Angiotensin Type 2 Receptor–Mediated Phosphorylation of eNOS in the Aortas of Mice With 2-Kidney, 1-Clip Hypertension

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Abstract—To evaluate the role of vascular angiotensin II (Ang II) type 2 (AT$_2$) receptor in renovascular hypertension, we investigated expressions of AT$_2$ receptor and endothelial nitric oxide synthase (eNOS) in thoracic aortas of mice with 2-kidney, 1-clip (2K1C) hypertension. The mRNA levels of AT$_2$ receptor in aortas, but not those of AT$_1$ and bradykinin B$_2$ receptors, increased 14 days but not 42 days after clipping. The contractile response to Ang II (≥0.1 μmol/L) was attenuated in aortic rings excised 14 days after clipping and was restored to that of rings from sham mice by antagonists of AT$_2$ receptor (PD123319) and B$_2$ receptor (icatibant). The aortic levels of total eNOS, phosphorylated eNOS at Ser$^{1177}$ (p-eNOS), total Akt, and phosphorylated Akt at Ser$^{473}$ (p-Akt) were increased in 2K1C mice on day 14, whereas only eNOS levels were increased on day 42. The aortic cGMP levels were ~20-fold greater in 2K1C mice on day 14 compared with sham mice. Administration of nicardipine for 4 days before the excision of aortas 14 days after clipping not only reduced blood pressure but also decreased the aortic levels of eNOS, p-eNOS, Akt, p-Akt, and cGMP to sham levels, whereas the administration of PD123319 or icatibant to 2K1C mice decreased p-eNOS and cGMP to sham levels without affecting blood pressure and the levels of eNOS, Akt and p-Akt. These results suggest that vascular NO production is enhanced by increased eNOS phosphorylation via the activation of AT$_2$ receptors in the course of 2K1C hypertension. (Hypertension. 2005;45:967-973.)

Key Words: angiotensin II ▪ bradykinin ▪ cyclic GMP ▪ nitric oxide synthase ▪ hypertension, renovascular

The 2-kidney, 1-clip (2K1C) Goldblatt model of hypertension is an experimental model that in many respects resembles human renovascular hypertension. It is well-documented that the increased activity of the renin-angiotensin system by the renal secretion of renin plays an important role in the development and maintenance of 2K1C hypertension.1 The critical role of the angiotensin II (Ang II) type 1 (AT$_1$) receptor in mediating 2K1C hypertension has been confirmed,2,3 whereas the role of the Ang II type 2 (AT$_2$) receptor in the development of 2K1C hypertension remains poorly understood. Recent studies have demonstrated that the acute and chronic AT$_2$ receptor blockade by PD123319 does not worsen the course of 2K1C hypertensive mice, suggesting that the AT$_2$ receptor does not play a major counterbalancing role against the vasoconstricting actions of Ang II in 2K1C hypertension.2,4

In a previous study using mice with abdominal aortic banding, we demonstrated the upregulation of AT$_2$ receptors in thoracic aortas, and that the stimulation of AT$_2$ receptors by Ang II attenuated the AT$_1$ receptor-mediated contractile response to Ang II in the aorta via the activation of the kinin-nitric oxide (NO)-cGMP cascade.5 In addition, we provided evidence that the elevation of plasma renin levels after the banding-induced reduction of renal blood pressure is associated with the upregulation of AT$_2$ receptors in the aortas by increased levels of circulating Ang II via the activation of AT$_1$ receptors.5,6

Thus, the first aim of the present study was to determine whether the expression of the vascular AT$_2$ receptor is altered in the course of 2K1C hypertension, and if it plays a vasoprotective role via the activation of the kinin-NO-cGMP cascade. The second aim was to investigate the expression of endothelial NO synthase (eNOS) in the vasculature of mice with 2K1C hypertension, because it is thought that the increased eNOS activity seen in Ang II–dependent models of hypertension serves as an important vasodilator counteracting system by modulating the magnitude of the blood pressure response.7

Methods

2K1C Hypertension in Mice

All animal experiments were performed according to the guidelines of the Kobe Gakuin University Experimental Animal Care and Use Committee. 2K1C hypertension was induced in male mice (9-week-old ICR; Japan SLC; Hamamatsu, Japan) by clipping the right renal
artery with a silver clip (0.14-mm internal gap), as described previously.\(^3\) Sham-operated mice, which underwent the same surgical procedure except for the placement of the renal artery clip, served as controls.

**Detections of AT\(_1\), AT\(_2\), and Bradykinin B\(_2\) Receptor mRNAs by the Reverse-Transcription Polymerase Chain Reaction**

Animals were euthanized under ether anesthesia 14 and 42 days after the sham operation or clipping of the renal artery. To detect AT\(_1\), AT\(_2\), and bradykinin B\(_2\) receptor mRNAs, we used the reverse-transcription polymerase chain reaction with specific primers, followed by Southern blotting, as described previously.\(^5,6\) For the semiquantitative analysis of AT\(_1\) receptor mRNA, the densities of the blots were normalized with GAPDH mRNA blots. To evaluate the roles of blood pressure and the AT\(_1\) receptor on the expression of AT\(_1\) receptor mRNA, nicardipine hydrochloride (2 mg/kg twice per day) or losartan potassium (1 mg/kg once per day) was intraperitoneally administered, respectively, for 15 days (from day 1–1 to day 13) after the sham and 2K1C operations.

**Measurement of the Contractile Response to Ang II in the Aortic Rings**

The thoracic aorta was excised 14 and 42 days after clipping and cut into 3-mm rings, as described previously.\(^6\) After equilibration, cumulative concentration–response curves were constructed for Ang II (0.1 mmol/L to 1 \(\mu\)mol/L). In some experiments, the AT\(_1\)-receptor antagonist, PD123319 (Sigma Aldrich, St. Louis, MO; 1 \(\mu\)mol/L), and the B\(_2\)-receptor antagonist, icatibant (Peptide Institute, Inc, Osaka, Japan; 1 \(\mu\)mol/L), were added to the bath 15 minutes before the cumulative addition of Ang II. Some rings were preincubated with the NO synthase inhibitor, \(N^0\)-nitro-L-arginine methyl ester hydrochloride (l-NAME) (Nacalai Tesque, Kyoto, Japan; 0.1 mmol/L) 30 minutes before the Ang II challenge.

**Immunoblot Analyses of eNOS, Phosphorylated eNOS, Akt, and Phosphorylated Akt**

The aortas were homogenized in lysis buffer (50 mmol/L \(\beta\)-glycerophosphate, 100 mmol/L NaVO\(_3\), 2 mmol/L MgCl\(_2\), 1 mmol/L EGTA, 0.5% Triton X-100, 1 mmol/L dl-dithiothreitol, 20 mmol/L pepstatin, 20 mmol/L leupeptin, 0.1 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Equal amounts of protein (20 \(\mu\)g/lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Hybond-P; Amersham Biosciences). The blots were then blocked with 5% skim milk and 2% bovine serum albumin in Tris-buffered saline/Tween 20. To specifically detect total eNOS, the blots were reprobed with monoclonal anti–\(\beta\)-actin antibodies available commercially (Santa Cruz Biotechnology and Cell Signaling Technology), then the bound antibodies were detected by peroxidase-conjugated anti-rabbit IgG antibodies and the ECL system (Amersham Biosciences). To adjust for loading differences, membranes were reprobed with monoclonal anti–\(\beta\)-actin antibodies (Sigma).

**Assay of the Aortic cGMP Content**

The cGMP contents were measured by radioimmunoassay\(^6\) in the thoracic aortas excised 14 days after the sham and 2K1C operations. To test a possible involvement of NO on the aortic cGMP content, l-NAME (10 mg/kg) was injected into the tail vein 1 hour before the excision of the aorta. Protein contents of the aortas were determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif).

**Effects of Nicardipine, PD123319, and Icatibant on the Aortic Contents of eNOS, p-eNOS, Akt, p-Akt, and cGMP in 2K1C Mice**

Nicardipine hydrochloride (25 mg/kg twice per day), PD123319 (1 mg/kg once per day), or icatibant (0.5 mg/kg once per day) was intraperitoneally administered for 4 days from days 10 to 13 after the sham or 2K1C operations, then the aortas were excised on day 14 for the measurements of eNOS, p-eNOS, Akt, p-Akt, and cGMP.

**Statistical Analysis**

All data are expressed as mean±SE. Statistical comparisons of the plasma renin concentration, blood pressure, AT\(_1\) receptor mRNA levels, and the aortic contents of eNOS, p-eNOS, Akt, p-Akt, and cGMP under various treatments were performed with the 1-way analysis of variance test, with pair-wise comparisons by the Bonferroni-Dunn method. Comparisons of the concentration–response curves of Ang II were performed by the repeated measures analysis of variance test followed by the Bonferroni-Dunn method. Differences were considered significant at \(P<0.05\).

**Results**

**2K1C-Induced Hypertension in Mice**

Systolic blood pressure significantly increased 7 days after the clipping of the right renal artery and this increase was maintained for 42 days, whereas it remained unchanged in sham-operated mice (Figure 1, see http://hypertension.ahajournals.org). The plasma renin concentration increased 7 days after clipping, peaked between days 14 and 28, and then returned to the sham level at 42 days (Figure II).

**Increased Expression of AT\(_1\) Receptor mRNAs in the Thoracic Aortas of 2K1C Mice**

There were no significant differences in AT\(_1\) and bradykinin B\(_2\) receptor mRNA levels in the thoracic aortas of sham and 2K1C mice on days 14 and 42, whereas AT\(_2\) receptor mRNA levels significantly increased in 2K1C mice on day 14, but not on day 42, compared with those in sham mice (Figure 1).

The administration of losartan, but not nicardipine, for 15 days (from days 1–1 to 13) after clipping inhibited the 2K1C-induced upregulation of AT\(_1\) receptor mRNA (Figure 2), whereas both drugs prevented systolic blood pressure elevation in 2K1C mice at 14 days (Figure III).

**Reduced Contractile Response to Ang II in Aortic Rings From 2K1C Mice**

The contractile response to cumulative concentrations of Ang II was studied in ring preparations of thoracic aortas 14 days after clipping (2K1C rings) in comparison to those of sham-operated mice (sham rings) in the organ bath. As shown in Figure 3A, Ang II-evoked contractions were significantly attenuated in 2K1C rings at higher concentrations of Ang II (\(>0.1 \mu\)mol/L) compared with sham rings. The addition of PD123319 (1 \(\mu\)mol/L) or icatibant (1 \(\mu\)mol/L) to the organ bath increased the contractile responses to Ang II of 2K1C rings to the levels observed in sham rings, but did not affect the Ang II responsiveness of sham rings (Figure 3A and 3B). Pretreatment with l-NAME increased the Ang II responsiveness of both sham and 2K1C rings, and no significant differences of the Ang II responsiveness were observed between these l-NAME–treated rings (Figure 3C).

In contrast to the aortic rings excised on day 14, the Ang II responsiveness of aortic rings excised from 2K1C mice on day 42 was not significantly different from that in sham mice (data not shown).
Increased Contents of eNOS, p-eNOS, Akt, and p-Akt, in the Thoracic Aortas of 2K1C Mice

We assayed the aortic contents of eNOS, p-eNOS on Ser1177, Akt, and p-Akt on Ser473 in 2K1C mice by Western blotting. The eNOS protein contents were significantly increased in the aortas of 2K1C mice on days 14 and 42 compared with those of sham mice (Figure 4). Protein contents of p-eNOS, Akt, and p-Akt were also increased in the aortas of 2K1C mice on day 14 compared with those of sham mice, but were not different from sham levels on day 42 (Figure 4).

To test whether the activation of AT2 receptors was responsible for the upregulations of eNOS and Akt, PD123319 or icatibant was administered for 4 days before the excision of aortas 14 days after clipping. Both PD123319 and icatibant decreased the p-eNOS contents to sham levels, but did not affect the upregulations of eNOS, Akt, and p-Akt (Figure 5). In contrast, nicardipine administration for 4 days before the excision of aortas inhibited the upregulations of eNOS, p-eNOS, Akt, and p-Akt (Figure 5).

Elevated systolic blood pressure levels of 2K1C mice at 14 days (129.3±4.5 mm Hg in 4 saline-treated 2K1C mice versus 111.0±3.3 mm Hg in 4 saline-treated sham-mice; P<0.001) were decreased to sham levels by nicardipine administration (109.5±4.3 mm Hg in 4 nicardipine-treated 2K1C mice versus 108.0±1.7 mm Hg in 4 nicardipine-treated sham-mice; P>0.1), but were unaffected by PD123319 (132.3±3.6 mm Hg in 4 PD123319-treated 2K1C mice versus 108.3±4.2 mm Hg in 4 PD123319-treated sham mice; P>0.001) and icatibant (125.6±3.5 mm Hg in 4 icatibant-treated 2K1C mice versus 109.8±1.7 mm Hg in 4 icatibant-treated sham mice; P<0.001).

Increased cGMP Contents of the Thoracic Aortas of 2K1C Mice

To evaluate vascular NO production in 2K1C mice in vivo, the cGMP contents of the aortas were assayed on day 14. The cGMP contents were ~20-fold greater in 2K1C mice compared with sham mice (23.40±1.7 pmol/mg protein in 9 2K1C mice versus 1.12±0.10 pmol/mg protein in 8 sham mice; P<0.001) (Figure 6). Elevated aortic cGMP contents of 2K1C mice declined below sham levels 1 hour after the intravenous injection of L-NAME (10 mg/kg) (Figure 6).
The elevation of cGMP in the aortas of 2K1C mice was inhibited by the administrations of nicardipine, PD123319, or icatibant for 4 days before the excision of aortas on day 14 (Figure 6).

Discussion

It has been demonstrated that the increased circulating Ang II plays the pivotal role in the development of 2K1C hypertension, whereas the augmentation of intrarenal Ang II levels by the renin-independent mechanisms plays the crucial role in the maintenance phase of 2K1C hypertension.1 Consistent with these characteristics of 2K1C hypertension, we observed elevated plasma renin concentrations in mice within 14 days after clipping, followed by their reduction to sham levels after 42 days, although the blood pressure was maintained at a high level. Concomitantly with these changes in plasma renin concentrations, the AT2 receptor mRNA levels revealed a 2-fold increase in the thoracic aortas of 2K1C mice on day 14, and then returned to sham levels by day 42. The administration of losartan, but not nicardipine, inhibited the 2K1C-induced upregulation of AT2 receptor mRNA, despite the inhibition of 2K1C-induced blood pressure elevation by either drug, suggesting that the increased level of circulating Ang II, and not mechanical stress on the aortic wall, stimulates the expression of AT2 receptor mRNA in the aorta via the activation of AT1 receptors. These results are comparable to our findings on the AT1 receptor-dependent upregulation of AT2 receptors in the thoracic aortas of rats and mice after abdominal aortic banding.5,6

The contractile responses to Ang II at concentrations >0.1 μmol/L were attenuated in aortic rings excised from 2K1C mice on day 14, but not on day 42, compared with those from sham mice. Because such decreased responses to Ang II were restored to those found in sham rings by PD123319 or icatibant, it is likely that the increased number of AT2 receptors in the aortic rings mediates the attenuation of AT1 receptor-induced contractile activity of Ang II via a signaling pathway involving the bradykinin B2 receptor. In addition, L-NAME treatment abolished differences in the Ang II responsiveness of sham and 2K1C rings, supporting the idea of the involvement of the kinin-NO cascade in AT2 receptor signaling. However, the finding that the attenuation of Ang II responsiveness in 2K1C rings was only observed at higher concentrations of Ang II than those reached in 2K1C hypertension9 may not support a relevant role of AT2 receptors in the development of 2K1C-induced blood pressure elevation. This notion is supported by previous observations that acute and chronic AT2 receptor antagonist administrations did not modify the course of 2K1C hypertension in mice.2,4

The contractile responses to Ang II in ring preparations of the thoracic aorta. Thoracic aortas were dissected from mice 14 days after sham and 2K1C operations, and the contractile responses to Ang II were compared by constructing cumulative concentration–response curves for Ang II. The results were expressed as the percentage of contraction evoked by 40 mmol/L KCl. A, Comparison of the cumulative concentration–response curves for Ang II of the aortic rings of sham-operated and 2K1C mice in the presence or absence of PD123319 (PD 1 μmol/L), which was added 15 minutes before the cumulative addition of Ang II. B, The effect of icatibant on the response to Ang II of the aortic rings of sham-operated and 2K1C mice. Icatibant (1 μmol/L) was added 15 minutes before the cumulative concentration–response curves for Ang II of the aortic rings of sham-operated and 2K1C mice in the presence or absence of PD123319 (PD 1 μmol/L), which was added 15 minutes before the cumulative addition of Ang II. C, The effect of L-NAME on the response to Ang II of the aortic rings of sham-operated and 2K1C mice. L-NAME (0.1 mmol/L) was added 30 minutes before the cumulative addition of Ang II. Values are the mean±SEM (n=4) for each point. *P<0.001 vs sham; †P<0.001 vs 2K1C.
We found increased basal contents of total eNOS protein in the aortas of 2K1C mice. Studies involving cell culture experiments and animal models have demonstrated that the mechanical forces on the vascular wall, such as blood pressure and shear stress, increase the eNOS expression of endothelial cells. In addition, a recent study using rat cardiomyocytes demonstrated that the AT2 receptor mediated the Ang II-induced increase in eNOS expression through a calcineurin-dependent pathway. Thus, there seemed to be 2 potential mechanisms for the upregulation of eNOS proteins in the aortas of 2K1C mice: increased mechanical forces and the activation of AT2 receptors. To test these hypotheses, nicardipine and PD123319 were administered for 4 days before the excision of aortas on day 14. We found that nicardipine, but not PD123319, blocked the 2K1C-induced upregulation of eNOS, suggesting that the increased mechanical forces, and not the activation of AT2 receptors, are responsible for the increased protein expression of eNOS in the aortas of 2K1C mice.

Besides eNOS protein levels, eNOS phosphorylation is thought to regulate enzyme activity in both a Ca2+-calmodulin–dependent and Ca2+-calmodulin–independent fashion. Among the 5 potential phosphorylation sites of eNOS, Ser1177 is considered to function as a sensor of shear stress, because the exposure of endothelial cells to laminar shear stress specifically increases phosphorylation at this site. In addition, various vasoactive substances, such as acetylcholine, bradykinin, insulin, and vascular endothelial growth factor, stimulate endothelial cells to elicit phosphorylation of the Ser1177 site of eNOS by Akt, protein kinase C, A, or G, and AMP-dependent protein kinase. The eNOS phosphorylation on Ser1177 results in an increase in eNOS activity, as well as in eNOS sensitivity to Ca2+-calmodulin, leading to enhanced NO production. In the present study, we found that the aortic levels of p-eNOS (Ser1177), Akt, and p-Akt (Ser473) increased in 2K1C mice, suggesting an enhanced eNOS phosphorylation on Ser1177 via phosphoinositide-3 kinase-dependent Akt activation in the aortas. However, the administration of PD123319 or icatibant blocked the 2K1C-induced increase of p-eNOS, but not of total eNOS, Akt, and p-Akt. These results suggest that the increased eNOS phosphorylation on Ser1177 is mediated by the activation of AT2 and bradykinin B2 receptors via Akt-independent mechanisms in 2K1C mice. In fact, a recent study provided evidence that bradykinin stimulated eNOS phosphorylation on Ser1177 via the activation of protein kinase A. Together, these data suggest that the increased number of AT2 receptors in the aortas of 2K1C mice mediates eNOS phosphorylation via the activation of B2 receptors. However, the administration of nicardipine for 4 days inhibited not only increases in total eNOS protein but also those in the p-eNOS, Akt, and p-Akt contents of the aortas of 2K1C mice, although the drug did not affect the 2K1C-induced upregulation of AT2 receptors. The mechanisms by which nicardipine inhibited the 2K1C-induced upregulation of p-eNOS are unknown, but the enhanced expression of eNOS protein under increased mechanical forces may link it to the AT2 receptor-mediated eNOS phosphorylation on Ser1177.

The cGMP contents of the thoracic aortas were significantly increased in 2K1C mice compared with sham mice.
and the increased levels were reduced below those in sham mice after L-NAME administration, indicating that basal NO production in the aorta was augmented in 2K1C hypertension. The administration of PD123319 or icatibant for 4 days not only blocked the 2K1C-induced increase in p-eNOS but also inhibited the elevation of aortic cGMP in 2K1C mice. Thus, it is likely that NO production in the aortas is increased in 2K1C hypertension by enhanced eNOS phosphorylation via the activation of AT2 receptors.

**Perspectives**

This study is the first to our knowledge to show that increased levels of circulating Ang II in the renovascular hypertension induce the upregulation and activation of vascular AT2 receptors that stimulate eNOS phosphorylation by Akt-independent mechanisms through the signaling pathway of B2 receptors, resulting in increased NO production. Thus, Ang II regulates NO production in vasculatures by the AT2 receptor-mediated eNOS phosphorylation during the development of renin-dependent hypertension, which may play a protective role in vascular disorders, including atherosclerosis. Evidence provided in this study will be helpful to understand a role of the vascular AT2 receptors in the renovascular hypertension.

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**References**


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