Angiotensin II Stimulates Endothelial NO Synthase Phosphorylation in Thoracic Aorta of Mice With Abdominal Aortic Banding Via Type 2 Receptor

Katsutoshi Yayama, Hiromi Hiyoshi, Daichi Imazu, Hiroshi Okamoto

Abstract—Abdominal aortic banding in mice induces upregulation of angiotensin II (Ang II) type 2 (AT\textsubscript{2}) receptors in the pressure-overloaded thoracic aorta. To clarify mechanisms underlying the vascular AT\textsubscript{2} receptor-dependent NO production, we measured aortic levels of endothelial NO synthase (eNOS), eNOS phosphorylated at Ser\textsuperscript{633} and Ser\textsuperscript{1177}, protein kinase B (Akt), and Akt phosphorylated at Ser\textsuperscript{73} in thoracic aortas of mice after banding. Total eNOS, both forms of phosphorylated eNOS, Akt, and phosphorylated Akt levels, as well as cGMP contents, were significantly increased 4 days after banding. The administration of PD123319 (an AT\textsubscript{2} receptor antagonist) or icatibant (a bradykinin B\textsubscript{2} receptor antagonist) abolished the banding-induced upregulation of both forms of phosphorylated eNOS, as well as elevation of cGMP, but did not affect the upregulation of eNOS, Akt, and phosphorylated Akt. In the in vitro experiments using aortic rings prepared from banded mice, Ang II produced significant increases in both forms of phosphorylated eNOS, as well as cGMP, and these effects were blocked by PD123319 and icatibant. Ang II–induced eNOS phosphorylation and cGMP elevation in aortic rings were inhibited by protein kinase A (PKA) inhibitors H89 and KT5720 but not by phosphatidylinositol 3-kinase inhibitors wortmannin and LY24002. The contractile response to Ang II was attenuated in aortic rings from banded mice via AT\textsubscript{2} receptor, and this attenuation was blocked by PKA inhibitors. These results suggest that the activation of AT\textsubscript{2} receptor by Ang II induces phosphorylation of eNOS at Ser\textsuperscript{633} and Ser\textsuperscript{1177} via a PKA-mediated signaling pathway, resulting in sustained activation of eNOS. (Hypertension. 2006;48:958-964.)

Key Words: angiotensin II type 2 receptor ■ endothelial nitric oxide synthase ■ bradykinin ■ signal transduction

N O is produced in the vascular system by endothelial NO synthase (eNOS). The enzymatic activity of eNOS is stimulated by a variety of mechanical forces, such as shear stress and stretching, as well as hormonal factors, including vascular endothelial growth factor, estrogen, acetylcholine, and bradykinin.\textsuperscript{1-6} The enzyme activity of eNOS is regulated by PKA, protein kinase B (Akt), and Akt phosphorylated at Thr\textsuperscript{273} in thoracic aortas of mice after banding. Total eNOS, both forms of phosphorylated eNOS, Akt, and phosphorylated Akt levels, as well as cGMP contents, were significantly increased 4 days after banding. The administration of PD123319 (an AT\textsubscript{2} receptor antagonist) or icatibant (a bradykinin B\textsubscript{2} receptor antagonist) abolished the banding-induced upregulation of both forms of phosphorylated eNOS, as well as elevation of cGMP, but did not affect the upregulation of eNOS, Akt, and phosphorylated Akt. In the in vitro experiments using aortic rings prepared from banded mice, Ang II produced significant increases in both forms of phosphorylated eNOS, as well as cGMP, and these effects were blocked by PD123319 and icatibant. Ang II–induced eNOS phosphorylation and cGMP elevation in aortic rings were inhibited by protein kinase A (PKA) inhibitors H89 and KT5720 but not by phosphatidylinositol 3-kinase inhibitors wortmannin and LY24002. The contractile response to Ang II was attenuated in aortic rings from banded mice via AT\textsubscript{2} receptor, and this attenuation was blocked by PKA inhibitors. These results suggest that the activation of AT\textsubscript{2} receptor by Ang II induces phosphorylation of eNOS at Ser\textsuperscript{633} and Ser\textsuperscript{1177} via a PKA-mediated signaling pathway, resulting in sustained activation of eNOS.
mediated eNOS phosphorylation, although the precise mechanisms are unknown.

Thus, the first aim of the present study was to confirm that the activation of AT2 receptors is associated with phosphorylation of eNOS at Ser633 and/or Ser1177 and results in increased NO production. The second aim was to determine which protein kinases are involved in AT2 receptor–dependent eNOS phosphorylation, because phosphorylation of eNOS at Ser633 and Ser1177 in intact cells by different kinases seems to be regulated by different stimuli.8 We used thoracic aortas of mice with abdominal aortic banding, because AT2 receptors are markedly upregulated in these aortas. Our results demonstrate that activation of AT2 receptors induces phosphorylation of eNOS at Ser633 and Ser1177 in an Akt-independent and PKA-dependent manner.

Methods

Animals and Aortic Banding

All of the animal experiments were performed according to the guidelines of the Kobe Gakuin University Experimental Animal Care and Use Committee. Male, 8-week-old ICR mice (Japan SLC, Hamamatsu, Japan) weighing 35 to 37 g were used for all of the studies. Aortic banding was performed by constriciting the abdominal aorta between the renal arteries just below the renal bifurcations as described previously.16

Western Blotting of eNOS, Phosphorylated eNOS, Akt, and Phosphorylated Akt

Protein contents of eNOS, Akt, and their phosphorylated forms in aortic tissues were measured by Western blotting, as described previously,15 using commercially available polyclonal antibodies specific for Akt, phosphorylated Akt (p-Akt) at Ser473 (p-Akt-Ser473) and phosphorylated eNOS (p-eNOS) at Ser1177 ([p-eNOS-Ser1177] Cell Signaling), eNOS (Santa Cruz Biotechnology), and eNOS phosphorylated at Ser633 ([p-eNOS-Ser633] Upstate). To adjust for loading differences, blots were reprobed with a monoclonal antibody to β-actin (Sigma).

Measurement of Contractile Response to Ang II in Aortic Rings

The thoracic aortas were excised 4 days after sham operation or banding and cut into 3-mm rings, as described previously.15 Aortic rings were equilibrated for 1.5 hours in an organ bath containing a Krebs–Henseleit solution (37°C, pH 7.4) under a resting tension of 0.7 g then treated with Ang II (0.1 μmol/L) for 10 minutes. To examine the effects of receptor antagonists or protein kinase inhibitors, aortic rings were equilibrated in the organ bath for 1 hour, then incubated for an additional 30 minutes with PD123319 (1 μmol/L; Sigma),14 icatibant (1 μmol/L; Sigma),15 wortmannin (1 μmol/L; Sigma),15 LY24002 (10 μmol/L; Calbiochem),16 H89 (10 μmol/L; Cell Signaling),16 or KT5720 (10 μmol/L; Calbiochem) before exposure to Ang II. Rings were frozen in dry ice acetone and kept at −80°C until they were analyzed.

Assay of Aortic cGMP Content

The cGMP content in thoracic aortas or aortic rings was measured by radioimmunoassay.15 Protein content of samples was determined by the Bio-Rad protein assay kit (Bio-Rad).

Statistical Analysis

All of the data are expressed as mean±SE. Statistical comparison of cGMP content under various treatments was performed using 1-way ANOVA and pairwise comparisons by the Bonferroni–Dunn method.

Results

Increased eNOS and p-eNOS Contents in Thoracic Aortas of Mice After Abdominal Aortic Banding

We determined the protein content of eNOS and p-eNOS in thoracic aortas of mice 4 days after abdominal aortic banding, because the AT2 receptor expression increases markedly in thoracic aortas of banded mice compared with sham animals.14 The eNOS content in aortas of banded mice was ≈3-fold greater than those of sham animals (Figure 1). Upregulation of eNOS by banding was not affected by the administration of either PD123319 (an AT2 receptor antagonist; 10 mg/kg IP, twice a day)20 or icatibant (a bradykinin B2 receptor antagonist; 0.5 mg/kg IP, once a day)21 for 4 days after banding (Figure 1). As shown in Figure 1, aortic contents of p-eNOS at Ser633 and at Ser1177 in banded mice were ≈3-fold greater than in sham animals, and these upregulations of p-eNOS were completely inhibited by administration of PD123319 or icatibant for 4 days. Neither PD123319 nor icatibant affected the levels of eNOS and p-eNOS in sham animals (data not shown).

Figure 1. The protein levels of eNOS, p-eNOS-Ser633, and p-eNOS-Ser1177 in thoracic aortas of mice 4 days after sham operation or aortic banding. PD123319 (PD; 10 mg/kg, twice a day) or icatibant (Icat; 0.5 mg/kg, once a day) were administered intraperitoneally for 4 days after sham operation or banding. Top, representative Western blots. Bottom, bar graph showing densitometric data for eNOS, eNOS phosphorylated at Ser633, and at Ser1177 as ratios relative to β-actin. Values are the mean±SEM (n=6). *P<0.001 vs saline-treated sham mice; #P<0.001 vs saline-treated banded mice.
Increased Akt and p-Akt Contents in Thoracic Aortas of Mice After Abdominal Aortic Banding

Akt, a serine/threonine kinase that is a major target of phosphatidylinositol 3-kinase (PI3K), phosphorylates eNOS at Ser1177, and this phosphorylation enhances eNOS activity and increases NO production in vascular endothelial cells.22,23 Protein levels of both Akt and p-Akt at Ser473 in thoracic aortas were significantly increased in aortas of banded mice compared with sham mice (Figure 2). The administration of PD123319 or icatibant for 4 days after banding did not affect the banding-induced upregulation of Akt and p-Akt (Figure 2). PD123319 and icatibant did not affect the aortic cGMP content in sham animals (data not shown).

Increased cGMP Content in Thoracic Aortas of Mice After Abdominal Aortic Banding

The cGMP contents in thoracic aortas of banded mice were 6-fold greater than those in sham mice (18.13 ± 1.87 fmol/mg of protein in 6 banded mice versus 3.05 ± 0.48 fmol/mg of protein in 6 sham-mice; \(P<0.001\); Figure 3). The banding-induced elevation of aortic cGMP was abolished by administration of PD123319 or icatibant for 4 days after banding (Figure 3). PD123319 and icatibant did not affect the aortic cGMP content in sham animals (data not shown).

Effects of Ang II on eNOS and p-eNOS Levels in Aortic Rings In Vitro

To determine the involvement of Ang II in eNOS phosphorylation, we studied the in vitro effects of Ang II on levels of eNOS and p-eNOS in aortic rings prepared from sham and banded mice. The basal levels of eNOS, p-eNOS-Ser633, and p-eNOS-Ser1177 were significantly greater in aortic rings from banded mice than those from sham mice, although aortic rings were incubated in vitro for 1.5 hours after the excision (Figure 4). Basal levels of these proteins in aortic rings from banded mice were not affected by an in vitro treatment with PD123319 (1 μmol/L) or icatibant (1 μmol/L) for 30 minutes (data not shown). Treatment with Ang II (0.1 μmol/L) for 10 minutes did not affect the contents of eNOS in aortic rings from banded mice (Figure 4) or sham mice (data not shown).

In contrast, the levels of both p-eNOS-Ser633 and p-eNOS-Ser1177 were further increased in aortic rings from banded mice after treatment with Ang II (Figure 4), whereas aortic rings from sham mice were not affected by Ang II (data not shown). The elevation of p-eNOS-Ser633 or p-eNOS-Ser1177 by Ang II was significantly inhibited by PD123319 (1 μmol/L) or icatibant (1 μmol/L; Figure 4).

Effects of Protein Kinase Inhibitors on Ang II–Induced Phosphorylation of eNOS in Aortic Rings

To determine which protein kinases are involved in AT1 receptor–mediated phosphorylation of eNOS, aortic rings pretreated with PI3K inhibitors (wortmannin, 1 μmol/L; 

![Figure 2. The protein levels of Akt and p-Akt-Ser473 in thoracic aortas of mice 4 days after sham operation or aortic banding. PD123319 (PD; 10 mg/kg; twice a day) or icatibant (Icat; 0.5 mg/kg, once a day) were administered intraperitoneally for 4 days after sham operation or banding. Top, representative Western blots. Bottom, bar graph showing densitometric data for Akt and Akt phosphorylated at Ser473 as ratios relative to β-actin. Values are the mean±SEM (n=6). *P<0.001 vs saline-treated sham mice.](http://hyper.ahajournals.org/)

![Figure 3. The cGMP levels in thoracic aortas of mice 4 days after sham operation or aortic banding. PD123319 (PD; 10 mg/kg; twice a day) or icatibant (Icat; 0.5 mg/kg; once a day) was administered intraperitoneally for 4 days after sham operation and aortic banding. The cGMP content in thoracic aortas was measured by radioimmunoassay. Values are mean±SEM, (n=6); *P<0.001 vs saline-treated sham mice; #P<0.001 vs saline-treated banded mice.](http://hyper.ahajournals.org/)
LY24002, 10 μmol/L) or PKA inhibitors (H89, 10 μmol/L; KT5720, 10 μmol/L) were exposed to Ang II for 10 minutes. Neither wortmannin nor LY24002 affected the Ang II–induced increases of p-eNOS-Ser1177 and p-eNOS-Ser633 in aortic rings from banded mice (Figure 5). In contrast, pre-treatment of aortic rings from banded mice with H89 or KT5720 resulted in a significant inhibition of Ang II–induced increases in both p-eNOS-Ser633 and p-eNOS-Ser1177 (Figure 5). These PKA inhibitors did not affect the basal levels of p-eNOS-Ser1177 and p-eNOS-Ser633 (data not shown). These protein kinase inhibitors did not affect the levels of total eNOS (Figure 5), Akt, and p-Akt (data not shown) in Ang II–treated or untreated aortic rings from banded mice.

Effect of AT2 and B2 Receptor Antagonists and Protein Kinase Inhibitors on Ang II–Induced Increases in cGMP Contents of Aortic Rings

The cGMP content of aortic rings from banded mice was significantly greater than in aortic rings from sham mice (10.05±1.12 fmol/mg of protein in 8 banding rings versus 2.33±0.32 fmol/mg of protein in 8 sham-rings; P<0.001; Figure 6). Basal levels of cGMP in rings from banded mice were not affected by an in vitro treatment with PD123319 (1 μmol/L), icatibant (1 μmol/L), wortmannin (1 μmol/L), LY24002 (10 μmol/L), H89 (10 μmol/L), or KT5720 (10 μmol/L) for 30 minutes (data not shown).

Treatment with Ang II (0.1 μmol/L) for 10 minutes resulted in a significant elevation of cGMP contents in aortic rings from banded mice compared with basal levels (30.11±1.55 fmol/mg of protein in 8 Ang II–treated rings versus 10.05±1.12 fmol/mg of protein in 8 untreated rings; P<0.001; Figure 6). Either

![Figure 4](image1.png)

Figure 4. In vitro effects of Ang II on levels of eNOS, p-eNOS-Ser633, and p-eNOS-Ser1177 in aortic rings prepared from mice 4 days after sham operation or aortic banding. After equilibration for 1 hour in an organ bath, aortic rings were incubated for 30 minutes with or without PD123319 (PD; 1 μmol/L) or icatibant (Icat; 1 μmol/L) and then treated with Ang II (0.1 μmol/L) for 10 minutes. Top, representative Western blots. Bottom, bar graph showing densitometric data for eNOS, p-eNOS-Ser633, and p-eNOS-Ser1177 as ratios relative to β-actin. Values are the mean±SEM (n=8). *P<0.001 vs sham rings; #P<0.001 vs untreated banded rings; §P<0.001 vs Ang II–treated banded rings.

![Figure 5](image2.png)

Figure 5. In vitro effects of protein kinase inhibitors on the Ang II–induced eNOS phosphorylation in aortic rings. Aortic rings were prepared from mice 4 days after sham operation or banding. After equilibration for 1 hour in an organ bath, aortic rings were incubated for 30 minutes with or without wortmannin (Wort; 1 μmol/L), LY24002 (LY; 10 μmol/L), H89 (10 μmol/L), or KT5720 (KT; 10 μmol/L), then treated with Ang II (0.1 μmol/L) for 10 minutes. Top, representative Western blots. Bottom, bar graph showing densitometric data for eNOS, p-eNOS-Ser633, and p-eNOS-Ser1177 as ratios relative to β-actin. Values are the mean±SEM (n=8). *P<0.001 vs sham rings; #P<0.001 vs untreated banded rings; §P<0.001 vs Ang II–treated banded rings.
PD123319 (1 μmol/L) or icatibant (1 μmol/L) almost completely inhibited the Ang II–induced increases in cGMP contents (Figure 6). The Ang II–induced increases in cGMP were not affected by wortmannin (1 μmol/L) and LY24002 (10 μmol/L) but were strongly inhibited by H89 (10 μmol/L; 18.02 ± 1.31 fmol/mg of protein in 8 rings) and KT5720 (10 μmol/L; 17.95 ± 1.41 fmol/mg of protein in 8 rings) versus Ang II alone (30.11 ± 1.55 fmol/mg of protein in 8 rings; *P<0.001; Figure 6).

**Effects of Protein Kinase Inhibitors on the AT2 Receptor–Mediated Attenuation of Ang II–Induced Contractile Response of Aortic Rings**

Ang II–induced contractile response was attenuated in aortic rings from banded mice as compared with those from sham mice, and this attenuation was inhibited by either PD123319 or icatibant (Figure 7A). Pretreatment with PKA inhibitors (H89, 10 μmol/L; KT5720, 10 μmol/L; Figure 7C) but not PI3K inhibitors (wortmannin, 1 μmol/L; LY24002, 10 μmol/L; Figure 7B) also significantly inhibited the attenuation of Ang II–induced contractile response in aortic rings from banded mice. These protein kinase inhibitors did not affect the Ang II–induced contractile response in aortic rings from sham mice (data not shown).

**Discussion**

As demonstrated in previous studies using the mouse and rat,13,14 abdominal aortic banding induces upregulation of AT2 receptors in the thoracic aorta, and the activation of aortic AT2 receptors in banded mice by Ang II results in enhanced NO production and increased cGMP.14 The upregulated AT2 receptors may be continuously stimulated by Ang II in banded animals, because the circulating levels of Ang II are elevated after banding,24 and the administration of PD123319 abolished the elevation of cGMP in thoracic aortas of animals with banding.14 Thus, it is likely that the AT2 receptors were upregulated by banding.
receptor functionally couples to the activation of the NO/cGMP system.

The phosphorylation of eNOS at Ser\(^{633}\) or Ser\(^{1177}\) results in reduced calcium dependence and sustained release of endothelial NO.\(^{8,9}\) The eNOS is phosphorylated at Ser\(^{1177}\) by Akt, which is phosphorylated and activated by PI3K.\(^{22-23}\) In the present studies, we found that abdominal aortic banding resulted in increased levels of eNOS, p-eNOS at Ser\(^{633}\) and Ser\(^{1177}\), Akt, and p-Akt at Ser\(^{473}\) in the thoracic aortas. The administration of PD123319 completely inhibited the banding-induced increases in p-eNOS, suggesting a mediation of AT\(_2\) receptors in eNOS phosphorylation. Like PD123319, the administration of icatibant abolished the banding-induced elevation of both p-eNOS and cGMP in aortas, confirming previous studies that the AT\(_2\) receptor–mediated vasodilator response to Ang II is mediated by a kinin/NO-dependent mechanism.\(^{14,15}\) In contrast, the banding-induced increases in total eNOS, total Akt, and p-Akt were not affected by PD123319 or icatibant, suggesting that upregulation of these proteins is independent of AT\(_2\) and B\(_2\) receptors and, hence, is not associated with increased production of NO in thoracic aortas of banded mice. The mechanisms underlying the banding-induced upregulation of these proteins are unknown, but changes in the mechanical forces on the vascular wall may be important.\(^{1-3}\)

To confirm further that the activation of AT\(_2\) receptors by Ang II is associated with eNOS phosphorylation, aortic rings were prepared from banded mice and treated with Ang II in vitro. Ang II produced increased levels of p-eNOS-Ser\(^{633}\) and p-eNOS-Ser\(^{1177}\), as well as cGMP. These effects of Ang II on both p-eNOS and cGMP levels were blocked by PD123319 or icatibant, suggesting that Ang II stimulates NO production through activation of eNOS by phosphorylation at Ser\(^{633}\) and/or at Ser\(^{1177}\) via the AT\(_2\) and B\(_2\) receptors. In contrast, p-Akt levels in aortic rings from banded mice were not affected by Ang II, suggesting that Akt is not a downstream effector of the AT\(_2\) receptor in eNOS phosphorylation.

The cGMP levels remained elevated in aortic rings from banded mice compared with sham rings, although the aortic rings were incubated without addition of Ang II for 1.5 hours after excision, suggesting that NO production remained elevated during the in vitro experiments. Because treatment with PD123319 did not affect the basal levels of cGMP in aortic rings from banded mice, it is unlikely that Ang II generated in aortic rings is responsible for the sustained NO production. Rather, it seems likely that the activation of eNOS before excision of aortas was maintained for ≥1.5 hours in vitro. This is similar to data showing that both p-eNOS-Ser\(^{633}\) and p-eNOS-Ser\(^{1177}\) after treatment with shear stress or 8-bromo-cAMP remain at elevated levels in bovine endothelial cells for ≥1 hour.\(^{25}\)

In this study, we found that the Ang II–induced elevations of p-eNOS-Ser\(^{633}\) and p-eNOS-Ser\(^{1177}\), as well as cGMP contents in aortic rings from banded mice, were inhibited by PKA inhibitors but not by PI3K inhibitors. PKA inhibitors also exhibited a significant inhibition on the AT\(_2\) receptor–dependent attenuation of Ang II–induced contractile response in aortic rings from banded mice. These results suggest that the AT\(_2\) receptor–dependent eNOS phosphorylation at Ser\(^{633}\) or Ser\(^{1177}\), as well as vasodilator response to Ang II, is mediated by PKA.

The stimulation of AT\(_1\) receptors causes local production of bradykinin in vessels and myocardium, which, in turn, stimulates B\(_2\) receptors to activate the NO/cGMP cascade.\(^{26,27}\) Our present study also supports B\(_2\) receptor–mediated cGMP production in aortas of banded mice, suggesting that bradykinin is an important mediator involving in the AT\(_2\) receptor–dependent phosphorylation of eNOS. Bradykinin can induce PKA-dependent eNOS phosphorylation at Ser\(^{633}\) and Akt-dependent phosphorylation at Ser\(^{1177}\).\(^{9}\) However, a recent study using bovine endothelial cells demonstrated that bradykinin-induced eNOS phosphorylation at Ser\(^{1177}\), as well as NO production, was blocked by a PKA inhibitor but not by a PI3K inhibitor.\(^{28}\) Our present results are consistent with these studies indicating that bradykinin stimulates eNOS phosphorylation at Ser\(^{633}\) and Ser\(^{1177}\) via PKA-dependent mechanisms. These data further support a potential role of bradykinin as a downstream effector of the AT\(_2\) receptor.

**Perspectives**

The present study demonstrates that the vascular AT\(_2\) receptor mediates the vasodilator action of Ang II through PKA-dependent phosphorylation of eNOS, which results in enhanced NO production in endothelial cells. Bradykinin is probably an important mediator involved in eNOS phosphorylation in a signaling pathway downstream of AT\(_2\) receptor. These data will help our understanding of the vasoprotective actions of AT\(_1\) receptor antagonists in cardiovascular diseases, in which the vascular AT\(_2\) receptor is upregulated.

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**Disclosures**

None.

**References**


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