Stimulation of Cyclic GMP Production via AT₂ and B₂ Receptors in the Pressure-Overloaded Aorta After Banding

Hiromi Hiyoshi, Katsutoshi Yayama, Masaoki Takano, Hiroshi Okamoto

Abstract—Abdominal aortic banding induces upregulation of the angiotensin II (Ang II) type-2 (AT₂) receptor, thereby decreasing the contractile response to Ang II in the thoracic aorta of the rat. The aim of this study was to use a mouse model to clarify the mechanisms by which the banding elicits upregulation of the aortic AT₂ receptor and the subsequent attenuation of Ang II responsiveness. Concomitantly with the elevation in blood pressure and plasma renin concentration after banding, AT₂-receptor mRNA levels in the thoracic aorta rapidly increased in mice within 4 days. Upregulation of the AT₂ receptor, as well as blood pressure elevation after banding, was abolished by losartan administration. The contractile response to Ang II was depressed in aortic rings of banding mice but not of sham mice, and was restored by either the AT₂-receptor antagonist PD123319 or the bradykinin B₂-receptor antagonist icatibant. cGMP content in the thoracic aorta of banding mice was 9-fold greater than that of sham mice, and the elevation was reduced to sham levels 1 hour after intravenous injection of PD123319 or icatibant. When aortic rings were incubated with Ang II, cGMP content increased in banding rings but not in sham rings; the pretreatment with PD123319 or icatibant inhibited Ang II-induced cGMP production. These results suggest that aortic banding induces upregulation of the AT₂ receptor through increased circulating Ang II via the AT₁ receptor, thereby activating a vasodilatory pathway in vessels through the AT₂ receptor via the kinin/cGMP system. (Hypertension. 2004;43:1258-1263.)

Key Words: receptors, angiotensin II ■ bradykinin ■ nitric oxide ■ cyclic GMP ■ vasodilation

Angiotensin II (Ang II) is a vasoactive peptide that regulates blood pressure and fluid homeostasis, and is likely to play a key role in the pathogenesis of cardiovascular diseases in humans. Ang II exerts its various actions through 2 receptor subtypes, type-1 (AT₁) and type-2 (AT₂). Disruption of the AT₂-receptor gene in the mouse increases basal blood pressure and induces pressure responsiveness to Ang II. In contrast, overexpression of the AT₂-receptor gene in vascular smooth muscle cells results in a depressed pressure-response to Ang II because of the activation of the nitric oxide (NO)-cGMP system via the AT₂-receptor–mediated vascular kallikrein-kinin system. In spontaneously hypertensive rats, administration of AT₂-receptor agonists induces a depressor response during simultaneous AT₁-receptor blockade. In normotensive rats, stimulation of AT₂ receptors causes vasodilation via the local production of bradykinin in resistant arteries of the rat mesentery in a flow-dependent manner. These observations suggest that the AT₂ receptor acts as a vasodilator pathway counterregulatory to AT₁-receptor–mediated vasoconstriction under normotensive and hypertensive conditions.

The AT₁ receptor is the predominant receptor subtype in the adult vasculature, whereas the AT₂ receptor is predominant during fetal development and declines after birth. Several studies showed that the AT₂ receptor is upregulated in cardiovascular tissues under pathological conditions such as myocardial infarction, heart failure, hypertension, and vascular injury. However, it is unknown whether increased numbers of AT₂ receptors function as a counterbalance to the AT₁-mediated actions of Ang II under these pathological conditions.

Recently, we showed in the rat that AT₂-receptor mRNA levels increase in the pressure-overloaded thoracic aorta after abdominal aortic banding. Because losartan administration abolishes banding-induced upregulation of the AT₁ receptor, it was suggested that Ang II enhances the expression of the AT₂-receptor gene via AT₁-receptor activation. Furthermore, the contractile response to Ang II in the pressure-overloaded thoracic aorta exhibits AT₂-receptor–dependent attenuation. However, it is unconfirmed that the kinin/cGMP system contributes to the signaling cascade of the AT₂ receptor in the rat aorta, because of a documented reduction in bradykinin-induced vasodilation responsiveness in the rat aorta.

The aim of the present study was to confirm the previous findings that the AT₂ receptor is upregulated in the pressure-overloaded aortas via the AT₁-receptor–mediated action of Ang II and to determine whether the action of Ang II via the AT₂ receptor is mediated by the kinin/NO/cGMP system. Therefore, we used a mouse model of the aortic banding,
because the mouse aorta exhibits a sensitive relaxation response to bradykinin.

**Methods**

**Abdominal Aortic Banding and Blood Pressure Measurement**

All animal experiments were performed according to the guidelines of the Kobe Gakuin University Experimental Animal Care and Use Committee. Aortic banding was performed in male 8-week-old ICR mice (Japan SLC, Hamamatsu, Japan) by constricting the abdominal aorta between the renal arteries just below the renal bifurcations as previously described.10,12 Systolic blood pressure was measured as reported in the expanded Methods section available online at http://www.hypertensionaha.org.

**Assay of Plasma Renin Concentration**

The plasma renin concentration was measured by radioimmunoassay of Ang I liberated in the presence of plasma from bilaterally nephrectomized rats as previously described.15

**Detection of AT1-, AT2-, and Bradykinin B2-Receptor mRNAs by Reverse Transcription-Polymerase Chain Reaction**

Animals were euthanized under ether anesthesia 2, 4, 7, or 28 days after aortic banding. Age-matched, sham-operated mice were also euthanized as controls. To detect AT1-, AT2-, and bradykinin B2-receptor mRNAs, we used reverse transcription-polymerase chain reaction (RT-PCR) with the specific primers, followed by Southern blotting as previously described.14 For semi-quantitative analysis of the AT2-receptor mRNA, the densities of the blots were normalized with GADPH mRNA blots. Expanded details are available online at http://www.hypertensionaha.org.

**Contractile Response of Aortic Rings to Ang II**

Mice were euthanized by bleeding from the carotid arteries under ether anesthesia 4 days after aortic banding. The thoracic aorta was excised and cut into 3-mm rings as previously described. To examine the in vivo effects of losartan (20 mg/kg), PD123319 (10 mg/kg), and icatibant (0.5 mg/kg), these drugs were injected into the tail-vein 1 hour before excision of the aorta. To determine the in vitro effects of Ang II, aortic rings prepared from the thoracic aorta 4 days after operation were equilibrated under a resting tension of 0.7 g for 1 hour, then incubated with isobutylmethylxanthine (IBMX, 50 μmol/L, Sigma-Aldrich, St. Louis, Mo) for 30 minutes. The rings were treated with Ang II (1 μmol/L) for 3 minutes, and then subjected to the assay for cGMP. Losartan, PD123319, or icatibant at the concentration of 1 μmol/L was added to the organ bath 10 minutes before, and t-NAME (0.1 mmol/L) 30 minutes before Ang II challenge.

**Elevation of cGMP in the Thoracic Aorta After the Abdominal Aortic Banding**

To evaluate whether elevated expression of the AT2 receptor in the pressure-overloaded aorta influences aortic cGMP production, cGMP content was assayed in the thoracic aoratas excised 4 days after sham-operation and banding. As shown in Figure 3, the content in banding mice was 9-fold greater than that in sham mice (20.14±3.89 fmol/mg protein in 4 banding-mice versus 2.45±0.51 fmol/mg protein in 4 sham-mice; P<0.001). Elevated cGMP in the aorta of banding mice declined to sham levels 1 hour after intravenous injection of the AT2-receptor antagonist PD123319 (10 mg/kg, 1.41±0.32 fmol/mg protein in 5 PD123319-treated banding mice; P<0.0001 versus saline-treated banding mice), but not after injection of losartan potassium (20 mg/kg). The bradykinin B2-receptor antagonist icatibant (0.5 mg/kg, IV) also reduced aortic cGMP content in banding animals to sham levels (1.75±0.20 fmol/mg protein in 4 icatibant-treated banding mice; P<0.0001 versus saline-treated banding mice). Neither PD123319 nor icatibant administration affected the aortic cGMP content in sham animals (Figure 3).

To elucidate the mechanisms responsible for the elevation of aortic cGMP content in banding mice, the thoracic aortas excised 4 days after banding were cut into rings and incubated with the phosphodiesterase inhibitor IBMX for 30

**Statistical Analysis**

All data were expressed as the mean±SEM. Statistical comparisons of plasma renin concentration, blood pressure, AT1-receptor mRNA levels, and cGMP content under various treatments were performed using a 1-way ANOVA with pairwise comparison by the Bonferroni-Dunn method. A comparison of the concentration-response curves of Ang II was performed by repeatedly measuring the analysis of variance followed by the Bonferroni-Dunn method. Differences were considered significant for P<0.05.

Results

### Changes in Blood Pressure and Plasma Renin Concentration After the Abdominal Aortic Banding

The systolic blood pressure at the carotid artery was significantly elevated in banding mice (129±5 mm Hg, n=10) as compared with sham-operated mice (106±2 mm Hg, n=4) by day 4, and the elevated blood pressure returned to sham levels by day 28 (Figure I; http://www.hypertensionaha.org). Intraperitoneal injection of losartan potassium (1 mg/kg, once a day) or nicardipine hydrochloride (2 mg/kg, twice a day) for 4 days after banding blocked the banding-induced hypertension (Figure I). The plasma renin concentration rapidly increased within 2 days after the aortic banding, peaked by day 4, then returned to the levels of sham animals by day 28 (Figure II; http://www.hypertensionaha.org). The administration of nicardipine did not affect the elevation of plasma renin concentration 4 days after banding (data not shown).

### Increased Expression of AT2-Receptor mRNA in the Thoracic Aorta After the Abdominal Aortic Banding

The mRNA levels of AT1-, AT2-, and bradykinin B2 receptors were determined by RT-PCR followed by Southern blotting in the thoracic aorta of sham-operated and banding mice. As shown by representative blots in Figure 1, there were no changes in AT1- and B2-receptor mRNA levels in sham-operated or banding mice, whereas the level of AT2-receptor mRNA was elevated 2, 4, and 7 days after banding, then returned to sham levels by day 28.

The administration of losartan but not of nicardipine for 4 days after banding completely inhibited the banding-induced upregulation of AT2-receptor mRNA (Figure 2).
minutes in an organ bath. As shown in Figure 4, cGMP content in the rings of banding mice (banding rings) was 10-fold greater than that in rings from sham mice (sham rings, 32.99±3.92 fmol/mg protein in 4 banding-rings versus 3.35±0.08 fmol/mg protein in 4 sham-rings; P<0.0001). High cGMP content in the banding rings was not affected by treatment with PD123319 (1 μmol/L) for 10 minutes, but was reduced by treatment with L-NAME (0.1 mmol/L) for 30 minutes (0.81±0.12 fmol/mg protein in 4 L-NAME–treated banding rings; P<0.0001 versus nontreated banding rings, Figure 4).

cGMP content in banding rings was further elevated by treatment with Ang II (1 μmol/L) for 3 minutes (61.93±5.95 fmol/mg protein in 4 Ang II–treated banding rings versus 32.99±3.92 fmol/mg protein in 4 nontreated banding rings; P<0.0001, Figure 4). In contrast, Ang II treatment did not affect cGMP content in sham rings (2.76±3.92 fmol/mg protein in 4 Ang II–treated sham rings versus 3.35±0.08 fmol/mg protein in 4 nontreated sham rings; P>0.1). The Ang II-induced elevation of cGMP contents in banding rings was completely inhibited by the addition of PD123319 (1 μmol/L) or icatibant (1 μmol/L), but not by losartan (1 μmol/L, 32.51±3.83 fmol/mg protein in 4 Ang II plus PD123319-treated banding rings and 30.63±2.39 fmol/mg protein in 4 Ang II plus icatibant-treated banding rings; P<0.0001 versus Ang II-treated banding rings, Figure 4). The depressed cGMP content in L-NAME–pretreated banding rings was not affected by treatment with Ang II (Figure 4).

Reduced Contractile Response to Ang II in Ring Preparations of the Thoracic Aorta After the Abdominal Aortic Banding

The contractile response to cumulative doses of Ang II was studied in aortic rings prepared from the thoracic aorta of sham-operated and banding mice 4 days after the operation. As shown in Figure 5A, Ang II-evoked contractility was significantly attenuated in banding rings at higher Ang II concentrations, such as 0.1 to 1 μmol/L, compared with sham rings. The addition of PD123319 (1 μmol/L) into the organ bath increased the contractile response to Ang II in banding rings to those levels observed in sham rings, while not affecting the Ang II responsiveness in sham rings (Figure 5B). Icatibant (1 μmol/L) also significantly augmented the
contractile response to Ang II in banding rings comparable to levels found in sham rings, while not affecting Ang II-responsiveness in sham rings (Figure 5C). Pretreatment with L-NAME for 30 minutes augmented the response to Ang II in sham and banding rings, and no significant difference in the Ang II responsiveness was observed between these L-NAME–pretreated rings (Figure 5D).

In contrast to aortic rings excised 4 days after banding, the contractile response to Ang II in aortic rings excised 28 days after banding was not significantly different from that seen in sham-operated mice (data not shown).

Discussion

After the abdominal aortic banding, the thoracic aorta is exposed to pressure-overload as a result of two potential mechanisms: mechanical obstruction by banding, and a vasopressor effect because of increased secretion of renin from the kidney. In the present study, we observed that systolic blood pressure at the carotid artery became elevated within 4 days after banding, then returned to levels found in sham-operated mice by day 28. The blood pressure elevation seemed to depend on the increase in circulating Ang II, because the plasma renin concentration coincidentally increased with blood pressure elevation, and the administration of losartan blocked the hypertensive response after aortic banding.

In parallel with the blood pressure elevation, the level of AT2-receptor mRNA in the thoracic aorta, but not that of AT1, and B2 receptors, was elevated after banding. The banding-induced elevation in blood pressure was blocked by the administration of losartan or nicardipine, whereas the increase in AT2-receptor mRNA was inhibited by losartan but not nicardipine. Thus, the banding-induced upregulation of the AT2 receptor seems to be mediated by Ang II through the AT1-receptor activation, but not by an increased mechanical load on the aorta. These results are comparable to the previous observations in the rat.10

Recent studies showed that the AT2-receptor–dependent vasodilator response to Ang II is mediated by the kinin/NO-dependent mechanisms in various vessels.4,15–19 In the present study, we observed that the basal cGMP level was elevated in the thoracic aorta 4 days after banding. Because elevated cGMP content in the aortas was reduced to sham levels in vivo by the administration of PD123319, the upregulation of the AT2 receptor in the aorta may be associated with enhanced cGMP production. In addition, the finding that the administration of icatibant abolished the elevation of cGMP in the thoracic aortas suggests the involvement of bradykinin in AT2-receptor–dependent cGMP production. In contrast, the basal level of cGMP in the aorta of transgenic mice which overexpressed the AT2 receptor is not different from that of wild-type mice.4 Increased plasma renin concentration in banding mice but not in transgenic mice accounts for the...
difference in the basal aortic cGMP levels between these two experimental models: increased levels of circulating Ang II in banding mice but not transgenic mice may probably contribute to increased basal levels of aortic cGMP in banding mice through activation of the AT2-receptor.

To explore the mechanisms underlying the above observations, we measured the cGMP content of aortic rings incubated with IBMX in an organ bath. The basal cGMP content in banding rings incubated without Ang II was 10-fold higher than that in sham rings and was depressed by L-NAME treatment. Because PD123319 was unable to influence the basal cGMP content of banding rings, it seems unlikely that the endogenous Ang II generated in banding rings is involved in the enhanced production of basal cGMP via AT2 receptors. These observations suggest that the enhanced generation of NO is sustained in vitro by the AT2-receptor–independent mechanisms and thereby inducing increased production of cGMP in banding rings. However, a recent study that elucidated the mechanism of ligand-independent signaling via the AT2 receptor does not preclude the potential contribution of the upregulated AT2 receptor to the enhancement of NO generation in banding rings. An alternative possibility is that NO synthase is upregulated in the pressure-overloaded aorta, as observed in the pressure-overloaded aorta of banding rats.

The addition of Ang II to the organ bath stimulated cGMP production in banding rings but not in sham rings, Ang II-dependent cGMP production was completely inhibited by PD123319 and icatibant, which suggests mediation occurred by AT2 and B2 receptors. These findings are compatible with in vivo observations that the administration of these antagonists reduced cGMP content in the thoracic aorta of banding mice but not sham mice. In addition, Ang II was unable to stimulate cGMP production in L-NAME–pretreated banding rings, suggesting that basal and Ang II–induced production of cGMP in banding rings entirely depends on the NO generation during incubation. Thus, Ang II likely stimulates cGMP production in banding rings through the kinin/NO system via activation of the AT2 receptor.

The contractile response to Ang II was attenuated in aortic rings from banding mice compared with that of sham-operated mice. Because this depressed response to Ang II was restored to the level found in sham rings by an in vitro treatment with PD123319 or icatibant, it is likely that stimulation of the AT2 receptor by Ang II in aortic rings results in the attenuation of the AT2-receptor–mediated contractile response through the activation of the B2-receptor. Furthermore, after the inhibition of NO synthesis by L-NAME treatment, there was no significant difference in the Ang II responsiveness between sham and banding rings, suggesting that NO plays a role in the AT2-receptor–mediated attenuation of contractile response to Ang II. Although a similar attenuation of Ang II responsiveness was observed in the aortic rings of banding rats, icatibant failed to restore Ang II responsiveness. The sensitive relaxation response to exogenous bradykinin observed in the thoracic aorta of the mouse...
which was not observed in the rat accounts for the difference in icatibant effect between these species.

Together, we have provided evidence that the banding-induced elevation of plasma renin concentration is associated with upregulation of the AT₂ receptor in the pressure-overloaded aorta via activation of the AT₁ receptor by elevated circulating levels of Ang II. Stimulation of this increased number of AT₂ receptors in the aorta by Ang II probably induces the generation of kinins. This in turn stimulates NO synthesis in the endothelium via the bradykini

Perspectives

Our results suggest that AT₂-receptor expression is upregulated by Ang II via activation of the AT₁-receptor to blunt the AT₁-receptor-mediated vasoconstriction. Although this explanation accounts for the regulation of vascular tone by Ang II, further studies under conditions of enhanced renin-angiotensin system, such as by salt depletion or renovascular hypertension, are required to confirm this hypothesis. This issue is important because the antihypertensive effect of AT₁-receptor blockers is, at least in part, dependent on AT₂-receptor activation.

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References

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Stimulation of Cyclic GMP Production via AT2- and B2- Receptors in the Pressure-Overloaded Aorta after Banding

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Blood pressure measurement

To assess the blood pressure elevation in the thoracic aorta after banding, systolic blood pressure was measured at the carotid artery using a PE-10 polyethylene catheter under anesthesia (pentobarbital sodium 50 mg/kg, intraperitoneally). Pulse wave forms were monitored by a polygraph system (Nihon Kohden, Tokyo, Japan). The effects of losartan and nicardipine on banding-induced blood pressure elevation were determined by the intraperitoneal injection of losartan potassium (Merck, Whitehouse Station, NJ; 1 mg/kg, once a day) or nicardipine hydrochloride (Wako Chemicals, Osaka, Japan; 2 mg/kg, twice a day) for 4 days after the sham operation or banding. The systolic blood pressure was then measured at the carotid arteries 24 hours after the last drug injection.

Detection of AT1-, AT2-, and bradykinin B2-receptor mRNAs by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the aorta using acid guanidium thiocyanate-phenol-chloroform as previously described. To detect AT1-, AT2- and bradykinin B2-receptor mRNA, we used RT-PCR with the following specific primers: sense 5’-CACCTATGTAAGATCGCTTC-3’ and antisense
5'-GCACAATCGCCATAATTATC-3' for the AT_1-receptor, sense

5'-CTGACTCTGAACATGTTTGCA-3' and antisense

5'-GGTGTCCTTTCTCTAAGAGA-3' for the AT_2-receptor, and

5'-ACCCATGTTGTGTTCAAGGA-3' and antisense

5'-GGTACACCTCTCGGACTTC-3' for the B_2-receptor, respectively. The RT-PCR products were analyzed by Southern blotting using cDNA primers specific for AT_1-, AT_2- and B_2-receptor mRNA as previously described.\textsuperscript{1} For semi-quantitative analysis of the AT_2-receptor mRNA, the densities of the blots were analyzed on a FUJIX BAS2000 (Fuji Film, Tokyo, Japan) phosphoimager system and normalized with glyceraldehyde phosphate dehydrogenase mRNA blots.

**Contractile response of aortic rings to Ang II**

The thoracic aorta was excised and immediately placed in Krebs-Henseleit solution (118.4 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl_, 1.2 mmol/L KH_2PO_, 1.2 mmol/L MgSO_, 25.0 mmol/L NaHCO_, and 11.1 mmol/L glucose). The aorta was cleaned of adherent tissue and cut into 3-mm rings. Each ring was vertically fixed under a resting tension of 0.7 g in a 5-mL organ bath filled with the solution (37 °C, pH 7.4)
described above. The bath solution was continuously aerated with a gas mixture of 95% 
O₂/ 5% CO₂, and then the rings were allowed to equilibrate for 90 minutes before starting 
the experiments. The isometric tension change was measured with a force-displacement 
transducer (Isometric Transducer UFER®; Medical Kishimoto, Kyoto, Japan) coupled to 
a dual-channel chart recorder (Mac Lab AD Instruments, Tokyo, Japan). After 
equilibration, Ang II was cumulatively added to a final concentrations of between 10 
pmol - 1 µmol/L to the bath solution. In some experiments, the AT₂-receptor antagonist, 
PD123319 (Sigma-Aldrich, St. Louis, MO; 1 µmol/L), and B₂-receptor antagonist, 
icatibant (Peptide Institute, Inc., Osaka, Japan; 1 µmol/L), were added into the bath 15 
minutes before the cumulative addition of Ang II. Some rings were preincubated with 
the NO synthase inhibitor N⁴-nitro-L-arginine methyl ester hydrochloride (L-NAME) 
(Nacalai Tesque, Kyoto, Japan; 0.1 mmol/L) 30 minutes before Ang II challenge. The 
contractile responses observed were expressed as a percentage of the maximal 
constriction evoked by 40 mmol/L KCl.
**Figure Legend**

**Figure I.**  A, Systolic blood pressure at the carotid arteries of mice 4 and 28 days after sham-operation and aortic banding.  B, Effects of losartan and nicardipine on the systolic blood pressure in mice 4 days after sham-operation and aortic banding.  After sham-operation or banding, mice were intraperitoneally injected with losartan potassium (1 mg/kg, once a day) or nicardipine hydrochloride (2 mg/kg, twice a day) for 4 days.  Then, systolic blood pressure was measured at the carotid arteries 24 hours after the last drug injection.  The control groups of sham and banding mice received saline.  Values represent mean ± SEM, and the numbers of animals used are shown in parentheses.

*P<0.0001 vs sham; #P<0.0001 vs saline-treated banding.

**Figure II.**  Plasma renin concentration in mice after sham-operation and aortic banding.  Values are mean ± SEM, n=3 to 4 animals at each point.  *P<0.001 vs sham.
References

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A) Systolic blood pressure (mmHg)

- **Sham**
  - 4 days: (4)
  - 28 days: (4)

- **Banding**
  - 4 days: (10)
  - 28 days: (4)

B) Comparison of blood pressure between Sham and Banding groups

- **Sham**
  - Saline: (4)
  - Losartan: (6)
  - Nicardipine: (4)

- **Banding**
  - Saline: (10)
  - Losartan: (7)
  - Nicardipine: (4)

*Significant difference between groups.

#Significant difference within groups.
Days after operation

Plasma renin concentration (µg Ang I/mL/h)

Sham

Banding

*