Angiotensin II–Induced Hypertension
Contribution of Ras GTPase/Mitogen-Activated Protein Kinase and Cytochrome P450 Metabolites


Abstract—We reported that norepinephrine and angiotensin II (Ang II) activate the Ras/mitogen-activated protein (MAP) kinase pathway primarily through the generation of cytochrome P450 (CYP450) metabolites. The purpose of the present study was to determine the contribution of Ras and CYP450 to Ang II–dependent hypertension in rats. Infusion of Ang II (350 ng/min for 6 days) elevated mean arterial blood pressure (MABP) (171 ± 3 mm Hg for Ang II versus 94 ± 5 for vehicle group, \(P < 0.05\)). Ras is activated on farnesylation by farnesyl protein transferase (FPT). When Ang II was infused in combination with FPT inhibitor FPT III (232 ng/min) or BMS-191563 (578 ng/min), the development of hypertension was attenuated (171 ± 3 mm Hg for Ang II plus vehicle versus 134 ± 5 mm Hg for Ang II plus FPT III and 116 ± 6 mm Hg for Ang II plus BMS-191563, \(P < 0.05\)). Treatment with the MAP kinase inhibitor PD-98059 (5 mg SC) reduced MABP. The CYP450 inhibitor aminobenzotriazole (50 mg/kg) also diminished the development of Ang II–induced hypertension to 113 ± 8 mm Hg. The activities of Ras, MAP kinase, and CYP450 measured in the kidney were elevated in hypertensive animals. The infusion of FPT III, BMS-191563, or aminobenzotriazole reduced the elevation in Ras and MAP kinase activity. Morphological studies of the kidney showed that FPT III treatment ameliorated the arterial injury, vascular lesions, fibrinoid necrosis, focal hemorrhage, and hypertrophy of muscle walls observed in hypertensive animals. These data suggest that the activation of Ras and CYP450 contributes to the development of Ang II–dependent hypertension and associated vascular pathology. (Hypertension. 2000;36:604-609.)

Key Words: angiotensin II ■ Ras ■ kinases ■ hypertension, experimental ■ cytochrome P450 ■ kidney

Angiotensin II (Ang II), the major biologically active component of the renin-angiotensin system, contributes to the regulation of vascular tone, salt and water balance, and blood pressure.\(^1,2\) It promotes vascular smooth muscle cell (VSMC) migration, hypertrophy, and delayed hyperplasia.\(^3,5\) Ang II also stimulates NADPH oxidase, p21 Ras, and phospholipase (PL)A\(_2\), PLC, and PLD.\(^6-9\) The activation of PLA\(_2\) and PLD by Ang II leads to the release of arachidonic acid,\(^9-11\) which is metabolized by cyclooxygenase to prostaglandins and thromboxane A\(_2\) and by lipoxygenase to hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (HETE). The cyclooxygenase products prostaglandin (PG)E\(_2\) and PGI\(_2\) attenuate the vascular and renal actions of Ang II and contribute to the antihypertensive mechanisms.\(^12\) On the other hand, the cyclooxygenase product thromboxane A\(_2\) and the lipoxygenase product 12-HPETE, which inhibits PGI\(_2\) synthase and thereby promotes the vasoconstrictor effect of endoperoxide PGH\(_2\), contribute to the prohypertensive mechanisms.\(^13\) The level of blood pressure appears to be determined by the balance between the antihypertensive and prohypertensive eicosanoids.\(^13\) Recently, it was reported that Ang II also promotes the metabolism of arachidonic acid into 20-HETE, which constricts blood vessels, causes VSMC hyperplasia, and contributes to high blood pressure.\(^14-17\) Moreover, it has been reported that 20-HETE stimulates Ras-GTPase (Ras) and mitogen-activated protein (MAP) kinase activity, which mediates VSMC proliferation.\(^16,18\) These observations and the demonstrations that Ang II stimulates the Ras/MAP kinase pathway\(^8,19\) and that MAP kinase is involved in Ang II–induced VSMC contraction\(^20,21\) raise the possibility that Ras/MAP kinase activated by the cytochrome P450 (CYP450) product of arachidonic acid, 20-HETE, contributes to hypertension, vascular injury, and hypertrophy in Ang II–dependent models of hypertension. To test this hypothesis, we investigated the activity of Ras, MAP kinase, and CYP450 and the effect of their inhibition on hypertension and associated renal vascular injury and structural alterations produced by Ang II.
Methods

Ang II was obtained from Bachem. Sodium pentobarbital, lauric acid, NAD, NADP, and heparin were from Sigma Chemical Co. The protein farnesyl transferase (FPT) inhibitors FPT III and BMS-191563 were from Calbiochem and a gift from Bristol-Myers Squibb, respectively. The osmotic minipump was from Alzet Corp. H-Ras antibody and anti-goat IgG were from Santa Cruz Biotechnology. Anti-glutathione S-transferase (GST) antibody was from Pharmacia Biotech and aminobenzotriazole (ABT) was from Acros. PD-98059, MAP kinase, and phosphospecific MAP kinase antibodies were from New England Biolabs. \([^{14}\text{C}]\)Lauric acid (55 mCi/mmol) was from Amersham.

Ang II–Induced Hypertension

All procedures were carried out in male Sprague-Dawley rats (Charles River Laboratories), weighing 300 to 350 g, in accordance with institutional guidelines for animal research. Ang II was infused with an Alzet osmotic minipump as described previously. Briefly, the animals were anesthetized with a ketamine-xylazine mixture (80 mg/kg ketamine, 8 mg/kg xylazine), a 1-cm midline incision was made in the abdominal cavity, and an osmotic minipump filled with Ang II dissolved in 0.001N acetic acid was inserted. Ang II was infused at a rate of 350 ng/min for 6 days. The sham control rats received 0.001N acetic acid, the vehicle of Ang II. Inhibitors of Ras farnesyl transferase (2 mg FPT III and 5 mg BMS-191563) were infused along with Ang II. The osmotic minipump delivered FPT III (232 ng/min) or BMS-191563 (578 ng/min) during a period of 6 days. The effect of the CYP450 inhibitor ABT (50 mg/kg) was evaluated by administering ABT intraperitoneally every second day for 6 days.

The MAP kinase kinase (MEK) inhibitor PD-98059 (5 mg dissolved in 300 μL DMSO) or its vehicle was administered subcutaneously on the sixth day after Ang II infusion. Mean arterial blood pressure (MABP; expressed in mm Hg) was measured via a catheter inserted in the femoral artery in animals anesthetized with pentobarbital sodium (60 mg/kg IP). Blood pressure was measured with a pressure transducer (Grass Instruments) and recorded on a polygraph (Grass Instruments).

Measurement of Ras, MAP Kinase, and Phosphospecific MAP Kinase Levels

Frozen kidney tissues were processed for Western blotting analysis as described previously. Proteins (200 to 400 μg) were resolved by SDS-PAGE (12%) and Western blotted with MAP kinase (1:1000 dilution) or Ras (1:200 dilution) or phosphospecific MAP kinase (1:1000 dilution) antibodies. The blots were developed with ECL Western blotting detection reagents (Amersham).

Measurement of Ras Activity

The Ras binding domain (RBD) of Raf-1, immobilized by fusion to GST and bound to glutathione beads, was used as an affinity reagent to precipitate Ras-GTP from cell lysates. The procedures for GST-RBD preparation and affinity precipitation of Ras-GTP were previously described with a few modifications. Two milligrams of GST-RBD preparation and affinity precipitation of Ras-GTP were performed with a pressure transducer (Grass Instruments) and recorded on a polygraph (Grass Instruments).

CYP450 4A Activity

CYP450 4A activity, measured as lauric acid 12-hydroxylase activity, was measured in kidney proteins according to a method described previously.

Results

Effects of Inhibitors of Ras Farnesyl Transferase and CYP450 on MABP in Rats With Ang II–Induced Hypertension

The blood pressure, measured as MABP, was significantly higher in rats infused with Ang II (171 ± 3 mm Hg) than in those infused with vehicle (95 ± 5 mm Hg; \(P<0.05\); Figure 1). Body weight was reduced in rats after Ang II infusion (60 ± 14 g). As previously reported, failure of the rats infused with Ang II to gain weight was associated with a reduction in the food intake, reduced sodium and potassium, and elevation in urinary volume (data not shown).

Infusion of Ras inhibitors FPT III and BMS-191563 with Ang II significantly reduced MABP (Figure 1). The decrease in body weight produced by Ang II infusion was not altered by the administration of FPT III and BMS-191563. The infusion of FPT III or BMS-191563 alone did not alter either MABP or body weight (data not shown). The MEK inhibitor PD-98059, when administered subcutaneously, reduced the blood pressure from 160 to 120 mm Hg within 30 to 60 minutes (\(n=4\)); blood pressure stayed at 120 mm Hg for an additional 30 minutes (Figure 2). The administration of DMSO (vehicle of PD-98059) did not alter the MABP in animals with Ang II–induced hypertension (data not shown).
The administration of the CYP450 inhibitor ABT, which is known to reduce CYP450 activity in spontaneously hypertensive rats, also significantly reduced MABP in animals infused with Ang II. The administration of ABT alone did not alter MABP (Figure 3).

CYP450 4A activity, measured as lauric acid hydroxylase activity, was elevated in the kidney of rats with Ang II–induced hypertension, and treatment with ABT attenuated this response (Figure 4). However, the administration of FPT III or BMS-191563, which are Ras farnesyl transferase inhibitors, did not alter the elevated levels of CYP450 activity in rats with Ang II–induced hypertension.

Effects of Inhibitors of Ras Farnesyl Transferase and CYP450 on Activation of Ras, MAP Kinase, and CYP450 in Rats With Ang II–Induced Hypertension

Protein extracts were obtained from the kidneys of rats with Ang II–induced hypertension, vehicle control animals, and hypertensive animals treated with inhibitors of Ras, MEK, and CYP450. Ras protein levels did not appear to be altered by the infusion of Ang II. Moreover, neither FPT III, BMS-191563, nor ABT altered Ras protein levels in the kidney of rats with Ang II–induced hypertension (Figure 5). Ras interacts with Raf and activates the MEK/MAP kinase signaling pathway. Moreover, it has been shown that residues 51 to 131 of mammalian Raf-1 (called the RBD) bind activated Ras or Ras-GTP but not Ras-GDP.26,27 The Ras activity, measured as Ras–GTP bound to the RBD of Raf, was increased in the Ang II–infused hypertensive rats (Figure 5). However, treatment of Ang II–induced hypertensive animals with FPT III, BMS-191563, or ABT reduced Ras activity in the kidneys (Figure 5). Treatment of normotensive rats with these inhibitors did not alter Ras protein and activity levels (data not shown).

Western blot analysis with the MAP kinase antibody showed that similar levels of ERK1 and ERK2 were present in kidney extracts from normotensive and Ang II–induced hypertensive animals. Moreover, treatment of hypertensive animals with inhibitors of Ras farnesyl transferase and CYP450 did not alter the protein levels (Figure 5). However, phosphorylated MAP kinase levels were elevated with Ang II treatment. This MAP kinase activity was attenuated in the

**Figure 2.** Effect of inhibitors of MEK (PD-98059) on MABP (mm Hg). Rats with Ang II–induced hypertension were treated with 5 mg PD-98059 subcutaneously, and the acute changes in MABP were monitored for 90 minutes (n=4).

**Figure 3.** Effects of inhibitors of CYP450 (ABT) on MABP (mm Hg) in rats with Ang II–induced hypertension. Data are presented as mean±SEM (n=3 to 16). *Significantly different from control. †Significantly different from animals infused with Ang II alone (Veh) (P<0.05).

**Figure 4.** Effects of inhibitors of Ras farnesyl transferase (FPT III and BMS-191563) and CYP450 (ABT) on CYP450 activity in rats with Ang II–induced hypertension. Control (lane 1) and Ang II–infused animals treated with vehicle (lanes 2 to 4), FPT III (lane 5), BMS-191563 (lane 6), and ABT (lane 7) were analyzed for CYP450 activity. The densities of the bands were quantified and presented as arbitrary units.

**Figure 5.** Effects of inhibitors of Ras farnesyl transferase (FPT III and BMS-191563) and CYP450 (ABT) on activation of Ras and MAP kinase. Control (lane 1) and Ang II–infused animals treated with vehicle (lane 2), FPT III (lane 3), BMS-191563 (lane 4), and ABT (lane 5). The blots were probed with Ras monoclonal antibody, MAP kinase antibody, or phosphospecific antibody. The experiments shown were performed 2 or 3 times, and a representative blot of these experiments is presented.
Effects of Ras Farnesyl Transferase and CYP450 Inhibitors on the Morphological Changes in the Kidney of Rats With Ang II–Induced Hypertension

Histological examination of the kidney sections was performed with light microscopy and observed only vascular, not glomerular, injury. There were no structural alterations in animals infused with the vehicle of Ang II or FPT III in normotensive untreated animals. Five animals made hypertensive with the infusion of Ang II showed considerable hypertension-induced vascular pathology in the form of concentric mural hypertrophy and the resultant narrowing of the intrarenal arteries and in “onion-skin” changes in the small arteries. A small branch of an interlobular artery displays transmural fibrinoid necrosis and endothelial cell hypertrophy (Figure 6A, arrow). The large interlobular artery shows hypertrophy of the myocytes and a few red blood cells within the wall. Focal fibrinoid necrosis was also apparent in other arteries of larger size. In addition, four of the five kidneys showed hemorrhage in the arteries, and 3 had fibrinoid necrosis. These lesions were widely distributed in that they were apparent in the large and small intrarenal arteries as well as in the extrarenal arteries located at the hilum of the kidney. Fibrinoid necrosis was found in 2 to 10 arteries per renal cross section (Figure 6).

On the other hand, 2 of 4 animals infused with Ang II plus FPT III had normal kidneys, as seen with light microscopy. Renal lesions in the other 2 animals were graded as mild and limited in terms of severity and the number of arteries with pathological lesions. A very mild and focal fibrinoid necrosis was detected in very few small arteries in these 2 kidneys (2 to 4 arteries with fibrinoid necrosis per cross section of the kidney). The interlobular artery at the cross section is essentially unremarkable except for the mild hypertrophy of the myocytes in the media of a few arteries (Figure 6).

Kidneys from 2 animals treated with Ang II plus ABT were also examined. Vascular lesions in both animals were limited and of mild intensity. One kidney showed focal hemorrhage in the wall of a single extrarenal artery, whereas the second kidney displayed circumferential fibrinoid degeneration in 1 small interlobular renal artery (data not shown).
Discussion
The vascular and renal actions of Ang II play an important role in its hypertensive mechanism. The cardiovascular and renal actions of Ang II are mediated through 1 or more intracellular signaling molecules.1–3,6,7 For example, arachidonic acid metabolites generated through lipoxygenase (12-HETE) or CYP450 (20-HETE) pathways have been reported to contribute to the vascular actions of Ang II.28,29 These products of arachidonic acid may exert their effects through activation of the Ras/MAP kinase pathway in the cardiovascular system.18,30,31 The present study demonstrates that the development of Ang II–induced hypertension in rats is mediated in part via activation of Ras/MAP kinase, probably via generation of arachidonic acid metabolites through CYP450, most likely 20-HETE.

The intraperitoneal infusion of Ang II (125 to 200 ng/min) during a period of 11 days has been shown to increase systolic blood pressure by ≈55 mm Hg in rats.22 However, in the present study, the infusion of Ang II at 350 ng/min during a period of 6 days increased MABP by 60 to 80 mm Hg. The increase in MABP produced by Ang II infusion was associated with an increase in Ras, MAP kinase, and CYP450 activities in the kidney. That Ras contributes to this model of hypertension was suggested by our demonstration that inhibitors of Ras farnesylation, which reduce the association of Ras with membranes and thereby its activity, significantly reduced MABP in animals infused with Ang II. The mechanism by which Ras activation leads to Ang II–induced hypertension most likely involves activation of the MEK/MAP kinase pathway. Ang II has been reported to increase MAP kinase activity through both Ras-dependent and Ras-independent pathways.18,32,33 Our finding that the Ras inhibitor FPT III decreased MAP kinase activity in the kidney of Ang II–infused hypertensive rats suggests the involvement of MAP kinase in this model of hypertension. Support for this view is our demonstration that administration of the MEK inhibitor PD-98059 reduces MABP and MAP kinase activity in the kidney of Ang II–infused hypertensive rats. From these observations, we conclude that the increase in Ras and MAP kinase activity caused by Ang II contributes to the development of hypertension. It is unlikely that the inhibitors of Ras and MEK had a nonspecific effect on the cardiovascular system, because they did not alter arterial blood pressure in normotensive rats. Whether the decrease in MABP produced by Ras/MAP kinase inhibitors in Ang II–infused rats is primarily due to a decrease in peripheral vascular resistance or in cardiac output remains to be determined.

It is well established that hypertrophy of VSMCs is an important feature of hypertension and that the structural changes in the vessel walls contribute to the increase in vascular resistance that promotes hypertension.34 Renin-Ang II–dependent hypertension is associated with pathological changes in the cardiovascular system, including vascular injury and remodeling.35–37 In the present study, in animals infused with Ang II, morphological and histological analyses of the kidney revealed extensive structural alterations, particularly hypertrophy of the vessel wall, which resulted in lumen compromise and an “onion-skin” pattern in small arteries. In our experiments, the infusion of FPT III in animals that received Ang II diminished the vascular pathological alterations, including the severity and number of vascular lesions and hypertrophy in the kidney. These results suggest that the increase in Ras activity also contributes to vascular damage associated with Ang II–induced hypertension. Neutralization of Ras with its antibody has been reported to inhibit Ang II–induced VSMC proliferation.38 These observations raise the possibility that activation of the Ras/MAP kinase pathway might initiate structural changes in the vasculature that contribute to the development of Ang II–induced hypertension.

The mechanism by which Ang II infusion increases Ras and MAP kinase activity in vivo is not known. Ang II has been shown to stimulate Ras/MAP kinase activity via metabolites of arachidonic acid generated through CYP450, mainly 20-HETE, and, to a lesser degree, by the lipoxygenase product 12-HETE.39 Ang II has also been shown to increase 20-HETE production in renal vessels, which contributes to the renal vasconstrictor and pressor action of the peptide.29 20-HETE stimulates Ras and MAP kinase activity in VSMCs and promotes VSMC contraction and proliferation. Therefore, it is possible that the structural changes in the vasculature and the hypertension caused by Ang II infusion are mediated via activation of the Ras/MAP kinase pathway by 20-HETE. Support for this view is our demonstration that ABT, which has been shown to inhibit CYP450 activity and to reduce 20-HETE production in spontaneously hypertensive rats and in the deoxycorticosterone-acetate salt models of hypertension,14,15 diminished the elevation in CYP450 activity and the development of Ang II–induced hypertension. Moreover, the effect of ABT to minimize the development of Ang II–induced hypertension in rats was associated with a decrease in Ras and MAP kinase activity in the kidney. Furthermore, in animals treated with ABT, the Ang II– induced renal vascular lesions were minimized. Although our data with ABT support the involvement of 20-HETE in an Ang II–induced increase in blood pressure and vascular lesions, we cannot exclude the contribution of other prohypertensive eicosanoids or mechanisms unrelated to eicosanoids. Moreover, our studies do not permit us to draw any conclusion as to whether the renal protective action of inhibitors of Ras farnesyl transferase and CYP450 is due to their direct effect on these pathways or a result of decrease in blood pressure. Additional studies with other antihypertensive agents would be required to address this issue.

In conclusion, the present study demonstrates that the activation of MAP kinase via Ras/MEK by metabolites of arachidonic acid generated through CYP450, most likely 20-HETE, contributes to the vascular injury, hypertrophy, and hypertension caused by Ang II in rats. Whether activation of Ras/MAP kinase also contributes to other models of hypertension and vascular structural alterations remains to be determined. Our recent studies indicate that Ras/MAP kinase also contributes to the development of high blood pressure in deoxycorticosterone acetate salt model of hypertension.23

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