Involvement of cytochrome P-450 1B1 in renal dysfunction, injury, and inflammation associated with angiotensin II-induced hypertension in rats

Brett L. Jennings,1 Larry J. Anderson,1 Anne M. Estes,1 Xiao R. Fang,1 Chi Young Song,1 William B. Campbell,2 and Kafaït U. Malik1

1Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee; and 2Department of Pharmacology, Medical College of Wisconsin, Milwaukee, Wisconsin

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Jennings BL, Anderson LJ, Estes AM, Fang XR, Song CY, Campbell WB, Malik KU. Involvement of cytochrome P-450 1B1 in renal dysfunction, injury, and inflammation associated with angiotensin II-induced hypertension in rats. Am J Physiol Renal Physiol 302: F408–F420, 2012. First published November 16, 2011; doi:10.1152/ajprenal.00542.2011.—We investigated the contribution of cytochrome P-450 1B1 (CYP1B1) to renal dysfunction and organ damage associated with ANG II-induced hypertension in rats. ANG II (300 ng·kg⁻¹·min⁻¹) or vehicle were infused for 2 wk, with daily injections of a selective CYP1B1 inhibitor, 2,4,3′,5′-tetramethoxystilbene (TMS; 300 μg/kg ip), or its vehicle. ANG II increased blood pressure and renal CYP1B1 activity that were prevented by TMS. ANG II also increased water intake and urine output, decreased glomerular filtration rate, increased urinary Na⁺ and K⁺ excretion, and caused proteinuria, all of which were prevented by TMS. ANG II infusion caused hypertrophy, endothelial dysfunction, and increased reactivity of renal and interlobar arteries to vasoconstrictor agents and renal vascular resistance and interstitial fibrosis as indicated by accumulation of α-smooth muscle actin, fibronectin, and collagen, and inflammation as indicated by increased infiltration of CD-3⁺ cells; these effects were inhibited by TMS. ANG II infusion also increased production of reactive oxygen species (ROS) and activities of NADPH oxidase, ERK1/2, p38 MAPK, and e-Src that were prevented by TMS. TMS alone had no effect on any of the above parameters. These data suggest that CYP1B1 contributes to the renal pathophysiological changes associated with ANG II-induced hypertension, most likely via increased ROS production and activation of ERK1/2, p38 MAPK, and e-Src and that CYP1B1 could serve as a novel target for treating renal disease associated with hypertension.

2,4,3′,5′-tetramethoxystilbene

The renin-angiotensin system contributes to cardiovascular and renal homeostasis by generating angiotensin peptides, mainly ANG II. This octapeptide constricts blood vessels and increases peripheral vascular resistance, stimulates aldosterone production, exerts direct tubular actions, and consequently increases peripheral vascular resistance, stimulates aldosterone production, exerts direct tubular actions, and consequently increases sympathetic tone and sustained hypertension in rats (33). More recently, it has been reported that the peripheral and central actions of ANG II cause an initial small elevation in blood pressure that results in T cell activation, inflammation, and sustained hypertension (23). In addition to its central actions, the renal actions of ANG II play an important role in chronically elevated blood pressure (12). Deletion of AT₁A receptors in the kidney or extrarenal tissues produces equal decreases in blood pressure (8). ANG II also promotes renal superoxide generation, inflammation, and damage, which are diminished in chemokine receptor 2-deficient mice (21). The increased renal perfusion pressure caused by ANG II-induced hypertension also stimulates renal superoxide production and end-organ damage (37).

The pathophysiological effects of ANG II are mediated by activation of one or more signaling molecules, including ROS and increased ERK1/2 and p38 MAPK activities (3, 31). ANG II also activates phospholipase A2 and releases arachidonic acid (AA) (30, 40). The metabolites of AA generated via lipooxygenase (12-HETE) and cytochrome P-450 (CYP) 4A (20-HETE) stimulate vascular smooth muscle cell (VSMC) migration, proliferation, and/or hypertrophy by activating ERK1/2 or p38 MAPK (42, 50, 52). AA and 20-HETE also stimulate ROS production in VSMCs and endothelial cells, respectively (58, 25). Moreover, 20-HETE mediates ANG II-induced renal vasoconstriction (2), and CYP4A has been implicated in various models of hypertension including ANG II-induced hypertension (31, 44, 45, 51). CYP1B1, which is highly expressed in cardiovascular tissues including VSMCs, can also metabolize AA into HETEs (6) and can generate ROS from this fatty acid in vitro (55). Recently, we reported that ANG II-induced VSMC migration, proliferation, and hypertrophy are mediated by ROS generated through CYP1B1 (55). Moreover, inhibition of CYP1B1 activity by a selective CYP1B1 inhibitor, 2,4,3′,5′-tetramethoxystilbene (TMS) (7), or Cyp1b1 gene disruption minimizes ANG II-induced hypertension (17). In view of the important role of the kidney in hypertension (8, 12), we conducted the current study to investigate the contribution of CYP1B1 to renal dysfunction and end-organ damage caused by ANG II-induced hypertension.

MATERIALS AND METHODS

Materials

ANG II and TMS were purchased from Bachem (Torrence, CA) and Cayman Chemical (Ann Arbor, MI), respectively. Dihydroethidium was from Invitrogen (Carlsbad, CA), the CYP1B1 antibody was purchased from BD Biosciences (Franklin Lakes, NJ), and antibodies against α-smooth muscle-specific actin, fibronectin, transforming growth factor (TGF)-β1, CD-3, NADPH oxidase (NOX-1), and...
NOX-4, ERK1/2, p38 MAPK and c-Src, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary phospho ERK1/2, phospho p38 MAPK, and phospho c-Src antibodies were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals were purchased from Sigma (St. Louis, MO).

**ANG II-Induced Hypertension in Rats**

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. Male Sprague-Dawley rats (250–300 g; Charles River Laboratories, Wilmington, MA) were anesthetized with ketamine (60 mg/kg ip) and xylazine (5 mg/kg ip), and miniosmotic pumps (model 2ML2, Alzet, Cupertino, CA) were implanted subcutaneously to infuse ANG II (300 ng·kg⁻¹·min⁻¹) or its vehicle, DMSO (100 μl) for 13 days. In one group of rats infused with ANG II, TMS (300 μg/kg) or its vehicle, DMSO (100 μl), was injected intraperitoneally (ip) every day beginning from day 1 of the experiment, and the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were measured using a noninvasive tail-cuff method (model XBP 1000, Kent Scientific, Torrington, CT). Before implantation of the miniosmotic pump, rats were acclimated to the blood pressure-measuring device for 1 wk.

**CYP1B1 Activity Assay**

CYP1B1 activity was determined using a P450-Glo Assay Kit (Promega, Madison, WI) as we have previously described (17). At the completion of the experiment, animals were anesthetized as described above, the left ventricle was punctured, and blood was flushed out by a blood pressure-measuring device for 1 wk. In one group of rats infused with ANG II, TMS (300 μg/kg) or its vehicle, DMSO (100 μl), was injected intraperitoneally (ip) every day beginning from day 1 of the experiment, and the systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were measured using a noninvasive tail-cuff method (model XBP 1000, Kent Scientific, Torrington, CT). Before implantation of the miniosmotic pump, rats were acclimated to the blood pressure-measuring device for 1 wk.

**Western Blot Analysis**

Animals were anesthetized and kidneys were 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 (17, 55). Blots were probed with different primary and corresponding secondary antibodies, and intensity of the bands was measured with ImageJ 1.42 software (National Institutes of Health, http://rsb.info.nih.gov/nih-image).

**Metabolic Cage Study or Analysis of Renal Function**

To assess renal function, individual rats were housed in metabolic cages for a period of 24 h after 13 days of ANG II infusion, 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 creatinine using a Vaporo vapor pressure osmometer (model 5520, Wescor, South Logan, UT), protein content by the standard Bradford method, and Na⁺ and K⁺ concentrations using a flame photometer (model 443, Instrumentation Laboratory, Lexington, MA). Creatinine clearance was calculated and used as an estimate of glomerular filtration rate (GFR) by measuring creatinine concentration in serum and urine samples with a QuantiChrom creatinine assay kit (BioAssay Systems, Hayward, CA). For serum collection, blood was obtained via left ventricular puncture following anesthesia of the animals. The blood was allowed to clot for ~30 min at room temperature, after which it was centrifuged 1,500 g for 15 min at 4°C. The serum was collected and stored at −80°C until further analysis. Albumin concentration in urine samples was measured using a rat albumin ELISA kit (Cayman Chemical) according to the manufacturer’s instructions.

**Measurement of Renal Hemodynamics**

**Renal blood flow.** Renal blood flow (RBF) was measured in rats using a 1.0-V Transonic renal flow probe which was attached to a TS420 perivascular flowmeter (Transonic Systems, Ithaca, NY) as described previously (10). Briefly, rats 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 allowed to clot for ~30 min at room temperature. For calculation of volume, urine was aliquoted and stored at −80°C until further analysis. Urine was analyzed for osmolality using a Vaporo vapor pressure osmometer (model 5520, Wescor, South Logan, UT), protein content by the standard Bradford method, and Na⁺ and K⁺ concentrations using a flame photometer (model 443, Instrumentation Laboratory, Lexington, MA). Creatinine clearance was calculated and used as an estimate of glomerular filtration rate (GFR) by measuring creatinine concentration in serum and urine samples with a QuantiChrom creatinine assay kit (BioAssay Systems, Hayward, CA). For serum collection, blood was obtained via left ventricular puncture following anesthesia of the animals. The blood was allowed to clot for ~30 min at room temperature, after which it was centrifuged 1,500 g for 15 min at 4°C. The serum was collected and stored at −80°C until further analysis. Albumin concentration in urine samples was measured using a rat albumin ELISA kit (Cayman Chemical) according to the manufacturer’s instructions.

**Kidney Tissue Levels of HETEs and Epoxysesicosatrienoic Acids**

AA metabolites generated in kidneys from rats in the different treatment groups were measured in kidney homogenate samples, which were prepared using the previously described method (36), with some modifications. Briefly, tissue was homogenized in acetonitrile, after which 5 μl of the appropriate internal standard was added. Samples were sonicated at 4°C for 10 min and stored at −20°C overnight. Samples were centrifuged, and 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. Before analysis, samples were resuspended in 20 μl of methanol and then separated on a C18 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-epoxyeicosatrienoic acids (EETs), were used. Samples were ionized by electron spray with a fragmentor voltage of 120 V and detected in a negative mode as described (32).

**Measurement of Renal Function**

**Renal vascular resistance.** Renal vascular resistance (RVR) was calculated as MAP/RBF.

**Measurement of Vascular Function**

**Vascular reactivity.** Following anesthesia (described above), the renal and interlobar arteries were quickly dissected free, cleaned of surrounding tissue, and ~2-mm rings were mounted in a wire myograph system (model 610M, Danish Myo Technology, Aarhus, Denmark). Vessels were continuously bathed in Krebs buffer (in mmol/l: 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 11.1 glucose, and 2.5 CaCl₂·2 H₂O) at 37°C, which was gassed with 95% O₂-5% CO₂ to maintain the pH at 7.4. Initial tensions of 9 and 5 mN were placed on renal and interlobar arteries, respectively, and the vessels were allowed to equilibrate for ~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 were measured as the force of contraction (in mN).

**Endothelium-dependent and -independent vasodilation.** Endothelial function was examined by constricting the renal and interlobar arteries with PE (20 μM) and then washes three times with fresh Krebs buffer. Cumulative concentration-response curves to nitroprusside (NP) were obtained, and responses were measured as the force of relaxation (in mN).
arteries with the concentration of PE that evoked a maximal response followed by addition of increasing concentrations of 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 and interlobar arteries 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.

**Media/lumen ratio.** Following anesthesia, renal and interlobar arteries were dissected free, cleaned of surrounding tissue, and incubated in 10% buffered formalin overnight. Vessels were dehydrated with graded ethanol followed by xylene (1 h) and embedded in paraffin. Embedded vessels were cut into 5-μm sections using a Microm microtome (model HM 315, GMI, Ramsey, MN) and stained with hematoxylin and eosin. Sections were viewed as described above, and images were analyzed using ImageJ 1.42 software from the National Institutes of Health.

**Measurement of Renal ROS Production**

To measure ROS production, kidney sections as well as sections of renal and interlobar arteries were exposed to dihydroethidium (DHE), following the previously described and validated method (27). Fresh, unfixed artery samples were placed in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) and frozen at −80°C. Ring segments were cut into 30-μm sections using a cryostat (model CM1850, Leica Microsystems, Bannockburn, IL) 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 (27) or 5 μM for kidney sections (11)] was topically applied. Coverslips 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 (model IX50, Olympus America, Melville, NY). Images were photographed using an Olympus digital camera (model DP71, Olympus America) and analyzed using ImageJ 1.42.

![Graphs showing changes in blood pressure and CYP1B1 activity](http://ajprenal.physiology.org/Downloadedfrom://ajprenal.physiology.org)
Table 1. TMS treatment prevented renal dysfunction 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>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake, g/24 h</td>
<td>30.72 ± 2.13</td>
<td>31.67 ± 2.85</td>
<td>31.46 ± 2.10</td>
<td>31.44 ± 3.17</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>39.88 ± 1.39</td>
<td>35.75 ± 1.96</td>
<td>57.75 ± 2.36*</td>
<td>42.50 ± 2.88†</td>
</tr>
<tr>
<td>Urine output, ml/24 h</td>
<td>13.94 ± 1.05</td>
<td>14.81 ± 0.92</td>
<td>27.50 ± 3.06*</td>
<td>16.70 ± 2.29†</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.58 ± 0.03</td>
<td>0.61 ± 0.02</td>
<td>0.89 ± 0.10*</td>
<td>0.61 ± 0.04†</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>1.06 ± 0.03</td>
<td>1.01 ± 0.02</td>
<td>0.72 ± 0.08*</td>
<td>1.00 ± 0.05†</td>
</tr>
<tr>
<td>Urinary Na⁺ excretion, μmol/min</td>
<td>0.23 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.46 ± 0.09*</td>
<td>0.29 ± 0.06†</td>
</tr>
<tr>
<td>Urinary K⁺ excretion, μmol/min</td>
<td>0.67 ± 0.09</td>
<td>0.65 ± 0.08</td>
<td>1.50 ± 0.35*</td>
<td>0.81 ± 0.18†</td>
</tr>
<tr>
<td>Osmolality, mosmol/kgH₂O</td>
<td>1,567 ± 86</td>
<td>1,481 ± 60</td>
<td>1,244 ± 31*</td>
<td>1,653 ± 74†</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>19.8 ± 1.8</td>
<td>18.2 ± 1.3</td>
<td>27.7 ± 2.1*</td>
<td>21.0 ± 1.2†</td>
</tr>
<tr>
<td>Albuminuria, mg/24 h</td>
<td>0.17 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>3.61 ± 1.15*</td>
<td>0.22 ± 0.04†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for all experiments. TMS, 2,4,3′,5′-tetramethoxystilbene Rats were infused with either ANG II or vehicle for 13 days and given daily intraperitoneal (ip) injections of TMS, and placed in metabolic cages for 24 h before completion of the experiment, and the parameters listed above were determined as described in MATERIALS AND METHODS. *P < 0.05 vehicle vs. ANG II. †P < 0.05 ANG II vs. ANG II+TMS.

Measurement of NADPH Oxidase Activity

NADPH oxidase activity was measured in kidney homogenates by measuring N,N′-dimethyl-9,9′-biacridinium dinitrate (lucigenin)-enhanced chemiluminescence, as described previously (57), 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 homogenized and sonicated in lysis buffer containing protease inhibitors (20 mmol/l phosphate buffer, 1 mmol/l EDTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 mmol/l PMSF, 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 was expressed as arbitrary units.

Statistical Analysis

Data were analyzed by one-way ANOVA or Student’s t-test. The values of three to six different experiments are expressed as means ± SE. P values <0.05 were considered statistically significant.

RESULTS

TMS Prevented ANG II-Induced Hypertension in Rats

Infusion of ANG II increased SBP (Fig. 1A), DBP (Fig. 1B), and MAP (Fig. 1C) in rats; these increases were prevented by daily injections of TMS. TMS alone and the vehicles of ANG II (0.9% saline) (Fig. 1) and TMS (DMSO; 100 μl; data not shown) had no effect on BP.

ANG II-Induced Hypertension Was Associated with Increased Renal CYP1B1 Activity But Not its Expression in Rats

Rats infused with ANG II showed an increase in renal CYP1B1 activity that was prevented by TMS (Fig. 2A). In the kidneys of rats given TMS alone, CYP1B1 activity was reduced compared with vehicle-treated animals (Fig. 2A). Renal CYP1B1 protein expression was unchanged in rats from any of the treatment groups (Fig. 2B).

TMS Prevented Increased Thirst and Renal Dysfunction Associated with ANG II-Induced Hypertension in Rats

ANG II-induced hypertension had no effect on food intake but was associated with an increase in water intake and urine output (Table 1). These changes were prevented in animals infused with ANG II and given concurrent injections of TMS.

Table 2. ANG II and TMS do not alter renal content of HETEs and EETs in rats

<table>
<thead>
<tr>
<th>Eicosanoid, pg/mg kidney wt</th>
<th>Vehicle</th>
<th>TMS</th>
<th>ANG II</th>
<th>ANG II+TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HETE</td>
<td>20.69 ± 6.07</td>
<td>20.11 ± 7.09</td>
<td>23.81 ± 14.31</td>
<td>18.22 ± 13.90</td>
</tr>
<tr>
<td>11-HETE</td>
<td>19.86 ± 2.11</td>
<td>19.92 ± 3.28</td>
<td>23.53 ± 7.20</td>
<td>23.98 ± 3.39</td>
</tr>
<tr>
<td>12-HETE</td>
<td>24.05 ± 3.50</td>
<td>31.35 ± 14.63</td>
<td>24.21 ± 3.98</td>
<td>27.04 ± 5.38</td>
</tr>
<tr>
<td>15-HETE</td>
<td>32.71 ± 3.87</td>
<td>29.94 ± 5.40</td>
<td>33.96 ± 5.48</td>
<td>37.48 ± 6.14</td>
</tr>
<tr>
<td>19-HETE</td>
<td>1.10 ± 0.18</td>
<td>1.29 ± 0.25</td>
<td>1.01 ± 0.43</td>
<td>1.50 ± 0.09</td>
</tr>
<tr>
<td>20-HETE</td>
<td>6.65 ± 0.89</td>
<td>7.08 ± 1.39</td>
<td>7.06 ± 2.70</td>
<td>6.58 ± 1.24</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>0.53 ± 0.12</td>
<td>0.68 ± 0.11</td>
<td>0.74 ± 0.11</td>
<td>1.15 ± 0.47</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>0.79 ± 0.21</td>
<td>0.77 ± 0.16</td>
<td>0.96 ± 0.22</td>
<td>1.37 ± 0.68</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4–6 for all experiments. Rats were infused with either ANG II or vehicle for 13 days and given daily ip injections of TMS, and kidney tissue was prepared and analyzed for contents of HETEs and epoxyeicosatrienoic acids (EETs) as described in MATERIALS AND METHODS.

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TMS alone had no effect on food intake, water intake, or urine output (Table 1). Infusion of ANG II decreased GFR, as indicated by an increase in serum creatinine and an associated decrease in creatinine clearance (Table 1). In addition, ANG II-induced hypertension resulted in increased urinary excretion of Na\(^+\) and K\(^+\), decreased urine osmolality, and increased proteinuria and albuminuria (Table 1). Administering TMS prevented these effects of ANG II (Table 1), but treatment with TMS alone had no effect on any of these renal parameters (Table 1).

**ANG II-Induced Renal Dysfunction Did Not Affect Renal Concentrations of Eicosanoids**

ANG II infusion or treatment with TMS did not alter the levels of HETEs or EETs in rat kidneys (Table 2).

**TMS Prevented Increased RVR, Vascular Reactivity, and Hypertrophy of Renal and Interlobar Arteries Associated with ANG II-Induced Hypertension**

After 2 wk of ANG II infusion, RVR was increased; this increase was prevented in rats given TMS (Table 3). The renal (Fig. 3, A and B) and interlobar (Fig. 3, C and D) arteries of rats infused with ANG II displayed increased reactivity to the vasoconstrictor agents PE and ET-1, respectively, that was prevented by TMS. TMS alone did not alter vascular reactivity of either artery to the vasoconstrictor agents (Fig. 3). The increased vascular reactivity of the renal and interlobar arteries from ANG II-treated rats was associated with an increase in the media/lumen ratio of the vessels that was prevented by TMS; this agent alone had no effect on the ratio (Table 4).

**TMS Prevented Endothelial Dysfunction and Increased Superoxide Production in Renal and Interlobar Arteries Associated with ANG II-Induced Hypertension**

Infusion of ANG II was also associated with endothelial dysfunction of renal (Fig. 4A) and interlobar (Fig. 4C) arteries of rats, as indicated by a decreased relaxation response to ACh; this decrease was prevented by concurrent administration of TMS (Fig. 4, A and C). Endothelium-independent relaxation to SNP was not different in renal (Fig. 4B) or interlobar (Fig. 4D) arteries from rats in any of the treatment groups. Infusion of ANG II also resulted in

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### Table 3. TMS treatment prevented changes in renal hemodynamics 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>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>106 ± 2</td>
<td>104 ± 4</td>
<td>173 ± 14*</td>
<td>112 ± 7†</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>8.36 ± 0.24</td>
<td>9.02 ± 0.86</td>
<td>10.73 ± 1.21*</td>
<td>9.00 ± 1.20</td>
</tr>
<tr>
<td>RVR, mmHg:ml−1·min</td>
<td>12.76 ± 0.54</td>
<td>11.90 ± 1.29</td>
<td>18.84 ± 2.30*</td>
<td>12.83 ± 1.10†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4–6 for all experiments. Rats were infused with either ANG II or vehicle for 13 days and given daily ip injections of TMS, and mean arterial pressure (MAP), renal blood flow (RBF), and renal vascular resistance (RVR) were determined as described in MATERIALS AND METHODS.

*P < 0.05 vehicle vs. ANG II. †P < 0.05 ANG II versus ANG II + TMS.
increased superoxide production in rat renal (Fig. 5A) and interlobar arteries (Fig. 5B), as indicated by increased 2-hydroxyethidium (2-OHE) fluorescence; this increase was again prevented by TMS (Fig. 5A and B, respectively). No difference was observed in the fluorescence intensity of either artery given TMS alone (Fig. 5A and B).

TMS Prevented Increased ROS Production and NADPH Oxidase Activity Associated with ANG II-Induced Hypertension in the Rat Kidney

ANG II-induced hypertension increased renal superoxide production, as indicated by increased 2-OHE fluorescence within kidney sections, specifically the glomerulus (Fig. 6A). TMS treatment prevented this increase in renal oxidative stress (Fig. 6A). NADPH oxidase activity also increased in the kidneys of rats infused with ANG II; this increase was prevented by TMS treatment (Fig. 6B). Moreover, increased protein expression of NOX-1 (Fig. 6C), but not NOX-4 (Fig. 6D) in the kidneys of ANG II-infused rats was also prevented by TMS.

TMS Prevented Increased Renal Activity of ERK1/2, p38 MAPK, and c-Src Associated with ANG II-Induced Hypertension

Infusion of ANG II increased activities of ERK1/2, p38 MAPK, and c-Src (Fig. 7, A–C, respectively), in rat kidneys, as measured by their phosphorylation. TMS alone had a minimal effect on the activities of these enzymes; however, it prevented the increase in their activities caused by ANG II (Fig. 7, A–C).

TMS Prevented Renal Damage and Inflammation Caused by ANG II Infusion

Infusion of ANG II increased vascular smooth muscle hypertrophy, as indicated by intrarenal blood vessels displaying greater media thickness compared with vehicle-treated animals (Fig. 8A); this hypertrophy was prevented by TMS. ANG II-induced hypertension was also associated with increased interstitial fibrosis, as indicated by increased interstitial α-smooth muscle actin (Fig. 8A), fibronectin (Fig. 8B), and TGF-β-positive staining in the kidney (Fig. 8C); these changes were prevented by TMS treatment (Figs. 8, A–C). Gomori
trichrome staining of the kidney revealed increased collagen deposition in the interstitial space of kidneys from ANG II-treated rats, further demonstrating interstitial fibrosis associated with ANG II infusion (Fig. 8D). This collagen deposition was absent in animals given TMS (Fig. 8D). Staining of kidney sections with standard hematoxylin and eosin revealed tubular dysfunction, as indicated by increased tubular dilation and protein cast formation in rats infused with ANG II (Fig. 8E), which was prevented by TMS. An increase in CD-3+/H11001 cells, indicating T lymphocyte infiltration, was also observed in the glomerulus of ANG II-infused animals (Fig. 8F). These CD-3+/H11001 cells were absent from the glomeruli of rats concurrently treated with TMS (Fig. 8F).

**DISCUSSION**

This study demonstrated that the renal water and electrolyte imbalance, proteinuria and albuminuria, vascular dysfunction and hypertrophy, and injury and inflammation associated with ANG II-induced hypertension in rats were mediated by CYP1B1 through ROS generation and activation of ERK1/2, p38 MAPK, and c-Src, independent of the production of HETEs and EETs. Previously, we showed that ANG II infusion at 150 ng·kg⁻¹·min⁻¹ for 2 wk in rats increased blood pressure and produced cardiac hypertrophy, fibrosis, and inflammation; aortic, mesenteric, and femoral vascular hypertrophy; and endothelial dysfunction that were prevented by the selective CYP1B1 inhibitor TMS (17). However, ANG II at 150 ng·kg⁻¹·min⁻¹ did not cause renal dysfunction, injury, or inflammation (our unpublished observations). However, ANG II infusion at 300 ng·kg⁻¹·min⁻¹ for 2 wk in the present study that increased SBP to a similar degree as that obtained with 150 ng·kg⁻¹·min⁻¹ (206 ± 13 mmHg, 300 ng·kg⁻¹·min⁻¹ ANG II vs. 185 ± 5 mmHg, 150 ng·kg⁻¹·min⁻¹ ANG II, P > 0.05) caused renal dysfunction, as indicated by a significant increase in water intake and urine output, increased Na⁺ and K⁺ excretion, decreased urine osmolality and GFR, as indicated by reduced creatinine clearance, and increased proteinuria and albuminuria. The concurrent administration of TMS prevented these effects of ANG II, indicating that they were mediated by CYP1B1. CYP1B1 has also been shown to contribute to renal water and electrolyte dysfunction, decreased GFR, and proteinuria associated with deoxycorticosterone acetate salt-induced hypertension in rats (46). In the present study, ANG II increased Na⁺ and K⁺ excretion. This could be due to elevated pressure that may have masked the direct tubular effects of ANG II to increase Na⁺ retention. An increase in Na⁺ excretion caused by ANG II infusion has been observed by other investigators in both rats (53) and mice (5, 13). Therefore, the effect of TMS to prevent the electrolyte and water imbalance caused by ANG II could result from a decrease in blood pressure; however, we cannot exclude any possible modification of the direct tubular effect of ANG II by TMS. In addition,
ANG II has also been shown to increase Na\(^+\) and K\(^+\) excretion by its central action (16). Therefore, it is possible that CYP1B1 might also be involved in the central actions of ANG II to increase thirst, and Na\(^+\) and K\(^+\) excretion, which is inhibited by TMS. Whether the increased urinary Na\(^+\) and K\(^+\) excretion caused by ANG II in the present study resulted in a negative balance of these cations remains to be determined. The effect of TMS to block ANG II-induced renal dysfunction is unlikely to be due to an alteration in ANG II receptor expression and/or its function, as TMS does not alter AT\(_1\) receptor expression or ANG II-induced increase in phosphorylation of PKC\(\alpha\) in VSMCs (55). The plasma half-life and metabolism of TMS is currently unknown, and whether TMS produces its effects directly and/or through one or more of its metabolites remains to be determined. Pharmacokinetic studies in rats have shown that TMS given intravenously as a bolus has a long terminal half-life and slow clearance rate (22). Although TMS has very low bioavailability (4.5%) when given by oral gavage to rats, plasma levels (85 ng/ml) obtained are far above its IC\(_{50}\) (1.5 ng) to inhibit the activity of human recombinant CYP1B1 (22). That CYP1B1 contributes to the renal dysfunction associated with ANG II infusion is supported by our observation that CYP1B1 is expressed in the kidney and is localized in human kidneys in both the nucleus and cytoplasm of tubular cells (29).

We have reported in cultured VSMCs that CYP1B1 is constitutively active and exposure to ANG II for 10 min does not increase its activity (55). However, ANG II increases ROS production that is prevented by inhibitors of cytosolic phospholipase A\(_2\) and AA metabolism, suggesting that the release of AA is required for CYP1B1 to generate ROS (55). CYP1B1 was also constitutively active in the kidney; however, ANG II infusion for 13 days further increased its activity without altering its expression. TMS inhibited both the basal and ANG II-induced increase in CYP1B1 activity. Whether the increase in renal CYP1B1 activity by ANG II in vivo is due to an increase in its reductase activity or some other biochemical modification or involves an endogenous substance generated by ANG II remains to be determined.

ANG II-induced hypertension was also associated with renal vascular hypertrophy, as indicated by an increased media/lumen ratio of renal and interlobar arteries, increased RVR, and increased vascular reactivity of renal and interlobar arteries to PE and ET-1. Additionally, in ANG II-treated animals, endothelium-dependent, ACh-induced relaxations were reduced, but not the relaxations caused by SNP, which acts directly on vascular smooth muscle. Because administration of TMS prevented all these effects associated with ANG II-induced hypertension, it appears that the renal vascular hypertrophy,
increased vascular reactivity, and endothelial dysfunction in renal and interlobar arteries depend on CYP1B1 activity. The loss of endothelial function in various models of experimental hypertension including ANG II-induced hypertension has been attributed to increased production of ROS that inactivates nitric oxide (17, 39, 47–49). The production of ROS, as indicated by 2-OHE fluorescence, in the renal and interlobar arteries was increased in animals infused with ANG II and was abolished in the vessels from TMS-treated rats. Thus it appears that they are dependent on CYP1B1 activity in the kidney. The mechanism by which CYP1B1 mediates renal water and electrolyte dysfunction, vascular dysfunction, injury, and inflammation, and ROS production associated with ANG II-induced hypertension could result from generation of HETEs and EETs. CYP1B1 metabolizes AA in vitro into HETEs and EETs (6), and 20-HETE and EETs stimulate salt and water excretion and alter renal vascular tone (15, 24, 43). In the present study, ANG II infusion increased renal CYP1B1 activity but did not alter renal production of HETEs or EETs. Moreover, TMS inhibited both the basal and ANG II-induced increase in renal CYP1B1 activity with 4% NaCl in the drinking water (28). Therefore, the effect of TMS in preventing renal dysfunction, injury, and inflammation could be due to its effect in preventing the rise in blood pressure caused by ANG II. However, infusion of ANG II at 150 ng·kg⁻¹·min⁻¹ given subcutaneously by miniosmotic pumps for 13 days without 4% NaCl produced a similar increase in blood pressure (17) as that by ANG II given at 2 ng·kg⁻¹·min⁻¹ with 4% NaCl in the drinking water (28), but it failed to cause renal injury or inflammation (data not shown). Therefore, it appears that a combination of salt and a low dose of ANG II contributes to pressure-induced renal damage in a manner similar to that observed with higher doses of ANG II alone. Whether infusion of ANG II (150 ng·kg⁻¹·min⁻¹) given over a prolonged period without salt produces renal injury and inflammation remains to be determined. Although we cannot exclude the effect of TMS in preventing ANG II-induced renal dysfunction, injury, and inflammation by preventing an increase in blood pressure, our demonstration that TMS inhibits VSMC growth in vitro via CYP1B1-generated ROS (55) suggests that ANG II may also produce renal damage via CYP1B1-mediated ROS production, independent of an increase in blood pressure. ANG II can cause renal damage without an increase in blood pressure. For example, in double transgenic rats carrying the human renin and angiotensinogen genes, triple drug treatment (hydralazine + reserpine + hydrochlorothiazide) prevented an ANG II-induced increase in blood pressure, and delayed, but did not prevent end-organ damage, inflammation, and cellular growth in the kidney (26). Also, renal damage and inflammation caused by ANG II+N⁵-nitro-L-arginine methyl ester-induced hypertension is independent of elevated renal perfusion pressure (38). Whether end-organ damage is prevented by TMS in these models of hypertension, or by triple drug therapy without TMS in animals given high doses of ANG II (300 ng·kg⁻¹·min⁻¹) in the present study remains to be determined.

The mechanism by which CYP1B1 mediates renal water and electrolyte dysfunction, vascular dysfunction, injury, and inflammation, and ROS production associated with ANG II-induced hypertension could also contribute to ROS production, because ANG II and renal perfusion pressure can independently increase ROS production (18, 54). The increase in renal perfusion pressure is the major cause of renal injury associated with hypertension by ANG II given at 2 ng·kg⁻¹·min⁻¹ with 4% NaCl in the drinking water (28). Therefore, the effect of TMS in preventing renal dysfunction, injury, and inflammation could be due to its effect in preventing the rise in blood pressure caused by ANG II. However, infusion of ANG II at 150 ng·kg⁻¹·min⁻¹ given subcutaneously by miniosmotic pumps for 13 days without 4% NaCl produced a similar increase in blood pressure (17) as that by ANG II given at 2 ng·kg⁻¹·min⁻¹ with 4% NaCl in the drinking water (28), but it failed to cause renal injury or inflammation (data not shown). Therefore, it appears that a combination of salt and a low dose of ANG II contributes to pressure-induced renal damage in a manner similar to that observed with higher doses of ANG II alone. Whether infusion of ANG II (150 ng·kg⁻¹·min⁻¹) given over a prolonged period without salt produces renal injury and inflammation remains to be determined. Although we cannot exclude the effect of TMS in preventing ANG II-induced renal dysfunction, injury, and inflammation by preventing an increase in blood pressure, our demonstration that TMS inhibits VSMC growth in vitro via CYP1B1-generated ROS (55) suggests that ANG II may also produce renal damage via CYP1B1-mediated ROS production, independent of an increase in blood pressure. ANG II can cause renal damage without an increase in blood pressure. For example, in double transgenic rats carrying the human renin and angiotensinogen genes, triple drug treatment (hydralazine + reserpine + hydrochlorothiazide) prevented an ANG II-induced increase in blood pressure, and delayed, but did not prevent end-organ damage, inflammation, and cellular growth in the kidney (26). Also, renal damage and inflammation caused by ANG II+N⁵-nitro-L-arginine methyl ester-induced hypertension is independent of elevated renal perfusion pressure (38). Whether end-organ damage is prevented by TMS in these models of hypertension, or by triple drug therapy without TMS in animals given high doses of ANG II (300 ng·kg⁻¹·min⁻¹) in the present study remains to be determined.

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Fig. 8. Renal damage and inflammation caused by ANG II infusion are prevented by TMS in rats. Rats were infused with either ANG II or vehicle for 13 days and given daily ip injections of TMS. Increased interstitial staining of α-smooth muscle actin (A; arrows), fibronectin (B), and transforming growth factor (TGF)-β (C), indicators of interstitial fibrosis, was observed in kidney sections from ANG II-treated rats that was prevented by TMS. D: Gomori trichrome staining revealed increased collagen deposition (intense red staining) in the interstitial space of kidneys from ANG II-treated rats but was absent in animals also treated with TMS. E: increased tubular dilation, which was prevented by TMS, was observed in the kidneys of ANG II-treated rats. F: CD-3⁺ cells (arrows), indicating T lymphocyte infiltration, are accumulated in the glomerulus of ANG II-infused animals; however, fewer CD-3⁺ cells were observed in animals concurrently treated with TMS.
production (55). Moreover, TMS inhibits the basal as well as ANG II- and AA-induced CYP1B1 activity in VSMCs. In contrast, TMS inhibited only ANG II- and AA-induced, but not basal ROS production, whereas apocynin inhibited ROS production, but not CYP1B1 activity in VSMCs (55). Although we cannot exclude the possibility that TMS prevents renal damage caused by ANG II by preventing an increase in blood pressure, this appears to be unlikely because TMS also prevents ANG II-induced migration, proliferation, and hypertrophy of VSMCs in culture by inhibiting ROS production (55). In the present study, treatment with TMS prevented the renal vascular and glomerular production of ROS, suggesting that the renal dysfunction, injury, and inflammation associated with ANG II-induced hypertension are mediated by ROS production consequent to activation of NADPH oxidase via CYP1B1 through AA metabolite(s), other than HETEs and EETs, possibly lipid peroxide(s) and/or isoprostanes. Supporting the view that CYP1B1 is involved in NADPH oxidase activation, we have found that TMS and adenosine CYP1B1 short-hairpin RNA inhibit ANG II- and AA-induced increases in NADPH oxidase activity in membranes of rat VSMCs (our unpublished observations).

The mechanism by which ANG II-generated ROS result in renal dysfunction and end-organ damage could be due to activation of one or more signaling pathways. ERK1/2, p38 MAPK, and c-Src have been implicated in the pathophysiological actions of ANG II (14). Our findings indicate that ANG II-induced hypertension is associated with renal activation of these signaling molecules and their activity was inhibited by TMS treatment. These findings suggest that one or more of these signaling molecules contribute to the renal water and electrolyte dysfunction, vascular dysfunction, albuminuria, and renal injury and inflammation associated with ANG II-induced hypertension.

In conclusion, this study demonstrates that CYP1B1 contributes to renal water and electrolyte imbalance, decreased GFR, renal vascular hypertrophy, and increased vascular reactivity, proteinuria, albuminuria, and renal fibrosis and inflammation associated with ANG II-induced hypertension. These renal pathophysiological changes caused by ANG II are most likely due to activation of various signaling molecules including ERK1/2, p38 MAPK, and c-Src through ROS generated by NOX-1 activation by AA metabolite(s)/lipid peroxides produced via CYP1B1, independent of HETEs and EETs. TMS, which inhibits CYP1B1 activity and prevents the renal pathophysiological changes that accompany ANG II-induced hypertension, could be useful in the treatment of renal disease associated with hypertension. However, further studies using models of renal injury that occur independently of pressure such as a chronic high-salt diet are required to determine whether the protective effects of TMS against renal damage caused by ANG II are secondary to a decrease in blood pressure.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.L.J. and K.U.M. provided the conception and design of research; B.L.J., L.J.A., A.M.E., X.R.F., C.Y.S., and W.B.C. performed experiments; B.L.J. analyzed data; B.L.J. and K.U.M. interpreted results of experiments; B.L.J. prepared Figs.; B.L.J. and K.U.M. drafted the manuscript; W.B.C. and K.U.M. edited and revised the manuscript; K.U.M. approved final version of the manuscript.

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