6β-Hydroxytestosterone Promotes Angiotensin II-Induced Hypertension via Enhanced Cytosolic Phospholipase A2α Activity

Purnima Singh, Chi Young Song, Shubha R. Dutta, Ajeeth Pingili, Ji Soo Shin, Frank J. Gonzalez, Joseph V. Bonventre, Kafait U. Malik

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 cPLA2α (cytosolic phospholipase A2α) and generation of prohypertensive eicosanoids in male mice. Eight-week-old male intact or orchidectomized cPLA2α+/Cyp1b1+/+ and cPLA2α−/Cyp1b1−/− and intact cPLA2α+/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 cPLA2α or Cyp1b1 genes or by administration of the arachidonic acid metabolism inhibitor 5,8,11,14-eicosatetraynoic acid to cPLA2α−/Cyp1b1−/− mice. 6β-hydroxytestosterone treatment restored these effects of angiotensin II in cPLA2α+/Cyp1b1+/+ mice but not in orchidectomized cPLA2α−/Cyp1b1−/− mice, which were lowered by 5,8,11,14-eicosatetraynoic acid in cPLA2α−/Cyp1b1−/− mice. Antagonists of prostaglandin E2-EP1/EP3 receptors and thromboxane A2-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 cPLA2α−/−/Cyp1b1−/− mice. These data suggest that 6β-hydroxytestosterone promotes angiotensin II-induced increase in systolic blood pressure and associated pathogenesis via cPLA2α activation and generation of eicosanoids, most likely prostaglandin E2 and thromboxane A2, 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,
**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 cPLA₂α (cytosolic phospholipase A₂α) activation, and production of the arachidonic acid metabolites prostaglandins (PG) E₂ and thromboxane (TXA₂) in male mice.
- PGE₂ by stimulating the EP1/EP3 receptors and TXA₂ 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 cPLA₂α activity. 6βOHT promotes Ang II-induced activation of cPLA₂α, leading to increased production of PGE₂ and TXA₂. Therefore, PGE₂ via EP1 and EP3, and TXA₂ via TP receptors, respectively contribute to the effect of 6βOHT to promote Ang II-induced hypertension and associated pathogenesis in male mice.

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**Nonstandard Abbreviations and Acronyms**

| Abbreviation | Description |
|--------------|-------------|
| 6βOHT        | 6β-hydroxytestosterone |
| AA           | arachidonic acid |
| Ang II       | angiotensin II |
| BP           | blood pressure |
| COX          | cyclooxygenase |
| cPLA₂α       | cytosolic phospholipase A₂α |
| CYP1B1       | cytochrome P450 1B1 |
| ERK1/2       | (extracellular signal-regulated kinase) |
| PGE₂         | prostaglandin E₂ |
| ROS          | reactive oxygen species |
| SBP          | systolic blood pressure |

production of reactive oxygen species (ROS), and cardiac and renal pathogenesis in male mice. 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 prostaglandins (PG) E₂ and thromboxane (TXA₂) in male mice. These include AA-COX (cyclooxygenase) derived thromboxane A₂ (TXA₂) that acts via prostanoid receptor (TP) and prostaglandin E₂ (PGE₂) that acts via the EP1/EP3 receptors. Since the testosterone-CYP1B1 generated metabolite 6βOHT is required for Ang II to cause hypertension, we performed this study to test the hypothesis that 6βOHT promotes Ang II-induced hypertension and its pathogenesis by enhancing cPLA₂α 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) cPLA₂α/cPLA₂α, cPLA₂α/cPLA₂α, and intact cPLA₂α/cPLA₂α (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) cPLA₂α gene disruption (cPLA₂α−/−/Cyp1b1+/+) (cPLA₂α−/−/Cyp1b1+/+) in intact mice; (2) treatment of cPLA₂α−/−/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 Orchic-CPLA\(\alpha^{+/+}\)/Cyp1b1\(+/+\) and cPLA\(\alpha^{+/+}\)/Cyp1b1\(+/+\) mice and in intact cPLA\(\alpha^{+/+}\)/Cyp1b1\(^{−/−}\) mice; (4) treatment with ETYA+6\(\beta\)OHT in cPLA\(\alpha^{+/+}\)/Cyp1b1\(^{−/−}\) mice; and (5) treatment with 6\(\beta\)OHT+EP1 (SC19220), EP3 (L-798106), \(\alpha\)

resistant collagen from the heart and kidney sections as described previously. 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. 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 TissueLyser 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\(\alpha\) and total (t)-cPLA\(\alpha\) antibodies and the corresponding secondary antibodies. The density of the bands was quantified by blinded investigators using ImageJ 1.42 analysis software (NIH: http://imagej.nih.gov/ij).

Urinary levels of proinflammatory cytokines

Aliquots of urine samples containing 10X protease/phosphatase inhibitor cocktail of AEBSF (4-benzene-sulfonyl fluoride hydrochloride), aprotilin, pepstatin, and leupeptin (Cell signaling, Catalog No. 5872S) in Tris base buffer (10 in 90 \(\mu\)L urine) were stored at \(-80^\circ\)C. Inflammatory cytokines IFN-\(\gamma\), IL-1\(\beta\), IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-\(\alpha\) 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-1\(\beta\), IL-6, IL-10, and TNF-\(\alpha\).

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 6\(\beta\)OHT-Treated Orchic-CPLA\(\alpha^{+/+}\)/Cyp1b1\(+/+\) and Intact cPLA\(\alpha^{+/+}\)/Cyp1b1\(^{−/−}\) but Not in Orchic-CPLA\(\alpha^{+/−}\)/Cyp1b1\(+/+\) Mice

CYP1B1 contributes to Ang II-induced hypertension in male mice and rats, and orchidectomy reduces the ability of Ang II to increase BP. Ang II activates cPLA\(\alpha\) and releases AA from tissue phospholipids. AA modulates one or more of the cardiovascular effects of Ang II and has been implicated in hypertension. 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 6\(\beta\)OHT-treated Orchic-CPLA\(\alpha^{+/+}\)/Cyp1b1\(+/+\) (Figure 1A, 1D through 1F) and intact cPLA\(\alpha^{+/+}\)/Cyp1b1\(^{−/−}\) (Figure 1B, 1D through 1F), but not in Orchic-CPLA\(\alpha^{+/−}\)/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)-cPLA₂α+/+ and intact male cPLA₂α+/+ but not in Orchi-cPLA₂α−/− mice. Measurement of SBP by tail-cuff in Orchi-cPLA₂α+/+ (A), intact male cPLA₂α+/+ (B), and Orchi-cPLA₂α−/− (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, $P<0.05 vs 6βOHT+Saline in the corresponding groups; #P<0.05 vs Orchi-cPLA₂α+/+. 
II (subcutaneous via osmotic pump) increased the SBP as measured by tail-cuff in intact cPLA$_2^{+/+}$/Cyp1b1+/- mice (Figure S1A in the Data Supplement). This effect was decreased in cPLA$_2^{+/+}$/Cyp1b1+/- mice (Figure S1B) and attenuated in cPLA$_2^{−/+}$/Cyp1b1+/- mice (Figure S1C). Ang II did not alter the heart rate (Figure S1D), pulse pressure (Figure S1E), or locomotor activity (Figure S1F) in 6βOHT-treated Orchi-cPLA$_2^{+/+}$/Cyp1b1+/-, intact cPLA$_2^{+/+}$/Cyp1b1+/-, and Orchi-cPLA$_2^{−/+}$/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. 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 Cyp1b1+/- and Cyp1b1−/− mice (Figure S3A through S3C). Ang II did not alter the heart rate (Figure S1D), consistent with our previous studies.10-12 Ang II produced cardiac and renal fibrosis in intact cPLA$_2^{α}$+/+ mice (Figure 2A through 2C). Consistent with our previous studies,10-12 Ang II increased cardiac and renal fibrosis as indicated by increased collagen deposition (blue color) in 6βOHT-treated Orchi-cPLA$_2^{α}$+/+ and intact cPLA$_2^{α}$+/+ mice (Figure 2A through 2C). Consistent with our previous studies,10-12 Ang II produced cardiac and renal fibrosis in intact cPLA$_2^{α}$+/+ and cPLA$_2^{−/+}$/Cyp1b1+/- mice (Figure S3A through S3C). These effects were decreased in cPLA$_2^{α}$+/+ and cPLA$_2^{−/+}$/Cyp1b1+/- mice (Figure S3A through S3C).

**Testosterone-CYP1B1 Generated Metabolite, 6βOHT Restored Ang II-Induced Cardiac and Renal Fibrosis in Orchi-cPLA$_2^{α}$+/+ and Intact cPLA$_2^{α}$+/+ but Not in Orchi-cPLA$_2^{α}$+/+ 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$_2^{α}$+/+ and intact cPLA$_2^{α}$+/+ mice (Figure 2A through 2C). Consistent with our previous studies,10-12 Ang II produced cardiac and renal fibrosis in intact cPLA$_2^{α}$+/+ and Cyp1b1+/- mice (Figure S3A through S3C). These effects were decreased in cPLA$_2^{α}$+/+ and cPLA$_2^{−/+}$/Cyp1b1+/- mice (Figure S3A through S3C).

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

Several BP regulating hormones, including Ang II, activate cPLA$_2$ and release AA.23-25 We observed that Ang II increased cPLA$_2^{α}$ activity in intact cPLA$_2^{α}$+/+ and Cyp1b1+/- but not in cPLA$_2^{α}$−/+ and Cyp1b1+/- mice, as measured by an increased ratio of p-cPLA$_2^{α}$/t-cPLA$_2^{α}$ expression in the kidney (Figure 3A). However, this effect was blunted in Orchi-cPLA$_2^{α}$+/+ and Cyp1b1+/- mice (Figure 3B).

**Testosterone-CYP1B1 Generated Metabolite, 6βOHT Restored Ang II-Induced Increased Urinary Excretion of PGE$_2$ and TXA$_2$ Metabolites in Orchi-cPLA$_2^{α}$+/+ and Intact cPLA$_2^{α}$+/+ but Not in Orchi-cPLA$_2^{α}$+/+ 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$_2$α, and 12-lipoxygenase (12-LOX). The vascular effects of PGE$_2$ and TXA$_2$ contribute to pro-hypertensive 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$_2^{α}$+/+ and intact cPLA$_2^{α}$+/+ 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$_2^{α}$+/+ mice. We observed that the effect of Ang II to increase urinary excretion of PGE$_2$ and TXA$_2$ metabolites was blunted in cPLA$_2^{α}$+/+ and 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$_2^{α}$+/+ and intact cPLA$_2^{α}$+/+ but not in Orchi-cPLA$_2^{α}$+/+ 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$_2^{α}$+/+ Mice**

Administration of ETYA blunted the Ang II-induced increase in SBP in cPLA$_2^{α}$+/+ and Cyp1b1+/- mice (Figure 4A), as described previously. In this study, ETYA attenuated the effect of 6βOHT to restore the Ang II-induced increase in SBP in intact cPLA$_2^{α}$+/+ 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$_2^{α}$+/+ and Cyp1b1+/- mice.
Antagonists of PGE2-EP1, EP3, and TXA2-TP Receptors Reduced the Effect of 6βOHT to Restore Ang II-Induced Increased SBP, and Cardiac and Renal Fibrosis in Intact cPLA2α+/+ Cyp1b1+/+ Mice

Antagonists of PGE2-EP1 (EP1RA), EP3 (EP3RA), and TXA2 (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 cPLA2α+/+ Cyp1b1+/+ mice.

6βOHT Promotes Ang II-Induced Cardiac and Renal ROS Production via cPLA2α in Male Mice

Oxidative stress has been implicated in various models of hypertension, including the Ang II model. Processes contributing to these phenomena have been attributed, in part, to increased generation of ROS, particularly superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), which function as important second messengers. We previously showed that Ang II-induced ROS production in male mice was abrogated by orchidectomy or by disruption of the Cyp1b1 or cPLA2α genes. In the present study, we show that treatment of intact cPLA2α+/+ 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 cPLA2α−/− 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-cPLA2α+/+ Cyp1b1+/+ but not in Orchi-cPLA2α−/− Cyp1b1+/+ mice (Figure S5D through S5F). However, Ang II failed to increase cardiac and renal ROS production in intact cPLA2α+/+ 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 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)-cPLA2α+/+ Cyp1b1+/+ and intact male cPLA2α+/+ Cyp1b1+/+ but not in Orchi-cPLA2α−/− 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.
Figure 3. Ang II (Angiotensin II) increased cPLA$_2$$\alpha$ (cytosolic phospholipase A$^{2+}$) activity measured by an increased ratio of phosphorylated (p) to total (t)-cPLA$_2$$\alpha$ expression in the kidney of cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{+/+}$ mice, an effect which was blunted in orchidectomized (Orchi)-cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{+/+}$ and intact male cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{-/-}$ mice and restored by 6$\beta$-hydroxytestosterone (6$\beta$OHT) treatment. cPLA$_2$$\alpha$ expression remained absent in intact or Orchi-cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{-/-}$ mice. Representative blots and quantitation of cPLA$_2$$\alpha$ expression in cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{+/+}$ and cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{-/-}$ (A), Orchi-cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{+/+}$ (B), cPLA$_2$$\alpha^{+/+}$/Cyp1b1 (C), and Orchi-cPLA$_2$$\alpha^{+/+}$/Cyp1b1$^{-/-}$ (D) mice. Saline was used as vehicle control for Ang II, and dimethyl sulfoxide (DMSO) as vehicle control for 6$\beta$OHT. 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 E2 (PGE2) metabolites and thromboxane A2 (TXA2) metabolite TXB2 in cPLA2α+/+ /Cyp1b1+/+ and 6β-hydroxytestosterone (6βOHT)-treated intact male cPLA2α+/+ /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 PGE2 metabolites. F, Quantitation of urinary excretion of TXA2 metabolite TXB2. 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 cPLA2α+/+ /Cyp1b1+/+; ‡P<0.05 vs 6βOHT+ETYA+Saline and ¶P<0.05 vs 6βOHT+Ang II in cPLA2α+/+ /Cyp1b1+.
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 cPLA₂α+/+ 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); aP<0.05 vs EP1RA+6βOHT+Saline, bP<0.05 vs EP1RA+6βOHT+Saline, cP<0.05 vs TPRA+6βOHT+Saline and dP<0.05 vs 6βOHT+Ang II in cPLA₂α+/+ 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 \(6βOHT\) 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 (C, representative images, and D, quantitation) ROS production. Effect of EP1RA, EP3RA, and TPRA on 6βOHT-treated Ang II-infused \(cPLA_2^{α^{+/+}}/Cyp1b1^{−/−}\) mice on cardiac (E, representative images, and F, quantitation) and renal (G, representative images, and H, 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 cPLA2α+/+/Cy p1b1−/− MICE (FIGURE 6D THROUGH 6F).

6βOHT PROMOTES ANG II-INDUCED SECRETION OF PROINFLAMMATORY CYTOKINES VIA cPLA2α IN MALE MICE

Disruption of the cPLA2α 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 cPLA2α+/−/Cyp1b1−/− mice. This effect was maintained after 6βOHT treatment in Orchi-cPLA2α+/−/Cyp1b1−/− and intact cPLA2α+/−/Cyp1b1−/− but not in Orchi-cPLA2α−/−/Cyp1b1−/− mice. Ang II also increased the urinary excretion of TNF-α (Figure S6A) and KC/GRO (Figure S6B) in intact cPLA2α+/−/Cyp1b1−/− and 6βOHT-treated Orchi-cPLA2α+/−/Cyp1b1−/−, intact cPLA2α−/−/Cyp1b1−/−, and in Orchi-cPLA2α−/−/Cyp1b1−/− mice. Among other cytokines, Ang II increased IFN-γ only in intact cPLA2α+/−/Cyp1b1−/− and IL-4 only in Orchi-cPLA2α+/−/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 cPLA2α activity and generation of AA-COX metabolites PGE2 and TXA2 in male mice. (2) PGE2, through the EP1/EP3 receptors, and TXA2 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 cPLA2α 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.12 In the present study, we demonstrated that disruption of the cPLA2α 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,12 suggests that 6βOHT must act upstream of cPLA2α. 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-cPLA2α+/−/Cyp1b1−/− and intact cPLA2α+/−/Cyp1b1−/− but not Orchi-cPLA2α−/−/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 cPLA2α. Ang II activates cPLA2α, which selectively catalyzes the release of AA from tissue phospholipids.23 Our finding that the Ang II-induced increase in cPLA2α activity induced by its phosphorylation in the kidney of intact cPLA2α+/−/Cyp1b1−/− mice was abolished in Orchi-cPLA2α+/−/Cyp1b1−/−, Orchi-cPLA2α−/−/Cyp1b1−/−, and intact cPLA2α−/−/Cyp1b1−/− mice, suggests that one or more CYP1B1-testosterone derived metabolites, most likely 6βOHT, is required for cPLA2α activation by Ang II. Supporting this hypothesis was our demonstration that 6βOHT treatment restored the effect of Ang II to cause cPLA2α phosphorylation without altering its expression in the kidneys of Orchi-cPLA2α−/−/Cyp1b1−/− and intact cPLA2α−/−/Cyp1b1−/− but not in cPLA2α−/−/Cyp1b1−/− mice. The mechanism by which 6βOHT promotes cPLA2α 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) activity23 that are involved in cPLA2α activation.23 Future studies will explore this mechanism.

Our findings that (1) Ang II increased the urinary excretion of PGE2, measured as its metabolites, and TXB2, the stable metabolite of TXA2, in cPLA2α+/−/Cyp1b1−/− mice and (2) Ang II increased the urinary excretion of PGE2 metabolites and TXB2 in 6βOHT-treated Orchi-cPLA2α+/−/Cyp1b1−/− and intact cPLA2α+/−/Cyp1b1−/−, but not Orchi-cPLA2α−/−/Cyp1b1−/− mice, suggests that AA released by cPLA2α 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 TXB2 level like on the SBP, in the intact cPLA2α+/−/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 ETYA12 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 PGE2 metabolites and TXB2 in intact cPLA2α+/−/Cyp1b1−/− mice. Ang II-induced cardiac hypertrophy and fibrosis and renal injury have been attributed to the increase in BP.25–28 Therefore, restoration by 6βOHT of Ang II-induced cardiac and renal fibrosis could also result from an increase in BP in intact cPLA2α+/−/Cyp1b1−/− mice.
Ang II infusion in intact cPLA_2α^{+/−}/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 and ROS. Therefore, hypertension-induced stretch in these mice might enhance cPLA_2α activation and eicosanoid 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 lipoxigenase-generated 12S-hydroxyeicosatetraenoic acid, and CYP450 A1 derived 20-hydroxyeicosatetraenoic acid. 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 and signaling of TXA_2 through TP receptor results in vasocostriction. The PGE_2 through EP1 and/or EP3, and TXA_2 via TP receptors, contributes to the vasoconstrictor and hypertensive effects of Ang II, PGE_2 via EP3 signaling pathway has also been implicated in Nω-nitro-L-arginine methyl ester hydrochloride/high salt-induced hypertension and impaired vasodilation and hypertension in the high salt diet-fed S-P467L mice with decreased peroxisome proliferator-activated receptor-gamma activity. In the present study, the selective antagonists of EP1 (SC19220), EP3 (L-798106), and TXA_2-TP (Terbutroban) 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_2α^{+/−}/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_2α 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, 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 and the role of calcium sensitization via Rho-kinase activation 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. 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 isolevuglandin protein adducts in spleen cells, production of proinflammatory cytokines, and renal fibrosis. PGE_2 also acts directly on dendritic cells via the EP1 receptor to increase isolevuglandin adducted proteins. 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.

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. 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_2α^{−/−}/Cyp1b1^{−/−} but not in 6βOHT-treated Orchi-cPLA_2α^{+/+}/Cyp1b1^{−/−} and intact cPLA_2α^{+/+}/Cyp1b1^{−/−} mice. Surprisingly, the Ang II-induced increase in excretion of TNF-α was similar in intact cPLA_2α^{+/+}/Cyp1b1^{−/−} and 6βOHT-treated Orchi-cPLA_2α^{+/+}/Cyp1b1^{−/−}, intact cPLA_2α^{+/+}/Cyp1b1^{−/−} and Orchi-cPLA_2α^{+/+}/Cyp1b1^{−/−} mice, suggesting that the effect of 6βOHT in the promotion of Ang II-induced excretion of TNF-α is independent of cPLA_2α 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_2α.

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_2α 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<sub>2</sub> in male mice. Moreover, testosterone-CYP1B1 generated metabolite 6βOHT also contributes to Ang II-induced hypertension and its pathogenesis. This study provides novel insight into the mechanism of interactions of cPLA<sub>2</sub> and testosterone metabolite 6βOHT. We show that 6βOHT acts upstream of cPLA<sub>2</sub> and by enhancing its activity in response to Ang II and production of PGE<sub>2</sub> and TXA<sub>2</sub> which via EP1 and EP3 and TXA<sub>2</sub> receptors, respectively promotes Ang II-induced hypertension and associated pathogenesis. However, the site of interaction of cPLA<sub>2</sub> 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, it would be important to determine the interaction of cPLA<sub>2</sub> and CYP1B1-generated 6βOHT in the paraventricular nucleus in Ang II-induced hypertension and cardiovascular and renal pathogenesis.

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Affiliations

Department of Pharmacology, Addiction Research, and Toxicology, College of Medicine, University of Tennessee Health Science Center, Memphis (P.S., C.Y.S., S.R.D., A.P., J.S.S., K.U.M.). Laboratory of Metabolism, National Cancer Institute, Bethesda, MD (F.J.G.). Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Harvard Institute of Medicine, Boston, MA (J.U.V.B.).

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