Cytochrome P450 1B1 Contributes to Renal Dysfunction and Damage Caused by Angiotensin II in Mice


Abstract—Cytochrome P450 1B1 contributes to the development of angiotensin II–induced hypertension and associated cardiovascular pathophysiology. In view of the critical role of angiotensin II in the kidney, as well as in salt and water homeostasis, and blood pressure regulation, we determined the contribution of cytochrome P450 1B1 to renal dysfunction and injury associated with angiotensin II–induced hypertension in male Cyp1b1+/+ and Cyp1b1−/− mice. Angiotensin II infusion (700 ng/kg per minute) given by miniosmotic pumps for 13 and 28 days increased systolic blood pressure in Cyp1b1+/+ mice; this increase was significantly reduced in Cyp1b1−/− mice. Angiotensin II increased renal Cyp1b1 activity, vascular resistance, and reactivity to vasoconstrictor agents and caused endothelial dysfunction in Cyp1b1+/+ but not Cyp1b1−/− mice. Angiotensin II increased water consumption and urine output, decreased urine osmolality, increased urinary Na⁺ and K⁺ excretion, and caused proteinuria and albuminuria in Cyp1b1+/+ mice that was diminished in Cyp1b1−/− mice. Infusion of angiotensin II for 28 but not 13 days caused renal fibrosis, tubular damage, and inflammation in Cyp1b1+/+ mice, which was minimized in Cyp1b1−/− mice. Angiotensin II increased levels of 12- and 20-hydroxyeicosatetraenoic acids; reactive oxygen species; and activity of NADPH oxidase, extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and c-Src in the kidneys of Cyp1b1+/+ but not Cyp1b1−/− mice. These data suggest that increased thirst, renal dysfunction, and injury and inflammation associated with angiotensin II–induced hypertension in mice depend on cytochrome P450 1B1 activity, thus indicating that cytochrome P450 1B1 could serve as a novel target for treating renal disease and hypertension. (Hypertension. 2012;59:348-354.) ● Online Data Supplement

Key Words: angiotensin II ■ CYP1B1 ■ Cyp1b1−/− mice ■ renal function ■ oxidative stress

Angiotensin II (Ang II) increases vascular tone, stimulates aldosterone synthesis, and acts directly on renal tubules promoting salt and water reabsorption. Ang II also exerts central actions and increases activity of the sympathetic nervous system; stimulates vasopressin secretion and thirst; and promotes cardiovascular hypertrophy, inflammation, generation of reactive oxygen species (ROS), endothelial dysfunction, and development of hypertension. Ang II activates cytosolic phospholipase A₂ and releases arachidonic acid (AA) from tissue phospholipids. AA metabolites produced through the lipoxigenase (12-hydroxyeicosatetraenoic acid [HETE]) and cytochrome P450 (CYP) 4A (20-HETE) pathways have been implicated in the actions of Ang II to promote vascular smooth muscle cell (VSMC) growth. Moreover, AA and 20-HETE increase ROS production in vascular cells, and 20-HETE mediates Ang II–induced renal vasoconstriction. CYP4A contributes to various models of experimental hypertension. However, Cyp1b1 that is expressed in cardiovascular tissues can also metabolize AA into HETEs in vitro. Ang II and AA stimulate VSMC migration, proliferation, and hypertrophy by generating ROS via CYP1B1. Cyp1b1 also contributes to the development and maintenance of Ang II–induced hypertension. In the kidney, Ang II plays an important role in regulating blood pressure (BP), and Ang II type 1A receptor gene deletion in the kidney or extrarenal tissues lowers BP to similar degrees. Moreover, Ang II–induced hypertension results in increased renal oxidative stress and end organ damage. The present study was conducted to determine the contribution of Cyp1b1 and underlying mechanism(s) involved in kidney dysfunction and injury associated with hypertension caused by Ang II.

Methods

Please see the online Data Supplement at http://hyper.aha.org.
Results

Cyp1b1 Gene Disruption Minimized the Hypertensive Effect of Ang II in Mice

Basal BPs were not different between Cyp1b1+/+ and Cyp1b1−/− mice; however, infusion of Ang II (700 ng/kg per minute) for 13 days increased systolic BP (Figure S1A), diastolic BP (Figure S1B), and mean arterial pressure (Figure S1C) in Cyp1b1+/+ mice; these increases were reduced in Cyp1b1−/− mice (Figure S1A through S1C, respectively). In Cyp1b1+/+ mice infused with Ang II for 28 days, systolic BP, diastolic BP, and mean arterial pressure increased for the first 13 days of infusion and then plateaued; this increase was significantly less in Cyp1b1−/− mice (Figure S2A through S2C, respectively). Infusion of vehicle (0.9% saline) for 13 or 28 days did not alter systolic BP, diastolic BP, or mean arterial pressure in either Cyp1b1+/+ or Cyp1b1−/− mice (Figures S1 and S2, respectively).

Ang II–Induced Hypertension Is Associated With Increased Renal Cyp1b1 Activity But Not Expression in Mice

Ang II infusion for 13 or 28 days increased Cyp1b1 activity compared with vehicle without altering its levels in the kidney of Cyp1b1+/+ mice (Figure S3). Cyp1b1 gene disruption and/or 13 days of Ang II infusion had no effect on protein expression of other AA metabolizing enzymes in kidney homogenates from Cyp1b1+/+ and Cyp1b1−/− mice (Figure S4A through S4G). Moreover, Cyp1b1 gene disruption and/or Ang II infusion for 28 days did not alter expression of AT1R protein in Cyp1b1+/+ or Cyp1b1−/− mice kidneys (Figure S4H).

Ang II–Induced Hypertension Is Associated With Increased Thirst and Renal Dysfunction in Cyp1b1+/+ But Not Cyp1b1−/− Mice

Basal food and water intake and urine output were not different between Cyp1b1+/+ and Cyp1b1−/− mice (Table). Urine analysis for sodium and potassium excretion, osmolality, proteinuria, and albuminuria from vehicle-treated animals showed no differences between Cyp1b1+/+ and Cyp1b1−/− mice (Table). However, after 28 days of Ang II infusion, an increase in 24-hour water consumption and urine output was observed in Cyp1b1+/+ mice, which was reduced in Cyp1b1−/− mice (Table). Infusion of Ang II for 28 days decreased urine osmolality and increased urinary excretion of Na+ and K+ and increased proteinuria and albuminuria to a greater degree in Cyp1b1+/+ than in Cyp1b1−/− mice (Table). Plasma levels of creatinine were increased to a greater degree in Cyp1b1+/+ than in Cyp1b1−/− mice infused with Ang II for 28 days, indicating a more pronounced decrease in glomerular filtration rate and impaired renal function in Cyp1b1+/+ than in Cyp1b1−/− mice (Table). Similar changes in the above parameters were observed in Cyp1b1+/+ mice after 13 days of Ang II infusion but were absent in Cyp1b1−/− mice (Table S1). Ang II infusion for 13 (Table S1) and 28 days (Table) increased plasma levels of aldosterone to a similar degree in both Cyp1b1+/+ and Cyp1b1−/− mice.

Ang II Infusion Increased 12- and 20-HETE Levels in the Kidneys of Cyp1b1+/+ But Not Cyp1b1−/− Mice

Basal levels of eicosanoids in kidneys of Cyp1b1+/+ and Cyp1b1−/− mice were not different (Table S2). Ang II infusion for 13 and 28 days increased levels of 12- and 20-HETE in kidneys of Cyp1b1+/+ but not Cyp1b1−/− mice (Table S2).

Infusion of Ang II Increased Renal Vascular Resistance in Cyp1b1+/+ But Not Cyp1b1−/− Mice

Basal renal vascular resistance was not different between Cyp1b1+/+ and Cyp1b1−/− mice (Table S3). Ang II infusion for 13 and 28 days was associated with increased renal vascular resistance in Cyp1b1+/+ mice but was significantly decreased in Cyp1b1−/− mice (Table S3).

Table. Cyp1b1 Gene Disruption Minimizes Renal Dysfunction Associated With 28 d of Ang II Infusion in Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Cyp1b1+/+</th>
<th>Cyp1b1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water intake, mL/24 h</td>
<td>7.50±0.36</td>
<td>13.43±1.02*</td>
<td>6.92±0.45</td>
</tr>
<tr>
<td>Urine output, mL/24 h</td>
<td>1.58±0.21</td>
<td>6.08±0.78*</td>
<td>1.20±0.16</td>
</tr>
<tr>
<td>Urinary Na+, mmol/24 h</td>
<td>0.045±0.008</td>
<td>0.349±0.053*</td>
<td>0.051±0.013</td>
</tr>
<tr>
<td>Urinary K+, mmol/24 h</td>
<td>0.27±0.06</td>
<td>1.39±0.30*</td>
<td>0.25±0.08</td>
</tr>
<tr>
<td>Osmolality, mOsmol/kg</td>
<td>2716±115</td>
<td>1281±205*</td>
<td>2630±79</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>3.80±0.46</td>
<td>10.36±1.16*</td>
<td>3.97±0.41</td>
</tr>
<tr>
<td>Albuminuria, mg/24 h</td>
<td>0.24±0.06</td>
<td>2.07±0.35*</td>
<td>0.27±0.07</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dL</td>
<td>0.070±0.006</td>
<td>0.121±0.014*</td>
<td>0.071±0.017</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/mL</td>
<td>221±92</td>
<td>719±169*</td>
<td>167±30</td>
</tr>
</tbody>
</table>

*Cyp1b1+/+ and Cyp1b1−/− mice were infused with angiotensin (Ang II) or vehicle for 28 d and placed in metabolic cages for 24 h before the end of the experiment for measurement of water intake and urine output. Urine and plasma were analyzed for the parameters listed as described in Methods. n = 4 to 6 for all experiments, and data are expressed as mean±SEM.

*P<0.05 vehicle vs corresponding value from Ang II–treated animal.
†P<0.05 Cyp1b1+/+ Ang II vs Cyp1b1−/− Ang II.
Ang II Infusion Is Associated With Increased Vascular Reactivity, Vascular Smooth Muscle Hypertrophy, and Endothelial Dysfunction in the Renal Artery of Cyp1b1+/+ But Not Cyp1b1−/− Mice

The response of the renal artery to phenylephrine (Figure S5A and S5C) and endothelin-1 (Figure S5B and S5D) was not different between vehicle-infused Cyp1b1+/+ or Cyp1b1−/− mice. Ang II infusion for 13 or 28 days increased response of the renal artery from Cyp1b1+/+ mice to phenylephrine and endothelin-1; the increased response was prevented in Cyp1b1−/− mice infused with Ang II for 13 (Figure S5A and S5B, respectively) or 28 days (Figure S5C and S5D, respectively). The increased vascular reactivity of the renal artery from Ang II–treated Cyp1b1+/+ mice correlated with an increase in the media:lumen ratio, an indicator of vascular smooth muscle hypertrophy (Table S4). Cyp1b1−/− mice infused with Ang II for 13 days showed no change in the media:lumen ratio of arterial vessels; Cyp1b1−/− mice infused with Ang II for 28 days showed a significantly reduced media:lumen ratio compared to Cyp1b1+/+ mice (Table S4).

Ang II infusion in Cyp1b1+/+ mice for 13 days was also associated with endothelial dysfunction of the renal artery, as demonstrated by decreased relaxation to acetylcholine (Figure S6A). Renal arteries from Cyp1b1+/+ mice infused with Ang II for 13 days displayed no change in relaxation to acetylcholine (Figure S6A). Endothelium-independent relaxation to sodium nitroprusside was not different in renal arteries from mice in any of the treatment groups (Figure S6B). Similar results were obtained in Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II or its vehicle for 28 days (Figure S6C and S6D, respectively).

Ang II–Induced Hypertension Is Associated With Increased ROS Production in Kidney and Renal Arteries and Renal NADPH Oxidase Activity in Cyp1b1+/+ But Not Cyp1b1−/− Mice

Infusion of Ang II for 13 and 28 days resulted in increased superoxide production in renal arteries of Cyp1b1+/+ mice, as demonstrated by increased 2-hydroxyethidium fluorescent intensity (Figure S7A and S7B, respectively). This increase was not found in Cyp1b1−/− mice infused with Ang II for 13 days (Figure S7A) and was significantly reduced in Cyp1b1−/− mice infused with Ang II for 28 days (Figure S7B). Ang II infusion for 13 days was also associated with increased superoxide production in kidney sections, specifically the glomerulus of Cyp1b1+/+ but not Cyp1b1−/− mice (Figure S8A). Correlating with the increased ROS production in the kidneys of Cyp1b1+/+ mice infused with Ang II for 13 days was an increase in NADPH oxidase activity that was absent in Cyp1b1−/− mice (Figure S8B). Similarly, 28 days of Ang II infusion caused an increase in glomerular superoxide production and renal NAPDH oxidase activity in Cyp1b1+/+ mice, both of which were significantly reduced in the kidneys of Cyp1b1−/− mice (Figure S8A and S8B, respectively).

Ang II Infusion for 4 But Not 2 Weeks Caused End Organ Damage in Mice

Cyp1b1+/+ mice that showed renal dysfunction after 13 days of Ang II infusion showed an increase in intrarenal vascular hypertrophy (Figure S9A) but no signs of renal damage, as demonstrated by a lack of interstitial fibrosis, proteinaceous cast formation, tubular dilation, or inflammation (Figure S9B through S9E, respectively). However, Cyp1b1−/− mice infused with Ang II for 28 days displayed increased intrarenal vascular hypertrophy (Figure A), interstitial fibrosis as demonstrated by increased α-smooth muscle actin staining (Figure B), tubular dilation (Figure C), proteinaceous cast formation (Figure D), and inflammation as demonstrated by increased CD-3+ cells in the glomerulus (Figure E). All of these pathophysiological changes were minimized in Cyp1b1−/− mice infused with Ang II for 28 days (Figure A through E, respectively).

Ang II–Induced Hypertension Is Associated With Increased Renal Activities of Extracellular Signal-Regulated Kinase 1/2, p38 Mitogen-Activated Protein Kinase, and c-Src in Cyp1b1+/+ But Not Cyp1b1−/− Mice

In the kidneys of Cyp1b1+/+ mice, infusion of Ang II for 13 and 28 days increased the activity of extracellular signal-regulated kinase (ERK) 1/2, p38 mitogen-activated protein kinase (MAPK), and c-Src, as measured by phosphorylation of these kinases; this increase was prevented in the kidneys of Cyp1b1−/− mice infused with Ang II for 13 days (Figure S10A through S10C, respectively) and was significantly reduced in the kidneys of Cyp1b1−/− mice infused with Ang II for 28 days (Figure S10D through S10F, respectively).

Discussion

The novel finding in the present study is that Cyp1b1 activity contributes to renal dysfunction, injury, and inflammation associated with Ang II–induced hypertension in mice, most likely via generation of 12- and 20-HETE, ROS and activation of ERK1/2, p38 MAPK, and c-Src. Infusion of Ang II (700 ng/kg per minute) for 13 and 28 days produced similar increases in systolic BP, diastolic BP, and mean arterial pressure and resulted in renal dysfunction as manifested by increased water intake, urine output, Na+ and K+ excretion, plasma creatinine (an indicator of decreased glomerular filtration rate), proteinuria, albuminuria, and decreased urine osmolality. Because the effect of Ang II to increase BP was significantly reduced but not abolished in Cyp1b1−/− mice, it appears that a component of the Ang II–induced increase in BP is independent of Cyp1b1 activity in the mice. Whether it involves any other metabolites of AA generated via enzymes other than Cyp1b1 or is attributed to its central actions remains to be determined. Our findings that renal dysfunction was prevented in Cyp1b1−/− mice infused with Ang II for 13 days and significantly diminished in Cyp1b1−/− mice infused with Ang II for 28 days suggest that Cyp1b1 contributes to the renal dysfunction associated with Ang II–induced hypertension. Moreover, the failure of Ang II to increase water intake in Cyp1b1−/− mice raises the possibility that Cyp1b1 might play a role in the central action of Ang II to stimulate thirst. The increase in urinary sodium excretion produced by Ang II infusion that has also been reported by other investigators could be attributed to increased BP that probably masks the
suggests that the aforementioned effects of Ang II were markedly diminished acetylcholine-induced but not sodium nitroprusside–induced relaxation of the renal artery. The fact that prusside–induced relaxation of the renal artery to phenylephrine and endothelin-1 in 

renal artery from

ished and that no endothelial dysfunction was found in the

dysfunction caused by Ang II. The endothelial dysfunction associated with Ang II–induced hypertension is most likely attributed to increased generation of ROS that inactivates NO.5,17,23–25 Supporting this view, infusion of Ang II increased production of ROS in the renal artery of Cyp1b1+/+ but not Cyp1b1−/− mice.

Ang II–induced hypertension is associated with renal damage.26 In the present study, infusion of Ang II for 13 days in Cyp1b1+/+ mice increased BP and produced renal electrolyte and vascular dysfunction, proteinuria, and impaired glomerular filtration rate, as indicated by increased plasma creatinine, but did not cause renal injury or inflammation. However, infusion of Ang II for 28 days caused renal injury, as observed by interstitial fibrosis because of increased α-smooth muscle actin accumulation, dilated tubules, proteinaceous cast formation, and inflammation as manifested by increased infiltration of T lymphocytes (CD-3+, CD-3− cells) in the interstitium. In Cyp1b1−/− mice, these effects of Ang II were minimized, suggesting that Cyp1b1 also contributes to renal injury and inflammation associated with Ang II–induced hypertension. The renal injury and inflammation in rats caused by Ang II (2 ng/kg per minute) with 4% NaCl in drinking water has been attributed mainly to increased renal perfusion pressure.26 In the present study, Ang II infusion for 13 days without NaCl produced a marked increase in BP and renal dysfunction without end organ damage in Cyp1b1+/+ mice. Infusion of Ang II for 28 days in our study produced a direct tubular effects of Ang II to stimulate sodium reabsorption.

Although Cyp1b1 is constitutively active in the kidney, and the rate-limiting step is most likely the release of AA, infusion of Ang II for 13 or 28 days markedly increased Cyp1b1 activity without altering its expression levels in the kidney of Cyp1b1+/+ mice. In contrast, in the kidney of Cyp1b1−/− mice, there was no Cyp1b1 expression, and, thus, Cyp1b1 activity was not altered by Ang II infusion. Although we observed no shift in Cyp1b1 bands on the Western blots in renal samples from Ang II–treated Cyp1b1+/+ mice, we cannot rule out any biochemical modification of this enzyme or involvement of some other endogenous factor(s), including increased P450 reductase and/or NADPH levels that are responsible for the increased activity.

In the present study, infusion of Ang II for 13 and 28 days also caused renal vascular hypertrophy, as demonstrated by increased media:lumen ratio of the renal artery, increased renal vascular resistance, and increased response of the renal artery to phenylephrine and endothelin-1 in Cyp1b1+/+ mice. Moreover, in Cyp1b1+/+ mice, Ang II infusion for 13 and 28 days diminished acetylcholine-induced but not sodium nitroprusside–induced relaxation of the renal artery. The fact that the aforementioned effects of Ang II were markedly diminished and that no endothelial dysfunction was found in the renal artery from Cyp1b1−/− mice infused with Ang II suggests that Cyp1b1 also contributes to renal vascular...
similar increase in BP as observed after 13 days of infusion, but additionally caused renal injury and inflammation. It appears that the addition of salt or a prolonged increase in renal perfusion pressure caused by Ang II is required for end organ damage.

Ang II and elevated renal perfusion pressure produce an independent increase in production of ROS,27,28 and ROS contributes to the development of hypertension and end organ damage.29,30 Although we cannot exclude the possibility of Ang II–induced ROS production secondary to increased renal perfusion pressure, Ang II may also exert renal actions independent of increased perfusion pressure. Ang II is known to stimulate VSMC migration, proliferation, and hypertrophy by increasing ROS production via Cyp1b1.16 In double-transgenic rats carrying the human renin and angiotensinogen genes, treatment with hydralazine plus reserpine plus hydrochlorothiazide prevents Ang II–induced increase in BP but not end organ damage, inflammation, or cellular growth in the kidney.31 Renal injury associated with Ang II plus Nω-nitro-L-arginine methyl ester–induced hypertension that is independent of elevated renal perfusion pressure has been proposed to be attributed to increased oxidative stress and inflammation caused by ischemia associated with increased renal vasoconstriction.32 The renal dysfunction caused after 13 and 28 days of Ang II infusion in Cyp1b1+/+ mice could be the result of increased production of ROS via Cyp1b1.16 In fact, Ang II infusion increased NADPH oxidase activity and ROS production, as indicated by increased 2-hydroxyethidium fluorescence in the glomeruli of the kidneys of Cyp1b1+/+ mice. This increase was abolished in Cyp1b1−/− mice infused for 13 days and significantly reduced in those infused for 28 days with Ang II. ROS produced by Ang II has been shown to promote sodium retention.33 However, in the present study, Ang II increased sodium excretion, and, as stated above, the increased water intake and BP caused by Ang II could have masked the effects of ROS to promote tubular reabsorption of sodium. Although Ang II infusion for 13 days increased ROS production, it did not cause renal injury or inflammation in Cyp1b1+/+ mice. Therefore, it appears that increased oxidative stress with a prolonged increase in BP for 28 days of Ang II infusion are required for injury and inflammation in the kidney of Cyp1b1+/+ mice. Whether these pathological changes are caused by a further decrease in NO production resulting in ischemia remains to be determined.

The mechanism of increased ROS production caused by Ang II–induced hypertension could be because of activation of NADPH oxidase by HETEs and/or eicosatrienoic acids generated by Cyp1b1 in the kidney. Cyp1b1 metabolizes AA, in vitro, into HETEs and eicosatrienoic acids,15 and 20-HETE and eicosatrienoic acids stimulate salt and water excretion and affect renal vascular tone.34–36 12-HETE and/or 20-HETE also mediate the vascular actions of Ang II or increase vascular reactivity to phenylephrine.11,37,38 In addition, these eicosanoids also increase NADPH oxidase activity or ROS production in endothelial cells,10 macrophages,39 and podocytes.40 The present demonstration that infusion of Ang II increased the levels of 12- and 20-HETE in the kidneys of Cyp1b1+/+ but not Cyp1b1−/− mice suggests that these

HETEs may contribute to renal electrolyte imbalance, proteinuria, injury, and inflammation associated with Ang II–induced hypertension by increasing ROS production and/or by direct tubular and vascular actions in the kidney. Although 20-HETE exerts prohypertensive effect by its vascular actions, it also produces an antihypertensive effect by its renal tubular effects, and the balance between these effects of 20-HETE could determine its effect on BP.35 However, in the present study, the level of 12-HETE, which produces renal vasoconstriction,41 was also increased. Moreover, there was also increased vascular ROS production, renal vascular hypertrophy, and increased vascular reactivity to vasoconstrictor agents. All of these effects could mask the renal antihypertensive effects of 20-HETE.

The increase in 12- and 20-HETE production in the kidney of Cyp1b1+/+ mice is unlikely to be because of increased expression of 12/15 lipoxigenase or CYP4A, because infusion of Ang II did not alter expression of these enzymes. In a previous study in rat VSMCs, we failed to observe an increase in either 12- or 20-HETE,42 suggesting species-specific and tissue-specific and/or in vitro versus in vivo differences in the production of these eicosanoids via Cyp1b1. Our previous observation that, in VSMCs, Ang II- and AA-induced production of ROS mediated via Cyp1b1, is independent of 12- and 20-HETE43 suggests that products of AA other than 12- and 20-HETE generated via Cyp1b1 could stimulate ROS production in vivo. Therefore, 12- and 20-HETE may contribute to the renal dysfunction, injury, and inflammation caused by Ang II in Cyp1b1−/− mice by a mechanism that is independent of ROS generated via Cyp1b1. The loss of Ang II–induced ROS production and improvement of renal dysfunction and end organ damage in Cyp1b1−/− mice is unlikely to be because of alterations in AT1R expression, because infusion of Ang II did not alter the expression of AT1R in the kidneys of Cyp1b1+/+ and Cyp1b1−/− mice. Moreover, the lack of these changes observed in Cyp1b1−/− mice is also unlikely to be because of an inability of Ang II to stimulate release of aldosterone in Cyp1b1−/− mice, because plasma levels of aldosterone were increased to a similar degree in Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II for 13 or 28 days.

The mechanisms by which 12- and 20-HETE and/or ROS generated via Cyp1b1 after infusion of Ang II cause renal dysfunction and end organ damage in Cyp1b1+/+ mice could be because of activation of ±1 signaling pathway. 12- and 20-HETE and ROS cause activation of ERK1/2, p38 MAPK, and c-Src,8,42–45 which have been implicated in many biological actions of Ang II.46,47 Our demonstration that Ang II infusion increased activities of ERK1/2, p38 MAPK, and c-Src in the kidney of Cyp1b1+/+ but not Cyp1b1−/− mice suggests that these signaling molecules contribute to the renal electrolyte and vascular dysfunction, proteinuria, and end organ damage.

In conclusion, the present study demonstrates for the first time that Cyp1b1 contributes to water and electrolyte dysfunction, proteinuria, increased renal vascular resistance and reactivity and endothelial dysfunction, and renal injury and inflammation associated with Ang II–induced hypertension in mice. These pathophysiological changes caused by Ang
II–induced hypertension are most likely mediated by 12- and 20-HETE and ROS generated via Cyp1b1 and activation of ERK1/2, p38 MAPK, and c-Src.

**Perspectives**

Hypertension is one of the leading causes of end-stage renal disease. Although substantial progress has been made in understanding the pathophysiology of kidney damage associated with hypertension, the underlying mechanisms are still not well understood, and elucidation of these mechanisms could provide a rational approach for treating renal disease associated with hypertension. The current study demonstrates a novel mechanism whereby Cyp1b1 plays an important role in renal electrolyte and water imbalance, proteinuria, vascular dysfunction, and injury, as well as inflammation, most likely by generating 12-and 20-HETE and/or ROS, associated with Ang II–induced hypertension and associated renal dysfunction and damage suggest that Cyp1b1 could serve as a novel target for developing therapeutic agents for treating renal disease associated with hypertension.

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We thank Dr Richard J. Roman for his valuable discussions and assistance with determining plasma creatinine by high-performance liquid chromatography and Dr David L. Armbruster for editorial assistance.

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**Disclosures**

None.

**References**


Cytochrome P450 1B1 Contributes to Renal Dysfunction and Damage Caused by Angiotensin II in Mice


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CYTOCHROME P450 1B1 CONTRIBUTES TO RENAL DYSFUNCTION AND DAMAGE CAUSED BY ANGIOTENSIN II IN MICE

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Short Title: Role of CYP1B1 in Ang II-induced renal dysfunction

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**Materials**

Ang II was purchased from Bachem (Torrance, CA), and dihydroethidium (DHE) was from Invitrogen™ (Carlsbad, CA). The cytochrome P450 (CYP) 1B1 antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Antibodies against mouse Cyp1a1, cyclooxygenase (COX) 1, COX 2, 12/15 lipoxygenase, Ang II type 1 receptor (AT1R), α-smooth muscle specific actin, CD-3, ERK1/2, p38 MAPK, and c-Src were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against rat Cyp4a1/a2/a3 (recognizes mouse orthologs Cyp4a10/a12/a14) and human CYP2B6 (recognizes mouse ortholog Cyp2b10) were from Millipore (Billerica, MA), and the phospho ERK1/2, phospho p38 MAPK and phospho c-Src antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The antibody against human CYP4F2 (most likely recognizes mouse orthologs Cyp4f13/f15) was from Research Diagnostics, Inc. (Flanders, NJ). 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 kindly provided by Dr. Frank Gonzalez, National Cancer Institute. Cyp1b1−/− mice were produced and validated as previously described (1), backcrossed 10 generations to a C57BL/6 background, and then brother:sister mated to generate a homozygous line. Male C57BL/6 (Cyp1b1+/+) (Jackson Laboratory, Bar Harbor, ME) were used as control animals throughout the experiment for comparison of drug effects, and all animals were 20 to 30 g and approximately 8 weeks of age at the beginning of the experiment. The genotype of both Cyp1b1−/− and Cyp1b1+/+ mice was routinely analyzed in our laboratory 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 saline for 13 or 28 days (Alzet®, Cupertino, CA; models 1002 and 1004, respectively), and blood pressure was measured 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. It should be noted that we chose these two time courses of Ang II infusion because, in a preliminary experiment, we found that, while 13 days of Ang II infusion caused renal dysfunction, it did not cause renal damage. Because we wanted to investigate the potential role of Cyp1b1 in end organ damage associated with Ang II-induced hypertension, we infused Ang II (700 ng/kg/min) for 28 days, as this time course of Ang II infusion has previously been shown to cause renal damage in mice (3, 4).

**Cyp1b1 activity assay**

Cyp1b1 activity was determined using the P450-Glo™ Assay Kit (Promega) as we previously described (5). 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). Kidneys were dissected free, cleaned of surrounding tissue, snap frozen in liquid N2, and...
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stored at -80°C until use. Kidney samples were homogenized (2 x 3 min) in ice-cold 0.1 M 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 µM L-CEE substrate and 0.1 M potassium phosphate buffer (pH 7.4) and incubated at 37°C for 10 min. 100 µM 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**

Animals were anesthetized, and kidneys removed as described above. Kidney 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 (6). 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).

**Analysis of renal function**

To assess renal function, individual mice were housed in metabolic cages for 24 h prior to the end of the experiment, allowing for the measurement of water consumption and the separation of urine from fecal material and food waste. Urine was collected in tubes that contained a small volume of mineral oil to prevent evaporation. Following calculation of volume, urine was aliquoted and stored at -80°C until further analysis. Urine was analyzed for osmolality using a Vapro® vapor pressure osmometer (Wescor, South Logan, UT; model 5520), protein content by the standard Bradford method and Na⁺ and K⁺ concentrations, using a flame photometer (Instrumentation Laboratory, Inc., Lexington, MA; model 443). Albumin concentration in urine samples was measured using a mouse albumin ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX) according to the manufacturer’s instructions. Creatinine was measured in plasma samples by HPLC at the HPLC/mass spectrometry core facility, University of Mississippi Medical Center, Department of Pharmacology and Toxicology, as described previously (7). Plasma aldosterone concentration was determined using a mouse aldosterone ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. For plasma collection, animals were anesthetized, and blood was withdrawn directly from the abdominal aorta and transferred to K⁺-EDTA tubes (BD Vacutainer®; 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.

**Kidney tissue levels of HETEs and EETs**

AA metabolites generated in kidneys from Cyp1b1+/+ and Cyp1b1−/− mice infused with vehicle or Ang II for 13 and 28 days were measured in kidney homogenate samples, which were prepared using the previously described method (8), with some modifications. Briefly, tissue was
homogenized in acetonitrile after which 5 µl of appropriate internal standard was added. Samples were sonicated at 4°C for 10 min and placed at -20°C overnight. Samples were centrifuged; the supernatant was transferred to a new tube and then dried under nitrogen. Samples were resuspended in 250-500 µl of methanol and dried under nitrogen. Prior to analysis, samples were resuspended in 20 µl of methanol and then separated on C_{18} reverse-phase columns using water and acetonitrile with 0.005% acetic acid as a mobile phase. Internal standards, 5-, 11-, 12-, 15-, 19-, and 20-HETEs and 11,12- and 14,15-EETs were used. Samples were ionized by electron spray with a fragmentor voltage of 120 V and detected in a negative mode as described (9).

**Measurement of renal hemodynamics**

**Renal blood flow:** Renal blood flow (RBF) was measured in mice using a 0.5-V Transonic renal flow probe attached to a TS420 perivascular flow meter (Transonic Systems, Ithaca, NY) as described (10). Briefly, mice were anesthetized as described above and placed on a 37°C heated surgery table. The left kidney was exposed from a subcostal flank incision, and the renal artery was isolated from the renal vein. After equilibrating for approximately 15 min, RBF was recorded for a 10-min period, and the average RBF over this time period was calculated and expressed in ml/min.

**Renal vascular resistance:** Renal vascular resistance was calculated as MAP/RBF.

**Measurement of vascular function**

**Vascular reactivity:** Following anesthesia (described above), the renal artery was quickly dissected free, cleaned of surrounding tissue, and approximately 2-mm rings were mounted in a 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 NaHCO_{3}, 1.2 MgSO_{4}, 1.2 KH_{2}PO_{4}, 11.1 glucose, 2.5 CaCl_{2}.2H_{2}O) at 37°C, which was gassed with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. An initial tension of 3 mN was placed on the renal artery 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 renal artery 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 renal artery 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, the renal artery was dissected free, cleaned of surrounding tissue, and incubated in 10% buffered formalin overnight. Arteries were dehydrated with graded ethanol followed by xylene (1 h) and embedded in paraffin. Embedded arteries were cut into 5-µm sections using a Microm microtome (GMI Inc., Ramsey, MN; model HM 315) and stained with hematoxylin and eosin. Sections were viewed as described above, and images were analyzed using ImageJ 1.42 (http://rsb.info.nih.gov/ij; National Institutes of Health).
**Measurement of renal ROS production**

To measure ROS production, kidney and renal artery sections were exposed to (DHE), following the previously described and validated method (10). Fresh, unfixed kidney and artery samples were placed in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek USA Inc., Torrance, CA) and frozen at -80°C. Kidney and vascular ring segments were cut into 30-µm sections using a cryostat (Leica Microsystems, Bannockburn, IL; model CM1850) and placed on a glass slide. Sections were incubated in PBS for 30 min at 37°C, and then DHE (2 µm for blood vessels sections (11) or 5 µm for kidney sections (12)) was topically applied. Cover slips were applied, and sections were further incubated at 37°C in a light-protected humidified chamber for 30 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 measured in kidney homogenates by measuring lucigenin (N,N′-dimethyl-9,9′-biacridinium dinitrate)-enhanced chemiluminescence, as described previously (13), with some modifications. Following anesthesia, the kidney was isolated, cleaned of surrounding tissue, snap frozen in liquid N2, and stored at -80°C until use. Kidney samples were ground to a fine powder in liquid N2, homogenized, and sonicated in 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). Samples were then centrifuged at 3,000 g for 10 min at 4°C, and supernatants were kept on ice until 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.

**Immunohistochemical analysis**

At the completion of the experiment, animals were anesthetized as described above, the carotid artery was cannulated, and the animals were perfused with saline (3 min). The kidney was dissected free and placed in O.C.T compound. Sections (10 µm) were processed for α-smooth muscle actin (interstitial fibrosis) and CD-3 (T cell infiltration) immunohistochemistry, Gomori trichrome staining (proteinaceous cast formation), and standard hematoxylin and eosin staining (tubular dilation) as described previously (14). The stained cells were viewed with an Olympus® inverted system microscope (Olympus America Inc., Melville, NY; model BX41) and photographed using a SPOT™ Insight™ digital camera (Diagnostic Instruments Inc., Sterling Heights, MI; model Insight 2MP Firewire).

**Statistical analysis**

Data were analyzed by one-way analysis of variance or Student’s t-test. The values of three to six different experiments are expressed as the mean ± SEM. P values < 0.05 were considered statistically significant.
References


Table S1. *Cyp1b1* gene disruption minimizes renal dysfunction associated with 13 days of Ang II infusion in 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>Water Intake (ml/24 hours)</td>
<td>7.92 ± 0.47</td>
<td>13.42 ± 1.09</td>
</tr>
<tr>
<td>Urine Output (ml/24 hours)</td>
<td>1.72 ± 0.22</td>
<td>6.32 ± 1.19&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urinary Na&lt;sup&gt;+&lt;/sup&gt; Excretion (mmol/24 hours)</td>
<td>0.050 ± 0.010</td>
<td>0.363 ± 0.036&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urinary K&lt;sup&gt;+&lt;/sup&gt; Excretion (mmol/24 hours)</td>
<td>0.26 ± 0.04</td>
<td>1.46 ± 0.30&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>2854 ± 231</td>
<td>1228 ± 240*</td>
</tr>
<tr>
<td>Proteinuria (mg/24 hours)</td>
<td>3.5 ± 0.3</td>
<td>10.3 ± 1.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Albuminuria (mg/24 hours)</td>
<td>0.29 ± 0.11</td>
<td>1.94 ± 0.56&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma Creatinine (mg/dl)</td>
<td>0.076 ± 0.003</td>
<td>0.108 ± 0.004*</td>
</tr>
<tr>
<td>Plasma Aldosterone (pg/ml)</td>
<td>228 ± 29</td>
<td>763 ± 135*</td>
</tr>
</tbody>
</table>

*Cyp1b1*<sup>+/+</sup> and *Cyp1b1*<sup>-/-</sup> mice were infused with Ang II or vehicle for 13 days, and placed in metabolic cages for 24 h prior to the end of the experiment for measurement of water intake and urine output. Urine and plasma were analyzed for the parameters listed as described in Methods. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 *Cyp1b1*<sup>+/+</sup> Ang II vs. *Cyp1b1*<sup>-/-</sup> Ang II. (n = 4-6 for all experiments, and data are expressed as mean ± SEM).
Table S2. Ang II-induced hypertension is associated with changes in renal concentrations of 12- and 20-HETE in Cyp1b1+/+ , but not Cyp1b1−/− mice.

<table>
<thead>
<tr>
<th>Eicosanoid (pg/mg kidney weight)</th>
<th>Cyp1b1+/+</th>
<th>Cyp1b1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Ang II</td>
</tr>
<tr>
<td>13 Days Ang II infusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HETE</td>
<td>5.44 ± 0.79</td>
<td>5.06 ± 0.97</td>
</tr>
<tr>
<td>11-HETE</td>
<td>3.91 ± 1.01</td>
<td>4.44 ± 1.28</td>
</tr>
<tr>
<td>12-HETE</td>
<td>30.28 ± 8.60</td>
<td>83.88 ± 19.11*</td>
</tr>
<tr>
<td>15-HETE</td>
<td>9.50 ± 3.41</td>
<td>8.04 ± 0.93</td>
</tr>
<tr>
<td>19-HETE</td>
<td>0.70 ± 0.18</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>20-HETE</td>
<td>0.75 ± 0.11</td>
<td>2.15 ± 0.47*</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>0.29 ± 0.05</td>
<td>0.39 ± 0.09</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>0.36 ± 0.10</td>
<td>0.45 ± 0.16</td>
</tr>
</tbody>
</table>

| 28 Days Ang II infusion          |            |           |           |           |
| 5-HETE                           | 7.52 ± 3.17| 6.72 ± 1.50| 6.77 ± 1.05| 7.92 ± 2.40|
| 11-HETE                          | 4.24 ± 1.89| 3.78 ± 1.97| 3.32 ± 0.75| 3.74 ± 1.65|
| 12-HETE                          | 29.91 ± 9.28| 79.34 ± 26.06*| 32.51 ± 7.04| 37.65 ± 3.56†|
| 15-HETE                          | 9.55 ± 2.85| 7.33 ± 1.08| 10.86 ± 1.95| 9.84 ± 2.78|
| 19-HETE                          | 0.79 ± 0.25| 0.70 ± 0.20| 0.64 ± 0.13| 0.73 ± 0.38|
| 20-HETE                          | 0.77 ± 0.17| 3.80 ± 1.73*| 0.88 ± 0.16| 1.40 ± 0.69†|
| 11,12-EET                        | 0.27 ± 0.05| 0.26 ± 0.05| 0.27 ± 0.05| 0.21 ± 0.02|
| 14,15-EET                        | 0.45 ± 0.09| 0.41 ± 0.10| 0.54 ± 0.21| 0.40 ± 0.09|

Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 or 28 days. Kidney tissue was prepared and analyzed for concentrations of the eicosanoids shown above as described in Methods. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II. (n = 4-6 for all experiments, and data are expressed as mean ± SEM).
Table S3. *Cyp1b1* gene disruption minimizes changes in renal hemodynamics associated with Ang II infusion in mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Ang II</th>
<th>Vehicle</th>
<th>Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>13 Days Ang II infusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>105 ± 3</td>
<td>177 ± 4*</td>
<td>105 ± 3</td>
<td>134 ± 6*†</td>
</tr>
<tr>
<td>RBF (ml/min)</td>
<td>0.93 ± 0.05</td>
<td>0.84 ± 0.03</td>
<td>0.95 ± 0.05</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>RVR (mmHg · ml⁻¹ · min)</td>
<td>114 ± 4</td>
<td>210 ± 3*</td>
<td>111 ± 4</td>
<td>140 ± 6*†</td>
</tr>
<tr>
<td><strong>28 Days Ang II infusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>103 ± 3</td>
<td>172 ± 10*</td>
<td>99 ± 1</td>
<td>131 ± 3*†</td>
</tr>
<tr>
<td>RBF (ml/min)</td>
<td>0.92 ± 0.10</td>
<td>0.85 ± 0.04</td>
<td>0.79 ± 0.06</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>RVR (mmHg · ml⁻¹ · min)</td>
<td>115 ± 12</td>
<td>201 ± 4*</td>
<td>127 ± 8</td>
<td>157 ± 20*†</td>
</tr>
</tbody>
</table>

*Cyp1b1*⁺/⁺ and *Cyp1b1*⁻/⁻ mice were infused with either Ang II or vehicle for 13 or 28 days. Mean arterial pressure (MAP), renal blood flow (RBF) and renal vascular resistance (RVR) were measured and/or calculated as described in Methods. *P* < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †*P* < 0.05 *Cyp1b1*⁺/⁺ Ang II vs. *Cyp1b1*⁻/⁻ Ang II. (n = 3-6 for all experiments, and data are expressed as mean ± SEM).
Table S4. *Cyp1b1* gene disruption prevents increased media:lumen ratio of the renal artery, an indicator of vascular smooth muscle hypertrophy, associated with 4 weeks of Ang II-infusion in mice.

<table>
<thead>
<tr>
<th>Cyp1b1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Cyp1b1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Vehicle</td>
</tr>
</tbody>
</table>

**13 Days Ang II infusion**

| Media:lumen | 4.82 ± 0.49 | 9.56 ± 1.12<sup>*</sup> | 5.17 ± 0.40 | 5.26 ± 0.50<sup>†</sup> |

**28 Days Ang II infusion**

| Media:lumen | 4.61 ± 0.90 | 8.63 ± 0.42<sup>*</sup> | 5.18 ± 0.47 | 6.08 ± 0.28<sup>‡</sup> |

*Cyp1b1<sup>+/+</sup>* and *Cyp1b1<sup>−/−</sup>* mice were infused with either Ang II or vehicle for 13 or 28 days. The media:lumen ratio of the renal artery from animals in each of the different treatment groups was calculated as described in Methods. *<sup>P</sup> < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †<sup>P</sup> < 0.05 *Cyp1b1<sup>+/+</sup>* Ang II vs. *Cyp1b1<sup>−/−</sup>* Ang II. (n = 4-5 for all experiments, and data are expressed as mean ± SEM).
Cyp1b1 gene disruption protects against Ang II-induced hypertension in mice. Cyp1b1\(^{+/+}\) and Cyp1b1\(^{-/-}\) mice were infused with either Ang II or vehicle for 13 days, and systolic blood pressure (SBP; A), diastolic BP (DBP; B), and mean arterial pressure (MAP; C) were measured as described in Methods. *\(P < 0.05\) vehicle vs. corresponding value from Ang II-treated animal; † \(P < 0.05\) Cyp1b1\(^{+/+}\) Ang II vs. Cyp1b1\(^{-/-}\) Ang II (n = 4-6 for all experiments and data are expressed as mean ± SEM).
Cyp1b1 gene disruption protects against Ang II-induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 28 days, and systolic blood pressure (SBP; A), diastolic BP (DBP; B), and mean arterial pressure (MAP; C) were measured as described in Methods. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II (n = 4-6 for all experiments and data are expressed as mean ± SEM).

Figure S2

28 Days Ang II infusion
Ang II-induced hypertension is associated with increased renal Cyp1b1 activity, but not expression in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 (A, B) or 28 (C, D) days. (A, C) At the completion of the experiment, kidney tissue was collected for measurement of Cyp1b1 activity using the P450-Glo™ assay as described in Methods. The activity of Cyp1b1 is expressed as relative luminescence units (RLU). (B, D) Cyp1b1 protein expression was measured by western blot in kidney tissue from Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II or its vehicle (saline) using approximately 10 µg of protein for loading as described in Methods. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal (n = 3-6 for all experiments and data are expressed as mean ± SEM).
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**Figure S4**

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
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<tbody>
<tr>
<td><strong>A</strong></td>
<td>1.00</td>
<td>0.97 ± 0.04</td>
<td>0.94 ± 0.06</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>1.00</td>
<td>0.98 ± 0.02</td>
<td>0.98 ± 0.05</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td><strong>C</strong></td>
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<td>1.01 ± 0.05</td>
<td>1.00 ± 0.09</td>
<td>0.99 ± 0.06</td>
</tr>
<tr>
<td><strong>D</strong></td>
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<td>1.07 ± 0.14</td>
<td>0.99 ± 0.06</td>
</tr>
<tr>
<td><strong>E</strong></td>
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<td><strong>F</strong></td>
<td>1.00</td>
<td>1.06 ± 0.09</td>
<td>1.03 ± 0.11</td>
<td>0.98 ± 0.10</td>
</tr>
<tr>
<td><strong>G</strong></td>
<td>1.00</td>
<td>1.02 ± 0.08</td>
<td>0.98 ± 0.17</td>
<td>1.09 ± 0.12</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>1.00</td>
<td>1.11 ± 0.15</td>
<td>0.95 ± 0.09</td>
<td>1.06 ± 0.14</td>
</tr>
</tbody>
</table>

**Cyp1b1** gene disruption and/or Ang II infusion do not alter renal expression of various AA metabolizing enzymes or AT1R in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 (A-G) or 28 (H) days. Protein expression of various AA metabolizing enzymes (A-G) or AT1R (H) were measured in kidney homogenates by western blot as described in Methods using approximately 10 µg of protein for loading. α-actin was used as a loading control and intensity of the bands was measured using ImageJ as described in Methods (n = 3 for all experiments and data are expressed as mean ± SEM).
Cyp1b1 gene disruption minimizes increased vascular reactivity of the renal artery that is associated with Ang II-induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 (A, B) or 28 (C, D) days. Vascular reactivity was measured as described in Methods. Responses of the renal artery from Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II or vehicle to increasing concentrations of phenylephrine (PE; A, C) and endothelin-1 (ET-1; B, D), respectively. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II. (n = 4-6 for all experiments and data are expressed as mean ± SEM).
Cyp1b1 gene disruption prevents endothelial dysfunction of the renal artery that is associated with Ang II-induced hypertension in mice. Cyp1b1<sup>+/+</sup> and Cyp1b1<sup>-/-</sup> mice were infused with either Ang II or vehicle for 13 (A, B) or 28 (C, D) days. Endothelial function was measured as described in Methods. Responses of the renal artery from Cyp1b1<sup>+/+</sup> and Cyp1b1<sup>-/-</sup> mice infused with Ang II or vehicle to increasing concentrations of acetylcholine (ACh A, C) and sodium nitroprusside (SNP; B, D), respectively. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1<sup>+/+</sup> Ang II vs. Cyp1b1<sup>-/-</sup> Ang II. (n = 4-6 for all experiments and data are expressed as mean ± SEM).
Cyp1b1 gene disruption minimizes increased oxidative stress in renal arteries that is associated with Ang II-induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 (A) or 28 (B) days. Superoxide production, as determined by fluorescence intensity of 2-OHE was measured in renal arteries as described in Methods. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II. (n = 4-6 for all experiments and data are expressed as mean ± SEM).
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**Cyp1b1** gene disruption minimizes increased renal oxidative stress and NADPH oxidase activity that is associated with Ang II-induced hypertension in mice. **Cyp1b1**+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 (A, B) or 28 days (C, D). (A, C) Superoxide production, as determined by 2-hydroxyethidium (2-OHE) fluorescence intensity, was measured as described in Methods. Arrows indicate increase fluorescent intensity observed in glomeruli of Ang II-infused animals. (B, D) NADPH oxidase activity was measured in kidney homogenates using a lucigenin-based luminescence assay as described in Methods. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II; ‡P < 0.05 Cyp1b1+/+ vehicle vs. Cyp1b1−/− vehicle (n = 4-6 for all experiments and data are expressed as mean ± SEM).
13 Days of Ang II infusion does not cause renal damage or inflammation in mice. Cyp1b1\(^{+/+}\) and Cyp1b1\(^{-/-}\) mice were infused with either Ang II or vehicle for 13 days. (A) Ang II infusion was associated with intra-renal vascular hypertrophy in Cyp1b1\(^{+/+}\), but not Cyp1b1\(^{-/-}\) mice. There was a lack of positive α-smooth muscle actin staining, an indicator of interstitial fibrosis (B), tubular dilation (C), proteinaceous cast formation (D), and CD-3\(^+\) cells, an indication of T-lymphocyte infiltration (E), observed in kidney sections from Cyp1b1\(^{+/+}\) and Cyp1b1\(^{-/-}\) mice infused with Ang II or vehicle.
Cyp1b1 gene disruption minimizes increased renal activities of ERK1/2, p38 MAPK and c-Src that are associated with Ang II-induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 (A-C) or 28 (D-F) days. Activities of ERK1/2 (A, D), p38 MAPK (B, E) and c-Src (C, F), as determined by phosphorylation of these kinases, were measured in kidney homogenates by western blot as described in Methods using approximately 10 µg of protein for loading. *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II (n = 4-6 for all experiments and data are expressed as mean ± SEM).