increases the expression of glutamate transporter-1 in the astrocytes, thereby increasing astrocytic glutamate uptake and thus reducing extracellular glutamate levels.[43] In the present study, the Ang II-induced increase in astrocyte proliferation and activation in PVN was reduced by ICV-E2 in OVX-*Cyp1b1*^{+/+}, and by ICV-2-ME, but not E2 in the OVX-*Cyp1b1*^{-/-} mice, as evidenced by the reduced number of GFAP+ cells and other morphological changes observed. Therefore, 2-ME could mediate the effect of E2 by increasing glutamate uptake in astrocytes.

While circulating E2 produced from ovaries can protect against hypertension by reducing oxidative stress and by increasing T-regulatory cells and/or inhibiting cytotoxic T-cells,[44,45] our experiments show that 2-ME produced locally in the brain from E2 by CYP1B1-COMT participates in mediating the protective effect of E2 against Ang II-induced hypertension (Graphical abstract, Figure S16). Our preliminary experiments show that CYP1B1 in the brain is also required for protection against DOCA-salt-induced hypertension in female mice. Whether it is mediated by E2-CYP1B1-COMT-generated 2-ME remains be investigated.

Perspectives

Our study provides further insights into the mechanism by which E2 via its central CYP1B1-COMT generated metabolite 2-ME in the hypothalamic PVN opposes Ang II-induced hypertension and improves autonomic dysfunction, and reduces neuroinflammation. Also, 2-ME in PVN serves as an antioxidative (increasing *nNos*-mRNA) and anti-inflammatory (increasing *Il10*-mRNA) agent. Therefore, OVX, a condition comparable to human ovarian failure, postmenopausal/hypoestrogenemic, and/or premenopausal/menstrual irregularities, or agents that interfere with central CYP1B1 or COMT activity, would accentuate the effects of Ang II in the development of neurogenic hypertension and neuroinflammatory diseases, which could be treated with 2-ME.

Disclosures

None.

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Novelty and Significance

What Is New?

- This is the first evidence that the central protective effect of E2 against Ang II-induced increases in blood pressure and sympathetic outflow is most likely mediated via CYP1B1-COMT-generated metabolite 2-ME locally in the PVN of the hypothalamus.
- 2-ME mediates the protective effects of E2 against Ang II-induced hypertension via genomic (ERα) and nongenomic (GPER1) estrogen receptors.
- Also, E2 minimizes reactive oxygen species production and neuroinflammation associated with Ang II-induced hypertension via CYP1B1-COMT-generated metabolite 2-ME in the PVN.
- Central CYP1B1 also contributes to protection against deoxycorticosterone acetate-salt-induced hypertension in female mice.

What Is Relevant?

- This study advances our understanding of the mechanism of the central action of E2 to protect against Ang II-induced hypertension. We demonstrate that E2 via its sequential metabolism by CYP1B1 and COMT to 2-ME locally in the brain, most likely in the PVN, by its action on ERα and GPER1 receptors ameliorates Ang II-induced hypertension, sympathoexcitation, and associated neuroinflammation in female mice.
- 2-ME generated from E2 by CYP1B1-COMT in the brain could protect premenopausal females against neurogenic hypertension-induced by deoxycorticosterone acetate-salt. The potential role of 2-ME in other models of neurogenic hypertension and neuroinflammatory diseases needs further exploration.

Summary

This study demonstrates that the E2-CYP1B1-COMT-generated metabolite 2-ME protects against Ang II-induced hypertension, reactive oxygen species production, and neuroinflammation in the female mice, most likely in the PVN via ERα and GPER1. This conclusion was supported by our findings that (1) ICV-E2 in OVX-*Cyp1b1*$^{+/+}$ but not in OVX-*Cyp1b1*$^{-/-}$ mice or ICV-injected siRNA-COMT in OVX-*Cyp1b1*$^{+/+}$ abrogated the Ang II-induced increase in blood pressure and sympathetic activity; (2) ICV-2-ME minimized Ang II-induced increase in blood pressure in OVX-*Cyp1b1*$^{-/-}$ mice, which was reversed by ICV-siRNA ERα and GPER1; (3) ICV-E2 in OVX-*Cyp1b1*$^{+/+}$ but not in OVX-*Cyp1b1*$^{-/-}$ mice and 2-ME in the OVX-*Cyp1b1*$^{-/-}$ mice, inhibited the Ang II-induced increase in reactive oxygen species production in the SFO and PVN, and activation of microglia, and astrocyte and neuroinflammation in PVN; and (4) transduction by Ad-GFP-CYP1B1-DNA in the PVN but not in SFO curtailed Ang II-induced increase in blood pressure in *Cyp1b1*$^{-/-}$ mice. Finally, we show that central CYP1B1 also protects against neurogenic hypertension induced by deoxycorticosterone acetate-salt, probably through a similar mechanism in female mice.