Sex differences in blood pressure (BP) level are well estab-
lished. Men are at an increased risk of hypertension and
cardiovascular disease versus premenopausal women of the
same age.1–5 The differences in the level of BP between women
and men have been attributed to sex steroids.5 Angiotensin II
(Ang II)–induced hypertension, female Cyp1b1−/− and Cyp1b1+/− mice were infused with Ang II (700 ng/kg per minute) or vehicle with
or without ovariectomy. In addition, mice were treated with the CYP1B1 inhibitor, 2,3′,4,5′-tetramethoxystilbene (TMS; 300 μg/kg IP, every third day), and 17-β estradiol metabolites, 2-hydroxyestradiol (2-OHE), 4-OHE, or 2-methoxyestradiol (1.5 mg/kg per day IP, for 2 weeks) and systolic blood pressure (SBP) measured. Ang II increased SBP more in Cyp1b1−/− than in Cyp1b1+/+ mice (119±3–171±11 versus 120±4–149±4 mm Hg; P<0.05). Ang II caused cardiovascular remodeling and endothelial dysfunction and increased vascular reactivity and oxidative stress in Cyp1b1−/− but not in Cyp1b1+/+ mice. The Ang II–induced increase in SBP was enhanced by ovariectomy and TMS in Cyp1b1+/+ but not in Cyp1b1−/− mice. 2-OHE did not alter Ang II–induced increase in SBP in Cyp1b1+/+ mice but minimized it in Cyp1b1−/− mice, whereas 4-OHE enhanced Ang II–induced increase in SBP in Cyp1b1+/+ mice but did not alter the increased SBP in Cyp1b1−/− mice. 2-OHE–derived catechol-O-methyltransferase metabolite, 2-methoxyestradiol, inhibited Ang II–induced increase in SBP in Cyp1b1+/+ mice. Ang II increased plasma levels of 2-methoxyestradiol in Cyp1b1+/+ but not in Cyp1b1−/− mice and increased activity of cardiac extracellular signal–regulated kinase 1/2, p38 mitogen-activated kinase, c-Src, and Akt in Cyp1b1+/+ but not in Cyp1b1−/− mice. These data suggest that CYP1B1 protects against Ang II–induced hypertension and associated cardiovascular changes in female mice, most likely mediated by 2-methoxyestradiol–inhibiting oxidative stress and the activity of these signaling molecules. (Hypertension. 2014;64:134-140.) • Online Data Supplement

Key Words: 2-methoxyestradiol ◼ cytochrome P450 1B1 ◼ hypertension ◼ ovariectomy ◼ oxidative stress

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From the Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis (B.L.J., L.W.G., A.K.P., N.S.K., A.M.E., X.R.F., K.U.M.); and Laboratory of Metabolism, National Cancer Institute, Bethesda, MD (F.J.G.).

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Correspondence to Kafait U. Malik, Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, 874 Union Ave, Memphis, TN 38163. E-mail kmalik@uthsc.edu

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Methods
For detailed Methods, see the online-only Data Supplement.

Results
Cyp1b1 Gene Disruption Enhanced the Hypertensive Effect of Ang II in Female Mice
Systolic BP (SBP) in Cyp1b1+/+ and Cyp1b1−/− mice was measured by the tail cuff method. Although this method has some limitations, infusion of Ang II caused a substantial increase in SBP in both Cyp1b1+/+ and Cyp1b1−/− mice during a period of 14 days; however, the increase was greater in Cyp1b1+/+ mice than in Cyp1b1−/− mice (Figure 1). We noted a consistent and highly significant difference in the SBP between these 2 groups without any change in basal pressure in the corresponding vehicle-treated controls measured twice weekly during a period of 2 weeks (Figure 1). Therefore, the differences observed in SBP measured by tail cuff in Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II are accurate and reproducible.

Infusion of Ang II Increased Cardiac CYP1B1 Activity and Expression in Female Mice
CYP1B1 activity and protein expression were increased in the hearts of Ang II–infused Cyp1b1+/+, but not in Cyp1b1−/− mice (Figure 2A and 2B, respectively).

Infusion of Ang II Increased Cardiac Hypertrophy and Fibrosis to a Greater Degree in Female Cyp1b1+/+ Mice Than in Cyp1b1−/− Mice
Infusion of Ang II increased heart weight:body weight ratio, an indicator of cardiac hypertrophy, in Cyp1b1+/+ and Cyp1b1−/− mice, but the increase was greater in Cyp1b1+/+ mice than in Cyp1b1−/− mice (Table S1 in the online-only Data Supplement). Hearts from Ang II–infused Cyp1b1−/− mice, also displayed fibrosis as indicated by α-smooth muscle actin-positive myofibroblasts and collagen deposition in the myocardium (Figure S1A and S1B, respectively).

Ang II Increased Vascular Reactivity and Remodeling, Promoted Endothelial Dysfunction,
and Increased Vascular Oxidative Stress in Female Cyp1b1+/+, but Not in Cyp1b1−/−, Mice
In Ang II–infused Cyp1b1+/+, but not Cyp1b1−/− mice, the response to phenylephrine and endothelin-1 was increased (Figure S2A and S2B, respectively). The increased vascular reactivity correlated with an increase in media:lumen ratio, an indicator of vascular remodeling (Table S2). In Cyp1b1−/− mice, infusion of Ang II had no effect on aortic endothelial function (Figure S2C). In contrast, Ang II caused endothelial dysfunction in aortas of Cyp1b1−/− mice, as demonstrated by a decreased relaxation response to acetylcholine (Figure S2C). Endothelium-independent relaxation to sodium nitroprusside did not differ in aortae from mice in any of the treatment groups (Figure S2D). Infusion of Ang II did not increase vascular superoxide production in Cyp1b1−/− mice (Figure S2E); however, in the aorta of Ang II–infused Cyp1b1−/− mice, elevated 2-hydroxyethidium fluorescence revealed increased superoxide production (Figure S2E).

Inhibition of CYP1B1 Activity With 2,3′,4,5′-Tetramethoxystilbene Increased the Hypertensive Effect of Ang II in Female Cyp1b1+/+ Mice
To further confirm involvement of CYP1B1 in protecting female Cyp1b1+/+ mice against Ang II–induced hypertension, we used 2,3′,4,5′-tetramethoxystilbene (TMS), a selective inhibitor of CYP1B1 activity. In Cyp1b1+/+ mice, Ang II infusion with concurrent injections of TMS every third day in doses shown to inhibit cardiovascular and renal CYP1B1 activity increased SBP; this increase was similar to that observed in Ang II–infused Cyp1b1−/− mice (Figure S3A and S3B). As expected, TMS did not alter the hypertensive effect of Ang II in Cyp1b1+/− mice (Figure S3B).

Depletion of Estrogen Increased the Hypertensive Effect of Ang II in Female Cyp1b1+/+ Mice
It is well established that infusion of Ang II produces a larger increase in BP in female mice that have undergone ovariectomy than in intact animals. To investigate whether estrogen metabolism by CYP1B1 may confer this resistance in female mice, we examined the effect of Ang II on SBP in ovariectomized Cyp1b1−/− and Cyp1b1+/+ mice. Ang II produced a larger increase in SBP in ovariecotomized Cyp1b1−/− mice than in intact Cyp1b1+/+ mice; this increase was similar to that observed in Cyp1b1+/− mice (Figure S4A and S4B). Ovariectomy did not alter the hypertensive effect of Ang II in Cyp1b1−/− mice (Figure S4B).

CYP1B1-Derived Metabolite of 17-β Estradiol, 2-OHE, Minimized the Hypertensive Effect of Ang II in Female Cyp1b1+/+ Mice
Because CYP1B1 is known to metabolize 17-β estradiol into hydroxysteriadiols, namely 2-OHE and 4-OHE, we next investigated whether these compounds influence Ang II–induced hypertension in female mice. In Cyp1b1+/+ mice, daily injections of 2-OHE did not alter the effect of Ang II to cause a small increase in BP (Figure S5A). In Ang II–infused Cyp1b1−/− mice, daily injections of 2-OHE markedly reduced SBP; the remaining extent of increase was similar to that observed in Cyp1b1+/+ mice (Figure S5A and S5B).
CYP1B1-Derived Metabolite of 17-β Estradiol, 4-OHE, Increased Ang II–Induced Hypertension in Female Cyp1b1−/− Mice

In Cyp1b1+/+ mice, daily injections of 4-OHE increased the hypertensive effect of Ang II to a level similar to that seen in Cyp1b1−/− mice (Figure S6A and S6B); this increase in SBP was also similar to that observed in TMS-treated or ovariectomized Cyp1b1−/− mice infused with Ang II. 4-OHE did not alter the hypertensive effect of Ang II in Cyp1b1−/− mice (Figure S6B).

2-MeE2, the Catechol-O-Methyltransferase (COMT)–Derived Metabolite of 2-OHE, Minimized the Hypertensive Effect of Ang II in Female Cyp1b1+/+ Mice, and its Levels Were Increased in Ang II–Infused Cyp1b1−/−, But Not in Cyp1b1−/−, Mice.

Methoxyestradiols, which are formed via COMT metabolism of hydroxyestradiols, are known to be more potent biological mediators of the CYP1B1/estrogen pathway. Because we found that 2-OHE, but not 4-OHE, protected against Ang II–induced hypertension, we investigated whether 2-MeE2 was responsible for the reduced hypertensive effect of Ang II in female Cyp1b1−/− mice; daily injections of 2-MeE2 did not alter the effect of Ang II to cause a small increase in BP (Figure 3A). In Cyp1b1+/+ mice infused with Ang II, concurrent daily injections of 2-MeE2 markedly reduced SBP to a level similar to that seen in Cyp1b1−/− mice (Figure 3A and 3B). In Cyp1b1−/− mice, infusion of Ang II was associated with increased plasma levels of 2-MeE2 (Figure 3C). In contrast, Cyp1b1+/+ mice displayed lower basal levels of 2-MeE2 compared with Cyp1b1−/− mice, and these levels were not altered by Ang II (Figure 3C).

Infusion of Ang II Was Associated With Increased Cardiac Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activity and Production of Reactive Oxygen Species in Female Cyp1b1−/− Mice

Reactive oxygen species (ROS) have been implicated in many biological actions of Ang II including hypertension. To determine whether protection against Ang II–induced hypertension was associated with increased cardiac cytochrome P450 (CYP) 1B1 activity and expression in female mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with vehicle or Ang II for 2 weeks. At the completion of the experiment, cardiac tissue was collected to measure CYP1B1 activity using the P450-Glo assay (A). The activity of Cyp1b1 is expressed as relative luminescence units (RLU). Cyp1b1 protein expression was measured by Western blot analysis in cardiac tissue from vehicle- and Ang II–infused Cyp1b1+/+ and Cyp1b1−/− mice using ≈10 μg of protein for loading (B). Cyp1b1 protein expression is normalized against α-actin, which was used as a loading control. *P<0.05 vehicle vs corresponding value from Ang II–infused animal (n=3–5 for all experiments; data are expressed as means±SEM).

Figure 2. Angiotensin II (Ang II)–induced hypertension was associated with increased cardiac cytochrome P450 (CYP) 1B1 activity and expression in female mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with vehicle or Ang II for 2 weeks. At the completion of the experiment, cardiac tissue was collected to measure CYP1B1 activity using the P450-Glo assay (A). The activity of Cyp1b1 is expressed as relative luminescence units (RLU). Cyp1b1 protein expression was measured by Western blot analysis in cardiac tissue from vehicle- and Ang II–infused Cyp1b1+/+ and Cyp1b1−/− mice using ≈10 μg of protein for loading (B). Cyp1b1 protein expression is normalized against α-actin, which was used as a loading control. *P<0.05 vehicle vs corresponding value from Ang II–infused animal (n=3–5 for all experiments; data are expressed as means±SEM).

Figure 3. 2-Methoxyestradiol (2-MeE2) minimized angiotensin II (Ang II)–induced hypertension in Cyp1b1+/+ mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with Ang II or vehicle and given intraperitoneal injections of the catechol-O-methyltransferase–derived metabolite of 2-hydroxyestradiol (2-OHE), 2-MeE2, daily for 2 weeks. A and B, Systolic blood pressure (SBP) was measured by tail cuff twice weekly. C, At the completion of the experiments, plasma was collected and analyzed for concentration of 2-MeE2 using a 2-MeE2 EIA kit. tP<0.05 Cyp1b1+/+ vehicle vs Cyp1b1−/− vehicle; tP<0.05 vehicle vs corresponding value from Ang II–infused animal; †P<0.05 Cyp1b1+/+ Ang II vs Cyp1b1−/− Ang II (n=5 for all experiments; data are expressed as means±SEM).

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hypertension in Cyp1b1+/+ mice was associated with decreased ROS production, we measured cardiac nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and ROS production. In Cyp1b1+/+ mice, infusion of Ang II had no effect on cardiac NADPH oxidase activity (Figure S7A); however, it produced a small, but significant, increase in cardiac superoxide production, as demonstrated by an increase in 2-hydroxyethidium fluorescence (Figure S7B and S7C). In Cyp1b1−/− mice, Ang II markedly increased cardiac NADPH oxidase activity (Figure S7A), which was associated with increased superoxide production (Figure S7A and S7B).

**Ang II Infusion Increased Cardiac Activity of ERK1/2, p38 MAPK, c-Src, and Akt in Female Cyp1b1−/−, but Not in Cyp1b1+/+, Mice**

Oxidative stress increases activity of several signaling molecules, including ERK1/2, p38 MAPK, c-Src, and Akt that have been implicated in Ang II–induced hypertension.15,16,19 To determine whether protection against Ang II–induced hypertension in Cyp1b1+/+ mice was associated with decreased ERK1/2, p38 MAPK, c-Src, and Akt activities, we determined cardiac activity of these signaling molecules by measuring their phosphorylation. Ang II did not alter the activities of these kinases in Cyp1b1+/+ mice, but they were increased in Cyp1b1−/− mice infused with Ang II (Figure S8A–S8D).

**Discussion**

The novel finding of this study was that increased CYP1B1 activity and expression were crucial for conferring resistance against Ang II–induced hypertension and associated pathological changes in female mice, most likely because of generation of 2-MeE2 that reduces cardiovascular oxidative stress and cardiac activities of signaling molecules ERK1/2, p38 MAPK, c-Src, and Akt.

Ang II increases SBP to a significantly greater degree in male than in female mice. Moreover, castration minimizes the effect of Ang II in males, whereas ovariectomy increases the effect of this peptide on SBP in female mice. We have previously reported that, in male rats and mice, CYP1B1 contributes to the development of Ang II–induced hypertension and associated pathophysiological changes that correlate with increased oxidative stress.2,21 However, our present study showed that, in female Cyp1b1+/− mice, the effect of Ang II to increase SBP was greater than in female Cyp1b1−/− mice; in fact, the increase in SBP produced by Ang II in female Cyp1b1+/− mice was different from that observed in male Cyp1b1+/− mice, suggesting that the lower increase in SBP produced by Ang II in female Cyp1b1+/− mice depends on CYP1B1. Supporting this conclusion was our demonstration that Ang II increased cardiac expression and activity of CYP1B1 in female Cyp1b1+/− mice. The diminished ability of Ang II to increase SBP in female Cyp1b1+/− mice was associated with decreased cardiac and vascular smooth muscle remodeling, reduced endothelial dysfunction, as indicated by improved relaxation to acetylcholine, and decreased vascular reactivity to phenylephrine and endothelin-1. Because the cardiovascular effects against Ang II were unmasked in Cyp1b1−/− female mice, CYP1B1 plays a protective role against Ang II–induced hypertension and its associated pathological changes. The possibility that protection against Ang II–induced cardiovascular remodeling, endothelial dysfunction, and increased vascular reactivity in female Cyp1b1−/− mice could also be a result of increased SBP cannot be excluded.

Our demonstration that the selective inhibitor of CYP1B1 activity, TMS, enhanced the effect of Ang II to increase SBP in Cyp1b1+/+ mice to a level similar to that observed in Cyp1b1−/− mice, which was not altered by TMS, also supports the view that protection against the increased pressor effect of Ang II depends on CYP1B1 activity in female mice. Furthermore, our finding that ovariectomy increased Ang II–induced hypertension in Cyp1b1+/+ mice, but not in Cyp1b1−/− mice, suggests that the protective effect of estrogen on Ang II–induced hypertension depends on CYP1B1 in female mice. Although Cyp1b1 gene disruption inhibited both the basal and the Ang II–induced increase in CYP1B1 activity, it enhanced Ang II–induced hypertension but did not alter basal SBP, suggesting that CYP1B1 activity is not required for maintaining basal SBP but rather for modulating the pressor effect of Ang II in female mice.

CYP1B1 is constitutively active, but it requires a substrate to generate the product(s) that attenuates Ang II–induced hypertension in female mice. Many substrates, including fatty acids, retinoids, melatonin, and steroids, are metabolized by CYP1B1,23–25 and ≥1 products generated from these substrates could modulate the cardiovascular effects of Ang II. CYP1B1 metabolizes 17-β estradiol into catecholestadiols, 2-OHE, and 4-OHE in hepatic and extrahepatic tissues. Therefore, it is possible that CYP1B1-dependent modulation of Ang II–induced hypertension in female mice is mediated by these metabolites. 4-OHE, which is known to cause cellular damage and has been implicated in estrogen carcinogenesis, increased Ang II–induced hypertension in Cyp1b1+/+ mice and did not alter the effect of Ang II to increase SBP in Cyp1b1−/− mice. However, 2-OHE did not alter the hypertensive effect of Ang II in Cyp1b1+/+ mice but minimized the increase in Ang II–induced hypertension in Cyp1b1−/− mice. Although 2-OHE is the preferential rat and mouse CYP1B1-derived 17-β estradiol metabolite,30 it is also formed in human tissues including cardiovascular tissues. 2-OHE in VSMCs, heart, kidney, and mesenteric blood vessels is subsequently converted into 2-MeE2 by COMT. Inhibitors of CYP1A1, CYP1B1, and COMT attenuate VSMC migration, proliferation, and collagen synthesis stimulated by serum. Although 2-OHE is formed mainly by the CYP1A family and to a lesser degree by CYP1B1, 21 we previously detected a significant amount of CYP1A1 in cardiovascular tissues. Moreover, the effect of Ang II to increase SBP is not different in male Cyp1l1+/+ and Cyp1l1−/− mice (B.L. Jennings et al, unpublished data, 2011). Therefore, 2-MeE2 most likely mediates the effect of 2-OHE to suppress Ang II–induced hypertension in Cyp1b1−/− mice. Supporting this view was our finding that 2-MeE2 minimized Ang II–induced hypertension in Cyp1b1−/− mice. 17-β estradiol and 2-OHE exert an anti-mitogenic effect in VSMCs from wild-type, but not COMT knockout, mice. These observations and the demonstration that 2-MeE2 exerts protective effects against ventricular
hypoertrophy, cardiac fibrosis, endothelial dysfunction, and vasoconstriction, as well as renal protective effects, suggest that 2-MeE2 mediates protection against Ang II–induced hypertension and associated pathophysiological changes in female mice. Supporting this view was our finding that Ang II increased plasma levels of 2-MeE2 in Cyp1b1+/− mice and that both basal and Ang II–induced increase in plasma 2-MeE2 levels were reduced in Cyp1b1−/− mice. The increase in plasma levels of 2-MeE2 produced by Ang II in Cyp1b1+/− mice could be because of an increase in CYP1B1 activity and increased metabolism of 2-OHE by COMT to 2-MeE2. Ang II has been shown to increase renal expression of COMT. Whether the Ang II–induced increase in hypertension seen in our study also involved increased activity and expression of COMT remains to be determined. Moreover, the significance of a small amount of 2-MeE2 observed in plasma of Cyp1b1−/− mice, which could be generated by CYP1A2, is not known. Because Cyp1b1 gene disruption and treatment with TMS or ovariectomy in Cyp1b1+/− mice and 2-OHE or 2-MeE2 in Cyp1b1+/− or Cyp1b1−/− mice did not alter basal SBP, it seems that 2-MeE2 modulates Ang II–induced hypertension by acting as a permissive factor. 2-MeE2 also minimizes hypertension and associated coronary vascular remodeling in male and ovariectomized spontaneously hypertensive rats and prevents DOCA-salt–induced hypertension in male rats.

The mechanism(s) by which 2-OHE and 2-MeE2 exert their protective cardiovascular effects seems to be independent of the classical genomic estrogen receptor. The protection against Ang II–induced hypertension in female rodents has been attributed to downregulation of Ang II type 1 receptors (AT₁) receptors and upregulation of Ang II type 2 receptors (AT₂) receptors, increased expression of angiotensin-converting enzyme-2 and increased levels of Ang-(1–7). Therefore, it is possible that the estrogen metabolites of CYP1B1 by regulating these components of the renin–Ang II system might contribute to protection against Ang II–induced hypertension and associated pathophysiological changes in Cyp1b1+/− mice. Supporting this view, it has been shown that 2-MeE2 by binding to GPR30 (G protein-coupled receptor 30) downregulates AT₁ receptor expression in the rat liver epithelial cell line. 2-OHE and 2-MeE2 also possess antioxidant properties, and ROS have been shown to contribute to several biological actions of Ang II, including VSMC growth and hypertension. A critical review of the literature indicates that ROS contribute to various models of hypertension in males but do not affect BP in females. We have reported previously that ROS generated via CYP1B1 contribute to Ang II–induced hypertension in male mice. However, estrogen upregulates manganese-superoxide dismutase and extracellular superoxide dismutase and decreases ROS production in VSMCs and inhibits Ang II–induced increase in NADPH oxidase expression in endothelial cells. Therefore, the mechanism by which 2-MeE2 suppresses Ang II–induced hypertension in female mice could be because of decreased production of ROS, probably because of alterations in the ratio of AT₁/AT₂ receptors and angiotensin-converting enzyme-2 expression. In the present study, Ang II did not significantly increase cardiac NADPH oxidase activity and produced a minimal increase in ROS production in female Cyp1b1+/− mice; however, it markedly increased them in Cyp1b1−/− mice.

The effect of peripheral Ang II to increase BP is mediated by increased oxidative stress in the subfornical organ of the brain. Because estrogen inhibits Ang II–induced ROS production in slices of the subfornical organ, it has been proposed that estrogen may inhibit the central actions of Ang II by decreasing ROS production in the subfornical organ. The central effect of estrogen also contributes to diminished effect of aldosterone-salt–induced hypertension in female compared with that in male rats, which is mediated via estrogen receptor-β and probably because of reduced oxidative stress in the paraventricular nucleus and rostroventrolateral medulla. Whether 2-MeE2 formed in peripheral tissues acts in these areas of the brain or is generated locally in circumventricular organs from brain-derived estrogen and CYP1B1 to suppress Ang II–induced ROS production and to contribute to protection against Ang II–induced and aldosterone-salt–induced hypertension in females remains to be determined. In preliminary experiments, we found that infusing Ang II results in increased CYP1B1 activity and decreased NADPH oxidase activity in the brain of female Cyp1b1+/− mice compared with that in Cyp1b1−/− mice (B.L. Jennings et al, unpublished data, 2013). Further studies will determine the site of relationship between CYP1B1 and NADPH oxidase in the brain. Moreover, in view of the critical role of innate and adaptive immune cell systems and inflammation in Ang II–induced hypertension and the findings that estrogen and 2-MeE2 inhibit activation of immune cells and inflammation suggest that these inhibitory actions of estrogen and 2-MeE2 might contribute to the diminished effect of Ang II to increase BP and associated cardiovascular pathological changes in female mice.

ROS generated by Ang II in the cardiovascular system are known to activate various signaling molecules, including ERK1/2, p38 MAPK, c-Src, and Akt that have been implicated in ≥1 biological actions of Ang II, including VSMC growth and hypertension in male mice. Therefore, it is possible that the decreased activity of ≥1 of these signaling molecules consequent to low oxidative stress by 2-MeE2 could contribute to the lower pressor effect of Ang II in female mice. Supporting this view is our observation that Ang II increased cardiac activity of ERK1/2, p38 MAPK, c-Src, and Akt in Cyp1b1+/−, but not in Cyp1b1−/− mice. 2-MeE2 has been shown to inhibit the activity of several signaling molecules including ERK1/2 and Akt in VSMCs. However, we cannot exclude the possibility that the effect of 2-MeE2 to decrease the activity of these signaling molecules by Ang II in vivo is because of a decrease in SBP or alteration in the ratio of AT₁/AT₂ receptors and angiotensin-converting enzyme-2 expression. In conclusion, this study demonstrates that protection against Ang II–induced hypertension and associated cardiovascular pathophysiological changes in female mice depends on CYPB1 activity. The attenuation of Ang II–induced hypertension in female mice, along with decreased Ang II–induced NADPH oxidase activity, ROS production, and activity of signaling molecules implicated in hypertension, including ERK1/2, p38 MAPK, c-Src, and Akt, is most likely because of CYPB1-mediated estrogen metabolism and downstream production of the estrogen metabolite, 2-MeE2.
Perspectives

Sex differences in BP in humans and various models of experimental hypertension have been attributed to female and male sex hormones. The present study provides the first evidence that increased CYP1B1 activity in female mice is critical in maintaining decreased Ang II–induced hypertension and associated cardiovascular pathophysiological effects, most likely because of increased metabolism of 17β-estradiol to 2-OHE, which is subsequently metabolized by COMT to 2-MeE2, that reduces oxidative stress and protects the cardiovascular system against the deleterious effects of Ang II. Therefore, 2-MeE2 could be useful in treating hypertension in men and in postmenopausal females and in women with polycystic ovary disease with high testosterone levels without producing estrogenic effects. Moreover, an important implication of this study is that inhibitors of CYP1B1 activity could be detrimental to the cardiovascular system and will promote hypertension and associated pathogenesis in females.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- Demonstration that increased cytochrome P450 (CYP) 1B1 activity is critical for maintaining the diminished effect of increased systolic blood pressure and oxidative stress caused by angiotensin II (Ang II) that result in pathophysiological changes in female mice.

- 17β-estradiol–derived metabolite, 2-hydroxyestradiol, and its catechol-O-methyltransferase (COMT)–derived metabolite, 2-methoxyestradiol (2-MeE2), inhibit the increase in Ang II–induced hypertension in Cyp1b1−/− female mice.

- Ang II increases plasma levels of 2-MeE2 in Cyp1b1−/−, but not in Cyp1b1+/−, female mice.

**What Is Relevant?**

- This study furthers our understanding of the mechanism whereby increased CYP1B1 activity via generation of the 17β-estradiol metabolite, 2-MeE2, plays a critical role in minimizing Ang II–induced hypertension and associated pathogenesis in female mice.

- Moreover, it has important implications for treating hypertension in postmenopausal women and in those with polycystic ovary disease. Notably, agents that inhibit CYP1B1 and COMT activity may exacerbate hypertension and cardiovascular pathology in females.

**Summary**

The present study shows that CYP1B1 gene disruption, inhibition of CYP1B1 activity with 2,3′,4,5′-tetramethoxystilbene, or ovariectomy in female Cyp1b1−/− mice increased Ang II–induced hypertension. The CYP1B1-derived 17β-estradiol metabolite, 2-hydroxyestradiol, and its COMT-derived metabolite, 2-MeE2, minimized Ang II–induced hypertension in female Cyp1b1−/− mice. Moreover, Ang II increased plasma levels of 2-MeE2 in Cyp1b1−/−, but not in Cyp1b1+/−, female mice. Ang II also increased oxidative stress and activities of extracellular signal–regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase, c-Src, and Akt in Cyp1b1−/− mice, which were minimized in Cyp1b1+/− mice. These data suggest that CYP1B1, via generation of 17β-estradiol metabolite, 2-MeE2, plays a crucial role in protecting female mice against Ang II–induced hypertension and associated pathogenesis by reducing oxidative stress and activity of ±1 signaling molecules.
Estrogen Metabolism by Cytochrome P450 1B1 Modulates the Hypertensive Effect of Angiotensin II in Female Mice

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/content/64/3/e2.full.pdf

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/04/29/HYPERTENSIONAHA.114.03275.DC1
In the online-only Data Supplement to the *Hypertension* article by Jennings et al (Jennings BL, George LW, Pingili AK, Khan NS, Estes AM, Fang XR, Gonzalez FJ, Malik KU. Estrogen Metabolism by Cytochrome P450 1B1 Modulates the Hypertensive Effect of Angiotensin II in Female Mice. *Hypertension*. 2014;64:134–140), a correction was needed.

The dosage information for 2-hydroxyestradiol, 4-hydroxyestradiol, and 2-methoxyestradiol was inadvertently left out. This information has been added on page 2 of the Data Supplement, under “Treatment groups,” to read:

c) 2-Hydroxyestradiol (2-OHE) and 4-OHE - Mice were infused with Ang II as described above. One group was injected i.p., daily with 2-OHE or 4-OHE, (1.5 mg/kg) the major metabolites of estrogen metabolism by CYP1B1 (5, 6). A separate group was injected with vehicle (DMSO, 20 µl).

d) 2-MeE2 - Mice were infused with Ang II as described above. One group of animals was injected i.p. daily with 2-MeOHE, (1.5 mg/kg) the resultant compound of COMT metabolism of 2-OHE (5). A separate group of animals was injected with vehicle (DMSO, 20 µl).

The authors apologize for the error.

This correction has been made to the online-only Data Supplement, which is available at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.114.03275/-/DC1.
ESTROGEN METABOLISM BY CYTOCHROME P450 1B1 MODULATES THE HYPERTENSIVE EFFECT OF ANGIOTENSIN II IN FEMALE MICE

Brett L. Jennings¹, L. Watson George¹, Ajeeth K. Pingili¹, Nayaab S. Khan¹, Anne M. Estes¹, Xiao R. Fang¹, Frank J. Gonzalez², and Kafait U. Malik¹

¹Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, and ²Laboratory of Metabolism, National Cancer Institute, Bethesda, MD 20892

Short Title: CYP1B1 protects female mice against hypertension

Corresponding Author: Kafait U. Malik, D.Sc., Ph.D., Professor of Pharmacology, Department of Pharmacology, College of Medicine, 874 Union Avenue, University of Tennessee Health Science Center, Memphis, TN 38163
E-mail: kmalik@uthsc.edu
Ph: (901)-448-6075; Fax: (901)-448-7206
Materials

Angiotensin (Ang) II was purchased from Bachem (Torrance, CA) and dihydroethidium (DHE) was purchased from Invitrogen™ (Carlsbad, CA). 2,3',4,5'-tetramethoxystilbene (TMS) was purchased from Cayman Chemical (Ann Arbor, MI). The cytochrome P450 (CYP) 1B1 antibody was purchased from BD Biosciences (Franklin Lakes, NJ), and antibodies against α-smooth muscle-specific actin, ERK1/2, p38 MAPK, c-Src, and Akt were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The primary phospho ERK1/2, phospho p38 MAPK, phospho c-Src, and phospho Akt antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). 2-Methoxyestradiol (2-MeE2) was purchased from Steraloids (Newport, RI). All other chemicals were purchased from Sigma (St. Louis, MO).

Animals

All experiments were performed according to the protocols approved by our Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Cyp1b1−/− mice were generated at the National Cancer Institute, transferred to the University of Tennessee, validated as previously described (1), backcrossed 10 generations to a C57BL/6J background, and then brother-sister mated to generate a homozygous line. Female C57BL/6J (Cyp1b1+/+) (Jackson Laboratory, Bar Harbor, ME) mice were used as control animals throughout the experiment for comparison of drug effects. All animals were 20 to 30 g and approximately 8 weeks of age at the beginning of the experiment. The genotype of Cyp1b1+/+ and Cyp1b1−/− mice was routinely assessed by PCR, as previously described (2). For PCR analysis, genomic DNA was obtained from tail snips using the Wizard® SV Genomic DNA Purification System (Promega, Madison, WI), according to the manufacturer’s instructions.

Ang II-induced hypertension in Cyp1b1+/+ and Cyp1b1−/− mice

Mice were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (50 mg/kg, i.p.), and miniosmotic pumps were implanted subcutaneously to infuse Ang II (700 ng/kg/min) or vehicle (0.9% saline) for 14 days (Alzet®, Cupertino, CA; model 1002), and blood pressure was measured twice weekly using a noninvasive tail cuff method (Kent Scientific; model XBP 1000). Prior to implantation of the miniosmotic pump, mice were acclimated to the blood pressure measuring device for 1 week.

Treatment groups

a) TMS - Mice were infused with Ang II as described above, and one group of animals was injected i.p., every 3rd day with the selective CYP1B1 inhibitor, TMS, (300 µg/kg) (3) or vehicle (DMSO, 20 µl).

b) Ovariectomy (OVX) - Mice underwent OVX as previously described (4). Following OVX, animals were left to recover for 7-10 days to ensure removal of all endogenous estrogen prior to infusion of Ang II or vehicle.

c) 2-Hydroxyestradiol (2-OHE) and 4-OHE - Mice were infused with Ang II as described above. One group was injected i.p., daily with 2-OHE or 4-OHE, (1.5 mg/kg) the major metabolites of estrogen metabolism by CYP1B1 (5, 6). A separate group was injected with vehicle (DMSO, 20 µl).

d) 2-MeE2 - Mice were infused with Ang II as described above. One group of animals was injected i.p. daily with 2-MeOHE, (1.5 mg/kg) the resultant compound of COMT
metabolism of 2-OHE (5). A separate group of animals was injected with vehicle (DMSO, 20 µl).

**Cyp1b1 activity assay**

*Cyp1b1* activity was determined using the P450-Glo™ Assay Kit (Promega) as described (7, 8). At the end of the experiment, animals were anesthetized as described above, the left ventricle was punctured, and blood was flushed out by perfusion with cold saline (3 min). The heart was dissected free, cleaned of surrounding tissue, snap frozen in liquid N₂, and stored at -80°C until use. Tissue samples were homogenized (2 x 3 min) in ice-cold 100 mmol/L potassium phosphate buffer (pH 7.4) using a TissueLyser II (QIAGEN, Valencia, CA). Following homogenization, samples were centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was collected and stored at -80°C until further use. Protein content in the samples was determined by the Bradford method, and 500 µg of protein was added to a reaction mixture containing 20 µmol/L L-CEE substrate and 100 mmol/L potassium phosphate buffer (pH 7.4) and incubated at 37°C for 10 min. 100 µmol/L of NADPH (final concentration) was added, and the solution was further incubated at 37°C for 45 min. Finally, a 1:1 volume of luciferin detection reagent was added to the samples, and they were mixed for 10 sec after which they were incubated at room temperature for 20 min. Luminescence was measured using a luminometer (Turner Designs, Sunny Vale, CA; model TD-20/20). Potassium phosphate buffer was used as a blank and subtracted from each reading; activity was expressed as relative luminescence units (RLU).

**Western blot analysis**

Mice were anesthetized and the heart removed as described above. Tissue samples were homogenized in lysis buffer, and protein content was determined by the Bradford method. Approximately 10 µg of protein was loaded and resolved on 8% SDS-polyacrylamide gels and processed for western blot analysis as described (9). Blots were probed with different primary and corresponding secondary antibodies, and intensity of the bands was measured with ImageJ 1.42 software (http://rsb.info.nih.gov/nih-image; National Institutes of Health). Protein expression of CYP1B1 was calculated as a ratio of expression of α-actin, and activities of signaling molecules (ERK1/2, p38 MAPK, c-Src, and Akt) were calculated as a ratio of phosphorylated protein to their corresponding non-phosphorylated levels.

**Measurement of cardiac hypertrophy**

*Heart weight:body weight ratio:* At the completion of the experiment and prior to sacrifice, animals were weighed. Immediately following sacrifice, hearts were removed and their wet weight determined. The ratio of heart weight (mg) to body weight (g) was calculated and used as a measure of cardiac hypertrophy.

**Immunohistochemical analysis**

At the completion of the experiments, mice were anesthetized as described above, the carotid artery was cannulated, and the animals perfused with saline (3 min). The heart was dissected free and placed in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek USA, Inc., Torrance, CA) and frozen at -80°C. Cardiac sections (5 µm) were cut using a cryostat (Leica Microsystems, Bannockburn, IL; model CM1850) and processed for α-smooth muscle actin (myofibroblasts) as described previously (10) or stained with Masson’s trichrome for...
collagen, according to the manufacturer’s instructions (Sigma). Sections were viewed with an Olympus® inverted system microscope (Olympus America, Inc., Melville, NY; model BX41), photographed using a SPOT™ Insight™ digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI; model Insight 2MP Firewire), and images were analyzed using ImageJ 1.42.

**Measurement of vascular function and structural changes**

*Vascular reactivity:* Following anesthesia (described above), the aorta was quickly dissected free, cleaned of surrounding tissue, and approximately 2 mm rings were mounted in a dual-wire myograph system (Danish Myo Technology, Aarhus, Denmark; model 610M). Vessels were continuously bathed in Krebs buffer (composition in mmol/L: 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 11.1 glucose, 2.5 CaCl₂.2H₂O) at 37°C, which was gassed with 95% O₂ and 5% CO₂ to maintain the pH at 7.4. An initial tension of 5 mN was placed on the vessels and allowed to equilibrate for approximately 30 min. To confirm the viability of the vessels, they were initially tested for constriction to 60 mmol/L KCl and then washed three times with fresh Krebs buffer. Cumulative concentration response curves to phenylephrine (PE) and endothelin-1 (ET-1) were obtained and responses measured as force of contraction in mN.

*Endothelium-dependent and -independent vasodilation:* Endothelial function was examined by constricting the aorta with the concentration of PE that evoked a maximal response followed by addition of increasing concentrations of acetylcholine (ACh). Changes in the response of vessels to ACh were measured and presented as a percentage of the PE-induced constriction. Endothelium-independent vasodilation was studied by constricting the aorta with the concentration of PE that evoked a maximal response followed by addition of increasing concentrations of sodium nitroprusside (SNP). Changes in the response of vessels to SNP were measured and presented as a percentage of the PE-induced constriction.

*Measurement of media:lumen ratio:* Following anesthesia (described above), the aorta was dissected free, cleaned of surrounding tissue, placed in O.C.T. compound, and frozen at -80°C. 10 µm sections were cut using a cryostat (Leica Microsystems, Bannockburn, IL; model CM1850) and stained with standard hematoxylin and eosin. Sections were viewed and photographed as described above and the media thickness and lumen diameter were quantified using ImageJ.

**Measurement of ROS production**

To measure ROS production, vascular and cardiac sections were exposed to dihydroethidium (DHE), following the previously described and validated method (11). Fresh, unfixed tissue samples were placed in O.C.T. compound and frozen at -80°C. Tissue segments were cut into 30 µm sections using a cryostat and placed on a glass slide. Sections were incubated in PBS for 30 min at 37°C, and then DHE, 2 µmol/L (11, 12) was topically applied. Cover slips were applied, and sections were further incubated at 37°C in a light-protected humidified chamber for 45 min. Sections were then rinsed in PBS, and fluorescence was detected using a 585-nm filter using an Olympus® inverted system microscope (Olympus America Inc., model IX50). Images were photographed with an Olympus® digital camera (Olympus America Inc., model DP71) and analyzed using ImageJ 1.42.

**Measurement of NADPH oxidase activity**

NADPH oxidase activity was determined in tissue homogenates by measuring lucigenin (N,N’-dimethyl-9,9’-biaacidinium dinitrate)-enhanced chemiluminescence, as described
previously (13), with some modifications (7). Following anesthesia (described above), the heart was isolated, cleaned of surrounding tissue, snap frozen in liquid N\textsubscript{2}, and stored at -80°C until use. Tissue samples were homogenized (2 x 3 min) in ice-cold lysis buffer containing protease inhibitors (20 mmol/l phosphate buffer, 1 mmol/l EGTA, 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 0.5 mmol/l phenylmethylsulphonylfluoride, and 150 mmol/l sucrose) using a TissueLyser II (QIAGEN). Following homogenization, samples were sonicated, centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was collected and stored at -80°C until further use. Protein content in the samples was determined by the Bradford method, and equal amounts of protein were combined 1:1 with a reaction mixture containing 5 µmol/l lucigenin (final concentration) and 100 µmol/l NADPH (final concentration). Luminescence was measured every minute for 10 min using a luminometer. Lysis buffer was used as a blank and subtracted from each reading and activity expressed as arbitrary units.

**Determination of plasma level of 2-MeE2**

*Plasma:* At the completion of the experiment, animals were anesthetized as described above, and blood was withdrawn directly from the abdominal aorta and transferred to K\textsuperscript{+}-EDTA tubes (BD Microtainer\textsuperscript{®}; BD Biosciences). Blood was centrifuged at 1,500 g for 15 min at 4°C, and the plasma was collected and stored at -80°C until further analysis.

*2-MeE2:* Plasma 2-MeE2 concentration was determined using a 2-MeE2 EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

**Statistical analysis**

Data were analyzed by one-way analysis of variance followed by Neuman-Keuls post-hoc test or Student’s t-test. The values of a minimum of three different experiments are expressed as the mean ± SEM. \( P \) values < 0.05 were considered statistically significant.
References


Table S1. Infusion of Ang II increases cardiac hypertrophy to a greater degree in female *Cyp1b1*<sup>−/−</sup> mice than in *Cyp1b1*<sup>+/+</sup> mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>Cyp1b1</em>&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th><em>Cyp1b1</em>&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Ang II</td>
</tr>
<tr>
<td>Body Weight (BW) (g)</td>
<td>20.1 ± 0.3</td>
<td>20.6 ± 1.2</td>
</tr>
<tr>
<td>Heart Weight (HW) (mg)</td>
<td>116.0 ± 6.7</td>
<td>134.0 ± 8.1*</td>
</tr>
<tr>
<td>HW (mg):BW (g)</td>
<td>5.75 ± 0.24</td>
<td>6.50 ± 0.24*</td>
</tr>
</tbody>
</table>

*Cyp1b1*<sup>+/+</sup> and *Cyp1b1*<sup>−/−</sup> mice were infused with vehicle or Ang II for 2 weeks as described in Methods. Heart weight:body weight ratio, an indicator of cardiac hypertrophy, was calculated. *P* < 0.05 vehicle vs. corresponding value from Ang II infused animal; †*P* < 0.05 *Cyp1b1*<sup>+/+</sup> Ang II vs. *Cyp1b1*<sup>−/−</sup> Ang II (n = 5 for all experiments and data are expressed as mean ± SEM).
Table S2. Infusion of Ang II increases media:lumen ratio, an indicator of vascular remodeling in female Cyp1b1<sup>−/−</sup>, but not Cyp1b1<sup>+/+</sup> mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cyp1b1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Cyp1b1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4.61 ± 0.90</td>
<td>5.18 ± 0.47</td>
</tr>
<tr>
<td>Ang II</td>
<td>5.85 ± 0.21</td>
<td>8.63 ± 0.42&lt;sup&gt;∗†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Cyp1b1<sup>+/+</sup> and Cyp1b1<sup>−/−</sup> mice were infused with vehicle or Ang II for 2 weeks. The media:lumen ratio of the aorta from animals in each of the different treatment groups was calculated. <sup>∗</sup>P < 0.05 vehicle vs. corresponding value from Ang II infused animal; <sup>†</sup>P < 0.05 Cyp1b1<sup>+/+</sup> Ang II vs. Cyp1b1<sup>−/−</sup> Ang II (n = 5 for all experiments and data are expressed as mean ± SEM).
Ang II-induced hypertension is associated with cardiac fibrosis in female \textit{Cyp1b1}^{+/−} mice. \textit{Cyp1b1}^{+/−} and \textit{Cyp1b1}^{−/−} mice were infused with vehicle or Ang II for 2 weeks as described in Methods. At the completion of the experiments, cardiac tissue was removed and processed for immunohistochemistry. (A) α-Smooth muscle actin (α-SMA)-positive myofibroblasts are present in the myocardium of Ang II-infused \textit{Cyp1b1}^{−/−} mice (arrows); these myofibroblasts are absent in vehicle-infused mice and Ang II infused \textit{Cyp1b1}^{+/−} mice. (B) Masson’s trichrome staining revealed increased collagen deposition (intense blue staining) in the perivascular space of Ang II-infused \textit{Cyp1b1}^{−/−} mice; however, this staining was reduced in \textit{Cyp1b1}^{+/−} mice and absent from vehicle-infused mice. Photomicrographs are representative images of at least three animals from each experimental group.
Infusion of Ang II is associated with increased vascular reactivity, endothelial dysfunction, and oxidative stress in Cyp1b1⁻/⁻, but not Cyp1b1⁺/⁺ mice. Cyp1b1⁺/⁺ and Cyp1b1⁻/⁻ mice were infused with vehicle or Ang II for 2 weeks as described in Methods. Vascular reactivity, endothelial function, and oxidative stress were measured. (A, B) The response of the aorta from vehicle- and Ang II-infused Cyp1b1⁺/⁺ and Cyp1b1⁻/⁻ mice to increasing concentrations of phenylephrine (PE) and endothelin-1 (ET-1), respectively. (C, D) The response of the aorta from vehicle- and Ang II-infused Cyp1b1⁺/⁺ and Cyp1b1⁻/⁻ mice to increasing concentrations of acetylcholine (ACh; endothelium-dependent relaxation) and sodium nitroprusside (SNP; endothelium-independent relaxation), respectively. (E) Representative photomicrographs of aorta from mice in each of the different treatment groups following incubation with dihydroethidium; graph depicts quantified data. *P < 0.05 vehicle vs. corresponding value from Ang II infused animal; †P < 0.05 Cyp1b1⁺/⁺ Ang II vs. Cyp1b1⁻/⁻ Ang II (n = 5 for all experiments and data are expressed as mean ± SEM).
Inhibition of CYP1B1 activity increased the hypertensive effect of Ang II in Cyp1b1+/+ mice. Cyp1b1+/+ and Cyp1b1-/− mice were infused with Ang II or vehicle, and given i.p. injections of the selective inhibitor of CYP1B1 activity, TMS, every 3rd day for 2 weeks. (A, B) Systolic blood pressure (SBP) was measured in Cyp1b1+/+ and Cyp1b1-/− mice, respectively, by tail cuff twice weekly. *P < 0.05 vehicle vs. corresponding value from Ang II infused animal; †P < 0.05 Ang II vs. corresponding value from Ang II + TMS treated animal (n = 5 for all experiments and data are expressed as mean ± SEM).
Endogenous estrogen is required to minimize the hypertensive effect of Ang II in Cyp1b1^{+/+} mice. Cyp1b1^{+/+} and Cyp1b1^{--} mice were sham operated or underwent ovariectomy (OVX), and were subsequently infused with Ang II for 2 weeks. (A, B) Systolic blood pressure (SBP) was measured in Cyp1b1^{+/+} and Cyp1b1^{--} mice, respectively, by tail cuff twice weekly. *P < 0.05 sham vs. corresponding value from Ang II infused animal; †P < 0.05 Ang II vs. corresponding value from Ang II + OVX animal (n = 5 for all experiments and data are expressed as mean ± SEM).
2-OHE, a CYP1B1-derived metabolite of 17-β estradiol, minimized Ang II-induced hypertension in Cyp1b1−/− mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with Ang II or vehicle, and given i.p. injections of the CYP1B1-derived metabolite of estrogen, 2-hydroxyestradiol (2-OHE), daily for 2 weeks. (A, B) Systolic blood pressure (SBP) was measured in Cyp1b1+/+ and Cyp1b1−/− mice, respectively, by tail cuff twice weekly. *P < 0.05 vehicle vs. corresponding value from Ang II infused animal; †P < 0.05 Ang II vs. corresponding value from Ang II + 2-OHE treated animal (n = 5 for all experiments and data are expressed as mean ± SEM).
4-OHE, a CYP1B1-derived metabolite of estrogen, enhances Ang II-induced hypertension in Cyp1b1+/+ mice, by decreasing cardiac CYP1B1 activity and increasing cardiac NADPH oxidase activity. Cyp1b1+/+ and Cyp1b1−/− mice were infused with Ang II or vehicle, and given i.p. injections of the CYP1B1-derived metabolite of estrogen, 4-hydroxyestraiol (4-OHE), daily for 2 weeks. (A, B) Systolic blood pressure (SBP) was measured in Cyp1b1+/+ and Cyp1b1−/− mice, respectively, by tail cuff twice weekly. * P < 0.05 vehicle vs. corresponding value from Ang II infused animal; † P < 0.05 Ang II vs. corresponding value from Ang II + 4-OHE treated animal (n = 5 for all experiments and data are expressed as mean ± SEM).
Ang II infusion increased cardiac NADPH oxidase activity and superoxide production to a greater degree in Cyp1b1⁻/⁻ mice compared to Cyp1b1⁺/⁺ mice. Cyp1b1⁺/⁺ and Cyp1b1⁻/⁻ mice were infused with vehicle or Ang II for 2 weeks. (A) NADPH oxidase activity was measured in cardiac homogenates. (B) Superoxide production, as determined by fluorescence intensity of 2-hydroxyethidium (2-OHE), was measured in the heart. Representative photomicrographs of hearts from mice in each of the different treatment groups following incubation with dihydroethidium; (C) graph depicts quantified data. *P < 0.05 vehicle vs. corresponding value from Ang II infused animal; †P < 0.05 Cyp1b1⁺/⁺ Ang II vs. Cyp1b1⁻/⁻ Ang II (n = 5 for all experiments and data are expressed as mean ± SEM).
Ang II infusion increased cardiac activities of ERK1/2, p38 MAPK, c-Src, and Akt in Cyp1b1\textsuperscript{+/+}, but not Cyp1b1\textsuperscript{−/−} mice. Cyp1b1\textsuperscript{+/+} and Cyp1b1\textsuperscript{−/−} mice were infused with vehicle or Ang II for 2 weeks as described in Methods. Activities of ERK1/2 (44/42 kDa) (A), p38 MAPK (38 kDa) (B), c-Src (72 kDa) (C), and Akt (60 kDa) (D), as determined by phosphorylation of these kinases, were measured in cardiac homogenates by western blot, using approximately 10 µg of protein for loading. Corresponding non-phospho antibodies were used as loading controls and intensity of the bands was measured using ImageJ. *P < 0.05 vehicle vs. corresponding value from Ang II infused animal; †P < 0.05 Cyp1b1\textsuperscript{+/+} Ang II vs. Cyp1b1\textsuperscript{−/−} Ang II (n = 3 for all experiments and data are expressed as mean ± SEM).