



2-Methoxyestradiol Ameliorates Angiotensin II–Induced Hypertension by Inhibiting Cytosolic Phospholipase A₂α Activity in Female Mice

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ABSTRACT: We tested the hypothesis that CYP1B1 (cytochrome P450 1B1)–17β-estradiol metabolite 2-methoxyestradiol protects against Ang II (angiotensin II)–induced hypertension by inhibiting group IV cPLA₂α (cytosolic phospholipase A₂α) activity and production of prohypertensive eicosanoids in female mice. Ang II (700 ng/kg per minute, SC) increased mean arterial blood pressure (BP), systolic and diastolic BP measured by radiotelemetry, renal fibrosis, and reactive oxygen species production in wild-type mice (*cPLA₂α^{+/+}/Cyp1b1^{+/+}*) that were enhanced by ovariectomy and abolished in intact and ovariectomized-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice. Ang II–induced increase in SBP measured by tail-cuff, renal fibrosis, reactive oxygen species production, and cPLA₂α activity measured by its phosphorylation in the kidney, and urinary excretion of prostaglandin E₂ and thromboxane A₂ metabolites were enhanced in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. 2-Methoxyestradiol and arachidonic acid metabolism inhibitor 5,8,11,14-eicosatetraenoic acid attenuated the Ang II–induced increase in SBP, renal fibrosis, reactive oxygen species production, and urinary excretion of prostaglandin E₂ and thromboxane A₂ metabolites in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. Antagonists of prostaglandin E₂ and thromboxane A₂ receptors EP1 and EP3 and TP, respectively, inhibited Ang II–induced increases in SBP and reactive oxygen species production and renal fibrosis in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. These data suggest that CYP1B1-generated metabolite 2-methoxyestradiol mitigates Ang II–induced hypertension and renal fibrosis by inhibiting cPLA₂α activity, reducing prostaglandin E₂ and thromboxane A₂ production and stimulating EP1 and EP3 and TP receptors, respectively. Thus, 2-methoxyestradiol and the drugs that selectively block EP1 and EP3 and TP receptors could be useful in treating hypertension and its pathogenesis in females. (**Hypertension**. 2021;78:1368–1381. DOI: 10.1161/HYPERTENSIONAHA.121.18181.) • [Data Supplement](#)

Key Words: blood pressure ■ fibrosis ■ ovariectomy ■ phosphorylation ■ renin

We reported previously that Ang II (angiotensin II), the main product of the renin-angiotensin system, produces hypertension by activating group IV cPLA₂α (cytosolic phospholipase A₂α), resulting in arachidonic acid (AA) release and the generation predominantly of eicosanoids with prohypertensive effects in male mice.^{1,2} The eicosanoids produced by COX (cyclooxygenase), PGE₂ (prostaglandin E₂) by acting on EP1 and EP3, and TXA₂ (thromboxane A₂) by acting on its TP (prostanoid receptor), contribute to the hypertensive

effect of Ang II.^{3–6} Also, AA metabolites generated via 12/15-lipoxygenase and cytochrome P450 A1, 12S- and 20-hydroxyeicosatetraenoic acids, respectively, participate in the vasoconstrictor effect of Ang II and contribute to its prohypertensive effect.^{7,8} There is sexual dimorphism in blood pressure (BP) levels in humans and various animal models of hypertension which is attributed to gonadal hormones and sex chromosomes affecting renin-angiotensin and immune cell system activity.^{9–11} Ang II produces a greater pressor effect in men than

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Novelty and Significance

What Is New?

- 17β-estradiol (E2)-CYP1B1 (cytochrome P450 1B1)-derived metabolite 2-ME (2-methoxyestradiol) protects against Ang II (angiotensin II)-induced hypertension, reactive oxygen species production, and renal fibrosis by inhibiting the activity of group IV cPLA₂α (cytosolic phospholipase A₂α) and generation of arachidonic acid metabolites prostaglandins (PG) E₂ and thromboxane (TX) A₂ in female mice.
- The decrease in PGE₂ and TXA₂ levels by reducing stimulation of the EP1/EP3 and TP receptors, respectively, ameliorates Ang II-induced hypertension, reactive oxygen species production, and renal fibrosis.

What Is Relevant?

- 2-ME and EP1, EP3, and TP receptor antagonists could be useful in treating hypertension and its pathogenesis in postmenopausal and hypoestrogenemic premenopausal women and women with menstrual irregularities ovarian failure as well as in males.

Summary

We have previously shown that E2 protects against Ang II-induced hypertension and associated cardiovascular and renal pathogenesis via CYP1B-generated metabolite 2-ME in female mice. The present study demonstrates that 2-ME exerts its protective effect against Ang II-induced hypertension and associated production of reactive oxygen species and renal fibrosis by inhibiting group IV cPLA₂α and generation of arachidonic acid metabolites PGE₂ and TXA₂ in female mice. The decrease in PGE₂ and TXA₂ production leads to decreased stimulation of the EP1/EP3 and TP receptors, respectively, extenuate Ang II-induced hypertension, reactive oxygen species production, and renal fibrosis. Therefore, 2-ME and EP1, EP3, and TP receptor antagonists could be beneficial in treating hypertension and associated pathogenesis in postmenopausal and hypoestrogenemic premenopausal women and women with menstrual irregularities caused by ovarian failure.

Nonstandard Abbreviations and Acronyms

2-ME	2-methoxyestradiol
AA	arachidonic acid
Ang II	angiotensin II
BP	blood pressure
COX	cyclooxygenase
cPLA₂α	cytosolic phospholipase A ₂ α
ETYA	5,8,11,14-eicosatetraenoic acid
NAME	Nω-nitro-L-arginine methyl ester hydrochloride
PGE₂	prostaglandin E ₂
PPARγ	peroxisome proliferator-activated receptor γ
ROS	reactive oxygen species
TXA₂	thromboxane A ₂

women.¹² In addition, Ang II infusion produces a higher increase in BP in males than in female animals.^{9–11} Ovariectomy enhances in female mice, and castration reduces the effect of Ang II in male mice to increase BP.¹³ However, whether the protection against Ang II-induced hypertension in the females depends on alteration in the activity of the cPLA₂α/AA system is unknown.

Previously, we showed that E2 (17β-estradiol)-CYP1B1 generated metabolite 2-hydroxyestradiol by its subsequent metabolism by catechol-*O*-methyltransferase to 2-ME (2-methoxyestradiol) protected against Ang

II-induced hypertension in female mice.^{14–17} However, the mechanism by which 2-ME protects against Ang II-induced hypertension is not known. Our preliminary observation that cPLA₂α gene disruption in females, as in male mice,¹ also prevents Ang II-induced hypertension led to the following hypothesis: CYP1B1-E2 generated metabolite 2-ME acts upstream by inhibiting cPLA₂α activity and reducing the generation of prohypertensive eicosanoids, protects against Ang II-induced hypertension and its pathogenesis.

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 of 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 female mice according to protocols approved by the University of Tennessee Health Science center Institutional Animal Care and Use Committee according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. *cPLA₂α^{+/+}/Cyp1b1^{+/+}*, *cPLA₂α^{-/-}/Cyp1b1^{+/+}*, and *cPLA₂α^{+/+}/Cyp1b1^{-/-}* female mice on the C57BL/6J background were used in this study. *cPLA₂α^{-/-}/Cyp1b1^{+/+}* and *cPLA₂α^{+/+}/Cyp1b1^{-/-}* female mice, respectively, were bred and genotyped as described previously^{18,19} and also as detailed in the [Data Supplement](#). The animals were randomly divided into various treatment groups and infused

subcutaneously with Ang II (700 ng/kg per minute) or saline for 14 days through implanted micro-osmotic pumps (Alzet, Cupertino, CA; model 1002). Mean arterial BP, systolic BP (SBP), and diastolic BP, and heart rate was measured by radiotelemetry. In some experiments, as described in the Results section, SBP was measured by the noninvasive tail-cuff method (Kent Scientific; model XBP 1000). To test our hypothesis, we investigated (1) the effect of *cPLA₂α* gene disruption on Ang II–induced hypertension and associated pathogenesis in intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice; (2) the effect of Ang II on renal *cPLA₂α* expression and activity in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice treated with E2 or 2-ME (1.5 mg/kg, IP, every third day), respectively, and their vehicle, dimethyl sulfoxide (DMSO); (3) the effect of Ang II on plasma levels of E2 and 2-ME in intact *cPLA₂α^{+/+}/Cyp1b1^{+/+}* and *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice, and in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice treated with exogenous E2 or 2-ME and their vehicle (DMSO); (4) the effect of AA metabolism inhibitor ETYA (5, 8, 11,14-eicosatetraenoic acid, 50 mg/kg, IP, every third day),²⁰ 2-ME, and antagonists of EP1 (SC19220, 10 μg/g, SC, second day),²¹ as well as EP3 (L-798106, 10 μg/g, SC, every second day),⁴ and (terutroban, 10 μg/g, SC, every second day)²² receptors and their vehicle (DMSO) on Ang II–induced increase in SBP in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. Administration of E2, 2-ME, ETYA, EP1, EP3, and TP receptor antagonists or their vehicle DMSO was initiated with the implantation of micro-osmotic pumps for infusion of Ang II or its vehicle (saline).

Histological Analysis

Kidney sections from various treatment groups were stained with Masson trichrome for collagen detection. The sections were viewed blinded with an Olympus inverted system microscope (Olympus America Inc, model BX41) and photographed using a SPOT Insight digital camera (Diagnostic Instruments Inc, model Insight 2MP Firewire). Images were quantified by ImageJ software version 1.53a (National Institutes of Health, Bethesda, MD).

Western Blot Analysis

Kidneys were lysed in TissueLyser II (Qiagen) and centrifuged; an equal amount of protein from each lysate was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were probed with anti-phospho (p)-*cPLA₂α* and *cPLA₂α* and corresponding secondary antibodies.

Urinary Levels of PGE₂ and TXB₂

Urine samples were collected by placing the mice in metabolic cages for 24 hours. PGE₂ is rapidly converted to its 13,14-dihydro-15-keto metabolite in vivo and further undergoes degradation to PGA products. We measured the concentration of its metabolites using a Prostaglandin E Metabolite ELISA kit (Cayman Catalog No. 514531) following the manufacturer's instructions. This kit enables the conversion of 13,14-dihydro-15-keto PGA₂ and 13,14-dihydro-15-keto PGE₂ to a single, stable derivative that can be quantified. TXA₂ is rapidly hydrolyzed nonenzymatically to form TXB₂ and excreted to urine. TXB₂ levels were measured using an ELISA kit (Cayman Catalog No. 501020).

Measurement of Renal Reactive Oxygen Species Production

Five micrometer frozen sections of the kidney were exposed to dihydroethidium, and the yellow fluorescence selective for 2-hydroxyethidium as an index of ROS was visualized on a fluorescence microscope (model IX50, Olympus America) with dual-wavelength filter, excitation at 375 nm and emission at 585 nm as described previously.²³ Images were quantified by ImageJ software version 1.53a.

Statistical Analysis

The data were analyzed by one-way ANOVA and Tukey test for multiple comparisons. BP data were analyzed by repeated measures 2-way ANOVA followed by Tukey multiple comparison test. The values of different experiments are expressed as mean±SEM, and *P*<0.05 was considered statistically significant. In most of our experiments, the main comparisons exceeded a power of 0.8 with the number of animals used (Data Supplement).

RESULTS

Ang II Increased Mean Arterial BP, SBP, and Diastolic BP in *cPLA₂α^{+/+}/Cyp1b1^{+/+}* But Not in Intact or Ovariectomized-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* Mice

Ang II activates *cPLA₂α* and releases AA from tissue phospholipids,²⁴ and AA metabolites with prohypertensive effects contribute to the development of Ang II–induced hypertension in male mice. Ang II produced a smaller increase in BP in females than in male animals,^{9–11} which is enhanced by ovariectomized.¹³ To determine if Ang II–induced hypertension in both intact and ovariectomized female mice is also dependent on *cPLA₂α*, we examined the effect of Ang II on BP in intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice. Ang II infusion for 2 weeks increased the mean arterial BP, and SBP and diastolic BP, as measured by radiotelemetry in intact (Figure 1A, Figure S1A and S1B in the Data Supplement) and to a much higher level, in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (Figure 1B, and Figure S1D and S1E), but not in intact and ovariectomized-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice (Figure 1A and 1B, and Figure S1A, S1B, S1D, and S1E); heart rate was not altered in these groups of mice (Figure S1C and S1F). The increase in SBP produced by Ang II, measured by tail-cuff and radiotelemetry at the same time of the day, are comparable. Therefore, we measured SBP in the rest of the experiments by tail-cuff.

Ang II Stimulated Renal Collagen Deposition and ROS Production in Intact and Ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* But Not Intact and Ovariectomized-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* Mice

Infusion of Ang II–induced collagen deposition as detected by Masson trichrome staining (Figure 1C and

1D), and ROS production, as determined by 2-hydroxyethidium fluorescence (Figure 1E and 1F) in the kidney of intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* but not intact and ovariectomized-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice.

Ang II–Induced Increase in Renal cPLA₂α Activity Was Enhanced in Ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and Intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* Mice and Attenuated by E2 Only in Ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}*, and by 2-ME in Both the Groups

Several vasoactive agents, including Ang II, increase cPLA₂α activity and release AA in the vascular smooth muscle cells.^{24–26} Infusion of Ang II did not alter cPLA₂α expression but increased cPLA₂α activity, as determined by a higher ratio of p-cPLA₂α to cPLA₂α in the kidneys of *cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice. To determine if E2-CYP1B1 generated metabolite 2-ME acts upstream or downstream of cPLA₂α, we examined the effect of Ang II on cPLA₂α activity and expression in the kidneys of intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice treated with E2 and 2-ME. Ang II–induced increase in the ratio of p-cPLA₂α was enhanced to a much greater degree in the kidneys of ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* compared with the intact *cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice (Figure 2A). E2 inhibited the ability of Ang II to increase p-cPLA₂α expression in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (Figure 2B) but not ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice (Figure 2C). Treatment with 2-ME attenuated the Ang II–induced increases in the ratio of p-cPLA₂α to cPLA₂α in both ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (Figure 2D) and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice (Figure 2E). As expected, cPLA₂α expression was not detected in the kidneys of *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice with the cPLA₂α antibody used in our experiment, indicating that this antibody detects only cPLA₂α (Figure 2F).

cPLA₂α Gene Disruption Did not Reduce Plasma Levels of E2 or 2-ME, and *Cyp1b1* Gene Disruption Inhibited Plasma Levels of 2-ME but not E2

To determine if *cPLA₂α* and *Cyp1b1* gene disruption affected the production of E2 and 2-ME, we examined the effect of Ang II infusion on plasma levels of E2 and 2-ME in intact *cPLA₂α^{+/+}/Cyp1b1^{+/+}* and *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice with exogenous E2 and 2-ME treatment or their vehicle DMSO. The plasma levels of E2 in intact *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice were not different

from that observed in *cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice and were not altered by Ang II (Figure S2A). Plasma levels of E2 and 2-ME were reduced in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice, and treatment with exogenous E2 but not 2-ME increased levels of E2 (Figure S2A and S2B). Ang II increased plasma levels of 2-ME in both intact *cPLA₂α^{+/+}/Cyp1b1^{+/+}* and *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice (Figure S2C). In ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice, 2-ME levels were reduced, and treatment with exogenous E2 raised the basal level of 2-ME that was increased by Ang II in the former but not in the latter groups of mice (Figure S2C and S2D). Administration of exogenous 2-ME increased its plasma levels in both ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice that were not altered by Ang II (Figure S2C and S2D).

2-ME Treatment Inhibited Ang II–Induced Increase in SBP, Renal Collagen Deposition, and ROS Production in Ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and Intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* Mice

We showed that 2-ME treatment inhibits Ang II–induced increase in SBP in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice.¹⁷ We confirmed this observation (Figure 3A and 3B) and show here that 2-ME also prevents the ability of Ang II to cause renal collagen deposition (Figure 3C and 3D) and ROS production in these mice (Figure 3E and 3F).

AA Metabolism Inhibitor ETYA Attenuated Ang II–Induced Increase in SBP, Renal Collagen Deposition, and ROS Production in Ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and Intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* Mice

Ang II failed to increase SBP (Figure 4A and 4B), produce renal collagen deposition (Figure 4C and 4D), and increase ROS production (Figure 4E and 4F) in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice treated with ETYA, an AA metabolism inhibitor.²⁰

cPLA₂α Gene Disruption, ETYA, and 2-ME Inhibited Ang II–Induced Increase in Urinary Excretion of PGE₂ and TXA₂ Metabolites

Ang II increased urinary excretion of stable metabolites of PGE₂ (13,14-dihydro-15-keto PGE₂/PGA₂) and TXA₂ (TXB₂), respectively, in intact *cPLA₂α^{+/+}/Cyp1b1^{+/+}* but not *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice (Figure

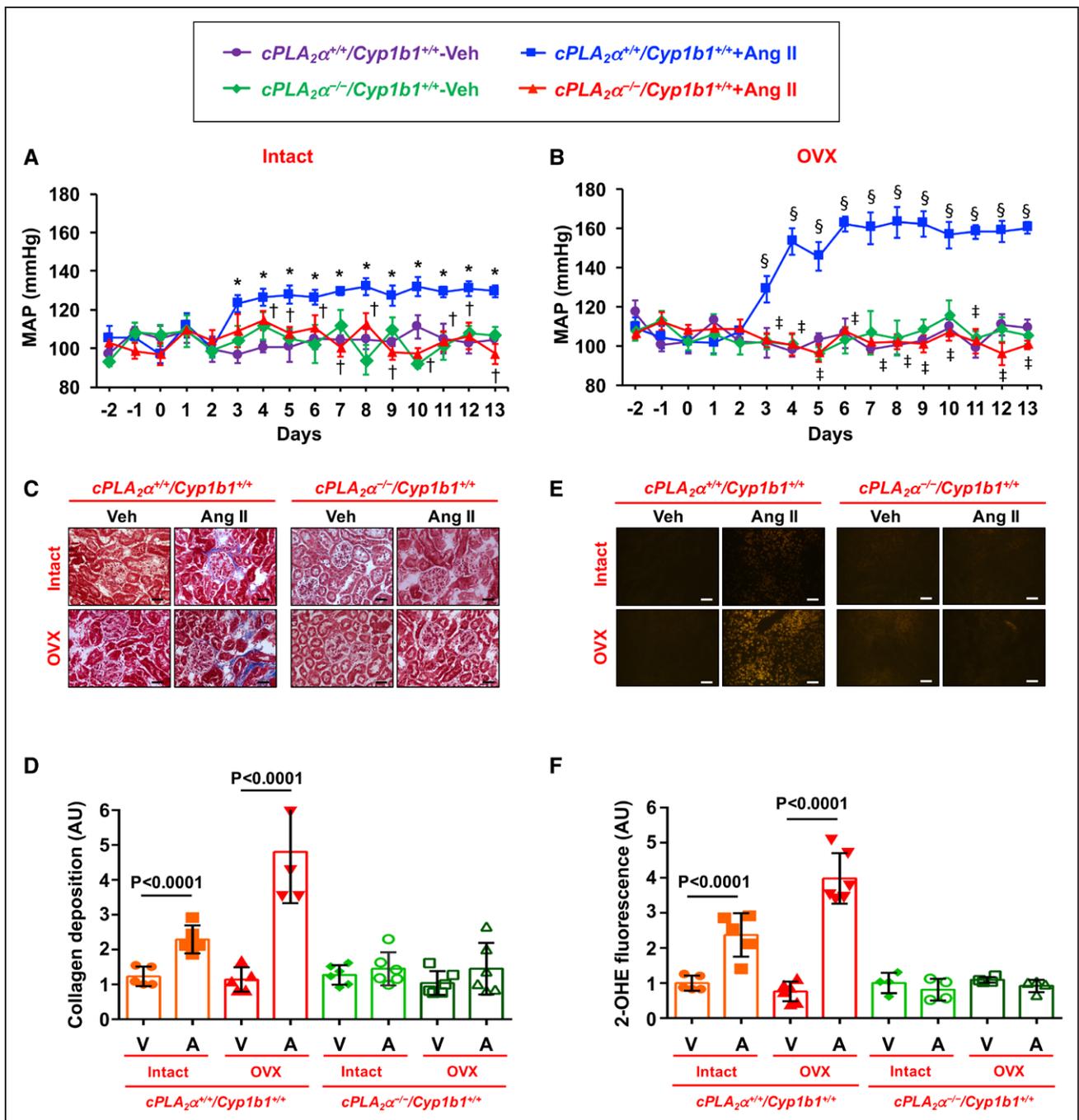


Figure 1. Ang II (angiotensin II) increased blood pressure (BP) and renal collagen deposition and 2-hydroxyethidium (2-OHE) fluorescence in intact and ovariectomized (OVX)-*cPLA₂ α ^{+/+}/Cyp1b1^{+/+}* but not intact *cPLA₂ α ^{-/-}/Cyp1b1^{+/+}* mice.

Mean arterial pressure (MAP; **A** and **B**) was measured by radiotelemetry. Saline was used as a vehicle (Veh) for Ang II. Collagen deposition was detected by Masson trichrome Staining (**C**), and reactive oxygen species were measured by DHE staining (**E**), and the quantitative values were expressed as arbitrary units (AU; **D** and **F**), respectively. Saline was used as a vehicle (Veh) for Ang II. Data are mean \pm SEM (n=5 per group) and analyzed by repeated measures 2-way ANOVA followed by Tukey multiple comparisons test. * P <0.05 and \S <0.05 vs day 0 value before osmotic pump with Ang II. † P <0.05 vs intact *cPLA₂ α ^{-/-}/Cyp1b1^{+/+}*; ‡<0.05, OVX-*cPLA₂ α ^{-/-}/Cyp1b1^{+/+}*. Data in (**D**) and (**F**) are mean \pm SEM (n=4–6 per group) and analyzed by 1-way ANOVA and Tukey multiple comparisons test. A indicates Ang II; cPLA₂ α , cytosolic phospholipase A2 α ; and V, Veh. Scale bars 50 μ m.

S3A and S3B). The AA metabolism inhibitor ETYA, E2, and 2-ME attenuated the ability of Ang II to increase the urinary excretion of metabolites of PGE₂ and TXB₂ in ovariectomized-*cPLA₂ α ^{+/+}/Cyp1b1^{+/+}* mice (Figure S3A and S3B), as well as ETYA and 2-ME

in intact *cPLA₂ α ^{+/+}/Cyp1b1^{-/-}* mice (Figure S3C and S3D). E2 failed to inhibit Ang II-induced increased urinary excretion of PGE₂ metabolites and TXB₂ in ovariectomized-*cPLA₂ α ^{+/+}/Cyp1b1^{-/-}* mice (Figure S3C and S3D).

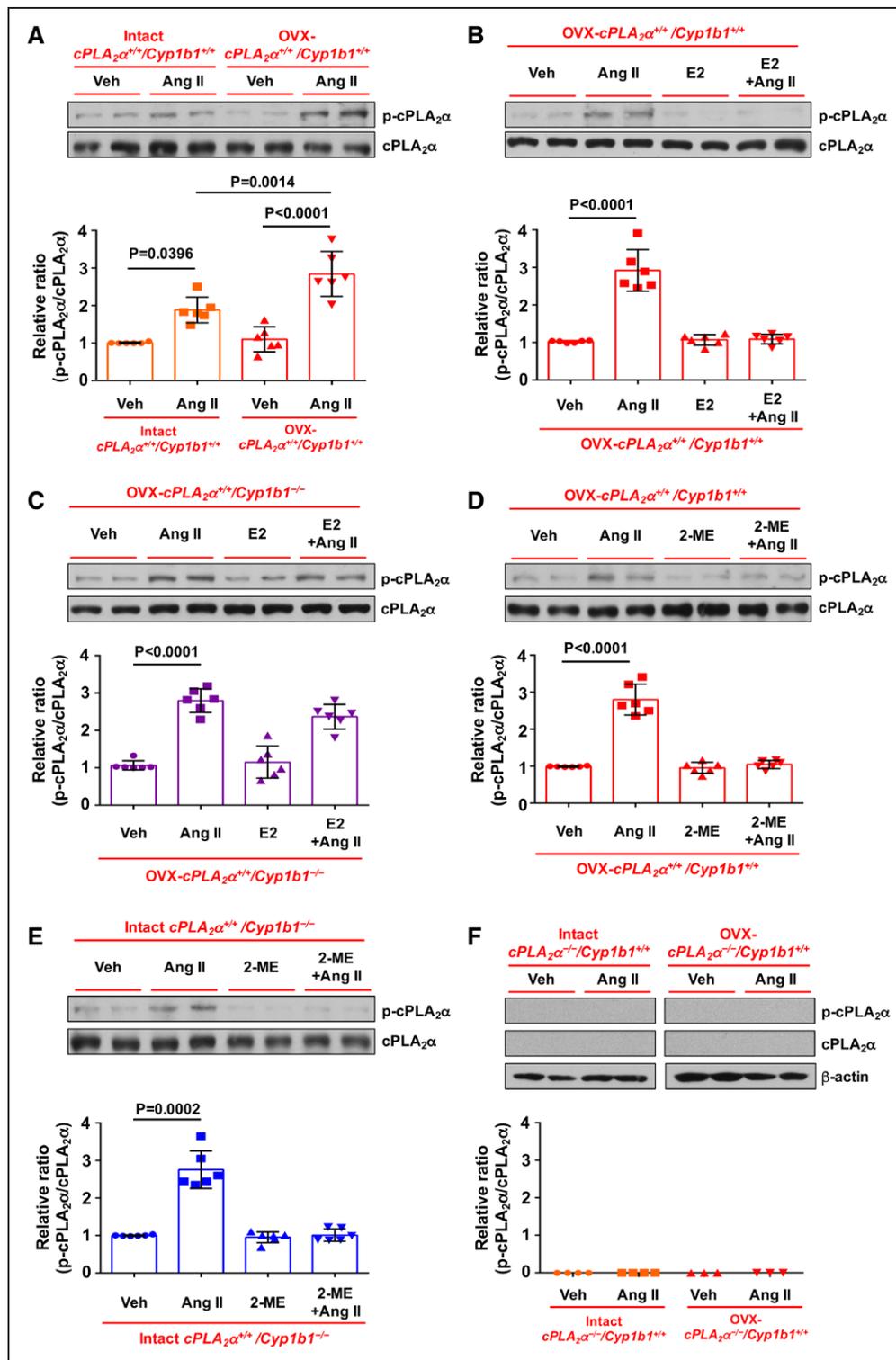


Figure 2. Ang II (angiotensin II) increased renal cPLA₂α (cytosolic phospholipase A₂α) activation in intact and ovariectomized (OVX)-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice.

OVX-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice expressed Ang II-induced phospho (p)-cPLA₂α, activation form of cPLA₂α, more than intact-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice (A). 17β-estradiol (E2) diminished Ang II-induced p-cPLA₂α expression in OVX-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (B) but not in OVX-*cPLA₂α^{-/-}/Cyp1b1^{-/-}* mice (C). 2-methoxyestradiol (2-ME) abolished Ang II-induced cPLA₂α activation in OVX-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (D) and in intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice (E). cPLA₂α proteins were not detected in OVX-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice (F). Saline was used as a vehicle (Veh) of Ang II (A and F) and dimethyl sulfoxide (B–D) for 2-ME. p-cPLA₂α and cPLA₂α expressions were analyzed by Western blot. Quantitative data were presented as the relative ratio, p-cPLA₂α, and cPLA₂α, and on the top of each panel is the representative blot for each group of experiments, respectively. β-actin was used for loading control in intact and OVX-*cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice (F). Data are mean±SEM (n=3–6 per group) and analyzed by 1-way ANOVA and Tukey multiple comparisons test.

PGE₂-EP1/EP3 and TXA₂-TP Receptor Antagonists Reduced the Ang II-Induced Increase in SBP, Renal Collagen Deposition, and ROS Production in Ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and *cPLA₂α^{+/+}/Cyp1b1^{-/-}* Mice

Receptor antagonists of PGE₂-EP1 (EP1RA, SC19220),²¹ EP3 (EP3RA, L-798106),⁴ and TXA₂ (terutroban)²² reduced Ang II-induced increases in SBP, renal collagen deposition, and ROS production in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (Figure 5A through 5C and Figure 6A and 6B) and in intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice (Figure 5D through 5F and Figure 6C and 6D).

DISCUSSION

The main findings of the present study are that (1) Ang II-induced hypertension and associated renal ROS production and fibrosis are dependent on cPLA₂α in female mice; (2) the E2-CYP1B1 derived metabolite 2-ME acts upstream of cPLA₂α and by inhibiting its activation by Ang II, prevented the production of the AA-generated COX metabolites PGE₂ and TXA₂; (3) ovariectomy and *Cyp1b1* gene disruption promote the Ang II-induced increases in cPLA₂α activation and production of PGE₂ and TXA₂, BP, renal fibrosis, and ROS production, all of which were mitigated by 2-ME; and (4) the AA metabolism inhibitor ETYA and antagonists of PGE₂ and TXA₂ receptors EP1, EP3, and TP, respectively, inhibited Ang II-induced hypertension and associated renal fibrosis and ROS production in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice.

Previously, we showed that the lower increase in BP by Ang II in females rather than in male mice is maintained by the E2 metabolite 2-ME, generated from its sequential metabolism by CYP1B1 and 2-hydroxyestradiol by catechol-*O*-methyl transferase.¹⁵ Moreover, by activating group IV cPLA₂α, Ang II releases AA, and generates prohypertensive eicosanoids that contribute to the development of hypertension and its associated pathogenesis in male mice.^{1,2} In the present study, cPLA₂α gene disruption also attenuated Ang II-induced increases in mean arterial BP, SBP, and diastolic BP, as measured by radiotelemetry, and increased associated renal fibrosis, which was determined by collagen deposition and ROS production as detected by 2-hydroxyethidium fluorescence in female mice. Our demonstration that (1) Ang II-induced increase in cPLA₂α activity measured by its phosphorylation was enhanced in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice; (2) treatment with E2 and 2-ME inhibited cPLA₂α activity in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* mice, and 2-ME but not E2 attenuated cPLA₂α activity in intact and

ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice, which suggests that the E2-CYP1B1-generated metabolite 2-ME acts upstream of cPLA₂α to inhibit its activity. Supporting this view was our demonstration that Ang II increased plasma levels of 2-ME but not E2 in intact mice, demonstrating that Ang II promotes the conversion of E2 to 2-ME. Moreover, this effect was absent in *Cyp1b1* but not *cPLA₂α* gene disrupted mice suggesting that this occurs by increased CYP1B1 activity. Supporting this notion were our findings that treatment with exogenous E2 increased both basal and Ang II-induced plasma levels of 2-ME in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* but not in intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. However, the administration of exogenous 2-ME increased its plasma levels in all these groups. Previously, we reported that Ang II increased renal CYP1B1 activity without altering its expression that was associated with increased plasma levels of 2-ME.¹⁵

The mechanism by which 2-ME inhibits Ang II-induced activation of cPLA₂α is not known. Since 2-ME treatment alone did not alter the activity of cPLA₂α, it appears that 2-ME acts as an inhibitory modulator of Ang II-induced activation of cPLA₂α. 2-ME, which could attenuate cPLA₂α activity via genomic E2 receptor and its nongenomic G-protein coupled estrogen receptor 1²⁷ by interfering with one or more signaling molecules including the influx of extracellular calcium, calcium-calmodulin-dependent kinase, and extracellular signal-regulated kinase 1/2 activities required for cPLA₂α stimulation by Ang II,^{24,25} remains to be determined. 2-ME was reported to inhibit an Ang II-induced rise in cytosolic calcium while also downregulating AT1 receptor in rat vascular smooth muscle cells via G-protein coupled estrogen receptor 1.²⁸ In our study, the ability of 2-ME to inhibit cPLA₂α activity is unlikely due to downregulation of the AT1a receptor because treatment with 2-ME did not alter AT1 receptor expression in the kidney.¹⁷

Ang II increased the urinary excretion of PGE₂ metabolites and TXB₂ in intact *cPLA₂α^{+/+}/Cyp1b1^{+/+}* but not in *cPLA₂α^{-/-}/Cyp1b1^{+/+}* mice, and these effects of Ang II were enhanced in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. Treatment with E2 and 2-ME in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and 2-ME but not E2 in intact and ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice inhibited Ang II-induced increases in urinary excretion of PGE₂ metabolites and TXB₂. These data suggest that 2-ME, by inhibiting cPLA₂α activity and AA-COX-generated metabolites of PGE₂ and TXA₂, ameliorates Ang II-induced hypertension, renal fibrosis, and ROS production. Supporting these conclusions are our findings that 2-ME and the AA metabolism inhibitor ETYA²⁰ attenuated the Ang II-induced increases in SBP, renal fibrosis, ROS production, and urinary excretion of PGE₂ metabolites and TXB₂ in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice.

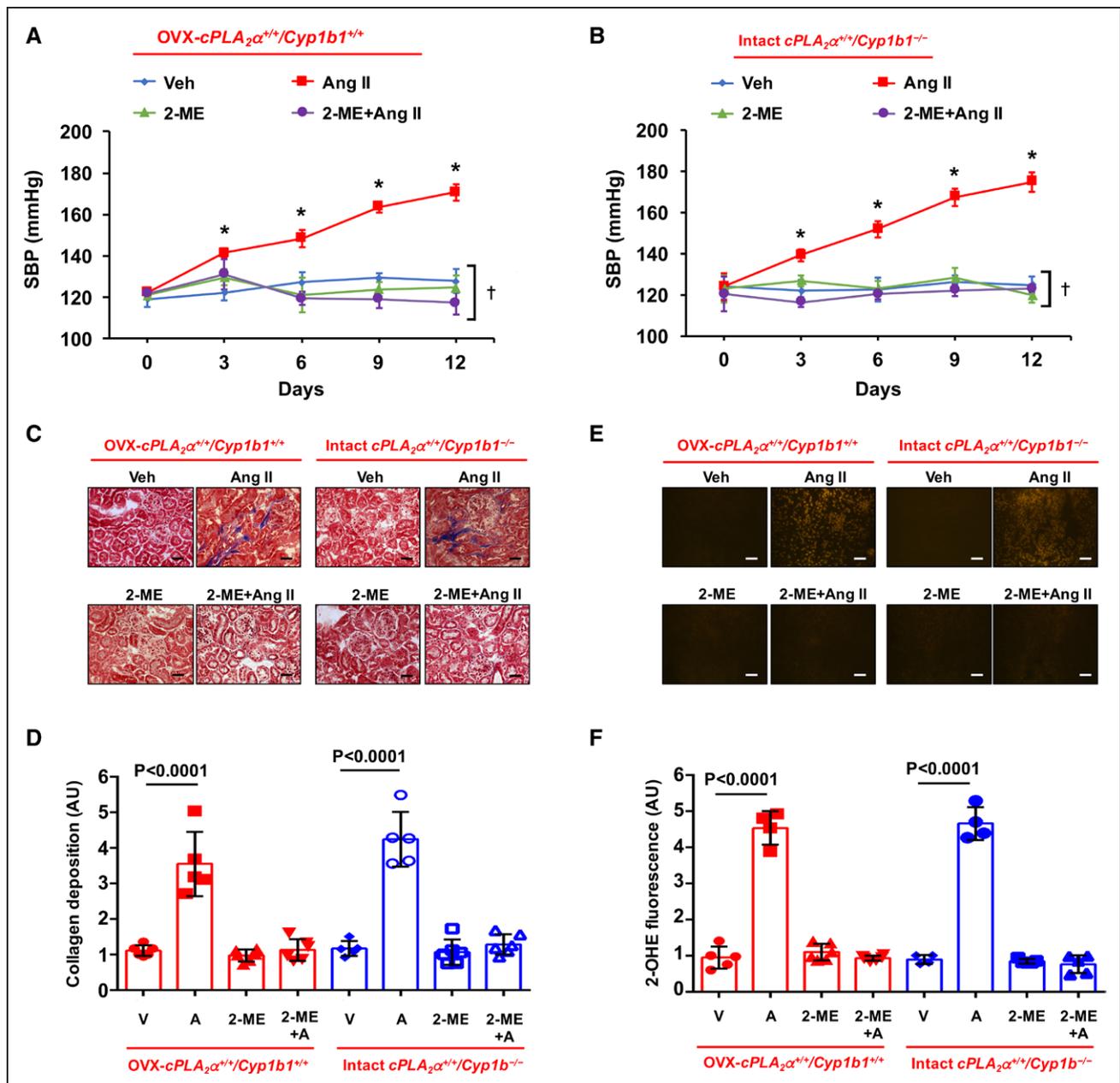


Figure 3. 2-Methoxyestradiol (2-ME) inhibited Ang II (angiotensin II)-induced increase in systolic blood pressure (SBP), renal collagen deposition, and 2-hydroxyethidium (2-OHE) fluorescence in ovariectomized (OVX)-cPLA₂α^{+/+}/Cyp1b1^{+/+} and intact cPLA₂α^{+/+}/Cyp1b1^{-/-} mice.

Systolic blood pressure (SBP) was measured by the tail-cuff method (A and B). Collagen deposition was detected by Masson trichrome staining (C), and reactive oxygen species were measured by DHE staining (E), and the quantitative values were expressed as arbitrary units (AU; D) and (F), respectively. Data are mean±SEM (n=4–6 per group). Data for SBP were analyzed using repeated measures 2-way ANOVA followed by Tukey multiple comparison test (A and B) and, data for collagen deposition and 2-OHE fluorescence by 1-way ANOVA followed by Tukey multiple comparison test (D and F). *P<0.05 vs day 0 value before osmotic pump with Ang II. †P<0.05 vs Ang II alone. A indicates Ang II; and V, vehicle. Saline was used as a vehicle of Ang II and DMSO for 2-ME. Scale bars 50 μm.

Ang II-induced increases in BP were reported to be responsible for cardiac hypertrophy and fibrosis, along with renal injury.^{29–32} Cyclic stretch via stress-induced calcium influx activates cPLA₂α and releases AA in rabbit proximal tubule cells³³ and increases ROS production in striated muscle.³⁴ Therefore, the mechanical stretch caused

by Ang II-induced increases in BP might also, by activating cPLA₂α, produce PGE₂ and TXA₂, and 2-ME, by inhibiting cPLA₂α activity, prevents the production of these eicosanoids, ROS production, and renal fibrosis in female mice. PGE₂ via EP1 and EP3,^{3,4} and TXA₂ through TP receptors,^{5,35} promote vasoconstrictor and hypertensive effects

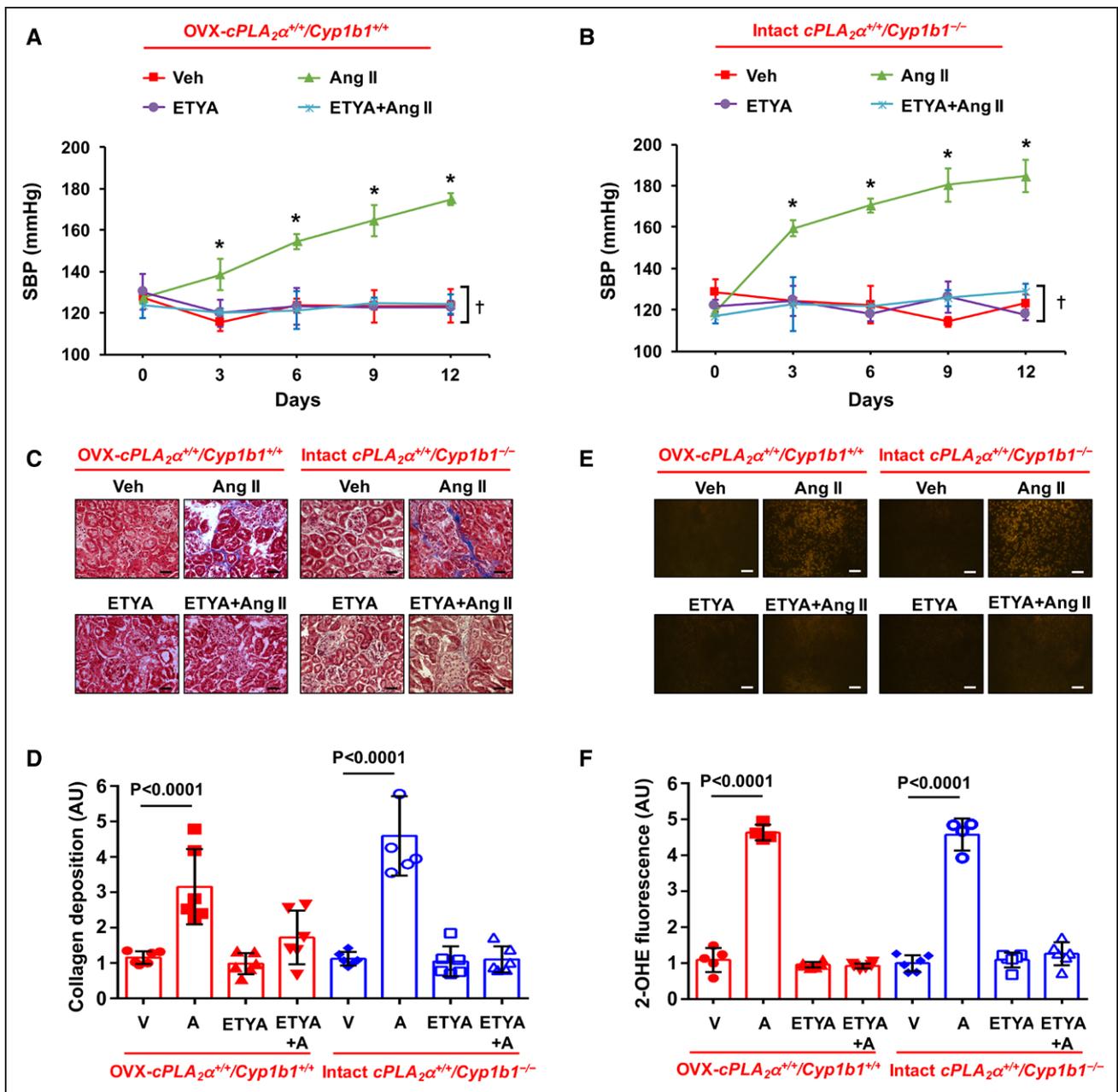


Figure 4. ETYA (5,8,11,14-eicosatetraynoic acid) inhibited Ang II (angiotensin II), increased systolic blood pressure (SBP), renal collagen deposition, and 2-hydroxyethidium (2-OHE) fluorescence in ovariectomized (OVX)-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice.

SBP was measured by the tail-cuff method in OVX-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (A) and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice (B), and collagen deposition was measured by Masson trichrome staining (C), and reactive oxygen species were measured by 2-OHE fluorescence (E), and the quantitative values were expressed as arbitrary units (AU; D and F), respectively. Saline was used as a vehicle (Veh) of Ang II and dimethyl sulfoxide for ETYA. Data are mean±SEM (n=5–6 per group). Statistical analysis was performed using repeated measures 2-way ANOVA and Tukey multiple comparisons test (A and B) and 1-way ANOVA and Tukey multiple comparisons test (D and F). *P<0.05 vs day 0 value before osmotic pump with Ang II; †P<0.05 vs Ang II alone. A indicates Ang II; and V, Veh. Scale bars 50 μm.

of Ang II.^{34,36} PGE₂/EP3 also contributes to L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride)³⁷ and high salt diet-fed S-P467L mice with decreased PPARγ (peroxisome proliferator-activated receptor γ) activity-induced hypertension.³⁸ In our study, antagonists

of EP1 (SC19220),²¹ EP3 (L-798106),⁴ and TXA₂-TP (terutroban)²² receptors reduced Ang II-induced increases in SBP, renal fibrosis, and ROS production in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. From these observations, it follows

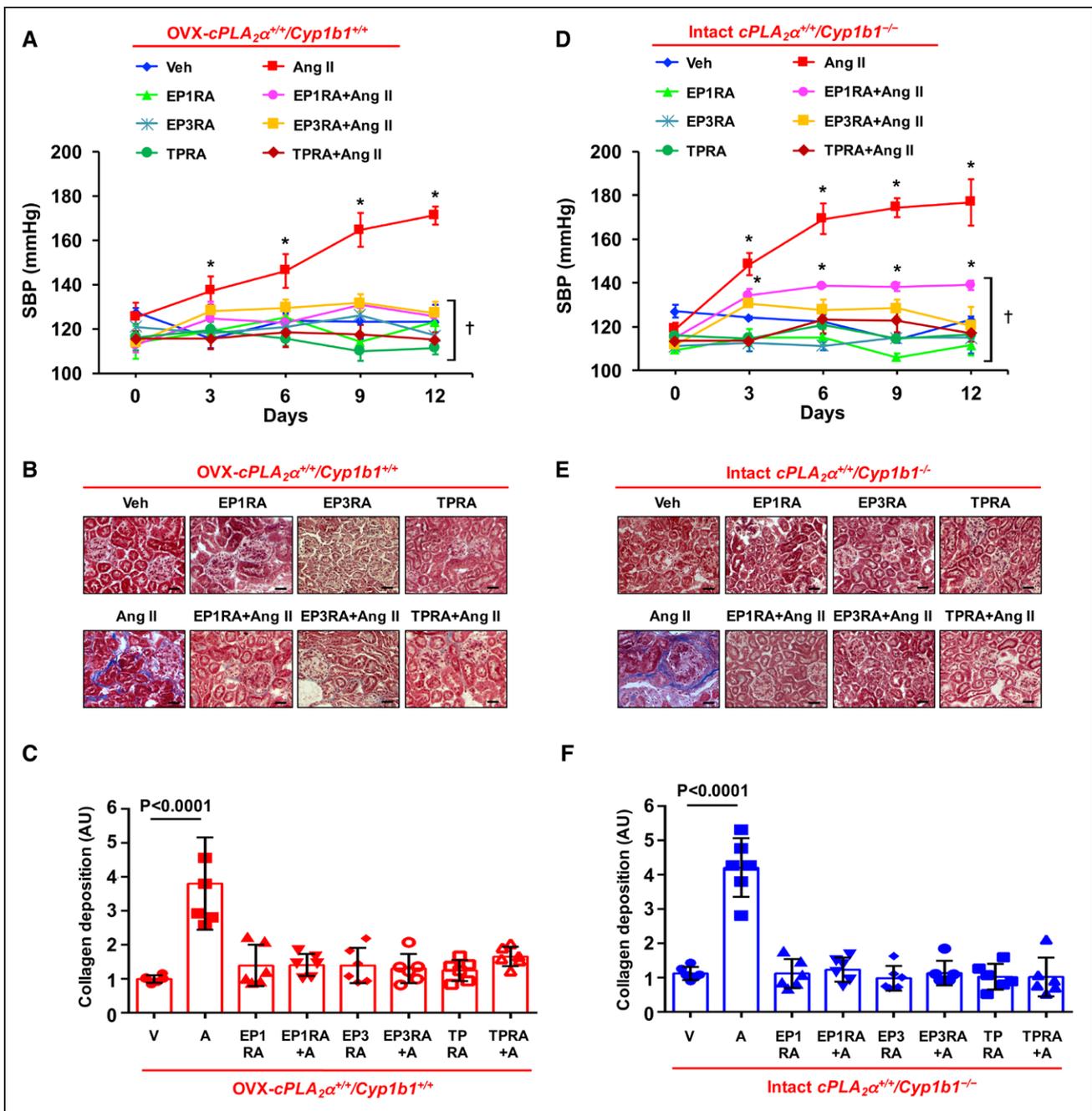


Figure 5. Prostaglandin E₂ receptor antagonist (RA) EP1, EP3, and thromboxane A₂ (TP) RA diminished Ang II (angiotensin II)-induced increased systolic blood pressure (SBP) and collagen deposition in ovariectomized (OVX)-cPLA₂α^{+/+}/Cyp1b1^{+/+} and intact cPLA₂α^{+/+}/Cyp1b1^{-/-} mice.

Blood pressure was measured by the tail-cuff method in OVX-cPLA₂α^{+/+}/Cyp1b1^{+/+} (A), and intact cPLA₂α^{+/+}/Cyp1b1^{-/-} mice (D), and the collagen deposition was detected by Masson trichrome staining (B and E), and the quantitative values expressed as an arbitrary unit (AU; C and F), respectively. Saline was used as a vehicle (Veh) of Ang II and dimethyl sulfoxide (DMSO) for EP1, EP3, and TP receptor antagonists. Data are mean±SEM (n=5–6 per group). Statistical analyses were performed using repeated-measures 2-way ANOVA followed by Tukey multiple comparison test (A and D) and 1-way ANOVA followed by Tukey multiple comparison test (C and F). *P<0.05 vs day 0 value before osmotic pump with Ang II; †P<0.05 vs Ang II alone. A indicates Ang II; and V, Veh. Scale bars 50 μm.

that the E₂-CYP1B1 generated metabolite 2-ME by inhibiting Ang II-induced cPLA₂α activation and production of PGE₂ and TXA₂, reduced stimulation of EP1 and EP3 and TP receptors, respectively, which ameliorates hypertension and associated renal fibrosis and

ROS production most likely consequent to decreased BP. Although EP3 and TP receptor antagonists attenuated Ang II-induced increase in BP in ovariectomized and intact *Cyp1b1* gene disrupted mice, EP1 receptor antagonist SC19220 abolished the effect of Ang II in

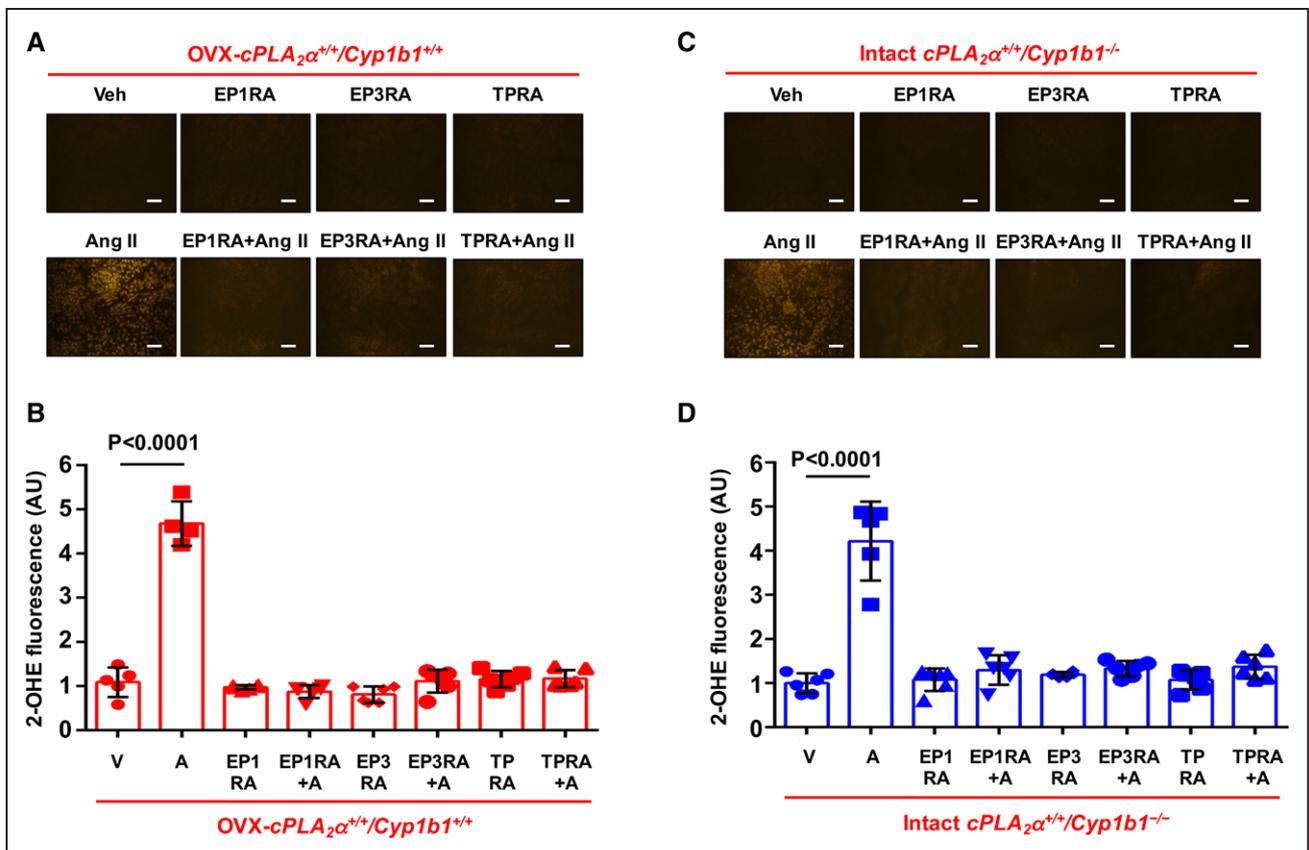


Figure 6. EP1, EP3, and TP receptor antagonists prevented Ang II (angiotensin II)-induced increase 2-hydroxyethidium (2-OHE) fluorescence in ovariectomized (OVX)-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice.

Reactive oxygen species were measured by 2-OHE fluorescence in the kidney section of OVX-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* (A), and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice (C), and quantitative values expressed as arbitrary units (AU), respectively (B and D). Saline was used as a vehicle (Veh) of Ang II and dimethyl sulfoxide for EP1, EP3, and TP receptor antagonists. Data are mean±SEM (n=5–6 per group) and analyzed using 1-way ANOVA and Tukey multiple comparisons test. A indicates Ang II; V, Veh; EP1RA, EP1 receptor antagonist; EP3RA, EP3 receptor antagonist; and TPRA, TP receptor antagonist. Scale bars 50 μm.

ovariectomized and significantly reduced it in *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice. EP1 receptor antagonist SC19220 used in our study has been reported not to alter the binding of [³H]PGE₂ to mouse cloned EP1 receptor expressed in Chinese hamster ovary cells,³⁹ but it attenuated the effect of PGE₂ to upregulate renin expression in mouse cortical CD-M1 cells expressing EP1 receptors²¹ and inhibited binding of [³H]PGE₂ to human cloned EP1 receptor expressed in COS cells.⁴⁰ One of the limitations of our study is that we did not confirm the selectivity of EP1, EP3, and TP receptor antagonists used in our study. Therefore, we cannot rule out their nonselective effects. Further studies using EP1, EP3, and TP receptor knockout mice are required to confirm our findings.

In the present study, EP1, EP3, and TP receptor antagonists did not reduce basal SBP in ovariectomized-*cPLA₂α^{+/+}/Cyp1b1^{+/+}* and intact *cPLA₂α^{+/+}/Cyp1b1^{-/-}* mice probably because the amount of PGE₂ and TXA₂ produced due to low basal *cPLA₂α* activity in the absence of Ang II, was insufficient to alter BP. Therefore, PGE₂ and TXA₂ via EP1 and EP3 and TP receptors, respectively,

appear to promote Ang II-induced increased BP, renal ROS production, and fibrosis by acting as permissive factors in ovariectomized- and *Cyp1b1* gene disrupted mice. Ang II promotes PGE₂-EP3 receptor-mediated constriction of the femoral artery by increasing cell calcium and activating proline-rich tyrosine kinase and Rho-kinase.⁴¹ However, as stated above, antagonists of EP1 and EP3 and TP receptors did not alter the basal BP. Therefore, activation of EP1, EP3, and TP receptors by PGE₂ and TXA₂, respectively, in our study could promote the effect of Ang II on vascular tone by facilitating the increase in cell calcium and sensitization to calcium by Rho-kinase activation.^{4,25} Ang II also increases ROS generation and isolevuglandin protein adducts in dendritic cells, T cells activation, and cytokine production that contribute to its hypertensive effect and renal fibrosis.⁴³ In the L-NAME/high salt-induced model of hypertension, ROS production, dendritic cell activation, accumulation of isolevuglandin protein adducts in spleen cells, and production of proinflammatory cytokines are dependent on PGE₂ activated EP3 receptors.³⁷ PGE₂ also via the EP1 receptor,

acts directly on dendritic cells to increase isolevuglandin adducted proteins.³⁷ The protection against Ang II–induced hypertension in females has been attributed to decreased proinflammatory T cells and increased Treg cell activation, along with increased AT₂ and Mas receptor expression and ACE2 in the kidneys of females compared with males.⁴⁴ Moreover, L-NAME, an inhibitor of the NOS system, increases BP and reduces the compensatory increase in Treg cells in female rats.⁴⁵ 2-ME stimulates NO production and inhibits L-NAME–induced increases in BP and cardiac and renal injury, collagen deposition, and infiltration of ED1⁺ macrophages in the hearts and kidneys.^{46,47} 2-ME also inhibits activation and proliferation of T and B cells and production of cytokines in vitro and in a model of autoimmune encephalomyelitis.⁴⁸ Since cPLA₂α is also required for L-NAME–induced hypertension and its associated endothelial dysfunction,⁴⁹ and deoxycorticosterone salt-induced hypertension (Song and Malik, unpublished work), isolevuglandins are formed from AA,⁵⁰ 2-ME, by inhibiting Ang II–induced cPLA₂α activation, AA release, and generation of PGE₂ and TXA₂ would reduce stimulation of EP1 and EP3 receptors. This, in turn, would decrease ROS production and isolevuglandin protein adducts in dendritic cells, activation of T cells and generation of cytokines, and increased BP and associated renal fibrosis. However, further studies are required to determine the effect of 2-ME on PGE₂/EP1 and EP3 and TXA₂/TP receptor-mediated Ang II- and L-NAME–induced isolevuglandin protein adducts formation in dendritic cells, and T lymphocytes and Treg cells activation. Furthermore, protection by 2-ME against eicosanoids with prohypertensive effects generated from AA via lipoxygenase and cytochrome P450 4A1 including 12S-HETE and 20-HETE, respectively, that contribute to Ang II–induced hypertension¹ are currently under investigation. Moreover, the contribution of renal sodium transport and renal vascular function to the eicosanoid-mediated effect of 2-ME in lowering Ang II–induced increased BP in ovariectomized- and *Cyp1b1* gene disrupted mice remains to be determined. Finally, the protective effects of 2-ME on pressure-dependent and independent cardiac hypertrophy and fibrosis and renal injury, lowering cholesterol levels, reducing neointimal growth, angiogenesis, pulmonary hypertension, and preeclampsia, magnesium insufficiency–induced salt-sensitive hypertension with reduced catechol-*O*-methyltransferase activity, and anticarcinogenic actions^{47,51–55} all of which could be due to its inhibitory effect on cPLA₂α activity and generation of eicosanoids that have been implicated in these disorders remains to be explored.

In conclusion, the E2-CYP1B1-generated metabolite 2-ME, by acting upstream of cPLA₂α and inhibiting its activation by Ang II, attenuates the COX-AA metabolites PGE₂ and TXA₂ and stimulation of EP1 and EP3, and TP receptors, respectively. This, in turn, reduces BP, and

renal fibrosis, and ROS production (Graphical Abstract, Figure S4). Therefore, selective inhibitors of EP1, EP3, and TP receptors and 2-ME could be useful for treating hypertension and its pathogenesis in postmenopausal females; hypoestrogenemic premenopausal women; women with menstrual irregularities due to ovarian failure; as well as in males.

Perspectives

The protection against Ang II–induced hypertension and associated cardiovascular and renal pathogenesis is mediated by the CYP1B1-E2 derived metabolite 2-ME in female mice.^{14–17} Moreover, Ang II–induced hypertension and its associated pathogenesis are mediated by prohypertensive eicosanoids produced by activation of cPLA₂α in male mice.¹ This study furthers our understanding of the mechanism by which the E2-CYP1B1 metabolite 2-ME protects against Ang II–induced hypertension and its pathogenesis by acting upstream of cPLA₂α and by inhibiting cPLA₂α activation, attenuates the generation of AA metabolites PGE₂ and TXA₂ and their prohypertensive effects mediated via stimulation of EP1 and EP3, and TP receptors, respectively. However, the site of interaction of 2-ME and cPLA₂α is unknown. We recently demonstrated that E2-CYP1B1 generated 2-ME in the paraventricular nucleus, which, by decreasing sympathetic activity, ameliorates Ang II–induced hypertension.²⁷ Hence, further studies are needed to determine the interaction of the CYP1B1-generated E2 metabolite 2-ME and cPLA₂α and AA metabolites in the paraventricular nucleus to elucidate the mechanism that contributes to the protective effect of E2 against Ang II–induced hypertension and its associated pathogenesis. Moreover, it was recently reported that there is an association of the CYP1B1 Leu432Val gene polymorphism with hypertension in a small group of Slovak midlife women.⁵⁶ Therefore, it would be important to explore CYP1B1 gene polymorphism further in a larger population of females and males to determine its impact on the contribution of cPLA₂α and AA-derived eicosanoids in hypertension and its pathogenesis. Thus, the regulation of CYP1B1-generated E2 metabolite 2-ME and cPLA₂α and AA metabolites in the kidney and the paraventricular nucleus is important to explore further how E2 protects against Ang II–induced hypertension and its associated pathogenesis.

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Disclosures

J.V. Bonventre is cofounder and holds equity in Goldfinch Bio. He is a co-inventor on KIM-1 and kidney organoid patents assigned to Mass General Brigham. He is a consultant and owns equity in Coegin Pharma. J.V. Bonventre's interests were reviewed and are managed by Brigham and Women's Hospital and Partners HealthCare International, Boston, MA, in accordance with their conflict of interest policies. The other authors report no conflicts.

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