Cytochrome P450 1B1 Contributes to Angiotensin II–Induced Hypertension and Associated Pathophysiology

Brett L. Jennings, Seyhan Sahran-Firat, Anne M. Estes, Kanak Das, Nasreen Farjana, Xiao R. Fang, Frank J. Gonzalez, Kafait U. Malik

Abstract—Hypertension is the leading cause of cardiovascular diseases, and angiotensin II is one of the major components of the mechanisms that contribute to the development of hypertension. However, the precise mechanisms for the development of hypertension are unknown. Our recent study showing that angiotensin II–induced vascular smooth muscle cell growth depends on cytochrome P450 1B1 led us to investigate its contribution to hypertension caused by this peptide. Angiotensin II was infused via miniosmotic pump into rats (150 ng/kg per minute) or mice (1000 µg/kg per day) for 13 days resulting in increased blood pressure, increased cardiac and vascular hypertrophy, increased vascular reactivity to vasoconstrictor agents, increased vascular reactive oxygen species production, and endothelial dysfunction in both species. The increase in blood pressure and associated pathophysiological changes were minimized by the cytochrome P450 1B1 inhibitor 2,3′,4,5′-tetramethoxystilbene in both species and was markedly reduced in Cyp1b1−/− mice. These data suggest that cytochrome P450 1B1 contributes to angiotensin II–induced hypertension and associated pathophysiological changes. Moreover, 2,3′,4,5′-tetramethoxystilbene, which prevents both cytochrome P450 1B1–dependent and –independent pathophysiological changes of angiotensin II–induced hypertension and inhibits associated pathophysiological changes could be clinically useful in the treatment of hypertension and associated cardiovascular and inflammatory diseases. (Hypertension. 2010;56:667-674.)

Key Words: angiotensin II ■ cytochrome P450 1B1 ■ Cyp1b1−/− mice ■ blood pressure ■ cardiac and vascular hypertrophy ■ vascular reactivity ■ endothelial function

Angiotensin II (Ang II) is a major component of the mechanisms regulating cardiovascular homeostasis by maintaining vascular tone and salt and water balance.1 Ang II also activates cytosolic phospholipase A2 and releases arachidonic acid (AA) from phospholipids.2 AA is metabolized by cyclooxygenase into prostaglandins and thromboxane A2, by lipoxygenase into 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs), by cytochrome P450 (CYP) α-hydroxylase into 20-HETE, and by epoxygenase into epoxyeicosatrienoic acids.3 Prostaglandins E2 and I2 and epoxyeicosatrienoic acids contribute to antihypertensive mechanisms,4,5 whereas prostaglandin precursor PGH2 and 20-HETE contribute to prohypertensive mechanisms.5–8 The balance between these antihypertensive and prohypertensive eicosanoids, together with other vasoactive agents, determines blood pressure levels. Products of AA generated via lipoxygenase (12-HETE) or CYP 4A (20-HETE) also promote vascular smooth muscle cell (VSMC) migration, proliferation, or hypertrophy by activating extracellular signal–regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK)9–13 and contribute to the vasoconstrictor action of Ang II.14 Moreover, inhibitors of lipoxygenase and CYP 4A minimize Ang II–dependent hypertension.5,15,16 Ang II and AA also stimulate production of reactive oxygen species (ROS) and activate c-Jun kinase and p38 MAPK but not ERK1/2 in VSMCs17–19 and Ang II–induced hypertension.20

In addition to cyclooxygenase, lipoxygenase, and CYP 4A, other CYP enzymes can also metabolize AA. CYP2 enzymes express mainly an epoxygenase activity, and CYP2B, 2C, and 2J are the major epoxygenases that metabolize AA into epoxyeicosatrienoic acids.8 CYP1 enzymes that metabolize xenobiotics can also metabolize endobiotics such as steroid hormones, retinoids, and fatty acids.21–24 CYP1A1 and CYP1B1 are expressed in several extrahepatic tissues, including cardiovascular tissues.25 CYP1A1-encoded enzymes are expressed in vascular endothelium and smooth muscle cells, with much higher levels of activity in endothelial cells, whereas CYP1B1 is highly expressed in VSMCs, with little expression in endothelial cells.26 However, shear stress up-regulates mRNA and protein levels of CYP1A1 and CYP1B1.
in endothelial cells. CYP1B1 can also metabolize AA in vitro mainly into midchain HETEs and to a lesser degree into terminal HETEs and epoxycisatrienoic acids. Moreover, bioactivation of procarcinogenic compounds such as aromatic hydrocarbons by CYP1B1 leads to formation of intermediates that form DNA adducts and polycyclic biphenyls which uncouple CYP1B1 resulting in the generation of ROS, lipid peroxidation, and DNA oxidation. Recently, we have shown that CYP1B1 mediates Ang II–induced VSMC migration and protein synthesis through ROS production. Therefore, it is possible that CYP1B1, through generation of AA metabolites and/or ROS, might activate ≥1 of the signaling molecules (e.g., ERK1/2 or p38 MAPK) that contribute to Ang II–induced hypertension. To test this hypothesis, we examined the effects of 2,3'-4,5'-tetramethoxystilbene (TMS), a selective inhibitor of CYP1B1, on the development and maintenance of Ang II–induced hypertension in rats and the effects of Ang II in wild-type (Cyp1b1+/−) and CYP1B1 knockout (Cyp1b1−/−) mice. The results of this study demonstrate that CYP1B1 contributes to the development and maintenance of hypertension, most likely by increased generation of ROS, ERK1/2, and p38 MAPK activity; vascular hypertrophy; endothelial dysfunction; and increased vascular reactivity.

Methods
Please see the online Data Supplement at http://hyper.ahajournals.org.

Results
TMS Counteracts the Hypertensive Effect of Ang II in Rats
Ang II infusion increased MAP that was prevented by TMS; TMS alone did not alter MAP (Figure S1A, available in the online Data Supplement). In hypertensive rats injected with TMS, MAP returned to basal levels (Figure S1B). For each treatment, appropriate vehicles were administered, and no difference was observed (data not shown).

TMS Protects Against Cardiac Hypertrophy, Fibrosis, and Inflammation Associated With Ang II–Induced Hypertension in Rats
Ang II infusion increased heart:body weight (HW/BW) ratio and brain natriuretic peptide mRNA expression, indicators of cardiac hypertrophy. TMS alone had no effect on HW/BW ratio and brain natriuretic peptide mRNA level but minimized the increase in these parameters caused by Ang II (Table S1, available in the online Data Supplement). The increase in myofibroblasts, as indicated by α-smooth muscle actin staining in the myocardium, and ED-1 positive cells, an index of macrophages, in the perivascular space of the heart caused by Ang II was reduced by TMS (Figure S2).

TMS Decreases CYP1B1 Activity But Not Its Expression or Plasma Levels of 12- and 20-HETE
CYP1B1 protein expression was not altered in the aorta (Figure S3A) or heart (Figure S3B) after the various treatments. However, in animals treated with TMS, CYP1B1 activity in the aorta and heart was reduced. CYP1B1 activity was not increased in animals infused with Ang II but was reduced by concurrent treatment with TMS (Figure S3C and S3D). In hypertensive rats given TMS, CYP1B1 activity was decreased in the aorta and heart (Figure S4). Plasma levels of 12- and 20-HETE were not changed in the different treatment groups (Table S2).

TMS Prevents Increased Vascular Reactivity and Vascular Smooth Muscle Hypertrophy and Improves Endothelial Dysfunction in Ang II–Treated Rats
Ang II–induced hypertension was associated with an increased response of aorta (Figure S5A) and mesenteric (Figure S5B) and femoral (Figure S5C) arteries to phenylephrine (PE), endothelin 1 (ET-1), and vasopressin, as well as the media:lumen ratio of these vessels (Table S3); these increases were prevented in rats treated with TMS. In rats given TMS alone, responses of these vessels to the above agents and their media:lumen ratio were not altered (Figure S5 and Table S3, respectively). In hypertensive rats given TMS, both vascular reactivity and media:lumen ratio were reduced in all of the blood vessels studied (Figure S6 and Table S3, respectively).

Ang II infusion caused endothelial dysfunction in the aorta and femoral artery but not the mesenteric artery, as determined by the dilatatory effect of acetylcholine (ACh) (Figure S7A). In animals infused with Ang II and given TMS, ACh- and sodium nitroprusside (SNP)–induced relaxations were not altered (Figure S7). After treatment with TMS to animals after they were made hypertensive with Ang II, ACh- and SNP-induced relaxations were not altered (Figure S8).

TMS Inhibits ROS Production, Activity, and Expression of NADPH Oxidase and ERK1/2 and p38 MAPK Activities in the Rat Aorta Caused by Ang II Infusion
ROS production, as determined by dihydroethidium (DHE) fluorescence, was increased in the aorta of animals infused with Ang II (Figure S9A and S9B). TMS treatment alone decreased ROS production in the aorta and abolished the increase caused by Ang II infusion (Figure S9A and S9B). To confirm the DHE fluorescence caused by the generation of 2-hydroxyethidium (2-OHE), as a specific indicator of superoxide production, we used a high-performance liquid chromatography method to separate 2-OHE formed from DHE generated in isolated aortic rings and measured using a fluorescence detector. As was observed with the DHE fluorescence, animals infused with Ang II showed a greater conversion of DHE to 2-OHE, which was inhibited with TMS (Figure S9C). TMS treatment alone had a minimal effect on basal conversion of DHE to 2-OHE (Figure S9C). ROS production was also decreased in animals made hypertensive and then given TMS, as determined by fluorescence microscopy after exposure of vessels to DHE (Figure S10). Expression of NADPH oxidase 1 (NOX 1), as measured by Western blot analysis (Figure S11A), and NADPH oxidase activity, as measured by a lucigenin-based luminescence assay (Figure S11B), were increased in the aorta of Ang II–infused animals.
but inhibited in animals treated with TMS. Expression of NOX 4 was not altered in any treatment group (Figure S11C).

Ang II is known to increase the activity of ERK1/2 and p38 MAPK in VSMCs, which contribute to hypertrophy and increased vascular reactivity. In this study, ERK1/2 and p38 MAPK activity, measured by phosphorylation of these kinases, were increased in the aorta of rats infused with Ang II that was attenuated in rats treated with TMS (Figure S12).

**CYP1B1 Contributes to the Development of Ang II–Induced Hypertension in Mice**

To further determine the contribution of CYP1B1 in the development of Ang II–induced hypertension, we examined the effect of Ang II in Cyp1b1−/− and Cyp1b1+/+ mice. Infusion of Ang II increased MAP in Cyp1b1+/+ and Cyp1b1−/− mice, but the increase was significantly less in Cyp1b1−/− than in Cyp1b1+/+ mice (Figure 1A). In Cyp1b1+/+ mice, TMS treatment alone had no effect on MAP, but the Ang II–induced increase in MAP was prevented by TMS (Figure 1B). The CYP1B1-independent component of Ang II–induced hypertension in Cyp1b1−/− mice was also abolished by TMS (Figure 1C).

**TMS and CYP1B1 Gene Disruption Reduce Cardiac Hypertrophy and CYP1B1 Activity in Ang II–Induced Hypertension in Mice**

Infusion of Ang II increased HW/BW ratio in Cyp1b1+/+ and Cyp1b1−/− mice; however, the increase in HW/BW ratio was significantly less in Cyp1b1−/− mice. In both strains of mice, the increase in HW/BW ratio associated with Ang II was prevented by TMS (Table 1).

CYP1B1 activity in the heart and kidney of Cyp1b1−/− mice was inhibited (Figure S13A and S13B). Infusion of Ang II did not increase CYP1B1 activity in tissues from Cyp1b1−/− or Cyp1b1+/+ mice; however, CYP1B1 activity was further reduced by TMS in both strains of mice (Figure S13A and S13B). The remaining activity in Cyp1b1−/− mice could result from CYP1A1 activity detected by the assay. Cyp1b1−/− mice showed no expression of CYP1B1 in the heart, and infusion of Ang II did not alter CYP1B1 protein expression in the heart of Cyp1b1+/+ mice (Figure S13C).

**Ang II–Induced Increase in Vascular Reactivity, and Hypertrophy and Endothelial Dysfunction Are Diminished in Cyp1b1−/− Mice**

In Cyp1b1+/+ and Cyp1b1−/− mice, infusion of Ang II increased vascular reactivity of the aorta to PE and ET-1 and media/lumen ratio; these increases were significantly less in Cyp1b1−/− than in Cyp1b1+/+ mice (Figure 2 and Table 2, respectively). The increased vascular reactivity to PE and ET-1 and media/lumen ratio associated with Ang II in Cyp1b1+/+ mice and that remaining in Cyp1b1−/− mice was prevented by TMS; TMS alone had no effect on these parameters (Figure S14 and Table 2).

Ang II infusion resulted in endothelial dysfunction, as indicated by decreased relaxation to ACh in the aorta from Cyp1b1+/+ but not Cyp1b1−/− mice (Figure S15A). Endothelium-independent relaxation of aorta to SNP in Cyp1b1+/+ and Cyp1b1−/− mice remained unaltered in all of the treatment groups (Figure S15B). The decrease in ACh-induced relaxation of aorta from Cyp1b1+/+ mice infused with Ang II was prevented by TMS (Figure S15C); SNP-induced relaxation of the aorta was not altered (Figure S15D). In Cyp1b1−/− mice infused with Ang II, ACh- and SNP-induced relaxation of the aorta remained unaltered (Figure S15E and S15F, respectively).
Table 1. TMS and Cyp1b1 Gene Disruption Reduce Cardiac Hypertrophy Associated With Ang II–Induced Hypertension in Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Vehicle</th>
<th>Ang II</th>
<th>TMS</th>
<th>Ang II + TMS</th>
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</thead>
<tbody>
<tr>
<td>Cyp1b1+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>24.2 ± 0.2</td>
<td>26.1 ± 0.2</td>
<td>25.2 ± 0.8</td>
<td>26.4 ± 0.3</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>112.7 ± 3.1</td>
<td>165.0 ± 3.5</td>
<td>123.7 ± 3.5</td>
<td>127.7 ± 3.8</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>4.7 ± 0.2</td>
<td>6.3 ± 0.1</td>
<td>4.9 ± 0.3</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Cyp1b1−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>25.6 ± 0.8</td>
<td>26.8 ± 0.1</td>
<td>25.5 ± 0.5</td>
<td>27.4 ± 0.6</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>112.0 ± 4.1</td>
<td>139.7 ± 2.3</td>
<td>115.7 ± 3.9</td>
<td>123.0 ± 3.6</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>4.4 ± 0.2</td>
<td>5.2 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>4.5 ± 0.1</td>
</tr>
</tbody>
</table>

Animals were treated and the ratio of HW/BW was calculated as described in the Methods section. n = 4 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.
‡P < 0.05 Cyp1b1+/+ Ang II vs Cyp1b1−/− Ang II + TMS.
§P < 0.05 Cyp1b1+/+ Ang II vs Cyp1b1−/− Ang II + TMS.

Ang II–Induced Increase in ROS Production Is Diminished in Cyp1b1+/− Mice and Inhibited by TMS in Cyp1b1+/− Mice

Infusion of Ang II in both Cyp1b1+/+ and Cyp1b1−/− mice increased aortic ROS production, as measured by fluorescence of 2-OHE generated after exposure of aorta to DHE, but the increase in fluorescence in Cyp1b1−/− mice was significantly less than that in Cyp1b1+/+ mice (Figure 3). In both Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II or its vehicle, TMS treatment decreased the intensity of fluorescence (Figure 3).

Ang II–Induced Increase in Expression of NOX 1 and NADPH Oxidase Activity Is Diminished in the Heart of Cyp1b1−/− Mice

Ang II infusion increased expression of NOX 1 in the heart of Cyp1b1+/+ mice; this increase was significantly greater than that observed in Cyp1b1−/− mice (Figure S16A). In addition, NADPH oxidase activity was increased in the hearts of Cyp1b1+/+ and Cyp1b1−/− mice, but the increase in Cyp1b1−/− mice was significantly less than that in Cyp1b1+/+ mice (Figure S16B).

Discussion

This is the first study to demonstrate a novel mechanism whereby CYP1B1 contributes to development and maintenance of Ang II–induced hypertension and associated vascular hypertrophy, endothelial dysfunction, increased vascular reactivity to vasoconstrictor agents, cardiac hypertrophy, fibrosis and inflammation and generation of ROS, increased expression of NOX 1 and activities of NADPH oxidase, ERK1/2, and p38MAPK.

Our finding that TMS, a selective inhibitor of CYP1B1,31 decreased CYP1B1 activity in the rat aorta and heart prevented Ang II–induced increase in MAP and normalized the MAP raised by Ang II infusion and minimized the associated increase in (1) HW/BW ratio and the expression of brain natriuretic peptide mRNA in the left ventricle; (2) actin staining of myofibroblasts and accumulation of ED-1–positive cells in the myocardium; and (3) the response of the aorta and mesenteric and femoral arteries to PE, ET-1, vasopressin, and the media:lumen ratio of these vessels, suggests that CYP1B1 contributes to the development and maintenance of hypertension in Mice and Inhibited by TMS in Cyp1b1+/− Mice

Figure 2. TMS and Cyp1b1 gene disruption reduce increased aortic response to vasoconstrictor agents associated with Ang II–induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 days and given IP injections of TMS as described in the Figure 1 legend. Vascular reactivity was measured in the aorta as described in the Methods section. A and B, The response of aorta of Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II or vehicle to increasing concentrations of phenylephrine (PE) and endothelin-1 (ET-1). *P < 0.05 vehicle vs corresponding value from Ang II–treated animal; †P < 0.05 Cyp1b1+/+ Ang II vs Cyp1b1−/− Ang II. n = 4 for all experiments, and data are expressed as mean ± SEM.

Table 2. TMS and Cyp1b1 Gene Disruption Reduce Media:Lumen Ratio Associated With Ang II–Induced Hypertension in Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Vehicle</th>
<th>Ang II</th>
<th>TMS</th>
<th>Ang II + TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp1b1+/+</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Thoracic Aorta</td>
<td>6.14 ± 0.09</td>
<td>9.83 ± 0.59</td>
<td>6.34 ± 0.45</td>
<td>6.27 ± 0.08</td>
</tr>
<tr>
<td>Cyp1b1−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thoracic Aorta</td>
<td>5.53 ± 0.93</td>
<td>7.09 ± 0.23</td>
<td>5.85 ± 0.58</td>
<td>5.96 ± 0.39</td>
</tr>
</tbody>
</table>

Animals were treated, and the media:lumen ratio of the aorta was calculated as described in the Methods section. n = 4 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.
‡P < 0.05 Cyp1b1+/+ Ang II vs Cyp1b1−/− Ang II + TMS.
§P < 0.05 Cyp1b1−/− Ang II vs Cyp1b1−/− Ang II + TMS.
hypertension and cardiac hypertrophy, fibrosis and inflammation, and vascular reactivity and hypertrophy. Because TMS treatment also reduced CYP1B1 activity in the heart and aorta of vehicle-treated animals and Ang II infusion did not increase the activity or expression of CYP1B1, it appears to be constitutively active in these tissues as shown in VSMCs.30 Although TMS treatment prevented the development and maintenance of hypertension caused by Ang II in rats, it did not completely inhibit CYP1B1 activity in the aorta or heart; the remaining activity could result from CYP1A1 activity detected by our assay or a related enzyme. In cultured rat VSMCs that do not express CYP1A1, TMS blocks the activity of CYP1B1, as measured by our assay.30

Recombinant CYP1B1 can metabolize AAs in vitro into midchain and terminal HETEs, including 12- and 20-HETE.24 Both 12- and 20-HETE contribute to VSMC proliferation and/or hypertrophy and vascular contraction caused by Ang II, and 20-HETE increases reactivity of mesenteric arteries to PE from spontaneously hypertensive and Wistar-Kyoto rats.32 Therefore, it is possible that the increased vascular reactivity and hypertrophy caused by Ang II could result from increased production of eicosanoids, 12- and 20-HETE. However, this appears to be unlikely, because plasma levels of 12- and 20-HETE were not altered in rats treated with Ang II or TMS. In rat VSMCs, TMS or adenovirus CYP1B1 short hairpin RNA also do not alter AA metabolism into HETEs.30 The conversion of AA to 12- and 20-HETEs examined in the femoral arteries of rats treated with TMS was also not altered (our unpublished data).

In the present study, infusion of Ang II in rats caused endothelial dysfunction in the aorta and femoral artery, as indicated by attenuation of relaxation to ACh but not to SNP that acts directly on vascular smooth muscle. Inasmuch as loss of ACh-induced relaxation caused by Ang II infusion

Figure 3. TMS and Cyp1b1 gene disruption reduce vascular oxidative stress associated with Ang II–induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 days and given IP injections of TMS as described in the Figure 1 legend. ROS production was determined in aortic sections by DHE fluorescence as described in the Methods section. §P<0.05 Cyp1b1+/+ vehicle vs Cyp1b1−/− vehicle; *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 vehicle vs corresponding value from TMS-treated animal; #P<0.05 Ang II vs corresponding value from Ang II+TMS-treated animal. n=4 for all experiments, and data are expressed as mean±SEM.
was restored by concurrent administration of TMS to rats, it appears that CYP1B1 participates in endothelial dysfunction in the aorta and femoral artery and may also contribute to increased vascular reactivity in Ang II–induced hypertension. Endothelium-dependent relaxation depends mainly on NO in larger arteries and on endothelium-derived hyperpolarizing factor(s) in small vessels. Chronic infusion of Ang II reduces NO-mediated relaxation in the aorta, whereas Ang II–infusion for 21 but not 14 days produces loss of the major component of relaxation dependent on endothelium-derived hyperpolarizing factor(s) and not the minor NO-dependent component of relaxation in the rat superior mesenteric artery. In our study, infusion of Ang II also failed to cause endothelial dysfunction in the small resistance mesenteric artery. From these observations, it follows that endothelial dysfunction in different vascular tissues depends on the duration of exposure to Ang II and that NO-dependent relaxation in larger vessels is more sensitive to Ang II and depends on CYP1B1 activity.

The endothelial dysfunction in different models of hypertension is in part attributed to the result of inactivation of NO by ROS. Therefore, in our study, restoration of endothelial impairment to ACh in the aorta and femoral artery, produced by concurrent treatment with TMS in Ang II–infused rats, most likely results from decreased production of ROS generated via CYP1B1. Supporting this view was our demonstration that Ang II infusion increased aortic superoxide production and expression of NOX 1 and NADPH oxidase activity, which was prevented in the aorta of rats treated with TMS. Recently, we reported that Ang II–induced increase in ROS production in rat VSMCs is mediated by CYP1B1. Because Ang II is known to stimulate ROS production by activating NADPH oxidase and it has been implicated in Ang II–induced hypertension, it is possible that ROS and/or AA metabolites generated by CYP1B1, independent of HETEs, result in activation of NADPH oxidase. ROS have been reported to amplify their own production by activating NADPH oxidase, xanthine oxidase, increasing intracellular uptake of iron and/or uncoupling endothelial NO synthase. Further studies are required to determine the relationship between CYP1B1 and NADPH oxidase and other ROS-producing systems. Moreover, we cannot exclude the possibility of an additional direct effect of TMS on NADPH oxidase or other ROS-producing systems.

The increase in ROS production from AA via CYP1B1 by Ang II infusion could result in increased vascular reactivity and hypertrophy through activation of ERK1/2 and p38 MAPK, which are known to mediate Ang II–induced hypertrophy of cultured VSMCs. Supporting this view was our finding that infusion of Ang II increased aortic ERK1/2 and p38 MAPK activity that was attenuated by treatment with TMS. Moreover, we have shown that, in cultured rat VSMCs or in cells transduced with adenovirus CYP1B1 short hairpin RNA, Ang II– and AA-induced ERK1/2 and p38 MAPK activities are inhibited by TMS without alterations in expression of Ang II type 1 receptor or its coupling to G proteins.

Further evidence that CYP1B1 contributes to the development of Ang II–induced hypertension and associated pathophysiological changes is derived from our studies in Cyp1b1−/− mice that do not express CYP1B1 protein. Our findings that the increase in MAP, HW/BW ratio, media: lumen ratio, the response to PE and ET-1, ROS production, and cardiac expression of NOX 1 and NADPH oxidase activity caused by Ang II infusion in Cyp1b1−/− mice were significantly decreased in Cyp1b1−/− mice strongly support our findings in the rat treated with TMS that CYP1B1 contributes to Ang II–induced hypertension and associated pathophysiological changes. Our finding that endothelial function remained unaltered in Cyp1b1−/− mice indicates that CYP1B1 is required for aortic endothelial dysfunction associated with Ang II–induced hypertension. CYP1B1 activity, measured in the heart and kidney of Cyp1b1−/− mice, was reduced but not abolished and was further reduced by TMS. The remaining activity could result from CYP1A1 or other related enzyme(s), as mentioned above. Inasmuch as administration of TMS abolished Ang II–induced increase in blood pressure and all of the associated cardiovascular changes in Cyp1b1−/− mice, as well as the CYP1B1-independent component of Ang II actions in Cyp1b1−/− mice, it appears that TMS exerts an additional protective effect against the deleterious effects of Ang II on the cardiovascular system. The mechanism of the protective effects of TMS against the CYP1B1-independent component of Ang II–induced hypertension and associated ROS generation and vascular changes, which could be attributed to its direct effect on ROS-generating systems, remains to be determined.

Ang II–induced hypertension also depends on its actions in the kidney and central nervous system, and superoxides have been implicated in hypertension caused by central actions of Ang II. Recently, it has been shown that T cells (T-helper 17 cells), via generation of superoxides, participate in Ang II–induced hypertension. Another recent report shows that Ang II, by increasing RhoA activity via Jak2-induced phosphorylation of Rho exchange factor Arhgef1, results in increased vascular contraction and hypertension. Therefore, it is possible that CYP1B1 expressed in the kidney, brain, and lymphocytes, via ROS generation and/or increased RhoA activity, might also contribute to Ang II–induced hypertension. Alternatively, ≥1 of these mechanisms might contribute to the CYP1B1-independent and TMS-sensitive component of Ang II–induced hypertension and associated pathophysiological changes. Whether CYP1B1 directly or indirectly also contributes to hypertension caused by increased activity of the sympathetic nervous system also remains to be determined.

Perspectives

Increased activity of the renin-angiotensin system is a major contributing factor in the development of various vascular diseases, including hypertension. Further understanding of the mechanisms that are involved in Ang II–dependent hypertension would provide a rationale approach for the development of new therapeutic agents for the treatment of cardiovascular diseases. This study provides evidence for the first time that CYP1B1 contributes to Ang II–induced hypertension and associated pathophysiological changes, including increased ROS production, vascular reactivity, endothelial dysfunction, and vascular and cardiac hypertrophy. In pre-
liminary experiments, we have found that CYP1B1 also contributes to deoxycorticosterone acetate/salt and N^\text{\textsubscript{ω}}-nitro-L-arginine methyl ester hydrochloride–induced hypertension and to spontaneous hypertension in rats. Moreover, TMS, which prevents both CYP1B1-dependent and -independent components of Ang II–induced hypertension and associated pathophysiological changes, could be clinically useful for treating hypertension and other cardiovascular and related inflammatory diseases.

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Disclosures
None.

References


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**CYTOCHROME P450 1B1 CONTRIBUTES TO ANGIOTENSIN II-INDUCED HYPERTENSION AND ASSOCIATED PATHOPHYSIOLOGY**

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**Short title:** Role of CYP1B1 in Ang II-induced hypertension

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Methods

Materials
Ang II and TMS were purchased from Bachem and Cayman Chemical, respectively. The primary antibody against ED-1 was from AbD serotec, and dihydroethidium was from Molecular Probes, Inc. The CYP1B1 antibody was purchased from BD Biosciences, and antibodies against \( \alpha \)-smooth muscle specific actin, ERK1/2, p38MAPK, NOX1, NOX4, were purchased from Santa Cruz Biotechnology, Inc. Phospho ERK1/2 and phospho p38 MAPK were purchased from Cell Signaling Technology, Inc. All other chemicals were purchased from Sigma.

Ang II-induced hypertension in rats
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. Male Sprague-Dawley rats (Charles River Laboratories, Willmington, MA) (250 to 350 grams) were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), and miniosmotic pumps (Alzet®, Cupertino, CA; model 2ML2) were implanted subcutaneously to infuse Ang II (150 ng/kg/min) or saline for 13 days. In one group of rats infused with Ang II, TMS (300 µg/kg) or its vehicle, DMSO (100 µl), was injected i.p. every third day beginning from day 1 of the experiment and blood pressure was measured using a non-invasive tail cuff method (Kent Scientific, Torrington, CT; model XBP 1000). Prior to implantation of the miniosmotic pump, rats were acclimated to the blood pressure measuring device for 1 week. To determine the effect of TMS on the maintenance of Ang II-induced hypertension, rats were implanted with a miniosmotic pump containing Ang II or saline as described above and were subsequently injected with TMS or DMSO every 2\textsuperscript{nd} day starting from the 8th day of Ang II infusion.

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 heart was dissected free and placed in O.C.T compound. Cardiac sections (5 \( \mu \)m) were processed for \( \alpha \)-smooth muscle actin (myofibroblasts) and ED1 (macrophages) as described previously (1). The stained cells were viewed with an Olympus® inverted system microscope (Olympus America Inc., model BX41) and photographed using a SPOT™ Insight™ digital camera (Diagnostic Instruments Inc., model Insight 2MP Firewire).

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.

BNP mRNA measurement: Total RNA was isolated from left ventricular homogenates using an RNeasy Mini Kit (QIAGEN, Valencia, CA) as per the manufacturer’s instructions, and deoxyribonuclelease was digested using an RNase-Free DNase Set (QIAGEN). Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) with 200 ng of total RNA and 60 \( \mu \)M of Random Primer (Roche) in a 20-\( \mu \)l volume, with time courses of 25°C for 10 min followed by 55°C for 30 min. Quantitative real-
time PCR was performed in 96-well plates with a LightCycler® (LC) 480 (Roche) using an LC480 Master Mix and a Universal Probe Library (UPL probe) (Roche) at a concentration of 10 µM with a final reaction volume of 10 µl, with the following conditions: 95°C for 5 min for activation, 45 cycles of 95°C for 10 sec, 60°C for 60 sec and 72°C for 10 sec for amplification. After testing 6 endogenous control genes, the TATA-binding protein (TBP) gene was used as endogenous control for BNP. The sequences of primers and the relevant probes (UPL) for BNP and TBP are described in Table 1. All samples were analyzed in triplicate. The relative amount of mRNA content of the target gene was normalized to the housekeeping gene mRNA content in the same sample of cDNA. Expression of BNP relative to TBP in each sample was calculated on the basis of the \( \Delta \Delta CT \) method, where \( \Delta CT \) is the difference in threshold cycle (Ct) values between the target and the endogenous control.

Table 1. Sequences of Primers and hybridization probes used

<table>
<thead>
<tr>
<th>Identification</th>
<th>Sequence</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP Forward</td>
<td>5' - GTC AGT CGC TTG GGC TGT - 3'</td>
<td>104</td>
</tr>
<tr>
<td>Reverse</td>
<td>5' - CAG AGC TGG GGA AAG AG - 3'</td>
<td></td>
</tr>
<tr>
<td>UPL Probe</td>
<td>5' - CTC TGC CT - 3'</td>
<td></td>
</tr>
<tr>
<td>TBP Forward</td>
<td>5' - CCC TAT CAC TCC TGC CAC A - 3'</td>
<td>98</td>
</tr>
<tr>
<td>Reverse</td>
<td>5' - GGT CAA GTT TAC AGC CAA GAT TC - 3'</td>
<td></td>
</tr>
<tr>
<td>UPL Probe</td>
<td>5' - AGC CTC TG - 3'</td>
<td></td>
</tr>
</tbody>
</table>

**CYP1B1 activity assay**

CYP1B1 activity was determined using the P450-Glo™ Assay Kit (Promega, Madison, WI) as recommended by the supplier. After 13 days of Ang II infusion, animals were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), the left ventricle was punctured and blood was flushed out by perfusion with cold saline (3 min). The heart and thoracic aorta were dissected free, cleaned of surrounding tissue, snap frozen in liquid N2 and stored until -80°C until use. Tissues were homogenized in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) using a TissueLyser II (2 x 3 min). Following homogenization, samples were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was removed and stored at -80°C until further use. Protein content in the samples was determined by 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) and expressed as relative luminescence units (RLU).

**Plasma levels of 12- and 20-HETE**

Plasma levels of 12-HETE were determined using a 12(S)-HETE ELISA kit (Abnova Corporation), and plasma levels of 20-HETE were determined using a 20-HETE ELISA kit (Detroit R&D, Inc.), as per the manufacturer’s instructions.
Measurement of vascular function

Vascular reactivity: Following anesthesia (described above), the thoracic aorta, superior mesenteric artery and femoral artery were 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 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 was placed on the vessels (thoracic aorta, 9 mN; mesenteric artery, 4 mN; femoral artery 5 mN) and allowed to equilibrate for approximately 30 min. To confirm the viability of the vessels, they were initially tested for the constriction to 60 mM KCl and then washed 3 times with fresh Krebs buffer. Cumulative concentration response curves to phenylephrine (PE), endothelin-1 (ET-1) and vasopressin were obtained and responses measured as force of contraction in mN.

Endothelium-dependent and -independent vasodilation: Endothelial function was examined by constricting the vessels with the concentration of PE that evoked a maximal response followed by the 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 vessels 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 thoracic aorta, mesenteric artery and femoral artery were dissected free, cleaned of surrounding tissue and incubated in 10% buffered formalin overnight. Tissues were dehydrated with graded ethanol followed by xylene (1 hr) and embedded in paraffin. Embedded tissues 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 using an Olympus® inverted system microscope (Olympus America Inc., Melville, NY; model IX50) and photographed using an Olympus® digital camera (Olympus America Inc.; model DP71). Images were analyzed using ImageJ 1.42 (http://rsb.info.nih.gov/nih-image; National Institutes of Health).

Measurement of vascular ROS production

To measure vascular ROS production, sections of thoracic aorta were exposed to dihydroethidium (DHE), following the previously described and validated method (2). Fresh, unfixed aorta samples were placed in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek USA Inc., Torrance, CA) and frozen at -80°C. Ring segments were cut into 30 µm sections using a cryostat (Bright Instrument Company, Huntingdon, Cambridgeshire, England; model OTF) and placed on a glass slide. Sections were incubated in PBS for 30 min at 37°C and then DHE (2 µm) 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 DP71). Images were photographed using an Olympus® digital camera (Olympus America Inc., model DP71) and analyzed using ImageJ 1.42.

Measurement of vascular superoxide production using HPLC
Vascular superoxide production was determined by measuring the oxidation of DHE to 2-hydroxyethidium (2-OHE) following the previously described method (3). Briefly, vascular rings were first incubated in Krebs buffer containing 50 µM DHE for 15 min at 37°C. Vessels were then washed and further incubated in Krebs buffer for 1 h at 37°C. Vessels were homogenized in 300 µl of cold methanol, filtered (0.22 µm) and analyzed using a Shimadzu HPLC system with a C-18 reverse phase column (Nucleosil 250, 4.5mm; Sigma). The mobile phase consisted of a gradient containing 60% acetonitrile and 0.1% trifluoroacetic acid and 2-OHE was separated by a linear increase in acetonitrile concentration from 37 to 47% over 23 min at a flow rate of 0.5 ml/min. Data was collected using LCSolution Chromatography Data System software (Shimadzu, Kyoto, Japan). Fluorescence detection at 580 nm (emission) and 480 nm (excitation) was used to monitor the production of 2-OHE.

**Measurement of NADPH oxidase activity**

NADPH oxidase activity was measured in rat aortic homogenates and mouse heart homogenates by measuring lucigenin (N,N′-dimethyl-9,9′-biacridinium dinitrate)-enhanced chemiluminescence, as described previously (4), with some modifications. Following anesthesia, the thoracic aorta or heart were isolated, cleaned of surrounding tissue, snap frozen in liquid N2 and stored until at -80°C until use. Tissues 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 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.

**Western blot analysis**

Heart and thoracic aorta samples were homogenized in lysis buffer, and protein content was determined by the Bradford method. For rat aorta and heart, 30 µg of protein and for mouse heart, 10 µg of protein were loaded and resolved on 8% SDS-polyacrylamide gels and processed for Western blot analysis as described (5). Blots were probed with different primary and corresponding secondary antibodies, and intensity of the bands was measured with ImageJ 1.42 software.

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

Male C57BL/6 (Cyp1b1+/+) mice were obtained from Jackson Laboratory (Bar Harbor, ME) Both Cyp1b1+/+ and Cyp1b1-/- male mice (25 to 30 g and approximately 8 weeks of age) were used for this study. Mice were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (50 mg/kg, i.p.), and miniosmotic pumps (Alzet®, model 1002) were implanted subcutaneously to infuse Ang II (1000 µg/kg/day) or saline for 13 days. In one group of Cyp1b1+/+ mice and one group of Cyp1b1-/- mice infused with Ang II, TMS (300 µg/kg) was injected i.p. every third day beginning from day 1 of the experiment and blood pressure was measured using a non-invasive 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.
All other parameters measured in $Cyp1b1^{+/+}$ and $Cyp1b1^{-/-}$ mice followed the same protocol as for the rat experiments.

**Statistical analysis**

Data were analyzed by one-way analysis of variance or Student’s $t$-test. The values of 3 to 6 different experiments are expressed as the mean ± SEM. $P$ values < 0.05 were considered statistically significant.

**References**

Table S1. TMS treatment prevents increased cardiac hypertrophy associated with Ang II-induced hypertension in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>TMS</th>
<th>Ang II</th>
<th>Ang II + TMS</th>
<th>Ang II HTN + TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW) (g)</td>
<td>398.7 ± 15.6</td>
<td>414.8 ± 37.1</td>
<td>425.8 ± 24.3</td>
<td>403.7 ± 15.8</td>
<td>428.8 ± 21.2</td>
</tr>
<tr>
<td>Heart weight (HW) (mg)</td>
<td>1380.0 ± 41.3</td>
<td>1490.0 ± 136.79</td>
<td>2040.0 ± 77.1*</td>
<td>1346.7 ± 117.2†</td>
<td>1578.3 ± 126.5‡</td>
</tr>
<tr>
<td>HW (mg)/BW (g)</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>4.8 ± 0.2*</td>
<td>3.3 ± 0.2†</td>
<td>3.7 ± 0.1‡</td>
</tr>
<tr>
<td>BNP:TBP mRNA (ΔΔCt)</td>
<td>1.0</td>
<td>0.7 ± 0.1</td>
<td>5.8 ± 0.4*</td>
<td>2.3 ± 0.7†</td>
<td>Not Determined</td>
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</table>

Animals were treated and results were obtained and calculated as described in Methods. *P < 0.05 Vehicle vs. Ang II; †P < 0.05 Ang II + TMS vs. Ang II; ‡P < 0.05 Ang II HTN + TMS vs. Ang II. Ang II HTN = Animals were infused with Ang II for 13 days and TMS was injected beginning 8th day of the experiment. (n = 6 for all experiments, and data are expressed as mean ± SEM).
Table S2. TMS and ANG II treatment do not alter plasma levels of 12- and 20-HETE in rats

<table>
<thead>
<tr>
<th>Eicosanoid (ng/ml)</th>
<th>Vehicle</th>
<th>TMS</th>
<th>Ang II</th>
<th>Ang II + TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-HETE</td>
<td>13.6 ± 4.6</td>
<td>18.7 ± 4.5</td>
<td>15.2 ± 4.3</td>
<td>13.7 ± 1.8</td>
</tr>
<tr>
<td>20-HETE</td>
<td>12.3 ± 0.3</td>
<td>13.0 ± 1.3</td>
<td>10.5 ± 2.0</td>
<td>11.1 ± 2.1</td>
</tr>
</tbody>
</table>

Plasma levels of 12- and 20-HETE were measured using commercially available kits (12-HETE, Abnova® Corporation; 20-HETE, Detroit R&D, Inc.), according to the manufacturer’s instructions. (n = 6 for all experiments, and data are expressed as mean ± SEM).
Table S3. TMS treatment prevents increased media: lumen ratio associated with Ang II-induced hypertension in rats

<table>
<thead>
<tr>
<th>Blood Vessel</th>
<th>Vehicle</th>
<th>TMS</th>
<th>Ang II</th>
<th>Ang II + TMS</th>
<th>Ang II HTN + TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic aorta</td>
<td>6.36 ± 0.33</td>
<td>6.10 ± 0.24</td>
<td>8.63 ± 0.24*</td>
<td>6.39 ± 0.28†</td>
<td>5.94 ± 0.24‡</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>6.22 ± 0.47</td>
<td>5.93 ± 0.49</td>
<td>8.85 ± 0.74*</td>
<td>6.16 ± 0.61†</td>
<td>6.38 ± 0.14‡</td>
</tr>
<tr>
<td>Femoral artery</td>
<td>6.45 ± 0.50</td>
<td>6.13 ± 0.24</td>
<td>7.85 ± 0.25*</td>
<td>5.80 ± 0.16†</td>
<td>6.36 ± 0.71‡</td>
</tr>
</tbody>
</table>

Animals were treated and the media: lumen ratio of the thoracic aorta, mesenteric artery and femoral artery was calculated as described in Methods. *P < 0.05 Vehicle vs. Ang II; †P < 0.05 Ang II vs. Ang II + TMS; ‡P < 0.05 Ang II vs. Ang II HTN + TMS. Ang II HTN = Animals were infused with Ang II for 13 days and TMS was injected beginning 8th day of the experiment. (n = 4 for all experiments, and data are expressed as mean ± SEM).
TMS counteracts the hypertensive effect of Ang II in rats. (A) Rats were infused with either Ang II (150 ng/kg/min) or vehicle (saline) with miniosmotic pumps for 13 days and given i.p. injections of the CYP1B1 inhibitor, TMS (300 µg/kg), or its vehicle (DMSO, 100 µl) every 3rd day starting day 1 of the experiment, and blood pressure was measured by tail cuff every 3rd day. (B) Rats were infused with Ang II or vehicle for 13 days, and, 8 days after beginning the experiment, one group was given TMS (300 µg/kg i.p.) or its vehicle every 2nd day for the remainder of the experiment, and blood pressure was measured by tail cuff every 3rd day. *P < 0.05 vehicle vs. Ang II; #P < 0.05 Ang II vs. Ang II + TMS; †P < 0.05 vehicle vs. Ang II HTN + TMS, ‡P < 0.05 Ang II vs. Ang II HTN + TMS. (n = 6 for all experiments, and data are expressed as mean ± SEM).
Figure S2

TMS decreases the presence of myofibroblasts and macrophages in the heart of Ang II-treated rats. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. At the completion of the experiment, cardiac tissue was removed and processed for α-Smooth muscle actin or ED-1 immunohistochemistry as described in Methods. (A) α-Smooth muscle actin-positive myofibroblasts are present in the perivascular space and the myocardium of Ang II-treated animals (arrows); these myofibroblasts are absent in the normal heart and in the heart of animals given TMS. (B) ED-1-positive macrophages are accumulated in the perivascular space of hearts from Ang II-treated animals (arrows); however, fewer macrophages are observed in animals treated concurrently with TMS.
TMS decreases CYP1B1 activity but not its expression in rat aorta and heart. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. (A, B) CYP1B1 protein expression was measured by Western blot analysis in the respective tissues from animals in the various treatment groups using 30 µg of protein for loading. (C, D) At the completion of the experiment, tissues were collected for analysis of CYP1B1 activity by P450-Glo™ assay as described in Methods. The activity of CYP1B1 in the thoracic aorta and heart, respectively, is expressed as relative luminescence units (RLU). ‖\( P < 0.05 \) Vehicle vs. TMS; #\( P < 0.05 \) Ang II vs. Ang II + TMS. (n = 6 for all experiments, and data are expressed as mean ± SEM).
Figure S4

TMS decreases CYP1B1 activity in hypertensive rats. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1B legend, after which tissues were collected for analysis of CYP1B1 activity with the P450-Glo™ Assay Kit as described in Methods. The activity of CYP1B1 in the thoracic aorta (A) and heart (B), respectively, is expressed as relative luminescence units (RLU). ‡P < 0.05 Ang II vs. Ang II HTN + TMS. (n = 6 for all experiments, and data are expressed as mean ± SEM).
Figure S5

**Thoracic Aorta**

- Vehicle
- TMS
- Ang II
- Ang II + TMS

**Mesenteric Artery**

- Vehicle
- TMS
- Ang II
- Ang II + TMS

**Femoral Artery**

- Vehicle
- TMS
- Ang II
- Ang II + TMS

**TMS prevents increased vascular reactivity to vasoconstrictor agents in Ang II-treated rats.** Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. Vascular reactivity was measured in the thoracic aorta, mesenteric artery and femoral artery as described in Methods. The vascular response to increasing concentrations of phenylephrine (PE), endothelin-1 (ET-1), and vasopressin (VP) in the thoracic aorta (A) mesenteric artery (B) and femoral artery (C), respectively, from animals in each of the groups. *P < 0.05 vehicle vs. Ang II; #P < 0.05 Ang II vs. Ang II + TMS. (n = 4 for all experiments, and data are expressed as mean ± SEM).
TMS prevents the increase in vascular reactivity to vasoconstrictor agents caused by Ang II infusion. Rats were infused with either Ang II or vehicle for 13 days, and given i.p. injections of TMS as described in Figure S1B legend. Vascular reactivity was measured in the thoracic aorta, mesenteric artery and femoral artery as described in the section of Methods. The vascular response to increasing concentrations of phenylephrine (PE), endothelin-1 (ET-1), and vasopressin (VP) in the thoracic aorta (A), mesenteric artery (B) and femoral artery (C), respectively, from animals in each of the groups. *P < 0.05 Vehicle vs. Ang II; ‡P < 0.05 Ang II vs. Ang II HTN + TMS. (n = 4 for all experiments, and data are expressed as mean ± SEM).
TMS prevents endothelial dysfunction in Ang II-treated rats. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. Endothelial function was measured in the thoracic aorta, mesenteric artery, and femoral artery as described in Methods. The vascular response to increasing concentrations of acetylcholine (ACh; endothelium-dependent relaxation) (A) and sodium nitroprusside (SNP; endothelium-independent relaxation) (B), respectively, was examined in the thoracic aorta, mesenteric artery, and femoral artery from animals in each of the groups. *P < 0.05 vehicle vs. Ang II; #P < 0.05 Ang II vs. Ang II + TMS. (n = 4 for all experiments, and data are expressed as mean ± SEM).
TMS treatment prevents endothelial dysfunction associated with Ang II-induced hypertension. Rats were infused with either Ang II or vehicle for 13 days, and given i.p. injections of TMS as described in Figure S1B legend. Endothelial function was measured in the thoracic aorta, mesenteric artery, and femoral artery as described in the section of Methods. The vascular response to increasing concentrations of acetylcholine (ACh; endothelium-dependent relaxation) (A) and sodium nitroprusside (SNP; endothelium-independent relaxation) (B), respectively, was examined in the thoracic aorta, mesenteric artery, and femoral artery from animals in each of the groups. *P < 0.05 Vehicle vs. Ang II; †P < 0.05 Ang II vs. Ang II HTN + TMS. (n = 4 for all experiments, and data are expressed as mean ± SEM).
Figure S9

TMS treatment prevents ROS production in the rat aorta associated with Ang II-induced hypertension. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. (A, B) Reactive oxygen species (ROS) production was determined in aortic sections by measuring fluorescence produced by 2-hydroxyethidium (2-OHE) after exposure to dihydroethidium (DHE) as described in Methods, and analyzed using ImageJ 1.42. (C) Superoxide production was measured by monitoring the conversion of DHE to 2-OHE using a HPLC-based protocol as described in Methods. *P < 0.05 vehicle vs. Ang II; **P < 0.05 Ang II vs. Ang II + TMS. (n = 3-6 for all experiments, and data are expressed as mean ± SEM).
TMS prevents ROS production in the aorta of rats made hypertensive by Ang II. Rats were infused with either Ang II or vehicle for 13 days, and given i.p. injections of TMS as described in Figure S1B legend. Reactive oxygen species (ROS) production was determined in aortic sections by DHE fluorescence as described in Methods. *P < 0.05 vehicle vs. Ang II; ‡P < 0.05 Ang II vs. Ang II HTN + TMS, (n = 6 for all experiments, and data are expressed as mean ± SEM).
Figure S11

TMS treatment prevents increased NOX 1 expression and NADPH oxidase activity in the rat aorta associated with Ang II-induced hypertension in rats. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. (A) NOX 1 expression was measured in aortic homogenates by Western blot as described in Methods using 30 µg of protein for loading. (B) NADPH oxidase activity was measured in aortic homogenates using a lucigenin-based luminescence assay as described in Methods. (C) NOX 4 expression was measured in aortic homogenates by Western blot as described in Methods using 30 µg of protein for loading. *P < 0.05 vehicle vs. Ang II; #P < 0.05 Ang II vs. Ang II + TMS. (n = 3-6 for all experiments, and data are expressed as mean ± SEM).
TMS decreases Ang II-induced ERK1/2 and p38 MAPK activities in the rat aorta. Rats were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S1A legend. ERK1/2 (A) and p38 MAPK activities (B) were measured in aortic homogenates by Western blot as described in Methods using 30 µg for loading. || P < 0.05 vehicle vs. TMS; * P < 0.05 vehicle vs. Ang II; ‖P < 0.05 Ang II vs. Ang II + TMS. (n = 3 for all experiments, and data are expressed as mean ± SEM).
Online Supplement

Figure S13

TMS and Cyp1b1 gene disruption decrease CYP1B1 activity in the heart and kidney of mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II (1 mg/kg/day) or vehicle (saline) with miniosmotic pumps for 13 days, and given i.p. injections of the CYP1B1 inhibitor, TMS (300 µg/kg), every 3rd day starting day 1 of the experiment. (A, B) At the completion of the experiment, tissues were collected for measurement of CYP1B1 activity by P450-Glo™ assay as described in the section of Methods. The activity of CYP1B1 in the heart and kidney is expressed as relative luminescence units (RLU). (C) CYP1B1 protein expression was measured by Western blot analysis in heart tissue from Cyp1b1+/+ and Cyp1b1−/− mice infused with Ang II or its vehicle (saline) using 10 µg of protein for loading.  †P < 0.05 Vehicle vs. corresponding value from TMS-treated animal; ‡P < 0.05 Ang II vs. corresponding value from Ang II + TMS-treated animal. (n = 4 for all experiments, and data are expressed as mean ± SEM).
TMS and Cyp1b1 gene disruption prevents the increased response of the thoracic aorta to vasoconstrictor agents associated with Ang II-induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S13 legend. Vascular reactivity was measured in the thoracic aorta as described in Methods. (A-D) The response of thoracic aorta of Cyp1b1+/+ and Cyp1b1−/− mice from different treatment groups to increasing concentrations of phenylephrine (PE) and endothelin-1 (ET-1). *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; #P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II + TMS; †P < 0.05 Cyp1b1−/− Ang II vs. Cyp1b1−/− Ang II + TMS. (n = 4 for all experiments, and data are expressed as mean ± SEM).
TMS and Cyp1b1 gene disruption prevent endothelial dysfunction in the thoracic aorta of Ang II-induced hypertension in mice. Cyp1b1+/+ and Cyp1b1−/− mice were infused with either Ang II or vehicle for 13 days and given i.p. injections of TMS as described in Figure S13 legend. Endothelial function was measured in the thoracic aorta as described in Methods. The response of thoracic aorta to increasing concentrations of acetylcholine (ACh; endothelium-dependent relaxation) (A, C, E) and sodium nitroprusside (SNP; endothelium-independent relaxation) (B, D, F). *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; #P < 0.05 Cyp1b1+/+ Ang II vs. Cyp1b1−/− Ang II + TMS. (n = 4 for all experiments, and data are expressed as mean ± SEM).
**Cyp1b1** gene disruption prevents NOX 1 expression and NADPH oxidase activity in the mouse heart associated with Ang II-induced hypertension. *Cyp1b1*+/+ and *Cyp1b1*−/− mice were infused with either Ang II or vehicle for 13 days as described in Figure S13 legend. (A) NOX 1 expression was measured in heart homogenates by Western blot as described in Methods using 10 µg of protein for loading. (B) NADPH oxidase activity was measured in heart homogenates using a lucigenin-based luminescence assay as described in Methods. NADPH activity in the aorta is expressed as relative luminescence units (RLU)/mg protein. §*P < 0.05 *Cyp1b1*+/+ vehicle vs. *Cyp1b1*−/− vehicle; *P < 0.05 vehicle vs. corresponding value from Ang II-treated animal; †*P < 0.05 *Cyp1b1*+/+ Ang II vs. *Cyp1b1*−/− Ang II (n = 4 for all experiments, and data are expressed as mean ± SEM).