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Upregulation of angiotensin II type-2 receptor in rat thoracic aorta by pressure-overload

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Running title: Upregulation of AT_2 receptor in pressure-loaded aorta

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Abbreviations

Ang II, angiotensin II; AT_1, angiotensin II type 1; AT_2, angiotensin II type 2; GAPDH; glyceraldehydes-3-phosphate dehydrogenase; NO, nitric oxide; L-NAME, N^G-nitro-L-arginine methyl ester; IBMX, isobutylmethylxanthine; cGMP, cyclic GMP; RT-PCR, reverse transcription-polymerase chain reaction; PRC, plasma renin concentration; PRA, plasma renin activity.

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Abstract

We have examined whether expression of angiotensin II (Ang II) type 1 (AT_1) and/or type 2 (AT_2) receptors are changed in thoracic aorta under pressure-overload by abdominal aortic banding in rats, and to determine whether their changes are accompanied by alteration in contractile response of thoracic aorta to Ang II. AT_2 receptor mRNA levels determined by reverse transcription-polymerase chain reaction (RT-PCR) or quantitative real-time PCR were increased by about 300% in aortas 4, 7, 14 and 28 days after banding without changes in AT_1 receptor mRNA levels. Contractile response of aortic rings to Ang II was decreased in thoracic aortas 7 days after banding, and AT_2 receptor antagonist PD123319 (10^{-6} M) increased the Ang II-responsiveness in pressure-loaded but not in sham rings. After removal of the endothelium or treatment with N^G-nitro-L-arginine methyl ester (L-NAME), no differences were observed in Ang II-responsiveness between sham and pressure-loaded rings. Either losartan (1 mg/kg/day, i.p.) or candesartan (2 mg/kg/day, p.o.) for 7 days after banding not only abolished the upregulation of AT_2 receptor mRNA in aortas but also recovered their Ang II-responsiveness. Basal cGMP levels were 2 times higher in pressure-loaded than in sham rings; both levels were not affected by Ang II (10^{-7} M, 5 min), but greatly decreased by L-NAME (10^{-4} M, 30 min). These results suggest that pressure-overload induces the upregulation of AT_2 receptor expression in aortas via AT_1 receptor and thereby negatively modulates the vasoconstrictor sensitivity to Ang II, probably mediated by the mechanisms independent of the NO-cGMP system.

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Introduction

Angiotensin II (Ang II), the most active component of the renin-angiotensin system, is a multifunctional hormone that plays an important role in the cardiovascular physiology and pathology (de Gasparo *et al*., 2000). Ang II production in the proximity to its receptors on the target cells constitutes the local renin-angiotensin system, which regulates the cardiovascular functions in both autocrine and paracrine fashions. The actions of Ang II are primarily mediated by two receptors, Ang II type 1 (AT_1) and type 2 (AT_2). The activation of the AT_1 receptor mediates vasoconstriction, proliferation of vascular smooth muscle cell, and production of extracellular matrix proteins by vascular smooth muscle cells (de Gasparo *et al*., 2000). In contrast, the AT_2 receptor has been considered to mediate vasodilation, antiproliferation and proapoptosis in the vasculature, presumably mediated by the activation of the nitric oxide (NO) system via bradykinin production (de Gasparo *et al*., 2000).

Although a small number of AT_2 receptors are present in the vessels, the physiological actions of Ang II via AT_2 receptors have been difficult to determine, because the AT_2 subtype has a low degree of expression compared with the AT_1 subtype (Viswanathan *et al*., 1991). Nevertheless, most previous studies using normotensive or hypertensive animals (Scheuer and Perrone, 1993; Siragy and Carey, 1999), as well as knockout or transgenic mice for the AT_2 receptor gene (Ichiki *et al*., 1995; Hein *et al*., 1995; Akishita *et al*., 1999; Tsutsumi *et al*., 1999), have demonstrated that the AT_2-receptor mediates a depressor response to Ang II. Recently, it became evident that the AT_2 receptor levels in the vasculature were increased under some pathological

conditions, such as hypertension and vascular injury (Otsuka *et al*., 1998; Touyz *et al*., 1999; Hutchinson *et al*., 1999). These evidence suggest that the upregulation of vascular AT_2 receptors under pathological conditions is one of the compensatory mechanisms of vessels counteracting the AT_1-mediated contractile response to Ang II to protect vessels from the mechanical overload. To test this hypothesis, we used a rat model of pressure-induced left ventricular hypertrophy (Doggrell and Brown, 1998) produced by suprarenal abdominal aortic coarctation (banding) to determine how the increased transmural pressure influences the vascular AT_2 receptor expression and thereby changes the contractile response to Ang II.

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Materials and Methods

Materials

The following materials were purchased from commercial sources: Ang II, bradykinin and icatibant from Peptide Institute, Inc. (Osaka, Japan), acetylcholine chloride, L-phenylephrine hydrochloride, and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) from Nacalai Tesque (Kyoto, Japan), PD123319 and isobutylmethylxanthine (IBMX) from Sigma (St. Louis, USA), [^{125}I]angiotensin I from PerkinElmer Life Sciences Japan (Tokyo, Japan). Losartan and candesartan were kindly supplied by Merck & Co. Inc. (New Jersey, USA) and Takeda Pharmaceutical Co. (Osaka, Japan), respectively.

Animals and operation

All animal experiments were performed according to the guidelines of the Kobe Gakuin University Experimental Animal Care and Use Committee. Male Wistar rats (4 weeks old; Japan SLC, Hamamatsu, Japan) were divided into 3 groups: (1) untreated, (2) sham-operated, and (3) pressure-overloaded rats. Pressure-overload was produced by abdominal aortic banding, which has been primarily used as a model of cardiac hypertrophy (Doggrell and Brown, 1998). Briefly, animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the aorta was exposed through a midline abdominal incision. For the banding model, a blunt 22-gage needle was placed adjacent to the abdominal aorta between the renal arteries just below the renal bifurcations, and a ligature was tightened around the aorta and adjacent needle. The

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sham procedure for control rats included injection of the same amount of anesthetic, in incision of approximately the same size, and the placement of a loosely tied ligature at the exact same position on the abdominal aorta.

Losartan was dissolved in saline and administered i.p. at a dose of 1 mg/kg once a day for 7 days. Candesartan was suspended in 10% gum Arabic and administered orally at a dose of 2 mg/kg once a day for 7 days.

Blood pressure measurement

The patency of the aortic banding was assessed by measuring the blood pressure of carotid artery under pentobarbital anesthesia (pentobarbital sodium 50 mg/kg, i.p.). To measure the blood pressure, the left carotid artery was cannulated with a PE-10 polyethylene catheter, and pulse wave forms were monitored by a polygraph system (Nihon Kohden, Tokyo, Japan).

Assays of plasma renin concentration (PRC) and activity (PRA)

Blood was collected from the abdominal aorta into a syringe containing 1/10 volume of 3.8% sodium citrate under ether anesthesia. Following centrifugation at 800 g for 15 min, plasma samples were collected and stored at –90˚C until assays. PRC or PRA in plasma samples were determined by radioimnmunoassay of Ang I liberated in the presence or absence of plasma from bilaterally nephrectomized rats, respectively, as described previously (Ohtani et al., 1989).

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Analysis of AT_1 and AT_2 receptor mRNAs by reverse transcription-polymerase chain reaction (RT-PCR)

Animals were sacrificed under ether anesthesia 4, 7, 14, and 28 days after aortic banding or sham-operation. Age-matched untreated rats were also sacrificed as a control. The excised thoracic aorta (approximately 10 mg) was stripped of adventitia, then homogenized in acid guanidinium-phenol-chloroform to extract total RNA, as described previously (Yayama *et al*., 2003). To detect AT_1 and AT_2 receptor mRNAs, we used two methods: one was RT-PCR followed by Southern blotting with respective specific probes, and second was quantitative real-time RT-PCR. Reverse transcription was performed in a reaction volume of 4 μL containing 0.3 μg RNA, 1.55 μL of dNTP (10 mmol/L), 0.8 μL of $MgCl_2$ (20 mmol/L), 0.4 μL of 10 x buffer (500 mmol/L KCl in 0.1 mol/L Tris-HCl, pH 8.3,), 0.05 μL of 10 unit enzyme (Moloney murine leukemia virus reverse transcriptase), and 0.2 μL of reverse-primer (50 pmol/μL). The mixture was incubated at 42˚C for 15 min, 95˚C for 5 min, then 4˚C for 5 min to allow synthesis of the first strand cDNA. The cDNA was amplified in a 20-μL reaction mixture containing 10 pmol of the forward primer, 1.6 μL of 10 x buffer, 4 μL of $MgCl_2$ (25 mmol/L), and 0.5 units of Taq DNA polymerase at 95˚C for 9 min, at 94˚C for 1 min, and annealing at 54˚C (AT_1 receptor), 56˚C (AT_2 receptor) or 60˚C (glyceraldehyde phosphate dehydrogenase; GAPDH) for 2 min, then extension at 72˚C for 3 min followed by at 72˚C for 10 min. The amplification cycles were 35 for the AT_1 receptor, 40 for the AT_2 receptor and 25 for GAPDH. After amplification, the PCR products were electrophoresed on a 1.5% agarose gel, then denatured, neutralized and transferred

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onto a nylon membrane by capillary blotting and cross-linked by UV irradiation. The transferred membranes were detected by Southern blot hybridization with ^{32}P-labeled cDNA probes specific for each DNA. The blot signals were detected using a Fujix Bio Imaging Analyzer BAS 2000 (Fuji Film, Tokyo, Japan). The forward and reverse primers for the AT_1 receptor were 5'-CACCTATGTAAGATCGCTTCT-3' and 5'-GCACAATCGCCATAATTATCC-3', for the AT_2 receptor 5'-CTGACCCTGAACATGTTTGCA-3' and 5'-GGTGTCCATTTCTCTAAGAGA-3', or for GAPDH 5'-GTGCCAAAAGGGTCATCATCT-3' and 5'-CAGCATCAAAGGTGGAGGAAT-3', respectively.

For quantitative measurement of the AT_2 receptor mRNA, 0.3 μg RNA samples from the thoracic aorta were reverse-transcribed using a reverse primer (5'-ATACCCATCCAGGTCAGAGCAT-3') as described above. The cDNA products in 2 μL were mixed with 18 μL of TaqMan Master Mixture (2 μL of 10 x buffer, 2.8 μL of $MgCl_2$ (20 mmol/L), 2 μL of reverse primer (3 μmol/L), 2 μL of forward primer (3 μmol/L), 2 μL of TaqMan probe (2 μmol/L), 0.4 μL of 10 mM dUTP, 0.1 μL of uracil N-glycosylase (1 unit/μl) and 0.1 μL of Taq DNA polymerase (5 unit/μl)). PCR was carried out in an ABI Prism 7700 system (Applied Biosystems, Tokyo, Japan) under the following conditions: 50˚C for 2 min. 95˚C for 10 min, 40 cycles at 95˚C for 15 sec, and 60˚C for 1 min. The PCR results were analyzed with the Sequence Detector 1.6 program (Perkin-Elmer, Norwalk, USA). For the AT_2-receptor, the following primers and probes were used: forward primer, 5'-CCCGTGACCAAGTCTTGAAGAT-3'; reverse primer, 5'-ATACCCATCCAGGTCAGAGCAT-3'; TaqMan probe,

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5'-FAM-TGGCATTCATCATTTGCTGGCTTCC-TAMRA-3'. For GAPDH: forward primer, 5'-CGTGTTCCTACCCCCAATGT-3'; reverse primer, 5'-TGATGTCATCATACTTGGCAGGTT-3'; TaqMan probe, 5'-FAM-CGTTGTGGATCTGACATGCCGCC-TAMRA-3'.

Organ chamber experiments

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), then the thoracic aorta was dissected free, excised, and placed in Krebs-Henseleit solution of the following composition (mmol/L): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1. The aortas were cleaned of adherent connective tissue and cut into rings (3 mm long). Each ring was fixed vertically under a resting tension of 1.0 g in a 10-mL organ bath filled with the solution (37°C, pH 7.4) described above. In some rings, the endothelium was mechanically removed by gentle rubbing with moistened cotton. The bath solution was continuously aerated with a gas mixture of 95% O_2/5% CO_2, and then the rings were allowed to equilibrate for 90 min before the start of the experiments. Isometric tension change was measured with a force-displacement transducer (model t-7; NEC San-Ei, Tokyo, Japan) coupled to a dual-channel chart recorder (model 8K21; NEC San-Ei). After reaching equilibrium, cumulative concentration-response curves were constructed for Ang II (10^{-10} – 10^{-7} M) in each ring. In some experiments, various agents, such as AT_2 receptor antagonist PD123319 (1 x 10^{-6} M), bradykinin type 2 (B_2) receptor antagonist icatibant (1 x 10^{-6} M), and L-NAME (1 x 10^{-4} M), were added 30 min before the cumulative addition of

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Ang II. The contractile responses obtained were expressed as a percentage of the maximal constriction evoked by 40 mM KCl. No significant changes in the contractile response to 40 mM KCl were observed in aortic rings from rats 1 week after banding as compared to those from age-matched untreated rats. Denudation of the endothelium was confirmed pharmacologically by the disappearance of the 1 x 10^{-7} M acetylcholine-induced relaxation response during constriction evoked by 3 x 10^{-7} M phenylephrine.

Assay of cyclic GMP (cGMP) in aortic rings

Aortic rings were fixed in a 10-mL organ bath filled with Krebs-Henseleit solution as described above. After allowing equilibration for 1 h, IBMX (5 x 10^{-5} M) was added to the organ bath. Twenty-five minutes after the addition of IBMX, the rings were stimulated with Ang II (1 x 10^{-7} M) for 5 min. In some experiments, L-NAME (1 x 10^{-4} M) was added at the same time as the IBMX addition. Thereafter, the rings were frozen in dry ice-acetone and homogenized in ice-cold 6% trichloroacetic acid. cGMP was extracted, acetylated and quantified by radioimmunoassay using the Cyclic GMP [^{125}I] Biotrak^{TM} assay system (Amersham Biosciences, Piscataway, NJ) and is expressed as picomoles per mg protein. Protein contents of the rings were measured by the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, USA) using gamma-globulin as a standard.

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Statistical analysis

All data are expressed as mean ± S.E. Statistical comparisons of PRC, PRA, blood pressure, AT_2 receptor mRNA levels and cGMP contents under various treatments were performed with one-way ANOVA with pairwise comparison by the Bonferroni-Dunn method. Comparison of concentration-response curves of Ang II was carried out by repeated measures ANOVA followed by the Bonferroni-Dunn method. Differences were considered significant for $p<0.05$.

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Results

Blood pressure in the thoracic aorta, PRC and PRA after aortic banding. The increased blood pressure in the thoracic aorta was monitored at the carotid artery following aortic banding. The systolic blood pressure was increased more than 30 mmHg 4 days after aortic banding, and the levels were maintained for 28 days: systolic blood pressures 4, 7, 14 and 28 days after banding were 127.6 ± 4.7, 128.2 ± 3.3, 129.3 ± 1.1, and 153.4 ± 7.8 mmHg, respectively, and were significantly higher than 97.2 ± 2.2, 97.5 ± 4.6, 95.9 ± 3.1, and 97.7 ± 5.9 mmHg in sham-operated rats, respectively, and 96.5 ± 3.1, 97.9 ± 3.7, 98.3 ± 4.5, and 98.1 ±4.6 mmHg in non-operated rats, respectively (n=5–6; $p<0.01$).

PRC and PRA were significantly increased at 2 and 4 days after aortic banding and returned to the levels of sham-operated animals at 7 days; both levels remained low 14 and 28 days after banding (Fig. 1).

AT_1 and AT_2 receptor mRNA levels in the thoracic aorta after aortic banding. To determine whether the pressure-overload alters the vascular expression of AT_1 and AT_2 receptor mRNAs, we examined the mRNA levels of these receptors in the thoracic aorta by RT-PCR. As shown in Fig. 2, signals corresponding to AT_1 receptor mRNA could easily be detected in the thoracic aorta of sham-operated animals, while only faint signals were detectable for AT_2 receptor mRNA. However, the levels of AT_2 receptor mRNA, but not AT_1 subtype, were increased 4, 7, 14 and 28 days after the banding of

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the abdominal aorta (Fig. 1). These observations were confirmed by quantitative real-time PCR: the levels of AT_2 receptor mRNA increased more than 300% after 4, 7, and 14 days compared with those of age-matched untreated rats or sham-operated rats (Fig. 2). High levels of AT_2 receptor mRNA were also observed after 28 days.

Involvement of the AT_1 receptor in the upregulation of AT_2 receptor mRNA in the thoracic aorta after aortic banding. To examine if the pressure-overload-induced increase in AT_2 receptor mRNA is mediated by Ang II itself, we studied the effects of the AT_1 receptor antagonists losartan and candesartan on the upregulation of AT_2 receptor mRNA in the thoracic aorta. As shown in Fig. 3, the upregulation of AT_2 receptor mRNA 7 days after aortic banding was completely inhibited by successive administration of losartan (1 mg/kg/day, i.p.) or candesartan (2 mg/kg, p.o.) for 7 days. In contrast, the administration of losartan or candesartan did not affect the blood pressure elevation in the thoracic aorta following aortic banding: 128.3 ± 5.9 mmHg in untreated rats with aortic banding (n=5) versus 126.7 ± 5.6 mmHg in losartan-treated rats with aortic banding (n=5; $p>0.1$) and 127.4 ± 5.2 mmHg in candesartan-treated rats with banding (n=5; $p>0.1$).

AT_2 receptor-dependent decrease in the contractile response to Ang II in ring preparations of pressure-overloaded thoracic aorta *in vitro*. The contractile response to Ang II was compared between the ring preparations of the thoracic aorta from sham-operated (control rings) and aortic banding rats (pressure-loaded rings).

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We examined thoracic aortas from rats 7 days after banding or sham-operation, because high levels of AT2 receptor mRNA were detected in thoracic aortas of banding rats during this period. The contractions evoked by Ang II were significantly decreased in the pressure-loaded rings at higher concentrations of Ang II, such as 3×10^{-8} and 1×10^{-7} M, compared to control rings (Fig. 4A). Blockade of the AT_2 receptor by PD123319 (1×10^{-6} M) increased the contractile response to Ang II in the pressure-loaded rings (Fig. 4C), but not in control rings (Fig. 4B).

Then we compared the contractile response to Ang II between the ring preparations from rats treated or untreated with losartan for 7 days after aortic banding, because of the observation that the administration of losartan abolished the upregulation of AT_2 receptor mRNA in the thoracic aorta after pressure-overload. Aortic rings were prepared from rats 24 hr after the final administration of losartan at day 6. As shown in Fig. 5, losartan administration to sham-operated animals did not alter responses to Ang II in aortic rings as compared to those from vehicle-treated sham animals. In contrast, the decrease in the response to Ang II by banding was significantly prevented in aortic rings from losartan-treated rats after banding as compared to vehicle-treated banding animals (Fig. 5).

Endothelium-dependent decrease in the contractile response to Ang II in ring preparations of pressure-overloaded thoracic aorta *in vitro*.

The contractile response to Ang II was markedly augmented by removal of the endothelium in either control (Fig. 6A) or pressure-loaded rings (Fig. 6B). When the

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concentration-response curves were compared between control and pressure-loaded rings after removal of endothelium, decreased response to Ang II in pressure-loaded rings was not observed, and rather the response was augmented significantly in the pressure-loaded rings at lower concentrations of Ang II between 1×10^{-10} and 1×10^{-9} M (Fig. 6C). PD123319 did not influence the response to Ang II in the endothelium-denuded rings of either control or pressure-loaded rings (data not shown).

To determine the involvement of endothelium-derived NO in the decreased response to Ang II in pressure-loaded rings, the ring preparations with intact endothelium were treated with a NO synthase inhibitor L-NAME (1×10^{-4} M) for 30 min before the cumulative addition of Ang II. As shown in Fig. 7, L-NAME pretreatment augmented the contractile response of Ang II in both control and pressure-loaded rings (Fig. 7A and 7B), and no significant differences were observed in the response to Ang II between these L-NAME-pretreated rings (Fig. 7C).

Previous studies have suggested a potential role of the bradykinin/NO system in AT_2 receptor-mediated aortic functions in mice (11). Therefore, we examined the effect of the bradykinin B_2 receptor antagonist icatibant on the decreased response to Ang II in the pressure-loaded rings with intact endothelium, and found that the pretreatment with icatibant (1×10^{-6} M) for 15 min did not influence the contractile response to Ang II in either control or pressure-loaded rings (data not shown). In both control and pressure-loaded rings, acetylcholine (1×10^{-8} M) exhibited a relaxation response during constriction evoked by 3×10^{-7} M phenylephrine, whereas no relaxation was observed by bradykinin up to the concentration of 3×10^{-6} M (data not shown).

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cGMP levels in aortic rings.

Basal cGMP levels were approximately 2 times greater in pressure-loaded rings than in control rings (8.46 ± 1.39 versus 4.83 ± 1.14 pmol/mg protein; n=4, $p<0.05$) (Fig. 7). Stimulation with Ang II (1 x 10^{-7} M) for 5 min did not significantly affect the cGMP levels in either control or pressure-loaded rings. Treatment with L-NAME (1 x 10^{-4} M) for 30 min markedly reduced the cGMP levels not only in control rings ($p<0.001$), but also in pressure-loaded rings ($p<0.001$). Ang II (1 x 10^{-7} M) did not influence the decreased levels of cGMP in L-NAME-pretreated rings.

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Discussion

In the present study, we found upregulation of the AT_2 receptor mRNA, but not of the AT_1 subtype, in the thoracic aorta under pressure-overload. Since either the administration of losartan or candesartan blunted the upregulation of AT_2 receptor mRNA without showing changes in the banding-induced pressure-overload, it seems likely that the mechanical load upregulates the aortic AT_2 receptor through the action of Ang II, probably via activation of AT_1 receptor. Although high levels of PRC and PRA were observed in animals 4 days after banding as a response of kidney to decreased blood pressure distal to the aortic banding, the levels returned to ranges of sham-operated animals after 7 days, suggesting that the pressure elevation at carotid artery seems to be related to the mechanical obstruction. In fact, the administration of AT_1 receptor antagonists did not decrease blood pressure at carotid artery in rats with aortic banding. These observations may be supported by a finding that enalapril did not reduce the carotid arterial pressure in rats with aortic banding (Baker *et al*., 1990).

Several lines of evidence have suggested the existence of a link between the effects of transmural pressure and Ang II in blood vessels. Noda *et al*. (1994) indicated that mechanical stretch and Ang II synergistically stimulated cultured rat aortic smooth muscle cells to induce a marked increase in the expression of parathyroid hormone-related peptide mRNA. Bardy *et al*. (1996) reported that the increased transmural pressure in the aorta might have caused the local generation of Ang II, which acted synergistically with the transmural pressure to enhance vascular fibronectin expression via the AT_1 receptor. Recently, Bonnet *et al*. (2001) demonstrated that the

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AT_2 receptor mRNA was upregulated in rat mesenteric arteries following a pressure dose of Ang II infusion for 2 weeks; they suggested the involvement of AT_1-receptor mediation in this Ang II effect, because AT_1 receptor antagonist inhibited the Ang II-induced upregulation of the AT_2 receptor. In the aortic banding model, the decreased blood pressure distal to the banding stimulates the kidney to release renin, resulting in increased circulating levels of Ang II. However, as shown in this study and by other investigators (Baker *et al*., 1990; Doggrell and Brown, 1998), the fact that the elevation of plasma renin is observed only within a few days after aortic banding does not account for the increased levels of the AT_2 receptor mRNA over three weeks. Therefore, a rapid increase in AT_2 receptor mRNA levels within 4 days seems to depend on a transient elevation of plasma renin, but the sustained increase in AT_2 receptor mRNA may probably be due to locally generated Ang II in the pressure-overloaded aorta.

In ring preparations of rat thoracic aortas that were dissected 7-days after aortic banding, the contractile response to Ang II was significantly decreased in comparison to that in control rings. The response to Ang II in the pressure-loaded rings was increased in the presence of the AT_2 receptor antagonist PD123319, but not in the control rings, suggesting a potential role of the AT_2 receptor in the decreased aortic response to Ang II. Indeed, the reduction of Ang II-responsiveness in pressure-loaded rings was prevented by the administration of losartan, which was found to inhibit the upregulation of AT_2 receptor mRNA after aortic banding. Since Ang II binds to the AT_1 and AT_2 receptor subtypes with similar affinity (Nouet and Nahmias, 2000), the contractile response of

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the aorta to Ang II seems to be dependent on the relative expression level and/or responsiveness of both receptors. Thus it seems likely that the decreased response to Ang II in the pressure-overloaded aortas depends on, at least in part, the upregulation of the AT_2 receptor.

There is evidence that Ang II binding to AT_2 receptors decreases the Gq-coupled phospholipase C activation by the AT_1 receptor (Gyurko *et al.*, 1992), suggesting that the activation of AT_2 receptors on aortic smooth muscle cells directly interacts with the signaling pathway of AT_1 receptors. However, a decreased response to Ang II was not observed in the endothelium-denuded rings of pressure-overloaded aortas, and PD123319 did not alter the response to Ang II in the pressure-loaded rings, as well as in the control rings, after removal of the endothelium. Thus it appears that the contraction of aortic smooth muscle cells by Ang II via the AT_1 receptor is attenuated by a vasorelaxation factor(s) derived from the endothelium via AT_2 receptor stimulation. A potential factor is NO, because of the observation that, after the inhibition of NO synthase by L-NAME, the response to Ang II became comparable in both control and pressure-loaded rings.

The basal levels of cGMP in pressure-loaded aortic rings were significantly higher than those in control rings. Since the cGMP levels in pressure-loaded rings were significantly reduced after L-NAME treatment, it is likely that the increased levels of basal cGMP in pressure-loaded rings result from the enhanced production of NO by the endothelium of pressure-loaded aortas. In fact, the protein and mRNA levels of endothelial NO synthase are upregulated in pressure-overloaded thoracic aortas after

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banding of the abdominal aorta (Bouloumie *et al*., 1997; Barton *et al*., 2001). Thus, the increased levels of basal cGMP in pressure-loaded rings may participate with, at least in part, the attenuation of the AT_1 receptor-mediated contractile response.

Given that the increased expression of AT_2 receptors in pressure-loaded aortas is functionally coupled to the NO-cGMP system, it is logical to expect that the stimulation by Ang II results in the elevation of cGMP levels in pressure-loaded rings, because of observations that the contractile response to Ang II in pressure-loaded rings was significantly increased by PD123319. However, stimulation by Ang II did not affect the cGMP levels in pressure-loaded rings, as in control rings. These results do not support the idea that the decreased response to Ang II observed in pressure-loaded rings simply depends on the NO-cGMP system via the activation of the AT_2 receptor. Thus it is reasonable to consider mechanisms other than the NO-cGMP system to explain the AT_2 receptor-mediated reduction of Ang II-responsiveness in pressure-loaded aortas. Taken together, the decreased contractile response to Ang II in thoracic aorta by pressure-overload appears to result from at least two different mechanisms: one is the pressure-overload-induced activation of the NO-cGMP system, and another the AT_2 receptor-dependent vasodilatory mechanisms, such as the activation of phospholipase A_2 and release of arachidonic acid (Zhu *et al*., 1998).

It has recently been demonstrated that the AT_2-receptor-mediated vasodilator response to Ang II is mediated by kinin in various vessels, such as the rat aorta (Gohlke *et al*., 1998), canine coronary microvessels (Seyedi *et al*., 1995) and rat mesenteric artery (Katada and Majima, 2002); these studies suggest that the AT_2 receptor is coupled

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to the local generation of kinin in the vascular wall, which stimulates NO production in endothelial cells via the bradykinin B_2 receptor. However, as shown in the present study, the decreased response to Ang II in pressure-loaded rings was not affected by the B_2 receptor antagonist icatibant. Furthermore, both control and pressure-loaded rings did not exhibit a relaxation response to exogenous bradykinin, even at high concentrations, in contrast to the sensitive relaxation by acetylcholine, indicating that the rat thoracic aorta is essentially insensitive to bradykinin. This observation is supported by a previous study (Wirth *et al*., 1996) that rat aorta shows vasorelaxation in response to acetycholine, but not to bradykinin. Thus it is unlikely that the kinin-NO-cGMP system plays a role in the signaling cascade of the AT_2-receptor in the rat thoracic aorta.

Recent studies on the vascular AT_2 receptor have focused on the pathophysiological roles under hypertensive conditions in which vascular AT_2 receptors are upregulated, such as in the mesenteric arteries of young spontaneously hypertensive rats (SHR) (Touyz *et al*., 1999) and in the thoracic aorta of SHR (Otsuka *et al*., 1998). Barber *et al*. (1999) demonstrated that an AT_2 receptor agonist induced a depressor response during simultaneous AT_1 receptor blockade in SHR, suggesting that the AT_2 receptor opposes the action of AT_1 receptor in blood pressure regulation, at least in SHR. Carey *et al*. (2000) demonstrated a depressor effect of Ang II in the presence of AT_1 receptor blocker via AT_2 receptor stimulation. These studies strongly suggest that the AT_2 receptor acts as a vasodilatory pathway counter-regulatory to the vasoconstrictor actions of Ang II through the AT_1 receptor. The present study supports this concept by

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the finding that the upregulation of the aortic AT_2 receptor under pressure-overload contributes to the attenuation of the AT_1 receptor-mediated aortic constriction. Thus the upregulation of the vascular AT_2 receptor under pressure-overload seems to be one of the compensatory responses of vessels counteracting the AT_1 receptor-dependent vasoconstriction to relieve the mechanical overload. However, the beneficial effects of AT_1 receptor antagonists through the activation of the AT_2 receptor (Liu *et al*., 1997; Gigante *et al*., 1998; Carey *et al*., 2001) may be limited under pathological conditions in which the expression of AT_2 receptors is controlled by the activation of the AT_1 receptor, as shown in the present study.

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Footnotes

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Legends for Figures

Fig. 1. Plasma renin concentration (PRC) and activity (PRA) in rats after the banding of the abdominal aorta. Plasma samples were collected from rats 2, 4, 7, 14 and 28 days after sham-operation or banding. PRC or PRA in plasma samples was determined by radioimmuoassay of Ang I after the incubation with or without bilaterally nephrectomized rat plasma, respectively. PRC in sham-operated rats (open circles) and in banding rats (closed circles), or PRA in sham-operated (open triangles) and in banding rats (closed triangles) were expressed as ng Ang I liberated by 1-ml plasma sample during 1-hour incubation with or without nephrectomized rat plasma. Each data point represents mean ± S.E. (n=4). ***Significantly different from sham-operated rats (p<0.001).

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Fig. 2. AT_1 and AT_2 receptor mRNA levels in the thoracic aorta after the banding of the abdominal aorta. Expression of AT_1 and AT_2 receptor mRNAs in thoracic aortas was determined by the RT-PCR Southern blotting (A) or the real-time PCR (B) 4, 7, 14 and 28 days after the banding of the abdominal aorta or in non-operated rats (day 0). Panel A shows representative blottings of RT-PCR products for AT_1 and AT_2 receptor mRNAs in 4 non-operated and 5 – 19 animals with aortic banding. In panel B, the amount of AT_2 receptor mRNA measured by real-time PCR is expressed as the ratio of AT_2 mRNA to GAPDH mRNA. Each column and vertical bar represent mean ± S.E. of age-matched non-operated rats (C; n=4), sham-operated rats (S; n=5) or banding rats (B; n=5). ***Significantly different from sham-operated rats ($p<0.001$).

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Fig. 3. Effects of losartan and candesartan administration on the levels of AT_1 and AT_2 receptor mRNAs in the thoracic aorta after the banding of the abdominal aorta. The aortic banding or sham-operation was carried out, then losartan (1 mg/kg, i.p.) or candesartan (2 mg/kg, p.o.) was administered once a day for 7 days to sham-operated and banding rats. The thoracic aortas dissected 7 days after operation were subjected to measurement of AT_1 and AT_2 receptor mRNAs by RT-PCR Southern blotting (panel A) and real-time PCR (panel B). Panel A shows representative blottings of RT-PCR products for AT_1 and AT_2 receptor mRNAs in sham-operated rats (sham; n=4), losartan-treated rats after sham-operation (losartan; n=4), aortic banding rats (banding; n=4), losartan treatment after banding (banding + losartan; n=4), candesartan treatment after sham-operation (candesartan; n=4), and candesartan treatment after banding (banding + candesartan; n=4). Panel B shows the amount of AT_2 receptor mRNA measured by real-time PCR as the ratio of AT_2 mRNA to GAPDH mRNA. Each column and vertical bar represent mean ± S.E.. ***Significantly different from groups of sham-operated rats, banding + losartan, and banding + candesartan (p<0.001).

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Fig. 4. Contractile response to Ang II in ring preparations of thoracic aortas. Thoracic aortas were dissected from rats 7 days after sham-operation or aortic banding, then the contractile response to Ang II was compared by the construction of cumulative concentration-response curves for Ang II. Data represent mean ± S.E.; results are expressed as percentage of contraction evoked by 40 mM KCl. (A) Comparison of the cumulative concentration-response curves for Ang II in aortic rings between sham-operated (open circles; n=5) and banding (closed circles; n=7) rats. Significantly different from rings from sham-operated rats (*p<0.05, **p<0.01). (B) Effect of PD123319 on the response to Ang II in rings from rats 7 days after sham operation (n=5). The cumulative concentration-response curves for Ang II were constructed before (open circles) or 15 min after the treatment with PD123319 (1 x 10^{-6} M) (closed circles). (C) Effect of PD123319 on the response to Ang II in rings from rats 7 days after aortic banding (n=7). The cumulative concentration-response curves for Ang II were constructed before (open circles) or 15 min after the treatment with PD123319 (1 x 10^{-6} M) (closed circles). Significantly different from untreated rings (*p<0.05, **p<0.01).

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Fig. 5. Effect of losartan administration after aortic banding on the contractile response to Ang II in ring preparations of thoracic aortas. After sham-operation or banding of the abdominal aorta, losartan (1 mg/kg, i.p.) was administered once a day for 7 days, then thoracic aortas were dissected 24 hr after the last administration of losartan. As control groups, saline was intraperitoneally administered once a day for 7 days into sham-operated or banding rats. The contractile responses to Ang II in thoracic aortas from losartan-treated rats with banding (open triangles; n=4) were significantly greater than those in vehicle-treated rats with banding (closed circles; n=4) (*p<0.05, **p<0.01), while those in the aortic rings from losartan-treated rats with sham-operation (closed triangles; n=4) were not significantly different from those in rings from vehicle-treated rats with sham-operation (open circles; n=4). Data represent mean ± S.E.; results are expressed as percentage of contraction evoked by 40 mM KCl.

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Fig. 6. Contractile response to Ang II in endothelium-denuded ring preparations of thoracic aortas. Thoracic aortas were dissected from rats 7 days after sham-operation or aortic banding, then the endothelium was removed before fixation in the organ bath. (A) Comparison of the cumulative concentration-response curves for Ang II between intact (open circles; n=5) and endothelium-denuded (closed circles; n=5) aortic rings from sham-operated rats. Significantly different from intact rings (*p<0.05, **p<0.01, ***p<0.001). (B) Comparison of the cumulative concentration-response curves for Ang II between intact (open circles; n=6) and endothelium-denuded (closed circles; n=6) aortic rings from banding rats. ***Significantly different from intact rings (p<0.001). (C) Comparison of the cumulative concentration-response curves for Ang II in endothelium-denuded rings between sham-operated (open circles; n=5) and banding (closed circles; n=6) rats. *Significantly different from sham-operated rats (p<0.05). Data represent mean ± S.E.; results are expressed as percentage of contraction evoked by 40 mM KCl.

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Fig. 7. Contractile response to Ang II in L-NAME-pretreated ring preparations of thoracic aortas. Aortic rings were prepared from thoracic aortas dissected from sham-operated rats (panel A; n=5) or rats 7 days after aortic banding (panel B; n=8), then cumulative concentration-response curves for Ang II were constructed before (open circles) or 30 min after the treatment with L-NAME (1×10^{-4} M) (closed circles). Significantly different from untreated rings (**$p<0.01$, ***$p<0.001$). Data on L-NAME-treated rings in both panel A and B are shown together in panel C, in which open or closed circles represent rings from sham-operated or banding rats, respectively. Data represent mean ± S.E.; results are expressed as percentage of contraction evoked by 40 mM.

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Fig. 8. Effects of Ang II and L-NAME on the cGMP levels in ring preparations of thoracic aortas *in vitro*. Aortic rings were prepared from thoracic aortas dissected from sham-operated rats or rats 7 days after aortic banding, then equilibrated in the organ bath for 1 hr. Thereafter, all rings were incubated with IBMX (5×10^{-5} M) for 30 min, then frozen in dry ice-acetone. Ang II (1×10^{-7} M) and/or L-NAME (1×10^{-4} M) was added into the bath 5 and/or 30 min before freezing the rings, respectively. cGMP was extracted from frozen tissues and assayed by radioimmunoassay as described in the text. The columns in sham and banding represent aortic rings from sham-operated and banding rats, respectively. Each column and vertical bar represent mean ± S.E. (n=4; $*p<0.05$).

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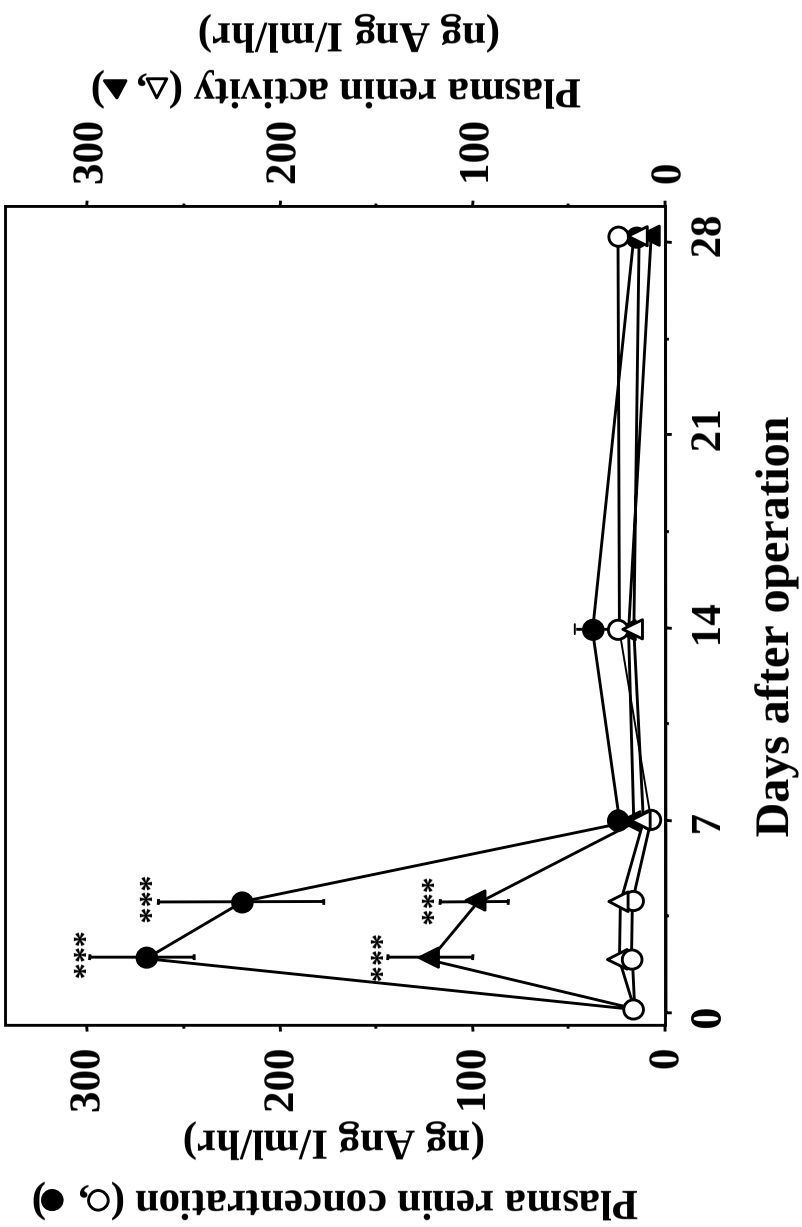

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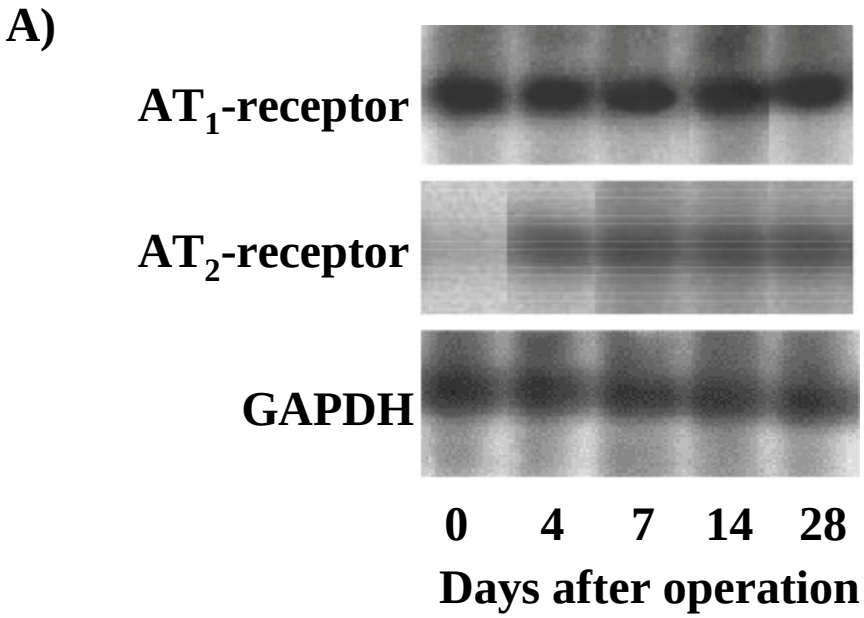

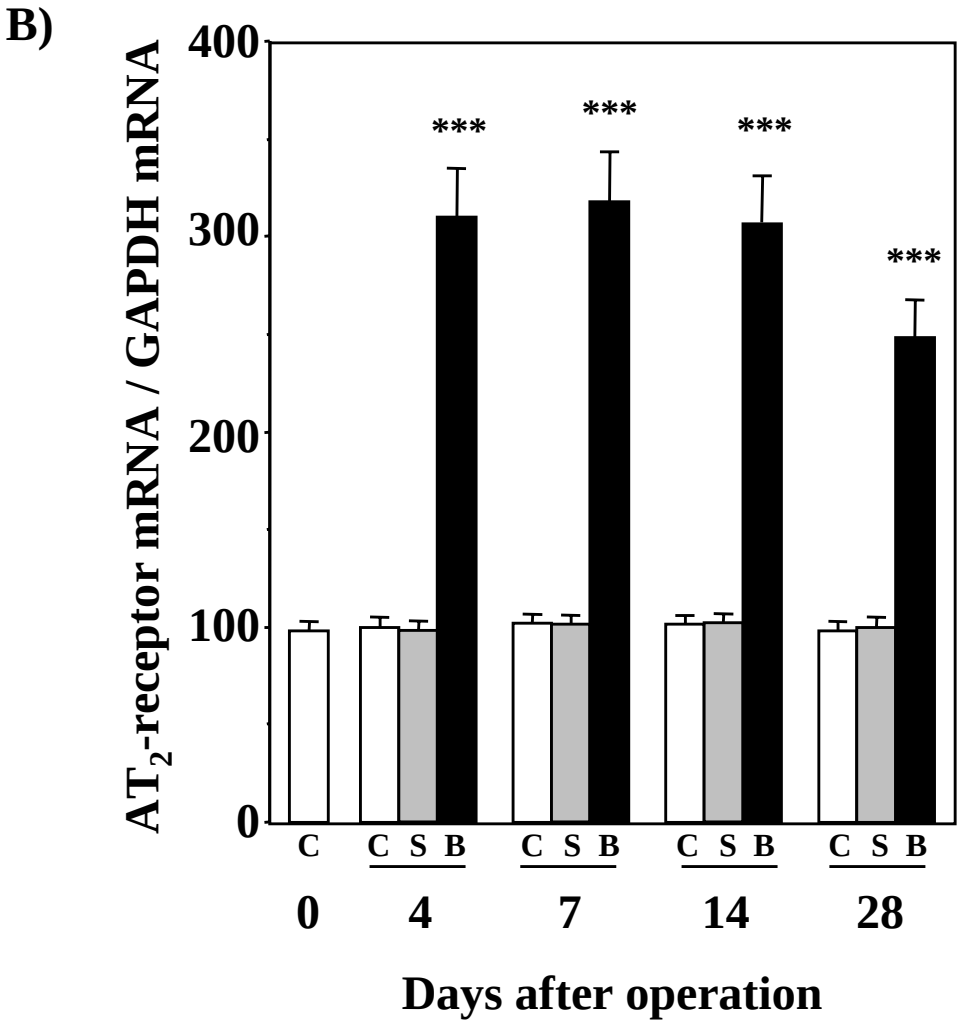

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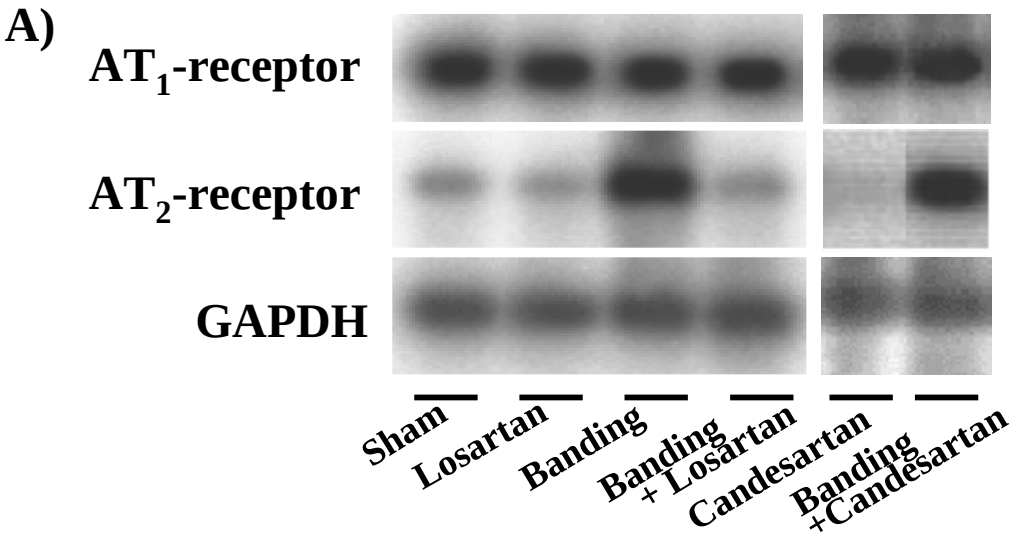

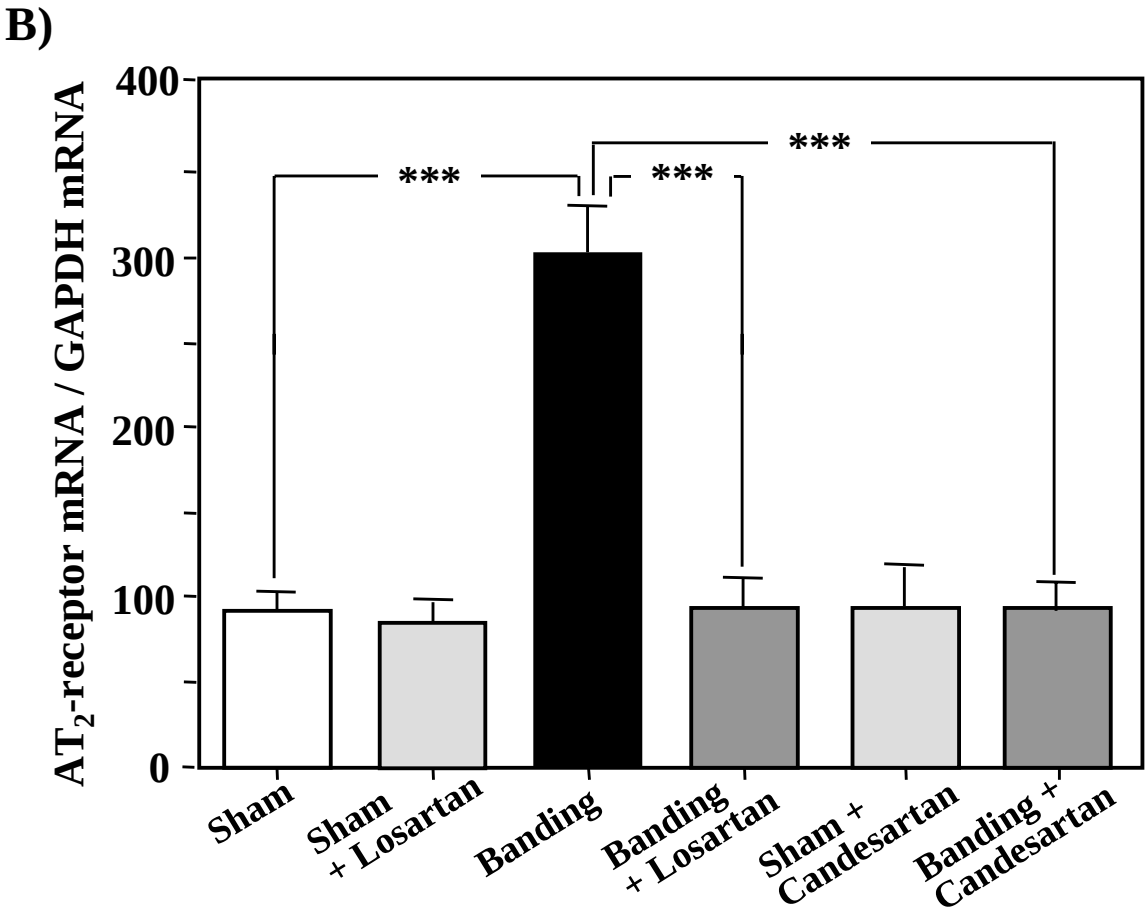

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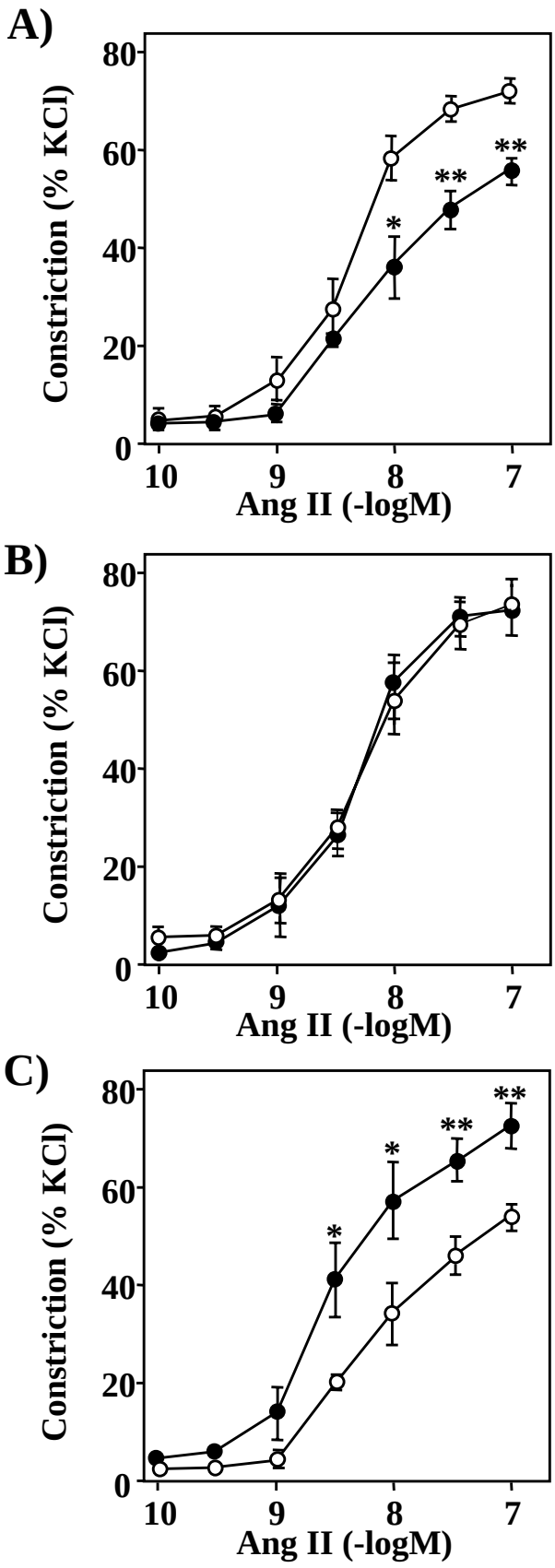

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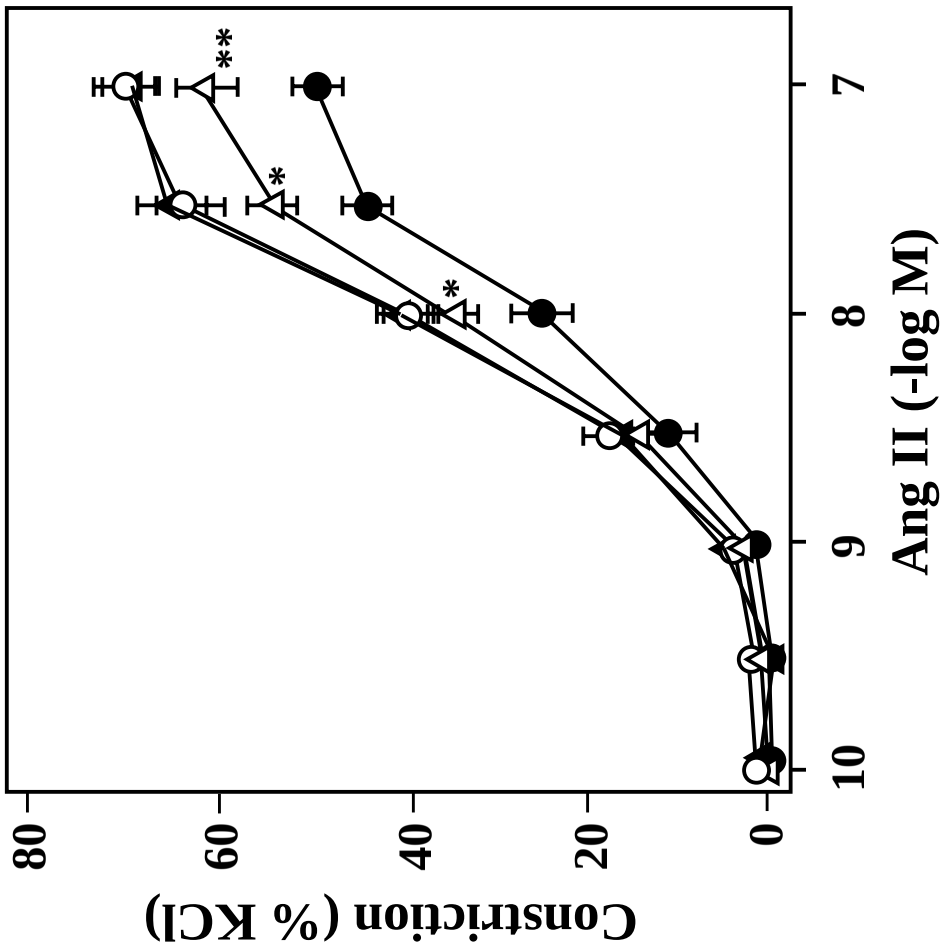

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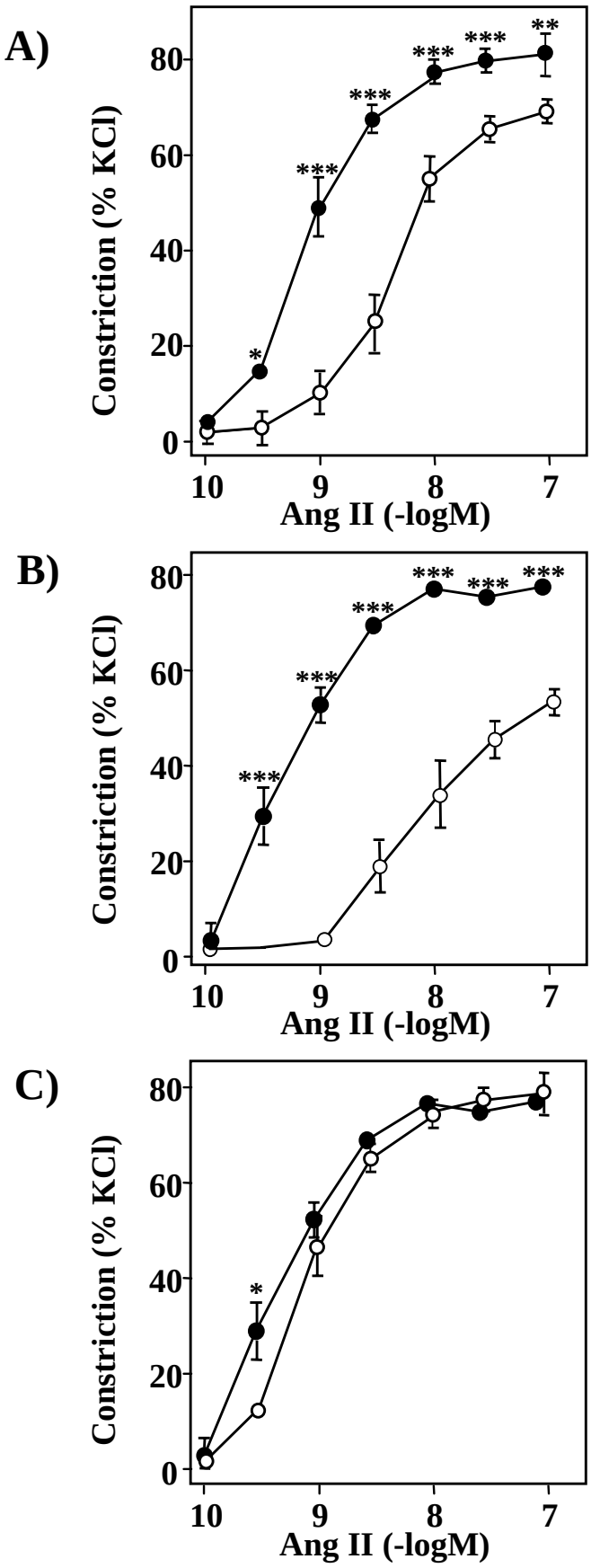

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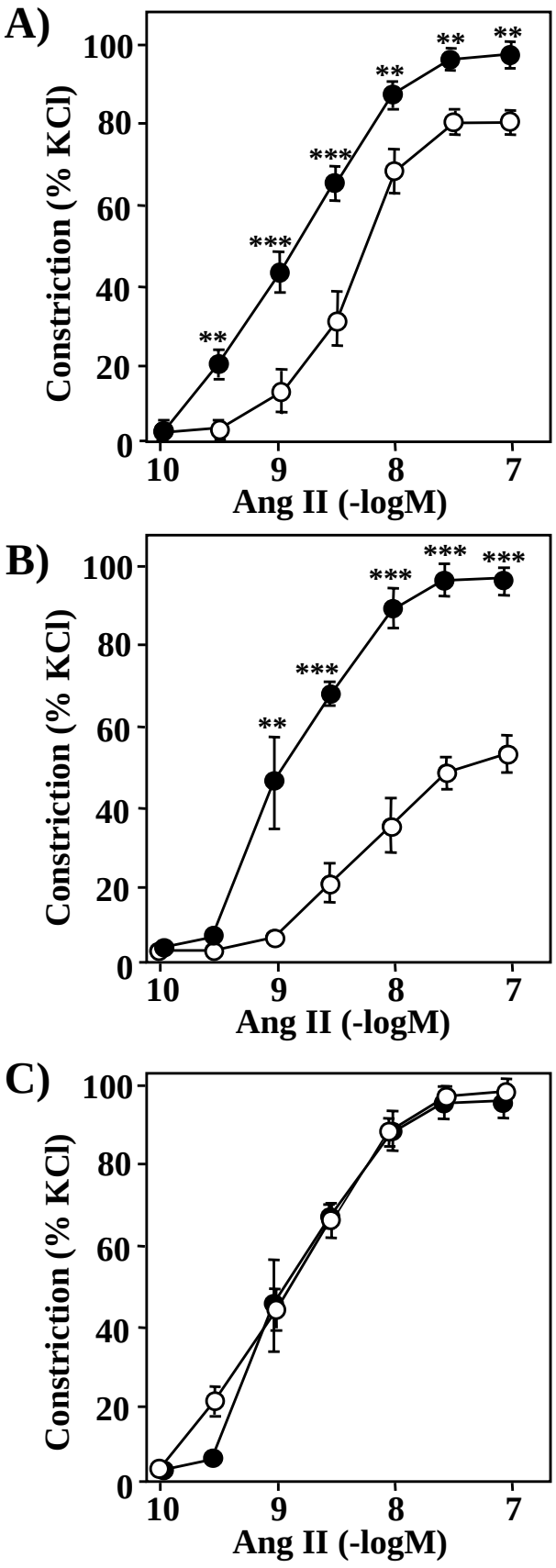

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