ANGIOTENSIN II-INDUCED HYPERTENSION

6β-Hydroxytestosterone Promotes Angiotensin II-Induced Hypertension via Enhanced Cytosolic Phospholipase $A_2\alpha$ Activity

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ABSTRACT: This study was conducted to test the hypothesis that the CYP1B1 (cytochrome P450 1B1)-testosterone metabolite 6β-hydroxytestosterone contributes to angiotensin II-induced hypertension by promoting activation of group IV cPLA$_2\alpha$ (cytosolic phospholipase A$_2\alpha$) and generation of prohypertensive eicosanoids in male mice. Eight-week-old male intact or orchidectomized *cPLA$_2\alpha^{+/+}$/Cyp1b1$^{+/+}$* and *cPLA$_2\alpha^{-/-}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2\alpha^{+/+}$/Cyp1b1$^{-/-}$* mice were infused with angiotensin II (700 ng/kg/min, subcutaneous) for 2 weeks and injected with 6β-hydroxytestosterone (15 μg/g/every third day, intraperitoneal). Systolic blood pressure was measured by tail-cuff and confirmed by radiotelemetry. Angiotensin II-induced increase in systolic blood pressure, cardiac and renal collagen deposition, and reactive oxygen species production were reduced by disruption of the *cPLA$_2\alpha$* or *Cyp1b1* genes or by administration of the arachidonic acid metabolism inhibitor 5,8,11,14-eicosatetraynoic acid to *cPLA$_2\alpha^{+/+}$/Cyp1b1$^{+/+}$* mice. 6β-hydroxytestosterone treatment restored these effects of angiotensin II in *cPLA$_2\alpha^{+/+}$/Cyp1b1$^{-/-}$* mice but not in orchidectomized *cPLA$_2\alpha^{-/-}$/Cyp1b1$^{+/+}$* mice, which were lowered by 5,8,11,14-eicosatetraynoic acid in *cPLA$_2\alpha^{+/+}$/Cyp1b1$^{-/-}$* mice. Antagonists of prostaglandin E$_2$-EP1/EP3 receptors and thromboxane A$_2$-TP receptors decreased the effect of 6β-hydroxytestosterone in restoring the angiotensin II-induced increase in systolic blood pressure, cardiac and renal collagen deposition, and reactive oxygen species production in *cPLA$_2\alpha^{+/+}$/Cyp1b1$^{-/-}$* mice. These data suggest that 6β-hydroxytestosterone promotes angiotensin II-induced increase in systolic blood pressure and associated pathogenesis via cPLA$_2\alpha$ activation and generation of eicosanoids, most likely prostaglandin E$_2$ and thromboxane A$_2$ that exerts prohypertensive effects by stimulating EP1/EP3 and TP receptors, respectively. Therefore, agents that selectively block these receptors could be useful in treating testosterone exacerbated angiotensin II-induced hypertension and its pathogenesis. **(*Hypertension*. 2021;78:1053–1066. DOI: 10.1161/HYPERTENSIONAHA.121.17927.)** • **Data Supplement**

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

Hypertension is the leading cause of cardiovascular diseases and mortality.[1] It has been well established that hypertension is associated with renal dysfunction and end-organ damage in various animal experimental models of hypertension, including Ang II (angiotensin II).[2,3] Testosterone is known to exert cardiovascular effects and has been implicated in the development of hypertension.[4–7] The expression of androgen receptor and metabolism of testosterone into various products that include 6β-hydroxytestosterone (6βOHT) are both increased in hypertrophic left ventricles from humans and spontaneously hypertensive rats and decreased in the presence of a human left ventricle–assistance device.[8] Testosterone is metabolized into 6βOHT in adult rat cultured myocytes.[9] Previously, we showed that the CYP1B1 (cytochrome P450 1B1)-testosterone-derived metabolite 6βOHT is required (acts as a permissive factor) for the development of Ang II-induced hypertension,

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Novelty and Significance

What Is New?

- 6β-hydroxytestosterone (6βOHT), a metabolite of testosterone formed by CYP1B1 (cytochrome P450 1B1), contributes to Ang II (angiotensin II)-induced hypertension, cardiac and renal fibrosis, and reactive oxygen species (ROS) production by promoting cPLA$_2$α (cytosolic phospholipase A$_2$α) activation, and production of the arachidonic acid metabolites prostaglandins (PG) E$_2$ and thromboxane (TXA$_2$) in male mice.
- PGE$_2$ by stimulating the EP1/EP3 receptors and TXA$_2$ via TP receptor contributes to the 6βOHT effect in promoting Ang II-induced hypertension and cardiac and renal fibrosis and producing reactive oxygen species.

What Is Relevant?

- Selective inhibitors of CYP1B1 that prevent the generation of 6βOHT from testosterone or EP1, EP3, and TP receptor antagonists that minimize the effects of 6βOHT could be useful in treating Ang II-dependent hypertension in hyperandrogenism or hypogonadal males on testosterone replacement therapy.

Summary

The testosterone-CYP1B1-generated metabolite 6βOHT contributes to the development of Ang II-induced hypertension, cardiac and renal fibrosis, reactive oxygen species production, and renal proinflammatory cytokine production in male mice, most likely via enhanced cPLA$_2$α activity. 6βOHT promotes Ang II-induced activation of cPLA$_2$α, leading to increased production of PGE$_2$ and TXA$_2$. Therefore, PGE$_2$ via EP1 and EP3, and TXA$_2$ via TP receptors, respectively contribute to the effect of 6βOHT to promote Ang II-induced hypertension and associated pathogenesis in male mice.

Nonstandard Abbreviations and Acronyms

6βOHT	6β-hydroxytestosterone
AA	arachidonic acid
Ang II	angiotensin II
BP	blood pressure
COX	cyclooxygenase
cPLA$_2$α	cytosolic phospholipase A$_2$α
CYP1B1	cytochrome P450 1B1
ERK1/2	(extracellular signal-regulated kinase)
PGE$_2$	prostaglandin E$_2$
ROS	reactive oxygen species
SBP	systolic blood pressure

production of reactive oxygen species (ROS), and cardiac and renal pathogenesis in male mice.[10,11] However, the mechanism by which 6βOHT promotes the effect of Ang II to increase blood pressure (BP) is not known.

Ang II-induced hypertension is mediated by group IV cPLA$_2$α (cytosolic phospholipase A$_2$α) activation, via the release of arachidonic acid (AA) and subsequent generation of prohypertensive eicosanoids.[12] These include AA-COX (cyclooxygenase) derived thromboxane A$_2$ (TXA$_2$) that acts via prostanoid receptor (TP) and prostaglandin E$_2$ (PGE$_2$) that acts via the EP1/EP3 receptors. Since the testosterone-CYP1B1 generated metabolite 6βOHT is required for Ang II to cause hypertension,[10] we performed this study to test the hypothesis that 6βOHT promotes Ang II-induced hypertension and its pathogenesis by enhancing cPLA$_2$α activity resulting in the generation of eicosanoids with prohypertensive effects.

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 by the corresponding authors 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 (IACUC). Intact or orchidectomized (Orchi) *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$*, *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$*, and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice (all on a C57BL/6J background) were randomly divided into various treatment groups and infused with Ang II (700 ng/kg per minute) or saline for 14 days with subcutaneously implanted osmotic pumps (Alzet, Cupertino, CA; model 1002). Systolic BP (SBP) was measured by the noninvasive tail-cuff method (Kent Scientific; model XBP 1000) and confirmed by radiotelemetry.

We examined the effects of the following genetic modifications or treatments on Ang II-induced hypertension and associated cardiac and renal pathogenesis: (1) cPLA$_2$α gene disruption (*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$*) in intact mice; (2) treatment of *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice with the AA metabolism inhibitor ETYA (5,8,11,14-eicosatetraynoic acid, 50 mg/kg, by intraperitoneal injection every third day)[12,13]; (3) treatment with the CYP1B1-testosterone generated metabolite, 6βOHT (15 μg/g

by intraperitoneal injection every third day) in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice and in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice; (4) treatment with ETYA+6βOHT in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice; and (5) treatment with 6βOHT+EP1 (SC19220),[14] EP3 (L-798106),[15] or TP (Terutroban)[16] receptor antagonists (10 µg/g, by subcutaneous injection every second day) in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice.

HISTOLOGICAL ANALYSIS

Heart and kidney sections from various treatment groups were stained using Masson's trichrome kit for collagen and dihydroethidium for ROS detection. We used a Pannoramic scanner (3DHISTECH, Budapest, Hungary) available in the UTHSC Core Facility for collagen detection in the heart and kidney sections as described previously.[17] The dihydroethidium-stained sections were visualized using a fluorescence microscope (model IX50, Olympus America) with a dual-wavelength filter, excitation at 375 nm, and emission at 585 nm described previously.[18] The images were viewed and quantified by blinded investigators using ImageJ 1.42 analysis software (NIH: http://imagej.nih.gov/ij).

WESTERN BLOT ANALYSIS

Kidneys were lysed in TissureLyser II (Qiagen) and centrifuged. An equal amount of protein from each lysate was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with primary anti-phospho (p)-cPLA$_2$α and total (t)-cPLA$_2$α antibodies and the corresponding secondary antibodies. The density of the bands was quantified by blinded investigators using ImageJ 1.42 software (NIH: http://imagej.nih.gov/ij).

URINARY LEVELS OF PGE$_2$ AND TXA$_2$ METABOLITES

Twenty-four hour urine samples were collected by placing the mice in metabolic cages. PGE$_2$ is rapidly converted in vivo to its 13, 14-dihydro-15-keto metabolite, which undergoes further degradation to yield PGA products. We measured the concentrations of PGE$_2$ metabolites in urine using a Prostaglandin E Metabolite EIA Kit (Cayman Catalog No. 514531), as per the manufacturer's instructions. This kit enables the conversion of 13, 14-dihydro-15-keto PGA$_2$ and 13, 14-dihydro-15-keto PGE$_2$ to a single, stable derivative that can be quantified. TXA$_2$ is rapidly hydrolyzed nonenzymatically to form TXB$_2$. We measured urinary TXB$_2$ levels using an ELISA kit (Cayman Catalog No. 501020).

URINARY LEVELS OF PROINFLAMMATORY CYTOKINES

Aliquots of urine samples containing 10X protease/phosphatase inhibitor cocktail of AEBSF (4-benzenesulfonyl fluoride hydrochloride), aprotinin, pepstatin, and leupeptin (Cell signaling, Catalog No. 5872S) in Tris base buffer (10 in 90 µL urine) were stored at −80 °C. Inflammatory cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α were measured using a V-PLEX Plus Proinflammatory Panel1 (mouse) Kit (catalog No. K15048G-1; Meso scale Discovery, MSD, Rockville, MD), as per the manufacturer's instructions. Measurement of urinary levels of these cytokines was used as a biomarker of renal inflammation. Prespecified cytokines of interest included in this panel were IL-1β, IL-6, IL-10, and TNF-α.

STATISTICAL ANALYSIS

The data were expressed as the mean±SEM with $P<0.05$ considered statistically significant. Statistical analysis was performed using Prism 6 (GraphPad Software). 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 followed by Tukey multiple comparisons test. In most experiments, the primary outcomes and main comparisons exceeded a power of 0.8 with the number of animals used.

RESULTS

Ang II Increased BP in 6βOHT-Treated Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and Intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but Not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* Mice

CYP1B1 contributes to Ang II-induced hypertension in male mice and rats,[19,20] and orchidectomy reduces the ability of Ang II to increase BP.[6] Ang II activates cPLA$_2$α and releases AA from tissue phospholipids.[21] AA modulates one or more of the cardiovascular effects of Ang II and has been implicated in hypertension.[12] In the present study, we observed that systemic infusion of Ang II for 2 weeks increased the SBP as measured by tail-cuff and increased mean arterial pressure, SBP, and diastolic BP as measured by radiotelemetry in 6βOHT-treated Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* (Figure 1A, 1D through 1F) and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* (Figure 1B, 1D through 1F), but not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure 1C through 1F). Consistent with our previous report, we observed that the 2-week infusion with Ang

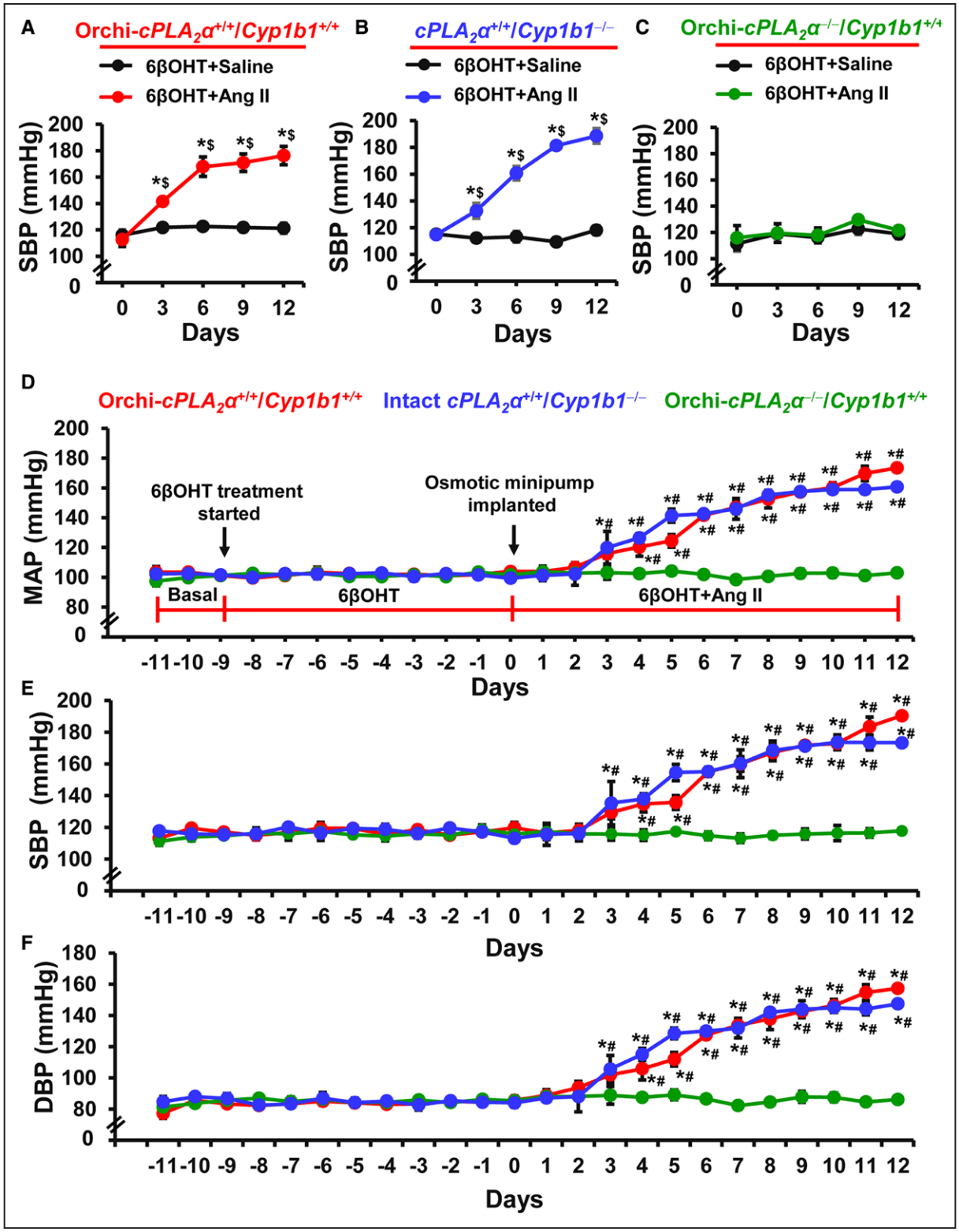

Figure 1. Ang II (Angiotensin II) increased the systolic blood pressure (SBP) in 6β-hydroxytestosterone (6βOHT)-treated orchidectomized (Orchi)-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact male *cPLA$_2$α$^{+/+}$/Cyp1b1^{l}* but not in Orchi-*cPLA$_2$α^{l}*/Cyp1b1$^{+/+}$ mice. Measurement of SBP by tail-cuff in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* (**A**), intact male *cPLA$_2$α$^{+/+}$/Cyp1b1^{l}* (**B**), and Orchi-*cPLA$_2$α^{l}/Cyp1b1$^{+/+}$* (**C**) mice. Measurement of mean arterial BP (MAP, **D**), SBP (**E**), and diastolic BP (DBP, **F**) by radiotelemetry showed similar effects of 6βOHT treatment on the action of Ang II in the mice mentioned above. Saline was used as vehicle control for Ang II. Data are Mean±SEM (n=4-5/group). A 2-way repeated measures ANOVA followed by Tukey multiple comparisons in **A** through **F**. *P<0.05 vs. Day 0 value (the day before implantation of the osmotic pump) within the group, $^{\$}P$<0.05 vs 6βOHT+Saline in the corresponding groups; $^{\#}P$<0.05 vs Orchi-*cPLA$_2$α^{l}/Cyp1b1$^{+/+}$*.

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II (subcutaneous via osmotic pump) increased the SBP as measured by tail-cuff in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice (Figure S1A in the Data Supplement). This effect was decreased in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice (Figure S1B) and attenuated in *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure S1C). Ang II did not alter the heart rate (Figure S1D), pulse pressure (Figure S1E), or locomotor activity (Figure S1F) in 6βOHT-treated Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$*, intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$*, and Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice as measured by radiotelemetry. We have observed that the increase in SBP by Ang II, measured by tail-cuff and radiotelemetry at the same time of the day are comparable.[18] Therefore, we measured the SBP in the remaining experiments in this study by tail-cuff.

To determine whether cPLA$_2$α affects testosterone levels, we examined the effect of cPLA$_2$α gene disruption on the effect of Ang II on plasma levels of testosterone. Plasma testosterone levels were similar in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$*mice and not altered by Ang II (Figure S2).

Testosterone-CYP1B1 Generated Metabolite, 6βOHT Restored Ang-II-Induced Cardiac and Renal Fibrosis in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and Intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but Not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* Mice

Orchidectomy and disruption of the *Cyp1b1* or *cPLA$_2$α* genes minimize the effect of Ang II to cause cardiac and renal hypertrophy and fibrosis in male mice.[10–12] In the present study, we observed that Ang II increased cardiac and renal fibrosis as indicated by increased collagen deposition (blue color) in 6βOHT-treated Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure 2A through 2C). Consistent with our previous studies,[10–12] Ang II produced cardiac and renal fibrosis in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice (Figure S3A through S3C). These effects were decreased in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice and further attenuated in *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure S3A through S3C).

Testosterone-CYP1B1 Generated Metabolite, 6βOHT Restored Ang II-Induced Increase in Renal cPLA$_2$α Activity in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and Intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but Not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* Mice

Several BP regulating hormones, including Ang II, activate cPLA$_2$ and release AA.[22–25] We observed that Ang II increased cPLA$_2$α activity in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* but not in *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice, as measured by an increased ratio of p-cPLA$_2$α to t-cPLA$_2$α expression in the kidney (Figure 3A). However, this effect was blunted in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* (Figure 3B), intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* (Figure 3C), and Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure 3D). 6βOHT treatment restored the ability of Ang II to increase cPLA$_2$α activity in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* (Figure 3B), intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* (Figure 3C) but not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure 3D).

Testosterone-CYP1B1 Generated Metabolite, 6βOHT Restored Ang II-Induced Increased Urinary Excretion of PGE$_2$ and TXA$_2$ Metabolites in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and Intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but Not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* Mice

On activation by Ang II, cPLA$_2$α releases AA from tissue phospholipids that are metabolized into TXA$_2$ and PGE$_2$, F$_2$α, D$_2$, and I$_2$ by COX enzymes.[21,26] The vascular effects of PGE$_2$ and TXA$_2$ contribute to prohypertensive mechanisms, PGE$_2$ via activation of the EP1/EP3 receptors and TXA$_2$ via activation of the TP receptor.[16,27] In the current study, Ang II increased the urinary excretion of PGE$_2$ metabolites and the TXA$_2$ metabolite TXB$_2$ in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice (Figure S4A and S4B). However, the Ang II-induced increase in urinary excretion of PGE$_2$ metabolites was abrogated (Figure S4A) and the increase in TXB$_2$ (Figure S4B) was markedly reduced in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice. We observed that the effect of Ang II to increase urinary excretion of PGE$_2$ and TXA$_2$ metabolites was blunted in *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure S4A and S4B), a finding similar to our previously published data.[12] Testosterone-CYP1B1 metabolite 6βOHT treatment restored the effect of Ang II to increase the urinary excretion of PGE$_2$ metabolites (Figure S4C) and TXB$_2$ (Figure S4D) in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice.

AA Metabolism Inhibitor Decreased the Effect of 6βOHT to Restore Ang II-Induced Increased SBP, Cardiac and Renal Fibrosis, and Urinary Excretion of PGE$_2$ and TXA$_2$ Metabolites in Intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* Mice

Administration of ETYA blunted the Ang II-induced increase in SBP in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice (Figure 4A), as described previously.[12] In this study, ETYA attenuated the effect of 6βOHT to restore the Ang II-induced increase in SBP in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice (Figure 4A). ETYA also decreased the Ang II-induced increase in cardiac and renal fibrosis (Figure 4B through 4D) as indicated by reduced collagen deposition, and reduced the urinary excretion of PGE$_2$ metabolites (Figure 4E) and TXB$_2$ (Figure 4F) in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice.

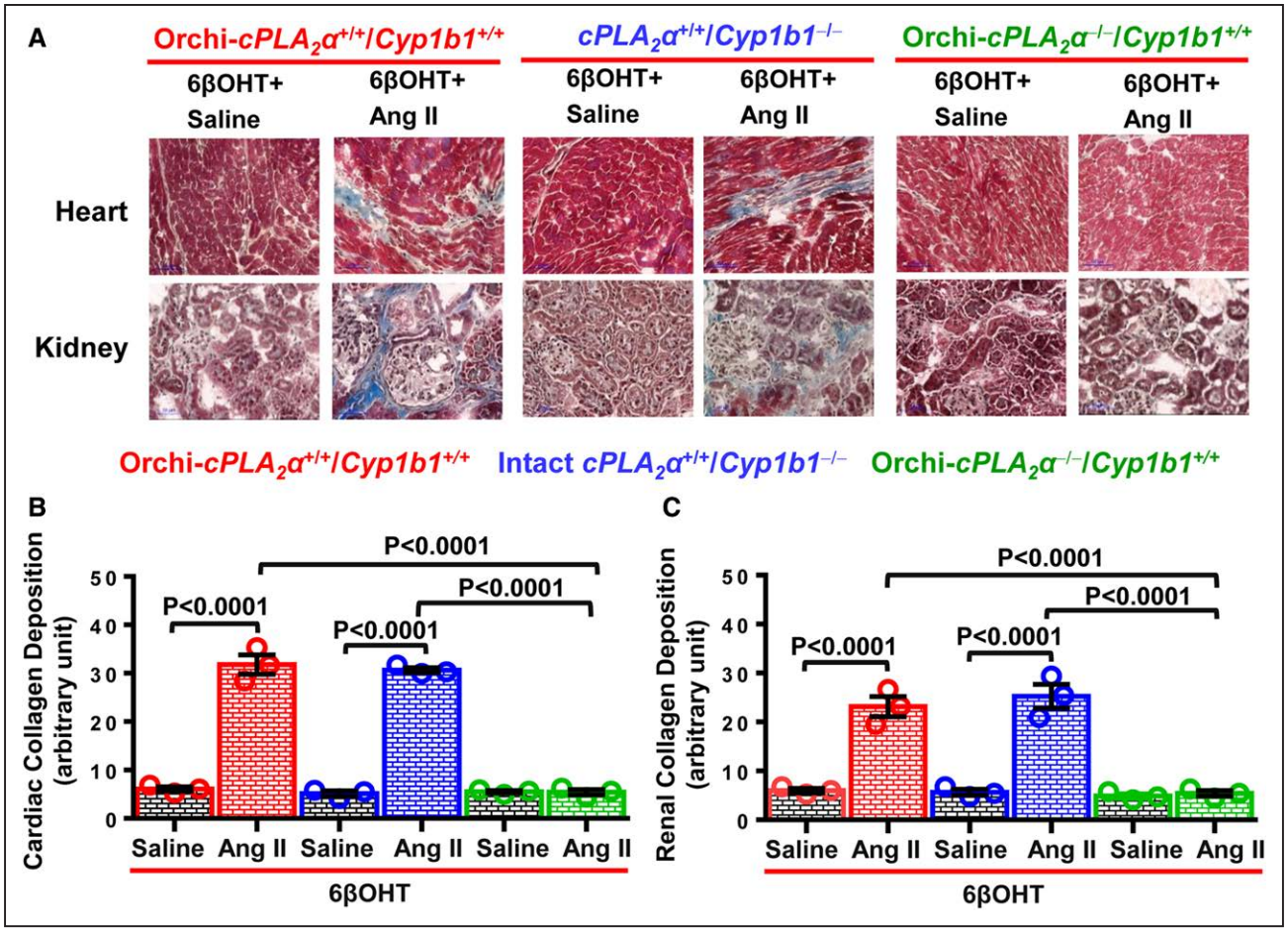

Figure 2. Ang II (angiotensin II) increased the cardiac and renal collagen deposition detected by Masson's Trichrome staining in 6β-hydroxytestosterone (6βOHT)-treated orchidectomized (Orchi)-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact male *cPLA$_2$α$^{+/+}$/Cyp1b1$^{/}$* but not in Orchi-*cPLA$_2$α//Cyp1b1$^{+/+}$* mice.

A, Representative images of heart and kidney. **B**, Quantitation of cardiac collagen deposition. **C**, Quantitation of renal collagen deposition. Saline was used as vehicle control for Ang II. Data are Mean±SEM (n=3/group). A 1-way ANOVA followed by Tukey multiple comparisons in **B** and **C**.

Antagonists of PGE$_2$-EP1, EP3, and TXA$_2$-TP Receptors Reduced the Effect of 6βOHT to Restore Ang II-Induced Increased SBP, and Cardiac and Renal Fibrosis in Intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* Mice

Antagonists of PGE$_2$-EP1 (EP1RA), EP3 (EP3RA), and TXA$_2$ (TPRA) receptors reduced the ability of 6βOHT to restore Ang II-induced increase in SBP (Figure 5A) and cardiac and renal fibrosis (Figure 6B through 6D) in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice.

6βOHT Promotes Ang II-Induced Cardiac and Renal ROS Production via cPLA$_2$α in Male Mice

Oxidative stress has been implicated in various models of hypertension, including the Ang II model.[28,29] Processes contributing to these phenomena have been attributed, in part, to increased generation of ROS, particularly superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2),[30,31] which function as important second messengers.[31,32] We previously showed that Ang II-induced ROS production in male mice was abrogated by orchidectomy or by disruption of the *Cyp1b1* or *cPLA$_2$α* genes.[10–12] In the present study, we show that treatment of intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice with the AA metabolism inhibitor ETYA reduced the effect of Ang II to increase cardiac and renal ROS production (Figure S5A through S5C) similar to that observed in intact *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice in response to Ang II. Moreover, the testosterone-CYP1B1 generated metabolite 6βOHT restored the effect of Ang II to increase cardiac and renal ROS production in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* but not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice (Figure S5D through S5F). However, Ang II failed to increase cardiac and renal ROS production in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice, which were restored by 6βOHT and decreased by ETYA treatment (Figure 6A through 6C). Furthermore, treatment with EP1RA, EP3RA, and TPRA decreased the effect of 6βOHT in restoring the Ang II-induced increase in

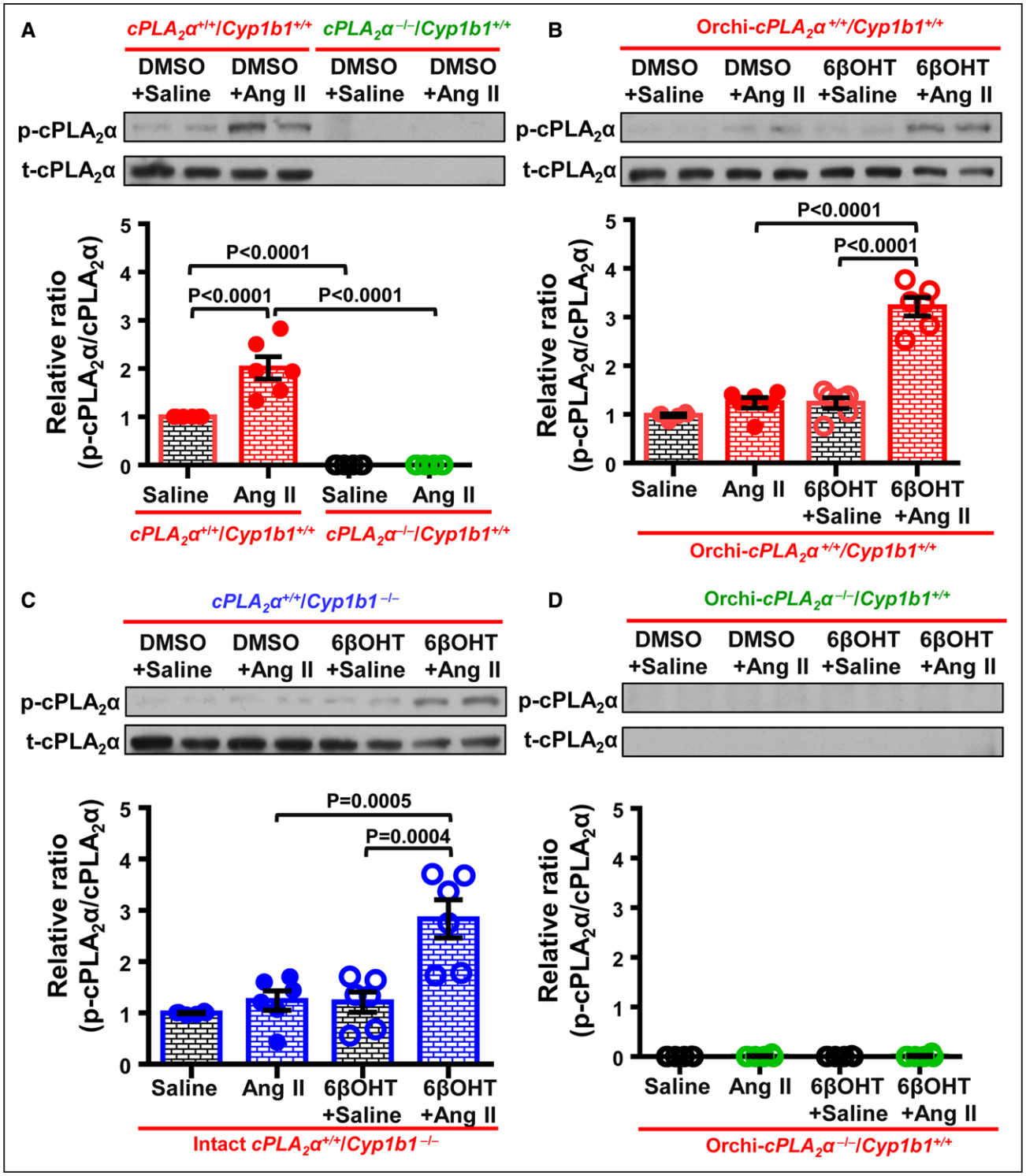

Figure 3. Ang II (Angiotensin II) increased cPLA$_2$α (cytosolic phospholipase A^{2}α) activity measured by an increased ratio of phosphorylated (p) to total (t)-cPLA$_2$α expression in the kidney of *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice, an effect which was blunted in orchidectomized (Orchi)-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact male *cPLA$_2$α$^{+/+}$/Cyp1b1$^{/}$* mice and restored by 6β-hydroxytestosterone (6βOHT) treatment.

cPLA$_2$α expression remained absent in intact or Orchi-*cPLA$_2$α$^{/}$/Cyp1b1$^{+/+}$* mice. Representative blots and quantitation of cPLA$_2$α expression in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and *cPLA$_2$α$^{/}$/Cyp1b1$^{+/+}$* (**A**), Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* (**B**), *cPLA$_2$α$^{+/+}$/Cyp1b1$^{/}$* (**C**), and Orchi-*cPLA$_2$α$^{/}$/Cyp1b1$^{+/+}$* (**D**) mice. Saline was used as vehicle control for Ang II, and dimethyl sulfoxide (DMSO) as vehicle control for 6βOHT. Top panel: representative blots; bottom panel: quantitation. Data are Mean±SEM (n=6/group). A 1-way ANOVA followed by Tukey multiple comparisons in **A** through **D**.

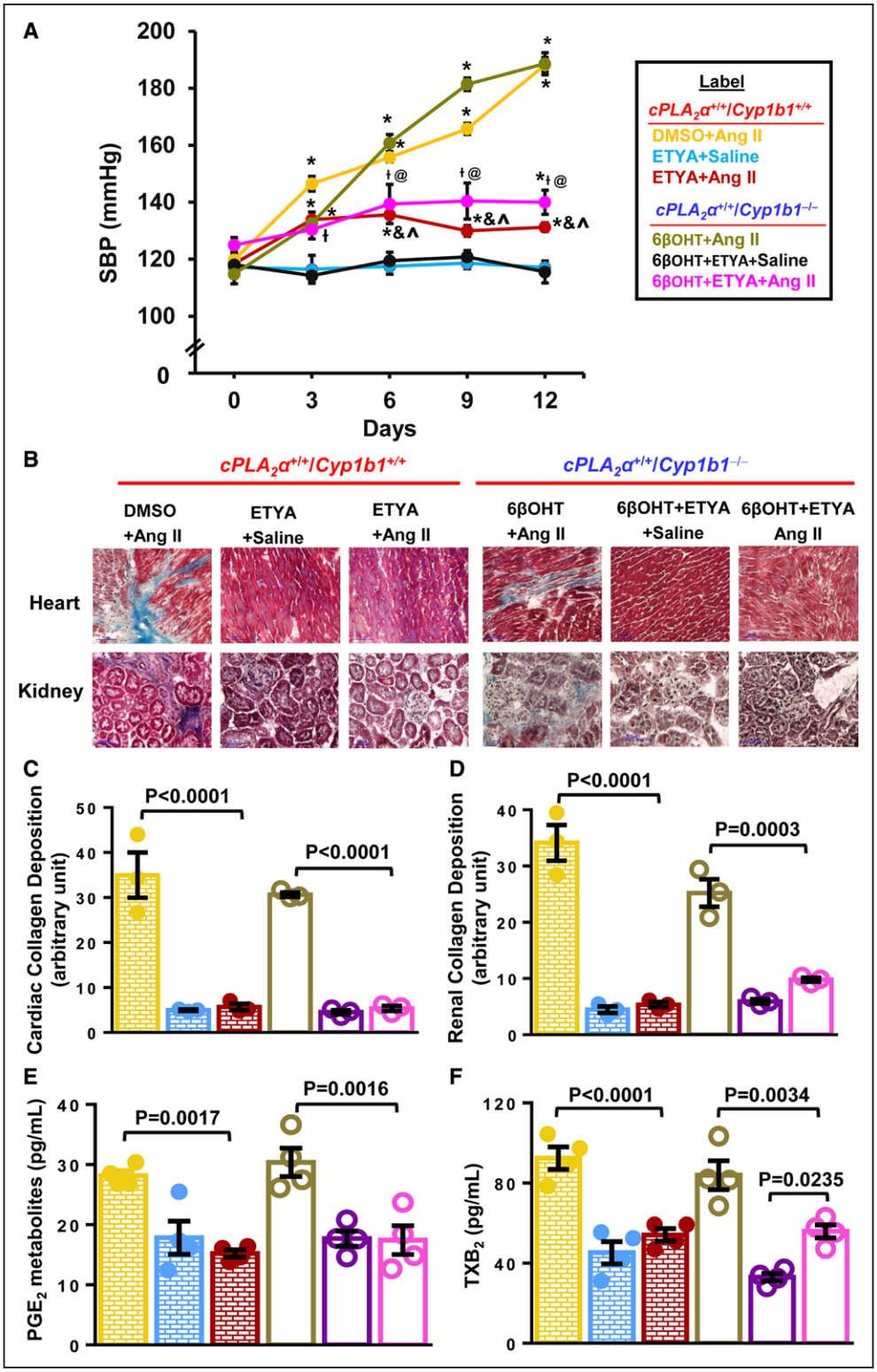

Figure 4. Arachidonic acid inhibitor 5, 8, 11, 14-eicosatetraynoic acid (ETYA) decreased the Ang II (angiotensin II)-induced increase in systolic blood pressure (SBP), cardiac, and renal collagen deposition, and urinary excretion of prostaglandin E^2 (PGE^2) metabolites and thromboxane A^2 (TXA^2) metabolite TXB^2 in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and 6β-hydroxytestosterone (6βOHT)-treated intact male *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice.

A, SBP measured by tail-cuff. **B**, Representative images of heart and kidney. **C**, Quantitation of cardiac collagen deposition. **D**, Quantitation of renal collagen deposition. **E**, Quantitation of urinary excretion of PGE^2 metabolites. **F**, Quantitation of urinary excretion of TXA^2 metabolite TXB_2. Saline was used as vehicle control for Ang II and dimethyl sulfoxide (DMSO) as vehicle control for 6βOHT and ETYA. Data are Mean±SEM (n=3-5/group). A 2-way repeated measures ANOVA followed by Tukey multiple comparisons in **A** and 1-way ANOVA followed by Tukey multiple comparisons in **C** through **F**. *P<0.05 vs Day 0 (Day of osmotic pump implantation for Ang II infusion); &P<0.05 vs ETYA+Saline and ^P vs DMSO+Ang II in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$*; ^1P<0.05 vs 6βOHT+ETYA+Saline and @P<0.05 vs 6βOHT+Ang II in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$*.

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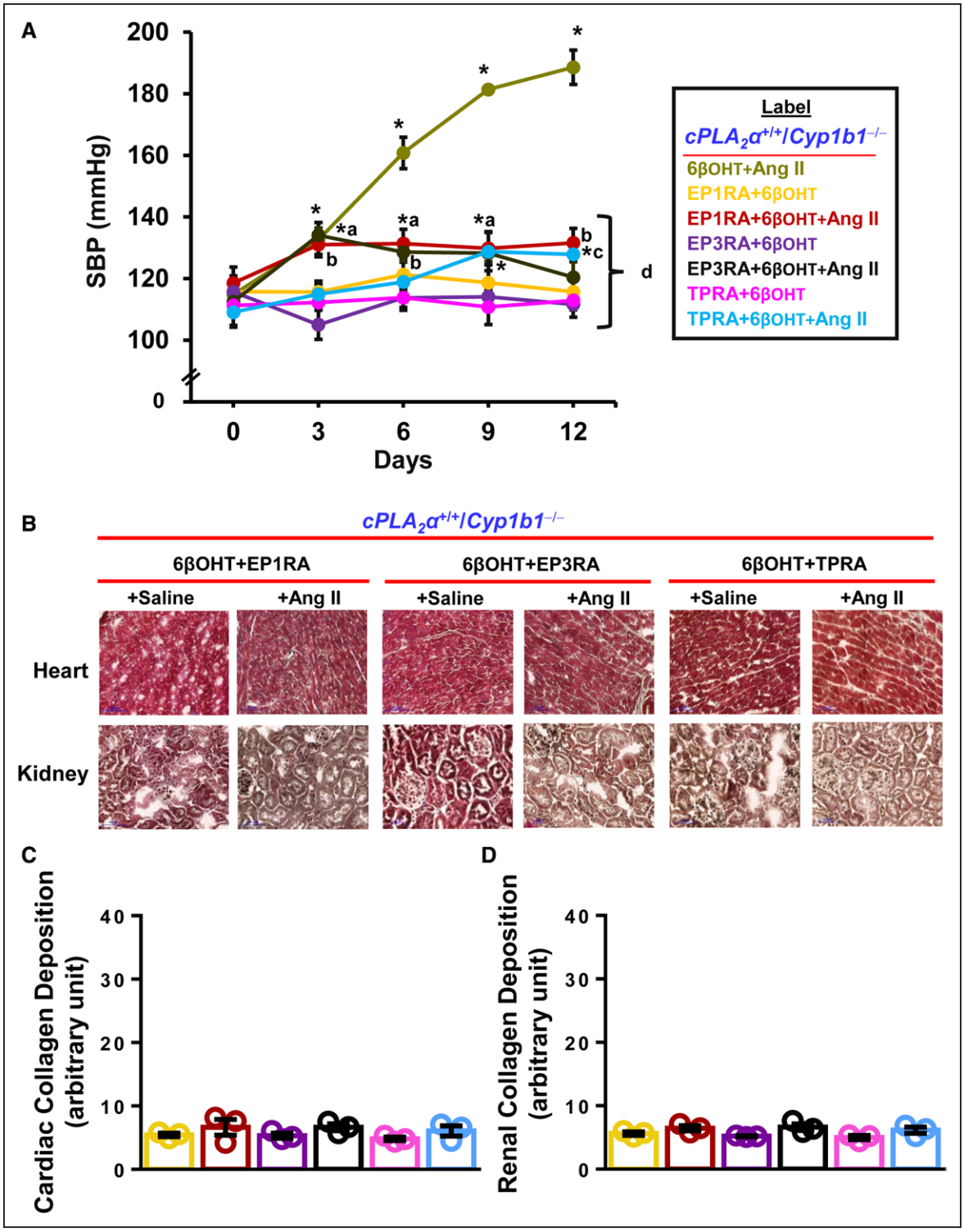

Figure 5. Receptor antagonists of prostaglandin E² (PGE²) EP1 (EP1RA), EP3 (EP3RA), and thromboxane A² (TXA²) (TPRA) decreased the effect of 6β-hydroxytestosterone (6βOHT) to restore Ang II (angiotensin II)-induced increase in systolic blood pressure (SBP), cardiac and renal collagen deposition detected by Masson's Trichrome staining in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice. **A**, SBP measured by tail-cuff. **B**, Representative images of heart and kidney. **C**, Quantitation of cardiac collagen deposition. **D**, Quantitation of renal collagen deposition. Saline was used as vehicle control for Ang II. Data are Mean±SEM (n=3-5/group). A 2-way repeated measures ANOVA followed by Tukey multiple comparisons in **A** and 1-way ANOVA followed by Tukey multiple comparisons in **C** and **D**. *P<0.05 vs Day 0 (Day of osmotic pump implantation for Ang II infusion); [a]P<0.05 vs EP1RA+6βOHT+Saline, [b]P<0.05 vs EP1RA+6βOHT+Saline, [c]P<0.05 vs TPRA+6βOHT+Saline and [d]P<0.05 vs 6βOHT+Ang II in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$*.

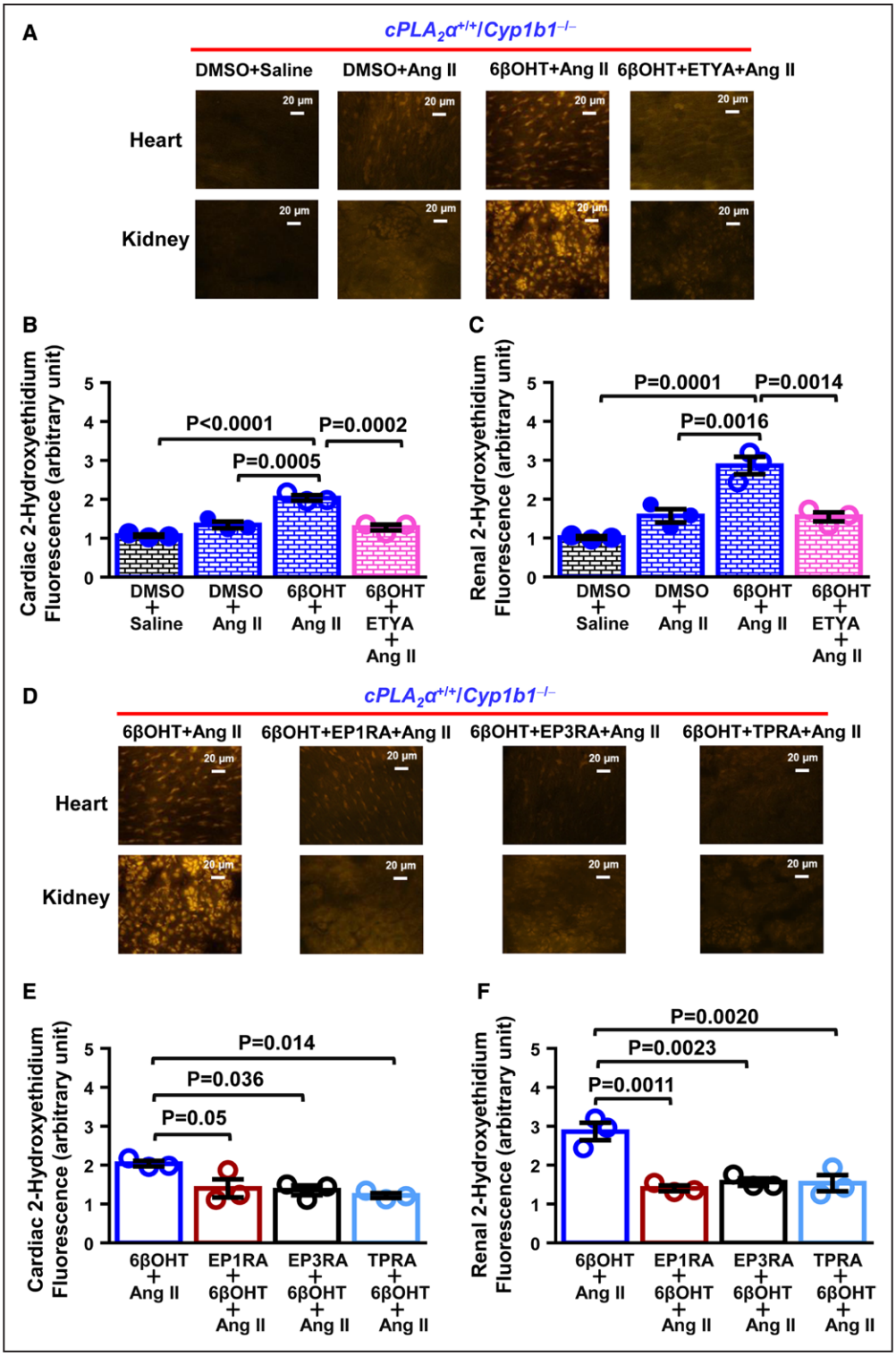

Figure 6. Ang II (Angiotensin II) failed to increase the production of reactive oxygen species (ROS) measured by the quantitation of 2-hydroxyethidium fluorescence in the heart and kidney in *cPLA$_2$α$^{+/+}$/Cyp1b1/* mice and restored by 6β-hydroxytestosterone (6βOHT) but decreased by the arachidonic acid metabolism inhibitor 5, 8, 11, 14-eicosatetraynoic acid (ETYA) and receptor antagonists of prostaglandin E^2 (PGE^2) EP1 (EP1RA), EP3 (EP3RA), and thromboxane (TX) A^2 (TPRA).
Effect of 6βOHT and ETYA in Ang II-infused *cPLA$_2$α$^{+/+}$/Cyp1b1/* mice on cardiac (**A**, representative images, and **B**, quantitation) and renal (**A**, representative images, and **C**, quantitation) ROS production. Effect of EP1RA, EP3RA, and TPRA on 6βOHT-treated Ang II-infused *cPLA$_2$α$^{+/+}$/Cyp1b1/* mice on cardiac (**D**, representative images, and **E**, quantitation) and renal (**C**, representative images, and **F**, quantitation) ROS production. Saline was used as vehicle control for Ang II, and dimethyl sulfoxide (DMSO) as vehicle control for 6βOHT, ETYA, EP1RA, EP3RA, and TPRA. Data are Mean±SEM (n=3/group). A 1-way ANOVA followed by Tukey multiple comparisons in **B, C, E** and **F**.

cardiac and renal ROS production in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice (Figure 6D through 6F).

6βOHT Promotes Ang II-Induced Excretion of Proinflammatory Cytokines via cPLA$_2$α in Male Mice

Disruption of the cPLA$_2$α gene protects male mice from the Ang II-induced increases in renal F4/80$^+$ macrophages and CD3$^+$ T lymphocytes infiltration.[12] To examine the effect of 6βOHT on Ang II-induced renal inflammation, we measured the urinary excretion of proinflammatory cytokines. Ang II increased the urinary excretion of IL-1β, IL-6, and IL-12p70 (Figure S6A) in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice. This effect was maintained after 6βOHT treatment in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but not in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice. Ang II also increased the urinary excretion of TNF-α (Figure S6A) and KC/GRO (Figure S6B) in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and 6βOHT-treated Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$*, intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$*, and in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice. Among other cytokines, Ang II increased IFN-γ only in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* and IL-4 only in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice (Figure S6B).

DISCUSSION

The present study provides the following novel information: (1) The CYP1B1-testosterone generated metabolite 6βOHT promoted Ang II-induced hypertension, cardiac and renal fibrosis, ROS production, and increased urinary levels of cytokines IL-1β, IL-6, and IL-12p70 by enhancing cPLA$_2$α activity and generation of AA-COX metabolites PGE$_2$ and TXA$_2$ in male mice, (2) PGE$_2$ through the EP1/EP3 receptors, and TXA$_2$ via TP receptors contribute to the effects of 6βOHT to promote Ang II-induced increase in BP and associated cardiac and renal fibrosis and ROS production in male mice.

Previously, we reported that cPLA$_2$α gene disruption prevents Ang II-induced hypertension, cardiac and renal fibrosis, and ROS production in male mice.[12] Moreover, we showed that Ang II-induced hypertension and associated pathogenesis are also decreased by disruption of the *Cyp1b1* gene, similar to the effect of orchidectomy, and restored by 6βOHT treatment.[10,11] In the present study, we demonstrated that disruption of the cPLA$_2$α gene does not alter the plasma testosterone levels in response to Ang II. This observation, together with our previous finding that Ang II stimulation of 6βOHT production was abolished by disruption of *Cyp1b1* gene,[10] suggests that 6βOHT must act upstream of cPLA$_2$α. Supporting this view was our finding that the effect of 6βOHT to restore the Ang II-induced increase in SBP measured by tail-cuff, or mean arterial pressure, SBP and diastolic BP measured by radiotelemetry, and cardiac and renal fibrosis as indicated by collagen deposition, and ROS production was ameliorated in Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* but not Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice. From these observations, it follows that the effect of 6βOHT to promote Ang II-induced increases in BP, cardiac and renal fibrosis, and ROS production are dependent on cPLA$_2$α. Ang II activates cPLA$_2$α, which selectively catalyzes the release of AA from tissue phospholipids.[23] Our finding that the Ang II-induced increase in cPLA$_2$α activity indicated by its phosphorylation in the kidney of intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice was abolished in Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$*, Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$*, and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice, suggests that one or more CYP1B1-testosterone derived metabolite, most likely 6βOHT, is required for cPLA$_2$α activation by Ang II. Supporting this hypothesis was our demonstration that 6βOHT treatment restored the effect of Ang II to cause cPLA$_2$α phosphorylation without altering its expression in the kidneys of Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$*, but not in *cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice. The mechanism by which 6βOHT promotes Ang II-induced cPLA$_2$α activation is not known. It is possible that 6βOHT acts on genomic androgen receptor or GPRC6A (nongenomic G protein-coupled receptor C6A) via independent pathways or by crosstalk between GPRC6A and nuclear androgen receptor to enhance the activity of one or more signaling molecules, including the cellular calcium levels and ERK1/2 (extracellular signal-regulated kinase) activity[33] that are involved in cPLA$_2$α activation.[34] Future studies will explore this mechanism.

Our findings that (1) Ang II increased the urinary excretion of PGE$_2$ measured as its metabolites, and TXB$_2$, the stable metabolite of TXA$_2$, in *cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* mice and (2) Ang II increased the urinary excretion of PGE$_2$ metabolites and TXB$_2$ in 6βOHT-treated Orchi-*cPLA$_2$α$^{+/+}$/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$*, but not Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice, suggests that AA released by cPLA$_2$α via COX-generated metabolite(s) mediate the action of 6βOHT to promote Ang II-induced hypertension, cardiac and renal fibrosis, and ROS production. However, the fact that the effect of Ang II on urinary TXB$_2$ level like on the SBP, in the intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice was reduced but not abolished suggests that testosterone and/or its other metabolites contribute to these effects of Ang II. Supporting this conclusion was our observation that AA metabolism inhibitor ETYA[12] decreased the effect of 6βOHT to restore the Ang II-induced increase in BP, and cardiac and renal fibrosis, ROS production, and the urinary output of PGE$_2$ metabolites and TXB$_2$ in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice. Ang II-induced cardiac hypertrophy and fibrosis and renal injury have been attributed to the increase in BP.[35–38] Therefore, restoration by 6βOHT of Ang II-induced cardiac and renal fibrosis could also result from an increase in BP in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice.

Ang II infusion in intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice in the absence of 6βOHT increased SBP by 10 to 20 mmHg but did not cause cardiac and renal fibrosis suggesting that this small increase in SBP was most likely insufficient to produce these effects. Mechanical stretch can also increase cPLA$_2$ activity and production of eicosanoids[39] and ROS.[40] Therefore, hypertension-induced stretch in these mice might enhance cPLA$_2$α activation and eicosanoids production that contributes to the effect of 6βOHT on restoring cardiac and renal fibrosis associated with Ang II-induced hypertension. However, the prohypertensive eicosanoids that contribute to Ang II-induced hypertension also include lipoxygenase generated 12S-hydroxyeicosatetraenoic acid, and CYP450 A1 derived 20-hydroxyeicosatetraenoic acid.[41,42] Therefore, the contribution of these eicosanoids to the observed effects of 6βOHT on restoring cardiac and renal fibrosis associated with Ang II-induced hypertension cannot be excluded, and it is a subject of current investigation.

COX-AA-derived metabolites PGE$_2$ activate EP1/EP3 receptors to produce vasoconstriction and activate EP2 and EP4 receptors to produce vasodilation[43] and signaling of TXA$_2$ through TP receptor results in vasoconstriction.[44] The PGE$_2$ through EP1 and/or EP3,[14,15] and TXA$_2$ via TP[45,46] receptors, contributes to the vasoconstrictor and hypertensive effects of Ang II.[14,15,47] PGE$_2$/EP3 signaling pathway has also been implicated in Nω-nitro-L-arginine methyl ester hydrochloride/high salt–induced hypertension[48] and impaired vasodilation and hypertension in the high salt diet-fed S-P467L mice with decreased peroxisome proliferator-activated receptor-gamma activity.[49] In the present study, the selective antagonists of EP1 (SC19220),[50] EP3 (L-798106),[15] and TXA$_2$-TP (Terutroban)[51] receptors inhibited the effect of 6βOHT to restore the Ang II-induced increase in SBP, cardiac and renal fibrosis, and ROS production in the intact *cPLA$_2$α$^{+/+}$/Cyp1b1$^{-/-}$* mice. These observations suggest that PGE$_2$ and TXA$_2$ contribute to these effects of 6βOHT via activation of PGE$_2$-EP1 and EP3, and TP receptors, respectively. Since EP1, EP3, and TP receptor antagonists did not alter the basal BP in 6βOHT-treated mice in the absence of Ang II, it appears that the amount of PGE$_2$ and TXA$_2$ formed by the low basal activity of cPLA$_2$α and AA release is insufficient to increase BP. Also, it is possible that PGE$_2$, via its effects on the EP2 and EP4 receptors that decrease the vascular tone,[43] masks the vasoconstrictor effect of PGE$_2$ that is mediated via EP1 and EP3 and TXA$_2$ through TP receptors.

The mechanism by which PGE$_2$ via EP1 and EP3 receptors, and TXA$_2$ via TP receptor participate in the effect of 6βOHT to promote Ang II-induced increase in SBP could involve an increase in vascular tone caused by the increase in cell calcium facilitated by these eicosanoids[14,44,52,53] and the role of calcium sensitization via Rho-kinase activation[52,53] in this process remains to be determined. Ang II increases the generation of ROS and isolevuglandin protein adducts in dendritic cells, the proliferation of T cells, and increased levels of cytokines that contribute to its hypertensive effect and renal fibrosis.[54] In the Nω-nitro-L-arginine methyl ester hydrochloride/high salt-induced model of hypertension, PGE$_2$ via EP3 receptors results in ROS production, dendritic cell activation, and accumulation of isolevoglandin protein adducts in spleen cells, production of proinflammatory cytokines, and renal fibrosis.[48] PGE$_2$ also acts directly on dendritic cells via the EP1 receptor to increase isolevuglandin adducted proteins.[48] PGE$_2$-EP3 receptors have also been implicated in the Ang II-induced increase in cardiac expression of NOX2 (NADPH oxidase), production of proinflammatory cytokines, cardiac dysfunction, and hypertrophy.[47]

We reported previously that *Cyp1b1* gene disruption in male mice inhibits Ang II-induced infiltration of renal CD4$^+$ T cells and was restored by 6βOHT treatment.[11] Therefore, it is possible that PGE$_2$ and TXA$_2$ via EP1 and EP3 and TP receptors, respectively, contribute to the effect of 6βOHT to promote Ang II-induced hypertension, cardiac and renal fibrosis by increasing ROS production, the activity of immune cells, and the generation of proinflammatory cytokines. Supporting this view were our data that Ang II-induced increased urinary excretion of proinflammatory cytokines IL-1β, IL-6, and IL-12p70 was reduced in 6βOHT-treated Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* but not in 6βOHT-treated Orchi-*cPLA$_2$α^{++}/Cyp1b1$^{+/+}$* and intact *cPLA$_2$α$^{+/+}$/ Cyp1b1$^{-/-}$* mice. Surprisingly, the Ang II-induced increase in excretion of TNF-α was similar in intact *cPLA$_2$α^{++}/Cyp1b1$^{+/+}$* and 6βOHT-treated Orchi-*cPLA$_2$α^{++}/Cyp1b1$^{+/+}$*, intact *cPLA$_2$α$^{+/+}$/ Cyp1b1$^{-/-}$* and Orchi-*cPLA$_2$α$^{-/-}$/Cyp1b1$^{+/+}$* mice, suggesting that the effect of 6βOHT in the promotion of Ang II-induced excretion of TNF-α is independent of cPLA$_2$α activity. Therefore, it seems that TNF-α does not contribute to the effect of 6βOHT in promoting Ang II-induced hypertension and associated pathogenesis. Further studies are required to elucidate the significance and the mechanism by which 6βOHT promotes Ang II-induced renal excretion of TNF-α independent of cPLA$_2$α.

In conclusion, 6βOHT, the metabolite of testosterone produced by CYP1B1, contributes to Ang II-induced hypertension, cardiac and renal fibrosis, production of ROS, and the cytokines IL-1β, IL-6, and IL-12p70 by promoting cPLA$_2$α activity and generating the AA-COX metabolites PGE$_2$ and TXA$_2$ in male mice (Graphical Abstract; Figure S7). PGE$_2$ and TXA$_2$ via EP1/EP3 and TP receptors, respectively, contribute to the effects of 6βOHT to promote Ang II-induced increase in BP and associated cardiac and renal fibrosis, ROS, and cytokine production in male mice. Therefore, selective inhibitors of EP1, EP3, and TXA$_2$ receptors and CYP1B1 activity would be useful for treating Ang II and testosterone-dependent hypertension and its pathogenesis.

PERSPECTIVES

We have shown that Ang II-induced hypertension and associated pathogenesis are mediated by prohypertensive eicosanoids produced by activation of cPLA$_2$α in male mice.[12] Moreover, testosterone-CYP1B1 generated metabolite 6βOHT also contributes to Ang II-induced hypertension and its pathogenesis.[10,11] This study provides novel insight into the mechanism of interactions of cPLA$_2$α and testosterone metabolite 6βOHT. We show that 6βOHT acts upstream of cPLA$_2$α and by enhancing its activity in response to Ang II and production of PGE$_2$ and TXA$_2$, which via EP1 and EP3 and TXA$_2$ receptors, respectively promotes Ang II-induced hypertension and associated pathogenesis. However, the site of interaction of cPLA$_2$α and CYP1B1 is not known. In view of our recent work that testosterone-CYP1B1 generated 6βOHT in the paraventricular nucleus by increasing sympathetic activity contributes to Ang II-induced hypertension,[18] it would be important to determine the interaction of cPLA$_2$α and CYP1B1-generated 6βOHT in the paraventricular nucleus in Ang II-induced hypertension and cardiovascular and renal pathogenesis.

ARTICLE INFORMATION

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Disclosures

J.V. Bonventre is cofounder and holds equity in Goldfinch Bio. He is co-inventor on KIM-1 and kidney organoid patents assigned to Mass General Brigham. He is 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.

REFERENCES

1. Ezzati M, Lopez AD, Rodgers A, Vander Hoorn S, Murray CJ. Comparative Risk Assessment Collaborating Group. Selected major risk factors and global and regional burden of disease. *Lancet*. 2002;360:1347–1360. doi: 10.1016/S0140-6736(02)11403-6
2. Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci USA*. 2006;103:17985–17990. doi: 10.1073/pnas.0605545103
3. Ruiz-Ortega M, Rupérez M, Esteban V, Rodríguez-Vita J, Sánchez-López E, Carvajal G, Egido J. Angiotensin II: a key factor in the inflammatory and fibrotic response in kidney diseases. *Nephrol Dial Transplant*. 2006;21:16–20. doi: 10.1093/ndt/gfi265
4. Reckelhoff JF. Gender differences in the regulation of blood pressure. *Hypertension*. 2001;37:1199–1208. doi: 10.1161/01.hyp.37.5.1199
5. Reckelhoff JF, Zhang H, Srivastava K, Granger JP. Gender differences in hypertension in spontaneously hypertensive rats: role of androgens and androgen receptor. *Hypertension*. 1999;34(4 pt 2):920–923. doi: 10.1161/01.hyp.34.4.920
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. Papamitsou T, Barlagiannis D, Papaliagkas V, Kotanidou E, Dermentzopoulou-Theodoridou M. Testosterone-induced hypertrophy, fibrosis and apoptosis of cardiac cells–an ultrastructural and immunohistochemical study. *Med Sci Monit*. 2011;17:BR266–BR273. doi: 10.12659/msm.881930
8. Thum T, Borlak J. Testosterone, cytochrome P450, and cardiac hypertrophy. *FASEB J*. 2002;16:1537–1549. doi: 10.1096/fj.02-0138com
9. Thum T, Borlak J. Cytochrome P450 mono-oxygenase gene expression and protein activity in cultures of adult cardiomyocytes of the rat. *Br J Pharmacol*. 2000;130:1745–1752. doi: 10.1038/sj.bjp.0703465
10. 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
11. Pingili AK, Thirunavukkarasu S, Kara M, Brand DD, Katsurada A, Majid DS, Navar LG, Gonzalez FJ, Malik KU. 6β-Hydroxytestosterone, a Cytochrome P450 1B1-Testosterone-metabolite, mediates Angiotensin II-induced renal dysfunction in male mice. *Hypertension*. 2016;67:916–926. doi: 10.1161/HYPERTENSIONAHA.115.06936
12. Khan NS, Song CY, Jennings BL, Estes AM, Fang XR, Bonventre JV, Malik KU. Cytosolic phospholipase A2α is critical for angiotensin II-induced hypertension and associated cardiovascular pathophysiology. *Hypertension*. 2015;65:784–792. doi: 10.1161/HYPERTENSIONAHA.114.04803
13. Sun FF, McGuire JC, Morton DR, Pike JE, Sprecher H, Kunau WH. Inhibition of platelet arachidonic acid 12-lipoxygenase by acetylenic acid compounds. *Prostaglandins*. 1981;21:333–343. doi: 10.1016/0090-6980(81)90151-9
14. Guan Y, Zhang Y, Wu J, Qi Z, Yang G, Dou D, Gao Y, Chen L, Zhang X, Davis LS, et al. Antihypertensive effects of selective prostaglandin E2 receptor subtype 1 targeting. *J Clin Invest*. 2007;117:2496–2505. doi: 10.1172/JCI29838
15. Chen L, Miao Y, Zhang Y, Dou D, Liu L, Tian X, Yang G, Pu D, Zhang X, Kang J, et al. Inactivation of the E-prostanoid 3 receptor attenuates the angiotensin II pressor response via decreasing arterial contractility. *Arterioscler Thromb Vasc Biol*. 2012;32:3024–3032. doi: 10.1161/ATVBAHA.112.254052
16. Francois H, Athirakul K, Mao L, Rockman H, Coffman TM. Role for thromboxane receptors in angiotensin-II-induced hypertension. *Hypertension*. 2004;43:364–369. doi: 10.1161/01.HYP.0000112225.27560.24
17. Mukherjee K, Pingili AK, Singh P, Dhodi AN, Dutta SR, Gonzalez FJ, Malik KU. Testosterone Metabolite metabolite 6β-Hydroxytestosterone Contributes contributes to Angiotensin II-Induced induced Abdominal abdominal Aortic aortic Aneurysms aneurysms in Apoe$^{-/-}$ Male male Micemice. *J Am Heart Assoc*. 2021;10:e018536. doi: 10.1161/JAHA.120.018536
18. Singh P, Dutta SR, Song CY, Oh S, Gonzalez FJ, Malik KU. Brain Testosterone-CYP1B1 (Cytochrome P450 1B1) Generated generated Metabolite metabolite 6β-Hydroxytestosterone Promotes promotes Neurogenic neurogenic Hypertension hypertension and Inflammationinflammation. *Hypertension*. 2020;76:1006–1018. doi: 10.1161/HYPERTENSIONAHA.120.15567
19. Jennings BL, Sahan-Firat S, Estes AM, Das K, Farjana N, Fang XR, Gonzalez FJ, Malik KU. Cytochrome P450 1B1 contributes to angiotensin II-induced hypertension and associated pathophysiology. *Hypertension*. 2010;56:667–674. doi: 10.1161/HYPERTENSIONAHA.110.154518
20. Jennings BL, Anderson LJ, Estes AM, Yaghini FA, Fang XR, Porter J, Gonzalez FJ, Campbell WB, Malik KU. Cytochrome P450 1B1 contributes to renal dysfunction and damage caused by angiotensin II in mice. *Hypertension*. 2012;59:348–354. doi: 10.1161/HYPERTENSIONAHA.111.183301
21. Rao GN, Lassègue B, Alexander RW, Griendling KK. Angiotensin II stimulates phosphorylation of high-molecular-mass cytosolic phospholipase A2 in vascular smooth-muscle cells. *Biochem J*. 1994;299:197–201. doi: 10.1042/bj2990197

22. Bonventre JV, Swidler M. Calcium dependency of prostaglandin E2 production in rat glomerular mesangial cells. Evidence that protein kinase C modulates the Ca2+-dependent activation of phospholipase A2. *J Clin Invest*. 1988;82:168–176. doi: 10.1172/JCI113566
23. Muthalif MM, Benter IF, Uddin MR, Harper JL, Malik KU. Signal transduction mechanisms involved in angiotensin-(1-7)-stimulated arachidonic acid release and prostanoid synthesis in rabbit aortic smooth muscle cells. *J Pharmacol Exp Ther*. 1998;284:388–398.
24. Trevisi L, Bova S, Cargnelli G, Ceolotto G, Luciani S. Endothelin-1-induced arachidonic acid release by cytosolic phospholipase A2 activation in rat vascular smooth muscle via extracellular signal-regulated kinases pathway. *Biochem Pharmacol*. 2002;64:425–431. doi: 10.1016/s0006-2952(02)01066-3
25. Muthalif MM, Benter IF, Uddin MR, Malik KU. Calcium/calmodulin-dependent protein kinase IIalpha mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. *J Biol Chem*. 1996;271:30149–30157. doi: 10.1074/jbc.271.47.30149
26. McGiff JC. Prostaglandins, prostacyclin, and thromboxanes. *Annu Rev Pharmacol Toxicol*. 1981;21:479–509. doi: 10.1146/annurev.pa.21.040181.002403
27. Zhang Y, Guan Y, Schneider A, Brandon S, Breyer RM, Breyer MD. Characterization of murine vasopressor and vasodepressor prostaglandin E(2) receptors. *Hypertension*. 2000;35:1129–1134. doi: 10.1161/01.hyp.35.5.1129
28. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension*. 2004;44:248–252. doi: 10.1161/01.HYP.0000138070.47616.9d
29. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest*. 1996;97:1916–1923. doi: 10.1172/JCI118623
30. Nakagami H, Takemoto M, Liao JK. NADPH oxidase-derived superoxide anion mediates angiotensin II-induced cardiac hypertrophy. *J Mol Cell Cardiol*. 2003;35:851–859. doi: 10.1016/s0022-2828(03)00145-7
31. Wenzel S, Taimor G, Piper HM, Schlüter KD. Redox-sensitive intermediates mediate angiotensin II-induced p38 MAP kinase activation, AP-1 binding activity, and TGF-beta expression in adult ventricular cardiomyocytes. *FASEB J*. 2001;15:2291–2293. doi: 10.1096/fj.00-0827fje
32. Touyz RM, Cruzado M, Tabet F, Yao G, Salomon S, Schiffrin EL. Redox-dependent MAP kinase signaling by Ang II in vascular smooth muscle cells: role of receptor tyrosine kinase transactivation. *Can J Physiol Pharmacol*. 2003;81:159–167. doi: 10.1139/y02-164
33. Benten WP, Lieberherr M, Sekeris CE, Wunderlich F. Testosterone induces Ca2+ influx via non-genomic surface receptors in activated T cells. *FEBS Lett*. 1997;407:211–214. doi: 10.1016/s0014-5793(97)00346-3
34. Leslie CC. Properties and regulation of cytosolic phospholipase A2. *J Biol Chem*. 1997;272:16709–16712. doi: 10.1074/jbc.272.27.16709
35. Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, et al. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation*. 1998;97:1952–1959. doi: 10.1161/01.cir.97.19.1952
36. Jacobi J, Schlaich MP, Delles C, Schobel HP, Schmieder RE. Angiotensin II stimulates left ventricular hypertrophy in hypertensive patients independently of blood pressure. *Am J Hypertens*. 1999;12:418–422.
37. Sparks MA, Rianto F, Diaz E, Revoori R, Hoang T, Bouknight L, Stegbauer J, Vivekanandan-Giri A, Ruiz P, Pennathur S, et al. Direct actions of AT1 (Type 1 Angiotensin) receptors in cardiomyocytes do not contribute to cardiac hypertrophy. *Hypertension*. 2021;77:393–404. doi: 10.1161/HYPERTENSIONAHA.119.14079
38. Mori T, Cowley AW Jr. Role of pressure in angiotensin II-induced renal injury: chronic servo-control of renal perfusion pressure in rats. *Hypertension*. 2004;43:752–759. doi: 10.1161/01.HYP.0000120971.49659.6a
39. Alexander LD, Alagarsamy S, Douglas JG. Cyclic stretch-induced cPLA2 mediates ERK 1/2 signaling in rabbit proximal tubule cells. *Kidney Int*. 2004;65:551–563. doi: 10.1111/j.1523-1755.2004.00405.x
40. Ward CW, Prosser BL, Lederer WJ. Mechanical stretch-induced activation of ROS/RNS signaling in striated muscle. *Antioxid Redox Signal*. 2014;20:929–936. doi: 10.1089/ars.2013.5517
41. Alonso-Galicia M, Maier KG, Greene AS, Cowley AW Jr, Roman RJ. Role of 20-hydroxyeicosatetraenoic acid in the renal and vasoconstrictor actions of angiotensin II. *Am J Physiol Regul Integr Comp Physiol*. 2002;283:R60–R68. doi: 10.1152/ajpregu.00664.2001
42. Yiu SS, Zhao X, Inscho EW, Imig JD. 12-Hydroxyeicosatetraenoic acid participates in angiotensin II afferent arteriolar vasoconstriction by activating L-type calcium channels. *J Lipid Res*. 2003;44:2391–2399. doi: 10.1194/jlr.M300183-JLR200
43. Breyer MD, Breyer RM. Prostaglandin E receptors and the kidney. *Am J Physiol Renal Physiol*. 2000;279:F12–F23. doi: 10.1152/ajprenal.2000.279.1.F12
44. Yamada K, Kubo K, Shuto K, Nakamizo N. Inhibition of thromboxane A2-induced vasocontraction by KF4939, a new anti-platelet agent, in rabbit mesenteric and dog coronary arteries. *Jpn J Pharmacol*. 1984;36:283–290. doi: 10.1254/jjp.36.283
45. Mistry M, Nasjletti A. Role of TXA2 in the pathogenesis of severe angiotensin II-salt hypertension. *Adv Prostaglandin Thromboxane Leukot Res*. 1989;19:207–210.
46. Wilcox CS, Welch WJ, Snellen H. Thromboxane mediates renal hemodynamic response to infused angiotensin II. *Kidney Int*. 1991;40:1090–1097. doi: 10.1038/ki.1991.319
47. Bryson TD, Pandrangi TS, Khan SZ, Xu J, Pavlov TS, Ortiz PA, Peterson E, Harding P. The deleterious role of the prostaglandin E2 EP3 receptor in angiotensin II hypertension. *Am J Physiol Heart Circ Physiol*. 2020;318:H867–H882. doi: 10.1152/ajpheart.00538.2019
48. Xiao L, Itani HA, do Carmo LS, Carver LS, Breyer RM, Harrison DG. Central EP3 (E Prostanoid 3) Receptors receptors Mediate mediate Saltsalt-Sensitive sensitive Hypertension hypertension and Immune immune Activationactivation. *Hypertension*. 2019;74:1507–1515. doi: 10.1161/HYPERTENSIONAHA.119.13850
49. Wu J, Fang S, Lu KT, Wackman K, Schwartzman ML, Dikalov SI, Grobe JL, Sigmund CD. EP3 (E-Prostanoid 3) Receptor receptor Mediates mediates Impaired impaired Vasodilation vasodilation in a Mouse mouse Model model of Saltsalt-Sensitive sensitive Hypertensionhypertension. *Hypertension*. 2021;77:1399–1411. doi: 10.1161/HYPERTENSIONAHA.120.16518
50. Sebeková K, Eifert T, Klassen A, Heidland A, Amann K. Renal effects of S18886 (Terutroban), a TP receptor antagonist, in an experimental model of type 2 diabetes. *Diabetes*. 2007;56:968–974. doi: 10.2337/db06-1136
51. Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM. Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. *J Biol Chem*. 1993;268:26767–26772
52. Kraemer MP, Choi H, Reese J, Lamb FS, Breyer RM. Regulation of arterial reactivity by concurrent signaling through the E-prostanoid receptor 3 and angiotensin receptor 1. *Vascul Pharmacol*. 2016;84:47–54. doi: 10.1016/j.vph.2016.05.015
53. Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca2+ entry and Ca2+ sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem J*. 2005;389(pt 3):763–774. doi: 10.1042/BJ20050237
54. Kirabo A, Fontana V, de Faria AP, Loperena R, Galindo CL, Wu J, Bikineyeva AT, Dikalov S, Xiao L, Chen W, et al. DC isoketal-modified proteins activate T cells and promote hypertension. *J Clin Invest*. 2014;124:4642–4656. doi: 10.1172/JCI74084