JCI The Journal of Clinical Investigation

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*J Clin Invest.* 1998;101(3):527-535. https://doi.org/10.1172/JCI1885.

**Research Article**

Angiotensin (Ang) II has two major receptor isoforms, AT1 and AT2. Currently, AT1 antagonists are undergoing clinical trials in patients with cardiovascular diseases. Treatment with AT1 antagonists causes elevation of plasma Ang II which selectively binds to AT2 and exerts as yet undefined effects. Cardiac AT2 level is low in adult hearts, whereas its distribution ratio is increased during cardiac remodeling and its action is enhanced by application of AT1 antagonists. Although in AT2 knock-out mice sensitivity to the pressor action of Ang II was increased, underlying mechanisms remain undefined. Here, we report the unexpected finding that cardiac-specific overexpression of the AT2 gene using alpha-myosin heavy chain promoter resulted in decreased sensitivity to AT1-mediated pressor and chronotropic actions. AT2 protein undetectable in the hearts of wild-type mice was overexpressed in atria and ventricles of the AT2 transgenic (TG) mice and the proportions of AT2 relative to AT1 were 41% in atria and 45% in ventricles. No obvious morphological change was observed in the myocardium and there was no significant difference in cardiac development or heart to body weight ratio between wild-type and TG mice. Infusion of Ang II to AT2 TG mice caused a significantly attenuated increase in blood pressure response and the change was completely blocked by pretreatment with AT2 antagonist. This decreased sensitivity to Ang II-induced pressor action […]



# Cardiac-specific Overexpression of Angiotensin II AT2 Receptor Causes Attenuated Response to AT1 Receptor–mediated Pressor and Chronotropic Effects

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## Abstract

**Angiotensin (Ang) II has two major receptor isoforms, AT1 and AT2. Currently, AT1 antagonists are undergoing clinical trials in patients with cardiovascular diseases. Treatment with AT1 antagonists causes elevation of plasma Ang II which selectively binds to AT2 and exerts as yet undefined effects. Cardiac AT2 level is low in adult hearts, whereas its distribution ratio is increased during cardiac remodeling and its action is enhanced by application of AT1 antagonists. Although in AT2 knock-out mice sensitivity to the pressor action of Ang II was increased, underlying mechanisms remain undefined. Here, we report the unexpected finding that cardiac-specific overexpression of the AT2 gene using α-myosin heavy chain promoter resulted in decreased sensitivity to AT1-mediated pressor and chronotropic actions. AT2 protein undetectable in the hearts of wild-type mice was overexpressed in atria and ventricles of the AT2 transgenic (TG) mice and the proportions of AT2 relative to AT1 were 41% in atria and 45% in ventricles. No obvious morphological change was observed in the myocardium and there was no significant difference in cardiac development or heart to body weight ratio between wild-type and TG mice. Infusion of Ang II to AT2 TG mice caused a significantly attenuated increase in blood pressure response and the change was completely blocked by pretreatment with AT2 antagonist. This decreased sensitivity to Ang II–induced pressor action was mainly due to the AT2-mediated strong negative chronotropic effect and exerted by circulating Ang II in a physiological range that did not stimulate catecholamine release. Isolated hearts of AT2 transgenic mice perfused using a Langendorff apparatus also showed decreased chronotropic responses to Ang II with no effects on left ventricular dp/dt max values, and Ang II–induced activity of mitogen-activated protein kinase was inhibited in left ventricles in the transgenic mice. Although transient outward $K^+$ current recorded in cardiomyocytes from AT2 TG mice was not influenced by AT2 activation, this study suggested that overexpression of AT2 decreases the sensitivity of pacemaker cells to Ang II. Our results demonstrate that stimulation of cardia AT2 exerts a novel antipressor action by inhibiting AT1-mediated chronotropic effects, and that application of AT1 antagonists to patients with cardiovascular diseases has beneficial pharmacotherapeutic effects of stimulating cardiac AT2. (*J. Clin. Invest.* 1998. 101:527–535.) Key words: angiotensin receptors • angiotensin II • transgenic mouse • inotropic action • $K^+$ current**

## Introduction

The presence of an endogenous renin-angiotensin (Ang)[1] system in the heart was established by detection of the expression of mRNAs encoding angiotensinogen and renin, Ang I converting enzyme (ACE) (1, 2), Ang II receptors (3–8), and the detection of Ang I and II immunoreactivities (9). Upregulation of angiotensinogen or ACE (1, 2) and Ang II receptor mRNAs (3–8) has been described in association with cardiac hypertrophy and acute myocardial infarction, suggesting that a cardiac renin–Ang system is activated in cardiac remodeling. At least two main Ang II receptor subtypes, AT1 and AT2, have been identified using receptor subtype–specific antagonists (10). Most of the well-known Ang II functions in the cardiovascular system are mediated by AT1, whereas there is little information regarding the physiological roles of AT2 and its signal transduction pathway (10). The AT2 has an antiproliferative effect upon neointimal formation after vascular injury (11) and upon coronary endothelial cells (12). Recent studies have indicated that AT2 associates with Gi proteins (13) and mediates inhibitory effects of mitogen-activated protein kinase (MAPK) activation in vascular smooth muscle cells (11), PC12W cells (14), and neuronal cultures (15). Consider-

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*Received for publication 30 September 1997 and accepted in revised form 8 December 1997.*

J. Clin. Invest.

0021-9738/98/02/0527/09 $2.00
Volume 101, Number 3, February 1998, 527–535
http://www.jci.org

1. *Abbreviations used in this paper:* ACE, angiotensin I converting enzyme; Ang, angiotensin; BP, blood pressure; HR, heart rate; IK, delayed rectifier; MAPK, mitogen activated protein kinases; MHC, myosin heavy chain; TG, transgenic.

ing that AT2 is abundantly and transiently expressed in fetal tissues (16, 17), this receptor may have an anti-AT1 effect on cellular proliferation and may also play a role in development and/or differentiation.

AT1 antagonists are currently undergoing clinical trials in patients with cardiovascular diseases. Treatment with AT1 antagonists causes elevation of plasma Ang II which selectively binds to AT2 and exerts undefined effects (18). Although the level of expression of AT2 is low in the adult cardiovascular system, it is increased in remodeling hearts such as in cardiac hypertrophy and infarction (4–6) or in mechanical stretch–induced hypertrophy of myocytes (19), and the distribution ratio of AT2 relative to AT1 is increased in failing human hearts by AT1 downregulation (20–23). Ichiki et al. (24) and Hein et al. (25) reported that disruption of the AT2 gene caused an increase in blood pressure (BP) and increased sensitivity to the pressor actions of Ang II. Interestingly, it was shown very recently that selective activation of AT2 by treatment with the AT1 antagonist losartan was associated with an unexpected lower risk of mortality than the ACE inhibitor captoril in elderly patients with heart failure (26). These findings suggest the physiological role of AT2 in blood pressure control or its cardioprotective effect, whereas the underlying mechanism remains poorly defined. We overexpressed AT2 in the heart where AT2 level is very low and thereby examined the effects of AT2 on Ang II–induced hemodynamic actions. Here, we report the unexpected finding that cardiac-specific overexpression of AT2 inhibits AT1-mediated positive chronotropic effects, resulting in attenuated sensitivity to the pressor actions of Ang II without affecting cardiac contractility, thereby indicating a novel hemodynamic effect on pathological conditions in which cardiac AT2 expression is increased.

## Methods

*Transgene constructs and screening of transgenic mice.* A 5.5-kb fragment of the mouse α-myosin heavy chain (MHC) promoter (kind gift from Dr. Jeffrey Robbins, University of Cincinnati, Cincinnati, OH) (27) and a mouse AT2 cDNA (28) were subcloned into pBsKs(−) plasmid. The resultant recombinant plasmid, pMHC-AT2, was digested with BamHI and XhoI to generate ~ 8.6 kb of DNA fragment consisting of the α-MHC promoter and AT2. The DNA fragment was then microinjected into the pronuclei of single cell fertilized mouse embryos to generate transgenic mice (C57BL/6 strain) (29). Samples of tail tissue were obtained and DNA was extracted using an easy DNA kit (Invitrogen Corp., San Diego, CA) To detect the transgenic mice, we designed sense and antisense PCR primers from the second and third exons of the mouse AT2-R gene, respectively. The resultant PCR product was 1180 bp long. The primers span about 1300 bp of second intron of genomic mouse AT2 and should only amplify the transgene.

*Northern analysis.* Total RNA was extracted by means of guanidium isothiocyanate–cesium chloride centrifugation, fractionated on a 1% agarose/formaldehyde gel, and transferred to a nitrocellulose membrane. Blots were then hybridized with a random-primer $^{32}$P-labeled DNA probe consisting of rat AT2 cDNA as described (3, 6).

*Membrane preparation and binding assay.* Atrium and left ventricles, including septum and free wall, were dissected, weighed, and minced with scissors and membrane fractions were prepared from pooled samples ($n$ = 10) as previously described (3, 30). Membrane fractions (60 μg of protein) were incubated with $^{125}$I-[Sar$^1$, Ile$^8$] Ang II (8–10 points in 0.05–5 nmol/liter for saturation experiment and 0.2 nmol/liter for competition experiment) in a total assay volume of 300 μl for 2 h at 22°C. The degradation rate of Ang II after 2 h incubation at 22°C was determined by reversed-phase HPLC. As much as 96.3± 4.2% ($n$ = 3) of the radioligand remained intact during incubation. Specific binding was determined from the difference between counts in the absence and presence of 3 μmol/liter unlabeled Ang II. All assays were conducted in duplicate. The Kd and Bmax values were estimated by Rosenthal analysis of the saturation data and Ki values were determined as previously described (30). AT1 and AT2 densities were calculated by nonlinear least-squares regression analysis on the basis of inhibition by CGP42112A, using the InPlot program (GraphPAD Software for Science, San Diego, CA).

*Measurements of BP, heart rate (HR), dp/dt, and infusion of Ang II.* Male mice (aged 16–20 wk) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). A cervical incision was made, the trachea was intubated, and the animal was connected to a volume-cycled ventilator. A PE10 flame-stretched, fluid-filled catheter was introduced into the left common carotid artery (for experiments with anesthetized mice) or into the femoral artery (for experiments with conscious mice) and attached to a transducer element (TXD-300; Digi-Med, Louisville, KY). The second cannula using a PE10 catheter was inserted into the left jugular vein for injection of drugs. For experiments with conscious mice, these catheters were threaded under the skin and exited at the nape of the neck, where the end of the cannula was sealed by heating. 24 h after surgery, a piece of PE50 tubing was connected to the femoral artery cannula. The other end of the tubing was connected to a swivel to allow free mobility of the mice. BP and HR were monitored continuously during the following protocol. Baseline values were recorded for 10–20 min until stable. Mice were then given a bolus administration of captoril (30 mg/kg body weight) followed by infusion of Ang II according to the method used in AT2 knock-out mice (24, 25). After 10 min of captoril administration, Ang II diluted in saline was infused at different doses in a volume of 10 μl directly into the catheter. Data were registered simultaneously on a chart recorder and transmitted online via an amplifier (model BPA-200; Digi-Med) and analyzed with a computer program (System Integrator Model 200; Digi-Med).

*Isolated perfused mouse heart using Langendorff preparation.* The mice were anesthetized with pentobarbital sodium (30 mg/kg) intraperitoneally and treated with 500 U/kg of heparin sodium to prevent blood coagulation. After thoracotomy, the hearts were isolated, suspended via the ascending aorta on a 20-gage cannula and were retrogradely perfused from a heated storage cylinder with 37°C oxygenated Krebs-Henseleit solution (KHS) (mmol/liter: $Na^+$, 145; $K^+$, 5.9; $Ca^{2+}$, 2.5; $MgSO_4$, 1.2; $Cl^-$, 119; $HCO^-$, 25; $H_2PO_4^-$, 1.2; EDTA, 0.5; and glucose, 5.5). The cylinder containing the buffer was oxygenated with 95% $O_2$–5% $CO_2$ and positioned above the heart to produce a constant pressure of 55 mmHg for retrograde perfusion of the coronary arteries. A catheter (PE50) was inserted into the left atrium through the pulmonary vein, guided through the mitral valve during opening, advanced into the left ventricle, and forced through the ventricular apex. The proximal end of the catheter remained in the left ventricle. The distal end of the catheter was connected through a larger catheter to a high-pressure transducer to record left ventricular (LV) pressure and its derivative, dP/dt. HR was continuously recorded over 20 min after injection of drugs and the maximal increase was used for data analyses.

*MAPK activity and immunoblotting.* Hearts stored at −80°C were powdered with a pestle under liquid nitrogen, homogenized and lysed with ice-cold buffer containing 10 mmol/liter Tris-HCl, pH 7.4, 20 mmol/liter NaCl, 1 mmol/liter sodium orthovanadate, 10 mmol/liter sodium pyrophosphate, 10 nmol/liter okadaic acid, 2 mmol/liter EGTA, 2 mmol/liter dithiothreitol, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin. After brief sonication, the homogenate was centrifuged for 5 min at 14,000 *g* and the supernatant was assayed for MAPK activity with an assay kit (Amersham Corp., Arlington Heights, IL) that measured the incorporation of [γ-$^{33}$P]ATP into a synthetic peptide (KRELVEPLTPAGEAPNQALLR) as a specific MAPK substrate. The reaction was carried out with the cell lysate (~ 10 μg of protein) in 75 mmol/liter

Hepes, pH 7.4, containing 1.2 mmol/liter $MgCl_2$, 2 mmol/liter substrate peptide, and 1.2 mmol/liter ATP, 1 μCi of [γ-$^{33}$P]ATP for 30 min at 30°C. The resultant solution was applied to a phosphocellulose membrane and extensively washed in 1% acetic acid and then in $H_2O$. The radioactivity was measured by liquid scintillation counting. For immunoblotting, the homogenized samples were suspended with SDS-polyacrylamide gel electrophoresis buffer, pH 6.8, containing 62.5 mmol/liter Tris-HCl, 2% SDS, 10% glycerol, 50 mmol/liter dithiothreitol, and 0.1% bromphenol blue. After brief sonication, samples were boiled for 5 min at 95°C and centrifuged and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gels were transferred onto Hybound-ECL membranes (Amersham Corp.) by electroblotting. The membrane was treated with rabbit polyclonal phospho-specific MAPK antibodies (New England Biolabs, Inc., Beverly, MA) that detect $p42^{MAPK}$ and $p44^{MAPK}$ only when catalytically activated by phosphorylation at Tyr-204. After incubation with secondary anti–rabbit antibodies, immunoreactive proteins were detected by the enhanced chemiluminescence reaction (ECL, Amersham Corp.). Thereafter, the protein on the filter was stripped and reprobed with MAPK antibody (New England Biolabs, Inc).

*Determination of plasma Ang II levels.* Blood samples were collected into the tubes containing 20 ml/liter ethanol, 24 mmol/liter phenanthroline, 125 mmol/liter disodium EDTA, and 2 g/liter neomycin. Plasma fraction was separated, extracted and plasma Ang II levels were measured with radioimmunoassay using rabbit polyclonal Ang II antibody (Chemicon International, Inc., Temecula, CA) after separation by reverse phase HPLC as reported (31).

*Preparation of myocytes.* Single ventricular myocytes were isolated from mice hearts using a previous described method (32). Briefly, the heart was perfused with $Ca^{2+}$ free Tyrode's solution containing collagenase type 1 (0.5 mg/ml; Worthington Biochemical Corp., Freehold, NJ) and BSA (1 mg/ml) for 20–30 min using the Langendorff apparatus at 37°C. At the end of the perfusion period, the heart was removed, passed through 200 μm nylon mesh, and centrifuged for 3 min at 100 *g*. The cells were stored in low $Cl^-$, high $K^+$ medium at room temperature (20–21°C).

*Electrophysiology.* Whole cell currents were recorded by patch-clamp techniques as previously described (32). The patch pipettes had a resistance of 2 MΩ or less. The experimental chamber (0.2 ml) was placed on a microscope stage, and the external solution changes were made using Y-tube techniques (32). To measure $K^+$ channel currents, depolarizing pulse were applied from a holding potential −80 mV. The patch pipettes solution contained (mmol/liter): 20 KCl, 110 K-aspartate, 2 $MgCl_2$, 5 ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 2 MgATP, and 0.1 GTP. The external solution was a Tyrode's solution (mM); 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 2 Hepes, 10 glucose.

*Reagents and statistical methods.* All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated below. Losartan was kindly provided by DuPont-Merck Pharmaceutical Co. (Wilmington, DE). PD123319 was provided by Parke-Davis and Warner-Lambert Co. (Ann Arbor, MI). The results are expressed as means±SEM. Analysis of variance and Fisher's PLSD test were used for multigroup comparisons, with $P < 0.05$ considered significant.

## Results

*Cardiac-specific overexpression of AT2 gene did not affect development and phenotype of myocardium.* The transgene construct contained the mouse α-MHC promoter (27) ligated to the coding sequence for the mouse AT2 (28). This promoter effects a pattern of transgene expression similar to that of endogenous α-MHC, which is the predominant heavy chain isoform in the adult mouse atria and ventricles. This isoform is not normally expressed in smooth or skeletal muscle, nor is it expressed in the ventricular chamber during fetal development. By PCR of genomic DNA, two founders containing the AT2 transgene were identified. Grossly, these founders demonstrated no phenotypic changes and two lines with confirmed transmission of the transgene (TG) were established (TG778 and TG788). Neonatal mortality in the transgenics did not differ from that in the nontransgenic animals, and there were no adult deaths. These animals had similar heart to body weight ratios relative to their control littermates designated as wild type and there was no evidence of developmental defects. No

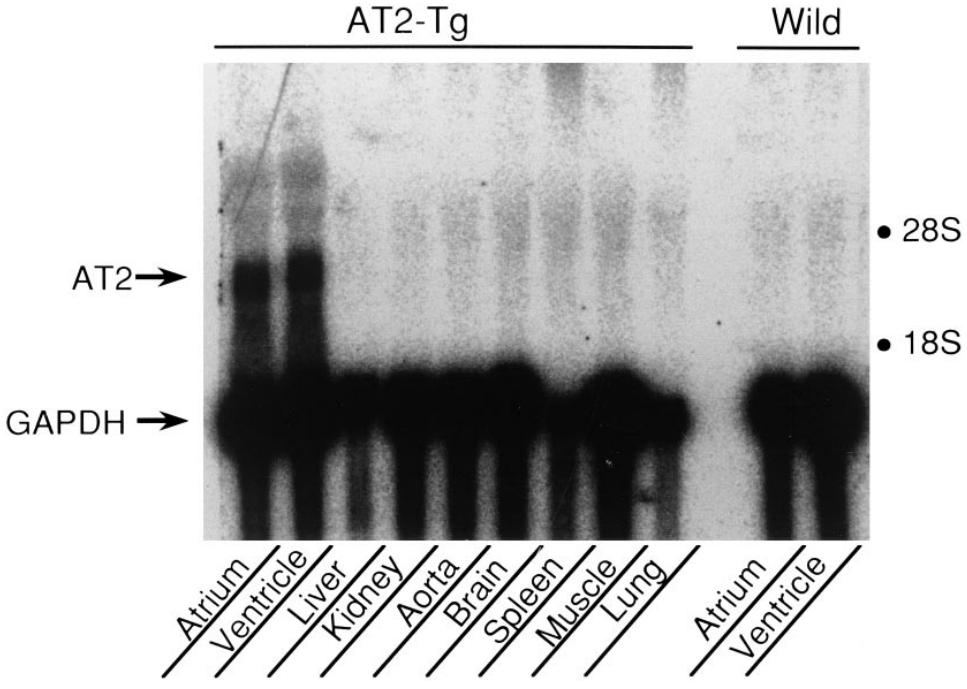


*Figure 1.* Northern blotting analysis of AT2 mRNA levels in various tissues from AT2 TG778 mice. Total RNA (20 μg) was fractionated on a 1% agarose/formaldehyde gel and transferred onto a nitrocellulose membrane. Blots were then hybridized with a random-primer $^{32}$P-labeled DNA probes consisted of mouse AT2 and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs. GAPDH mRNA levels were used as an internal RNA control. The exposure time was 14 d at −70°C.

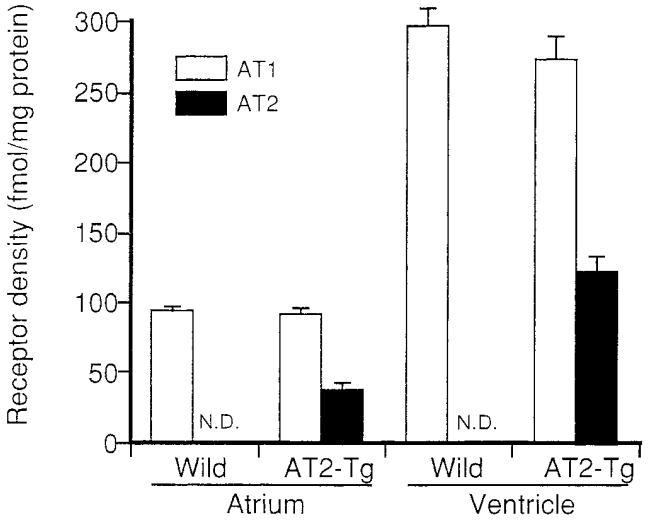


*Figure 2.* Transgene expression by radioligand binding. Ligand binding assays were performed on membrane fractions prepared from the pooled hearts from 10 wild-type or AT2 TG778 mice ~ 12–16 wk of age (*Wild*, $n = 4$; *AT2 Tg*, $n = 4$). AT1 and AT2 densities were calculated by nonlinear least-squares regression analysis on the basis of inhibition by CGP42112A, using the InPlot program (GraphPAD Software for Science). *N.D.*, not detected.

obvious morphological changes, such as myocyte necrosis or fibrosis, were observed in the atrial or ventricular myocardium, electrocardiograms were normal, and no arrhythmia or any conduction block was seen (data not shown).

Transgene expression assessed by Northern blotting on total RNA from various tissues of adult mice revealed that AT2 was exclusively and abundantly expressed in both atria and ventricles, but not in other tissues (Fig. 1). Saturation and competitive inhibition experiments using [$^{125}$I]-Sar$^{1}$ Ile$^{8}$–Ang II as a ligand revealed that AT2 protein was overexpressed in both atria and ventricles of the two transgenic lines, and the proportions of AT2 relative to AT1 were 41% in atria and 45% in ventricles of TG778 mice (Fig. 2). The proportions of AT2 in TG788 mice were 34% in atria and 37% in ventricles. The presence of AT2 was not detected in atria or ventricles of wild-type mice and there were no significant changes in cardiac AT1 numbers between TG and wild-type animals (Fig. 2). Ki values (nmol/liter) of cardiac AT1 were similar between TG778 and wild-type mice (AT1:27±2.4, AT2:29±2.1, $n = 4$) on the basis of inhibition by losartan. The kD value (nmol/liter) of AT2 in left ventricles of TG778 mice was 0.19±0.04 ($n = 4$) when saturation binding experiments were performed with AT2 selective ligand [$^{125}$I]-CGP42112A, in good agreement with the native binding affinity of AT2 (6). The level of expression of AT2 was high in both TG lines and therefore cannot be attributed to the site of genomic integration. The following experiments were performed using TG778 mice.

*Cardiac-specific overexpression of AT2 gene attenuated AT1-mediated hemodynamic actions.* It was reported that the targeted disruption of the mouse AT2 gene resulted in a significant elevation in basal BP and increased sensitivity to the pressor action of Ang II (24, 25), although the mechanism of this hemodynamic response remains to be determined. Changes of arterial BP and HR in response to Ang II infusion were simul-

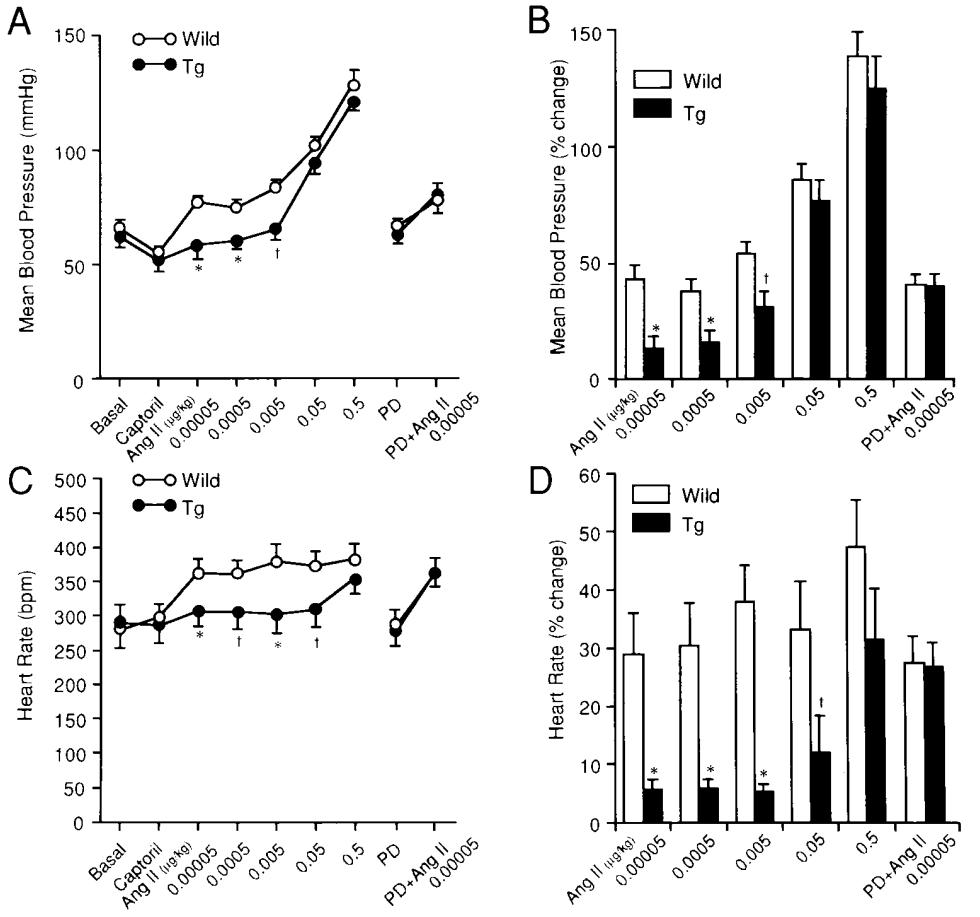


*Figure 3.* Changes in mean blood pressure and heart rate afer Ang II infusion in anesthetized AT2 TG778 mice. Mice (12–16 wk, $n = 15$ for wild and TG mice) were anesthetized with pentobarbital, and blood pressure and heart rate were simultaneously measured with catheters placed in the carotid artery. Mice were then given a bolus administration of captoril (30 mg/kg body weight) followed by infusion of Ang II at similar doses used in the experiments in AT2 knock-out mice (24, 25). After 10 min of captoril administration, Ang II diluted in saline was infused at different doses in a volume of 10 μl directly into the catheter. In the experiment to examine the effect of PD123319, PD123319 (10 mg per kg) was infused into mice ($n = 8$) pretreated with captoril and after 20 min Ang II was infused. Changes (percent) in blood pressure (*B*) and heart rate (*D*) are shown relative to the baseline levels after captoril treatment. The results are expressed as means±SEM. $^{*}P < 0.01$; $^{\dagger}P < 0.05$, versus the levels in wild-type mice.

taneously measured in both anesthetized and conscious TG mice using a catheter placed in the left carotid artery. Similar doses of Ang II to the range used in the experiments with AT2 knock-out mice were infused into TG and wild-type mice (24, 25). As shown in Fig. 3, *A* and *B*, the basal levels of BP and HR did not differ significantly between anesthetized TG and wild-type mice. Hemodynamic response to Ang II was examined after administration of the ACE inhibitor captoril to eliminate endogenous Ang II. Infusion of pharmacological doses of Ang II (from 0.05 to 0.5 μg/kg) resulted in a dose-dependent increase in absolute values and percent changes of BP from the basal level in both TG and wild-type groups, whereas infusion of lower doses of Ang II (0.00005–0.005 μg/kg) caused a significantly lower increase in BP level in TG mice compared with that in wild-type controls. It should be noted that this result was the converse of the increased sensitivity of BP after Ang II infusion observed in AT2 knock-out mice (24, 25). Interestingly, the response of HR to Ang II infusion was markedly suppressed in TG mice; both absolute values and percent changes of HR after infusion of Ang II were markedly attenuated from lower doses even at pharmacological doses of Ang II (Fig. 3, *C* and *D*). These attenuated responses observed in TG mice were completely inhibited by pretreatment with the AT2-R antagonist PD123319 (Fig. 3). Similar results were obtained from both TG778 and TG788 lines (only data for TG778 are shown).

Since the changes in BP and HR described above were measured in anesthetized mice to diminish the influence of surgical stress or baroreceptor reflex via the central sympathetic nervous system (32–34), we next performed the same experiments using conscious, unrestrained mice. There were no significant changes in basal BP levels between TG and wild-type mice (Fig. 4, *A* and *B*). The infusion of Ang II elevated BP levels dose dependently in wild-type mice, whereas the pressor effect of Ang II at lower doses (0.00005–0.0005 μg/kg) was significantly attenuated in TG mice (Fig. 4, *A* and *B*). Compared with the results in anesthetized mice, the response of HR after Ang II infusion was complex possibly due to the central sympathetic nervous reflex via arterial baroreceptors in response to BP elevation (Fig. 4, *C* and *D*). HR levels were dose dependently decreased in both TG and wild-type mice, whereas at lower doses of Ang II (0.00005 and 0.0005 μg/kg) at which a significant decrease in BP level was observed in TG mice, changes of HR were markedly inhibited in TG mice relative to wild-type controls (Fig. 4 *D*). Decreased responses of BP and HR observed in TG mice were completely abolished by pretreatment with PD123319 (Fig. 4). Thus, decreased sensitivity to pressor and chronotropic actions of Ang II was observed in both anesthetized and conscious TG mice, although the response in conscious mice was less obvious because of the central nerve reflex via arterial baroreceptors.

Ang II–mediated increases of BP and HR in wild-type mice were completely blocked by pretreatment with the AT1 antagonist losartan (Fig. 5, *A* and *B*), indicating that in wild-type mice the hemodynamic response after infusion of Ang II was exclusively mediated by AT1-dependent pathway. On the other hand, infusion of Ang II (0.00005–0.5 μg/kg) to TG mice pretreated with losartan did not cause significant changes in the responses of BP and HR compared with those in the wild-type mice (data not shown). As endogenous Ang II was elimi-

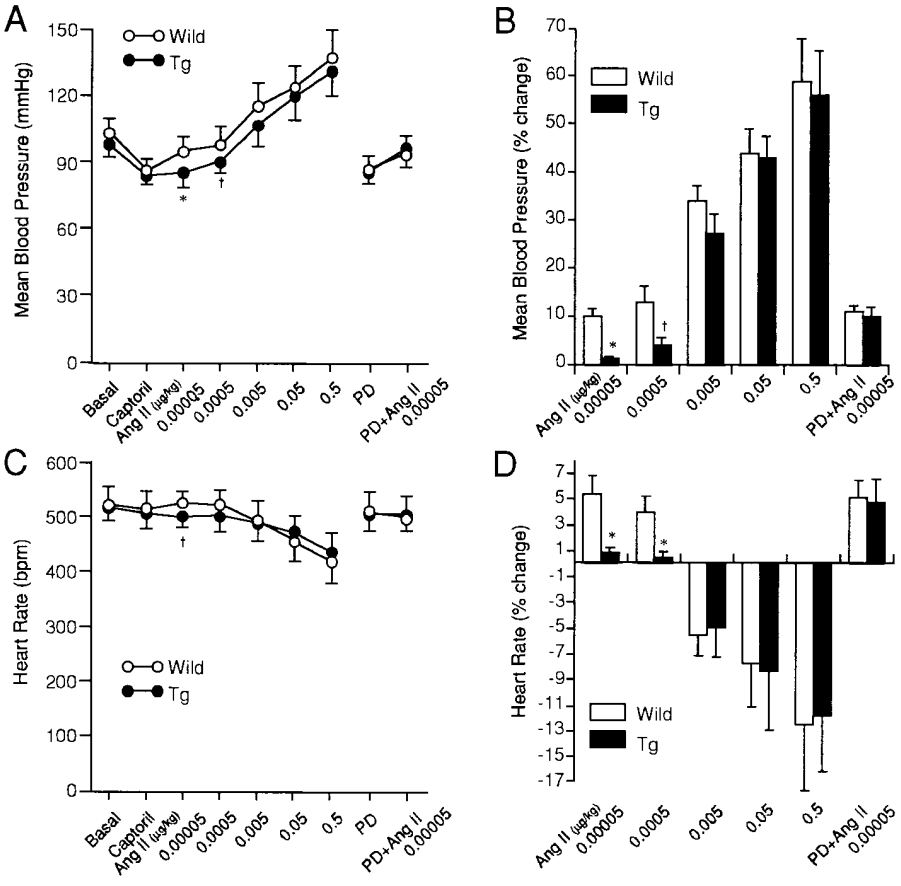


*Figure 4.* Changes in mean blood pressure and heart rate after Ang II infusion in conscious, unrestrained AT2 TG778 mice. Blood pressure and heart rate in mice (12–16 wk, $n = 12$ for both wild-type and TG mice) were simultaneously measured with catheters placed in the femoral artery at 24 h after surgery. Experimental conditions were the same as in Fig. 3. $^{*}P < 0.01$, $^{\dagger}P < 0.05$ versus the levels in wild-type mice.

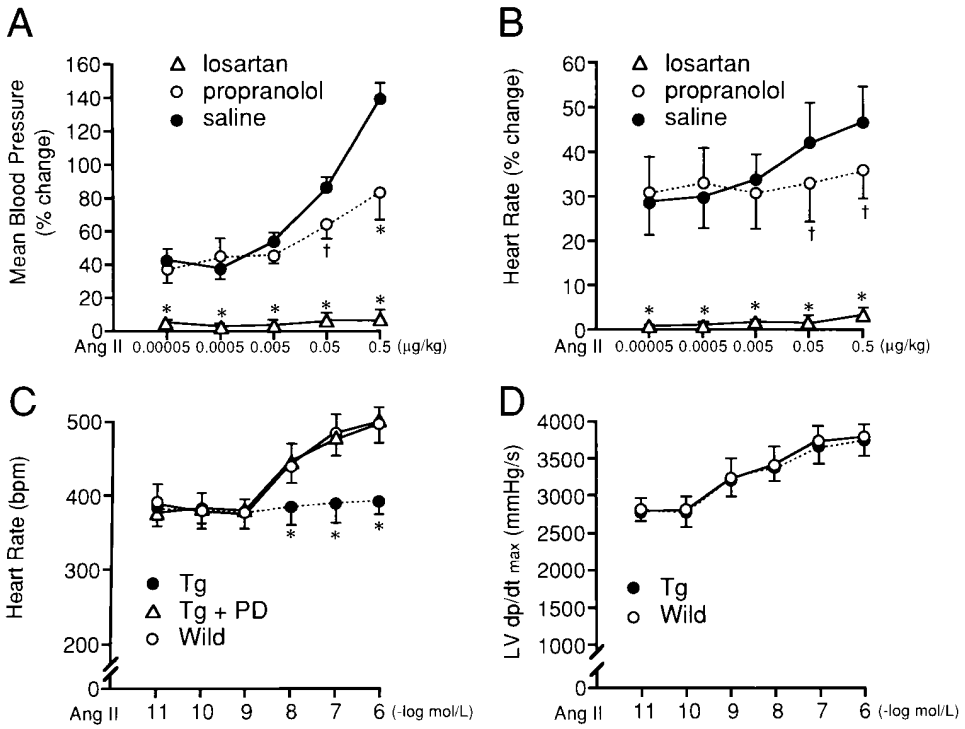


*Figure 5.* Effects of the AT1 antagonist losartan and propranolol on blood pressure (*A*) and heart rate (*B*) in anesthetized wild-type mice, and Ang II-induced effects on heart rate (*C*) and left ventricular dp/dt max (*D*) in isolated Langendorff hearts. Wild-type mice (12–16 wk, $n = 10$) were anesthetized with pentobarbital, and blood pressure and heart rate were directly measured with catheters placed in the carotid artery. Mice were then given a bolus injection of captoril (30 mg/kg body weight) and after 10 min losartan (10 mg/kg), propranolol (3 μmol/kg) or saline was infused in a volume of 10 μl, followed by infusion of increasing doses of Ang II. Hearts isolated from mice (12–16 wk, $n = 10$, for both wild type and TG mice) were perfused for 10 min with KHS buffer containing captoril (10 μmol/liter) using the Langendorff apparatus, and changes in heart rate or left ventricular dp/dt max values to Ang II were measured. In the experiment to examine the effects of PD123319 ($n = 10$), hearts were perfused for 20 min with KHS buffer containing PD123319 (50 μmol/liter) after captoril treatment and then the buffer was changed to the same solution containing Ang II.

nated by administration of captoril as performed in the Ang II infusion experiments in Figs. 3 and 4, these data suggest that the inhibitory effect of AT2 is significantly exerted on the pressor and chronotropic responses induced by AT1 stimulation rather than in the absence of AT1-mediated hemodynamic actions.

*Pressor and chronotropic actions after Ang II infusion at lower doses were mediated by AT1 but not by catecholamine receptors.* Since it was shown that Ang II induced a chronotropic effect by stimulating the release of catecholamines from the sympathetic ganglia or adrenal glands (33–35), we next examined whether pressor and chronotropic actions of Ang II were associated with Ang II–mediated catecholamine release. As shown in Fig. 5, *A* and *B*, BP and HR were dose dependently increased after Ang II infusion in anesthetized wild-type mice pretreated with vehicle saline solution, and pretreatment with the AT1 antagonist losartan (10 mg/kg) completely inhibited the changes in BP and HR after infusion of Ang II at doses from 0.00005 to 0.5 μg/kg. In wild-type mice pretreated with the β-adrenergic receptor blocker propranolol (3 μmol/kg), the increases in BP and HR after Ang II infusion at doses from 0.00005 to 0.005 μg/kg did not differ from those in vehicle-treated mice, whereas at higher doses of Ang II (0.05 and 0.5 μg/kg) the changes were significantly inhibited by propranolol (Fig. 5, *A* and *B*). The α1- and α2-adrenergic receptor antagonists prazosin (0.5 μmol/kg) and yohimbine (3 μmol/kg), respectively, did not significantly influence the increases in BP and HR after Ang II infusion at doses of 0.05 and 0.5 μg/kg (data not shown). The doses of these drugs had been established to be sufficient for substantial blockade of α- or β-adrenergic receptors (33, 35). These results demonstrate that hemodynamic actions of Ang II exerted at doses below 0.005 μg/kg were mainly mediated by AT1 and the response after Ang II infusion at doses over 0.05 μg/kg was due to additive effects of AT1 and β-adrenergic receptor stimulation.

*Chronotropic response to Ang II was decreased in isolated hearts from AT2 transgenic mice without affecting cardiac contractility.* To further confirm the AT2-mediated inhibitory effect on AT1-induced chronotropic responses and examine the effects of AT2 overexpression on cardiac contractility, we used isolated Langendorff heart preparations (Fig. 5 *C*). In hearts isolated from TG mice, HR did not significantly differ from those in wild-type mice at circulating Ang II concentrations from $10^{-11}$ to $10^{-9}$ mol/liter. HR in hearts from wild-type mice was significantly increased by 22–34% by perfusion with $10^{-8}$ to $10^{-6}$ mol/liter of circulating Ang II (Fig. 5 *C*) and this was completely inhibited by pretreatment with losartan (data not shown). The increases in HR level induced by $10^{-8}$ to $10^{-6}$ mol/liter Ang II were significantly inhibited in TG mice, and pretreatment with PD123319 completely blocked the response (Fig. 5 *C*). No significant changes in cardiac contractility as assessed by left ventricular (LV) $dp/dt_{max}$ values were observed during perfusion with increasing concentrations of Ang II (Fig. 5 *D*) between TG and wild-type mice.

*AT2-mediated hemodynamic effects were mediated by circulating Ang II at physiological concentrations.* We measured plasma Ang II levels after infusion of the lowest dose of Ang II (0.00005 μg/kg) causing negative chronotropic changes in AT2-TG mice. Basal plasma Ang II levels in conscious wild-type and TG mice were in the range from 61 to 87 pg/ml (74± 4.7 pg/ml, $n = 23$) and there was no significant difference between these two groups (wild type: 73±4.4, $n = 12$; TG: 75± 4.7, $n = 11$). After 0.00005 μg/kg of Ang II infusion, the range

of plasma Ang II was from 68 to 103 pg/ml (83±5.4 pg/ml, $n$ = 20) and there was no significant change relative to the basal level. Since treatment with the AT1 antagonist losartan was reported to elevate plasma Ang II levels by four- to fivefold in humans (18), it was considered that plasma Ang II levels after 0.00005 μg/kg of Ang II infusion changed within the physiological range.

*MAPK activity induced by Ang II was attenuated in hearts from AT2 TG mice.* Studies using cells expressing AT2 showed that AT2 exerted its physiological actions by inhibiting the activities of MAPKs (11, 14, 15). Therefore, next we examined, using isolated Langendorff hearts, whether AT2 overexpressed in the heart plays a functional role in MAPK activity. MAPK activities were determined by measuring the incorporation of [γ-$^{33}$P]ATP into a specific MAPK substrate using homogenized LV tissue samples and the phosphorylation of MAPK was analyzed with Western blotting using antibodies against phospho-specific MAPKs that detect p42$^{MAPK}$ and p44$^{MAPK}$. MAPKs were slightly phosphorylated even in left ventricles of both wild-type and TG mice perfused with a solution containing no Ang II, and perfusion of control hearts with Ang II (10 nmol/liter) for 10 min caused a maximal increase (2.6-fold relative to the control level) in MAPK activities that was completely blocked by pretreatment with losartan (100 nmol/liter) (Fig. 6 *A*). There were no significant differences in the basal activities of MAPK between TG and wild-type mice.

Perfusion with Ang II of hearts from TG mice also caused a significant increase (1.9-fold relative to the control level) in MAPK activity but this increase was lower ($P < 0.05$) than that in wild-type mice. Pretreatment with PD123319 (100 nmol/liter) resulted in a further marked increase (3.1-fold relative to the control level) in Ang II–induced MAPK activity, while pretreatment with losartan (100 nmol/liter) tended to reduce Ang II–induced MAPK activity compared with the basal level but this change was not significant. These findings established that in hearts from AT2 TG mice, AT1-mediated MAPK activities were inhibited by overexpression of AT2.

## Discussion

The major new finding in this study was that TG mice with cardiac-specific AT2 gene expression had a decreased sensitivity to AT1-mediated pressor action without any effect on cardiac contractility, which was mainly due to the inhibitory effect of AT2 on the AT1-mediated chronotropic effect. This novel effect of AT2 was exerted by circulating Ang II in the range that did not stimulate the release of catecholamines. Moreover, this negative chronotropic action was also confirmed in isolated Langendorff hearts. Circulating Ang II levels that evoked differences in BP and HR between AT2 TG mice and wild-type mice were in the physiological range, suggesting that under pathological conditions, such as cardiac hypertrophy or infarction (3–8) or failing hearts (20–23), in which the relative distribution ratio of AT2 is increased, these AT2-mediated effects may play an important role in the control of the cardiovascular system by Ang II. However, considering that the cardiac AT2

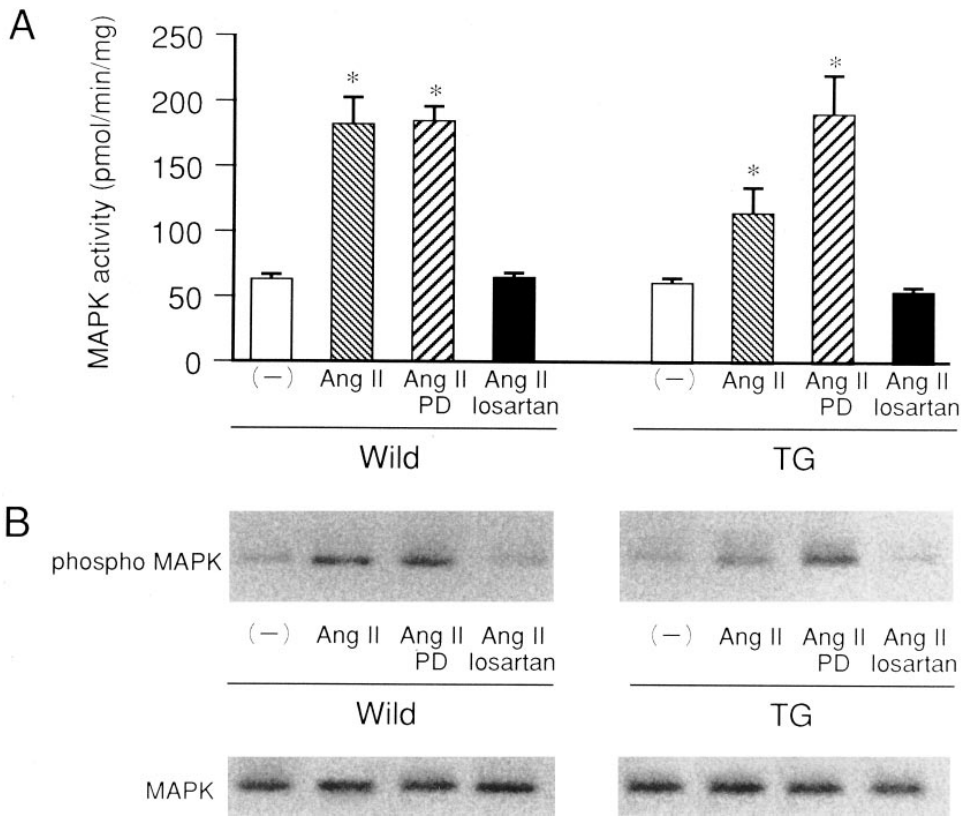


*Figure 6.* Changes of Ang II-induced MAPK activity in left ventricles from wild-type or AT2 TG mice. Isolated hearts from mice (12–16 wk) were perfused for 10 min with KHS buffer containing captoril (10 μmol/liter) using the Langendorff apparatus and then exposed to the same buffer containing Ang II (1 μmol/liter). Hearts were rapidly frozen with liquid nitrogen and stored at −80°C. Frozen hearts were homogenized, and solubilized samples were used for measurement of MAPK activity using an assay kit (Amersham Corp.) that measures the incorporation of [γ-$^{33}$P]ATP into a synthetic peptide as a specific MAPK substrate. For immunoblotting, the samples were subjected to SDS-PAGE, electroblotted onto polyvinylidene membranes and treated with rabbit polyclonal phospho-specific MAPK antibodies that detect p42$^{MAPK}$ and p44$^{MAPK}$ followed by incubation with secondary anti–rabbit antibodies and reprobed with MAPK antibody. In the experiment to examine the effects of PD123319 or losartan ($n$ = 10), hearts were perfused for 20 min with KHS buffer containing these drugs (50 μmol/liter each) after captoril treatment and then the buffer was changed to the same solution containing Ang II. Numbers of tested heart samples: control in the absence of Ang II ($n$ = 9), Ang II alone ($n$ = 8), Ang II + PD123319 ($n$ = 8), Ang II + losartan ($n$ = 8). The results are expressed as means±SEM. *$P < 0.01$ versus the control in the absence of Ang II.

level in humans with heart disease was relatively lower than cardiac AT2 level in TG mice (20–23) and that the AT2-mediated effect observed in conscious TG mice was modest, we should be more cautious in speculating about the potential relevance of the finding observed in AT2 TG mice to clinical situations. In this study, we also found that ventricular contractility of TG mice assessed by LV $dp/dt_{max}$ values did not significantly differ from those in wild-type mice at the baseline or after treatment with Ang II. Both cardiac output and total peripheral resistance of the arteries determine BP levels, and cardiac output depends on HR and stroke volume (36). As total peripheral resistance of arteries and vascular sensitivity to Ang II are likely to be similar between AT2 TG mice with cardiac-specific overexpression and wild-type mice, it is conceivable that a decrease in cardiac output due to the AT2-mediated negative chronotropic action is involved in the decreased sensitivity to the pressor action of Ang II in AT2 TG mice.

Ichiki et al. (24) found that HR was generally higher in AT2 knock-out mice at baseline and after Ang II infusion, consistent with our data showing AT2-mediated chronotropic action. In contrast, Hein et al. (25) reported that there was no difference in HR value after Ang II infusion between mutant and control mice. Ichiki et al. crossed the chimeric males with C57BL/6 females as in this study and administrated captoril before Ang II infusion. Hein et al. used FVB/N females and measured the changes in HR after Ang II infusion without captoril pretreatment. As Hein et al. found increased response of BP to Ang II infusion only after treatment with captoril, the differences in the experimental conditions rather than in the genetic background may account for this discrepancy. Thus, increased sensitivity to the pressor action of Ang II reported in AT2 knock-out mice could be at least partially explained by the lack of AT2-mediated negative chronotropic action leading to inhibition of AT1-mediated increase in cardiac output.

Activation of AT2 was reported to elicit stimulation of outward $K^+$ currents, $I_K$ (delayed rectifier) and Ito (transient outward), in neurons (37, 38) and to inhibit T-type $Ca^{2+}$ current in nondifferentiated NG108-15 cells (neuron like cell) (39), while in cardiomyocytes AT2-mediated effects on these channel activities remain undefined. Although we examined the effects of Ang II on Ito $K^+$ currents recorded from ventricular myocytes, no significant difference was seen in current–density and current–voltage relationship after exposure to Ang II between TG and wild-type mice (data not shown). $I_K$ currents were undetectable from both wild-type and AT2 TG mice, consistent with the previous report that $I_K$ currents were too small to be detected in myocytes isolated from mouse hearts (40). T-type $Ca^{2+}$ channels are mainly localized in pacemaker cells in the sinoatrial node and then play an important role in configuration of action potentials in pacemaker cells (41). It was reported that cardiac pacemaking in the sinoatrial node was primarily determined by time-dependent currents such as T type $Ca^{2+}$ currents and hyperpolarization-activated If current, and time-independent currents such as $Na^+$–$Ca^{2+}$ exchange current and $Na^+$–$K^+$ pump current (41). In this study, we could not detect T type $Ca^{2+}$ current from atrial myocytes despite repeated experiments probably because of the difficulty in isolating the cells from the sinoatrial node of mice. If the inhibitory effect by AT2 stimulation was exerted on the T type $Ca^{2+}$ current in pacemaker cells as observed in NG108-15 cells (39), the negative chronotropic response observed in AT2 TG mice might be partially explained by this mechanism.

In this study, we also found that the increase in MAPK activity after Ang II infusion was significantly inhibited in the hearts from AT2 TG mice, in agreement with previous observations in cells expressing AT2 such as vascular smooth muscle cells (11), PC12W cells (14), or neuronal cultures (15). Recent studies (42–44) also reported that AT2-mediated action opposed the growth action of AT1 on isolated cardiac myocytes and fibroblasts, most likely at the level of signal transduction. These findings demonstrate that the AT2 overexpressed in the heart plays a functional role in Ang II-mediated signal transduction systems, and may raise the possibility that AT2-mediated inhibition of MAPK activity was involved in the decreased sensitivity of pacemaker cells in response to Ang II. Very recently, we have reported that cardiac specific overexpression mice of AT1a using the same α-MHC promoter show conduction abnormality such as heart block (45). Together with the possibility that overexpression of AT2 affects the activity of pacemaker cells, the pacemaker cells might be one of the preferential sites of overexpression in α-MHC transgenic. Further studies are needed to define the mechanism responsible for the AT2-mediated negative chronotropic effect.

The findings in this study have important pharmacotherapeutic implications for the predicted actions of AT1 antagonists that have been used very recently for patients with cardiovascular diseases. Accumulated evidence indicated that the expression of cardiac AT2 was increased in cardiac hypertrophy or infarction (3–7) and that the distribution ratio of AT2 relative to AT1 was increased by downregulation of AT1 in failing human hearts (20–23). In addition, we also found that mechanical stretch-induced hypertrophy of cardiomyocytes resulted in enhanced AT2 gene expression (19). As circulating Ang II levels are increased by administration of AT1 antagonists (18) and Ang II preferentially binds to the cardiac AT2 in the presence of AT1 antagonists, AT2-mediated actions are expected to be further activated under these pathological conditions. This study demonstrated that AT2 stimulation has a novel hemodynamic effect by inhibiting AT1-mediated pressor and chronotropic actions without affecting cardiac contractility. Very recently, it was shown that selective activation of AT2 by treatment with the AT1 antagonist losartan was associated with an unexpected lower risk of mortality than the ACE inhibitor captoril in elderly patients with heart failure (26). The novel hemodynamic action via AT2, such as an inhibitory effect on AT1-mediated pressor and positive chronotropic actions, might be partially involved in this unexpected efficiency of losartan as a pharmacotherapeutic agent, raising the possibility that the AT2 have a cardioprotective action. Since blockade of the renin-angiotensin system is essential for the management of patients with heart failure (46) and it is expected that AT1 antagonists will be widely used for treatment of patients with cardiovascular diseases in the near future to a similar extent as ACE inhibitors, this novel cardioprotective effect of AT2 should be confirmed in clinical studies.

## Acknowledgments

We thank Dr. Atsuko Yatani, Division of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, for patch clamp experiments.

This study was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, the Study Group of Molecular Cardiology, The Naito Foundation, the Clinical Pharma-

cology Foundation in Japan, and Japan Medical Association and Japan Smoking Foundation.

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