Angiotensin II Type 2 Receptor Is Upregulated in Human Heart With Interstitial Fibrosis, and Cardiac Fibroblasts Are the Major Cell Type for Its Expression

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Abstract—The expression pattern of angiotensin (Ang) II type 2 receptor (AT2-R) in the remodeling process of human left ventricles (LVs) remains poorly defined. We analyzed its expression at protein, mRNA, and cellular levels using autopsy, biopsy, or operation LV samples from patients with failing hearts caused by acute (AMI) or old (OMI) myocardial infarction and idiopathic dilated cardiomyopathy (DCM) and also examined functional biochemical responses of failing hearts to Ang II. In autopsy samples from the nonfailing heart group, the ratio of AT1-R and AT2-R was 59% and 41%, respectively. The expression of AT2-R was markedly increased in DCM hearts at protein (3.5-fold) and mRNA (3.1-fold) levels compared with AMI or OMI. AT1-R protein and mRNA levels in AMI hearts showed 1.5- and 2.1-fold increases, respectively, whereas in OM and DCM hearts, AT1-R expression was significantly downregulated. AT1-R–mediated response in inositol phosphate production was significantly attenuated in LV homogenate from failing hearts compared with nonfailing hearts. AT2-R sites were highly localized in the interstitial region in either nonfailing or failing heart, whereas AT1-R was evenly distributed over myocardium at lower densities. Mitogen-activated protein kinase (MAPK) activation by Ang II was significantly decreased in fibroblast compartment from the failing hearts, and pretreatment with AT2-R antagonist caused an additional significant increase in Ang II–induced MAPK activity (36%). Cardiac hypertrophy suggested by atrial and brain natriuretic peptide levels was comparably increased in OM and DCM, whereas accumulation of matrix proteins such as collagen type 1 and fibronectin was much more prominent in DCM than in OM. These findings demonstrate that (1) AT2-R expression is upregulated in failing hearts, and fibroblasts present in the interstitial regions are the major cell type responsible for its expression, (2) AT2-R present in the fibroblasts exerts an inhibitory effect on Ang II–induced mitogen signals, and (3) AT1-R in atrial and LV tissues was downregulated during chronic heart failure, and AT1-R–mediated functional biochemical responsiveness was decreased in the failing hearts. Thus, the expression level of AT2-R is likely determined by the extent of interstitial fibrosis associated with heart failure, and the expression and function of AT1-R and AT2-R are differentially regulated in failing human hearts. (Circ Res. 1998;83:1035–1046.)

Key Words: angiotensin II type 2 receptor • AT2 receptor • angiotensin II type 1 receptor • AT1 receptor, angiotensin II

The presence of 2 isoforms of angiotensin (Ang) II receptor was originally proposed on the basis of differences in sensitivity of receptor–ligand binding to dithiothreitol. Ang type 2 receptor (AT2-R), which is insensitive to dithiothreitol and has a high affinity for PD123319 and CGP42112A, was isolated, and this receptor was shown to have the same seven-transmembrane domain of AT1-R but only minimal homology (see Review in References 1 and 2). Most of Ang II functions in the cardiovascular system are mediated by AT1-R, whereas there is little information regarding the physiological roles of AT2-R. Mice lacking AT2-R suggested an antipressor action of AT2-R, and cardiac-specific overexpression mice of AT2-R showed attenuated response to Ang II–induced chronotropic action. The AT2-R also has an antigrowth effect upon neointimal formation after vascular injury and on coronary endothelial cells. Recent evidence has indicated that AT2-R associates with Gi proteins and mediates inhibitory effects of mitogen-activated protein kinase (MAPK) activation. Considering that AT2-R is abundantly and transiently expressed in fetal tissues, this receptor may have an anti–AT1-R effect on cellular growth and may also play a role in development and/or differentiation.

The first study that examined human myocardium showed that there were no significant changes in total Ang II receptor numbers between failing and nonfailing hearts. Recent studies using human failing hearts indicated selective downregulation of AT1-R but not AT2-R, leading to an...
increase in the distribution ratio of AT₂-R relative to AT₁-R. Clinical treatment with AT₁-R antagonists causes elevation of plasma Ang II, which selectively binds to AT₂-R, and may exert as-yet undefined cardiac effects. In the present study, we report using left ventricle (LV) samples from failing human hearts that AT₂-R expression is upregulated in the fibroblasts present in fibrous regions, and the increased AT₂-R has an ability to inhibit Ang II–induced mitogen signals, whereas the expression of AT₁-R was downregulated and AT₁-R–mediated functional biochemical response was significantly attenuated.

**Materials and Methods**

**Patients**

LV tissues were obtained at autopsy from 17 subjects, 13 of whom had ischemic heart disease (n = 8) or idiopathic dilated cardiomyopathy (DCM, n = 9). Four normally functioning hearts (non-failing group) were obtained from patients with brain death who had no history of cardiac disease or pulmonary disease (age, 52 ± 7.5 years, range, 29 to 61; n = 4). LVs obtained during operation for aneurysmectomy from 4 old myocardial infarction patients and biopsy LV specimens from 5 DCM patients were also included in the present study. Diagnosis of ischemic heart disease or DCM had been performed by coronary angiography and histological examinations of biopsy samples. Patients with ischemic heart disease were further divided into 2 groups: those who died of acute myocardial infarction (AMI, n = 3) within 5 days after onset of first heart attack, and those who died of ischemic cardiomyopathy (n = 5) mainly as a result of OMI (n = 5). Two subjects in the OMI group (patient No. 4 and No. 5 in the Table) had received coronary bypass surgery. There were no significant differences in mean age between AMI, OMI, and DCM groups and the nonfailing control group (Table). Although we examined gender-dependent difference in the AT₁-R and AT₂-R densities, there appeared to be no specific change between men and women (Table). Pulmonary artery diastolic pressure (PAd) was measured by right heart catheterization (Swan-Ganz), and the worst values recorded within 24 hours before death were selected. Samples were obtained from the base of LV including ventricular septum, and in AMI and OMI patients, noninfarced portions of LV were used for preparation of membrane fractions. Biopsy specimens were obtained from the subendocardium of LV free wall using biotome.

Nine of the patients were pretreated with the angiotensin-converting enzyme (ACE) inhibitors (25 to 50 mg/d of captopril) (Table). This low-dose treatment resulted in no significant difference in receptor numbers (B_max) or in dissociation constant (K_d) in patients with and without ACE inhibitor therapy (B_max: ACE inhibitors, 8.4 ± 1.3 fmol/mg protein; no ACE inhibitors, 9.3 ± 1.7 fmol/mg protein, K_d 0.90 ± 0.2 and 0.89 ± 0.2 mmol/L, respectively). Complications of hypertension, diabetic mellitus, or hyperlipidemia also did not affect B_max values. The study was approved by the Ethical Committee of Kansai Medical University.

**Preparation of Plasma Membrane Fractions**

Autopsy samples (10 to 30 g) excised within 1 hour after death, and tissue samples obtained during operation were immediately placed in iced, oxygenated Tyrode’s solution and then homogenized by a Polytron at 4°C in 2 volumes of buffer A composed of ice-cold 0.32 mol/L sucrose, 0.5 mmol/L EDTA, and 25 mmol/L Tris (pH 7.5), including protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 mg/L bacitracin, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 40 U/mL trastylol, and antipain, phosphoramidon, amastatin, and bestatin, each at 1 μg/mL). The homogenates were centrifuged at 500 g for 10 minutes at 4°C, and the supernatant fractions were recentrifuged at 48 000g for 30 minutes at 4°C. The pellets were resuspended in a solution containing 0.6 mol/L KCl and 30 mmol/L histidine at pH 7.0 (including protease inhibitors mentioned above) to solubilize actin and myosin filaments and were centrifuged at 48 000g for 30 minutes at 4°C. The pellets obtained after the final centrifugation were resuspended in binding buffer containing protease inhibitors mentioned above and immediately used for the binding assay. Thus, in the present study, the tissue samples and membrane preparations were not subject to the frozen storage process, because we had found that during the freezing process of tissue samples, AT₁-R and AT₂-R numbers were decreased by 30% ± 2.7% and 8.2 ± 1.4% (n = 4), respectively. Ligand binding assay was performed whenever each membrane fraction was prepared, and interassay variations were very low (4.8 ± 0.7, n = 10).

**BindingAssay and Analysis of Data**

The binding assay of cardiac AT₁-R, AT₂-R, and AT₃-R was performed as previously described. Briefly, membrane fractions (~500 μg of protein) were incubated with [125I]-[Sar²,Ile⁸] Ang II for the saturation experiment (0.2 mmol/L [125I]-[Sar²,Ile⁸] Ang II for the competition experiment) in a total assay volume of 300 μL for 90 minutes at 21°C. The assay buffer contained 50 mmol/L Tris (pH 7.6), 100 mmol/L NaCl, 10 mmol/L MgCl₂, 1 mmol/L EGTA, 0.25 mg/mL BSA, and a variety of protease inhibitors used for membrane preparation. The degradation rate of [Sar²,Ile⁸] Ang II after 90 minutes of incubation at 21°C was determined by reverse-phase HPLC. The results showed that under the incubation conditions, as much as 97.1 ± 2.4% (n = 3) of the radioligand remained intact. Specific [125I]-[Sar²,Ile⁸] Ang II binding was determined from the difference between counts in the absence and presence of 3 mmol/L unlabeled Ang II. K_d was calculated from the equation K_d = IC₅₀/(1 + L/K_d), where L is the concentration of [125I]-[Sar²,Ile⁸] Ang II. Total Ang II receptor numbers were determined from Scatchard analysis, and AT₁-R and AT₂-R were separately calculated from total Ang II receptor numbers on the basis of distribution ratio determined with nonlinear least-squares regression analysis by inhibition of CGB42112A using the GraphPad InPlot computer program (GraphPad).

**Na⁺,K⁺-ATPase Activity**

The assay was performed as previously reported in rat hearts. Briefly, membranes were preincubated with or without 5 mmol/L ouabain at 37°C for 5 minutes. The reaction was initiated by the addition of 2 mmol/L ATP, and released P, was measured according to the modification of the method of Parvin and Smith.

**RNase Protection Analyses and Northern Blotting**

RNase protection assays were performed with 40 μg of total RNA as previously described. DNA was digested with DNase in the presence of RNase inhibitor to remove contaminating genomic DNA. cDNAs encoding human AT₁-R and AT₂-R were subcloned into pBS vector. An RNA probe for the AT₁-R (270 bp) was produced from Drai-digested EcoRI and PsI cDNA fragment of human AT₁-R (nucleotides = 280 to +1140). An AT₂-R RNA probe (100 bp) was generated from AvoII-digested EcoRI and Kpnl fragment of human AT₂-R cDNA (nucleotides = 189 to +425). Antisense cRNA was transcribed using RNA polymerase and hybridized with total RNA at 45°C and digested with RNase T1 and A, resulting in 210- and 90-bp protected signals for the AT₁-R and AT₂-R mRNAs. The difference between the size of the protected signal and the RNA probe used was due to the digestion of an additional part of RNA probe transcribed from DNA sequence present in the linker site of pBS vector. For quantitative analyses, the signal intensities were measured by laser densitometry and normalized relative to GAPDH mRNA levels quantified by Northern blotting.

When AT₁-R mRNA levels in nonfailing LV tissues were quantified by this analysis, the inter- and intraassay variations were less than 10% (7.6%, n = 5; 6.4%, n = 7, respectively), suggesting that the validity of the data in mRNA quantification was very high. Human atrial and brain natriuretic peptides (ANPs and BNP) cDNA probes were kindly provided by Dr Y. Saitoh (Kyoto University), and rat α₁(I) collagen cDNA and fibronectin cDNA were kind gifts from Dr D. Rowe (Connecticut Health Center) and Dr R.O. Hynes (Massachusetts Institute of Technology).

**Inositol Phosphate Assays**

Inositol phosphate measurement using cardiac homogenate from LV tissues was performed according to the method described by Berridge.

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with a slight modification. LV tissues kept in oxygenated Tyrode’s solution were minced finely with scissors and homogenized to small pieces in the buffer A with the use of a homogenizer. Cardiac homogenate was centrifuged at 500g for 10 minutes at 4°C, and the pellet was suspended in 10-volume of DMEM containing 200 μCi [3H]-myoinositol and incubated for 3 hours at ~22°C. At the end of incubation, cardiac homogenate was rinsed 3 times with the buffer containing 20 mMol/L HEPES (pH 7.4) and 10 mMol/L LiCl and subsequently incubated with various concentrations of Ang II in the same medium for 30 minutes at ~22°C. The reactions were stopped by addition of 5-volume of ice-cold chloroform/methanol (1:2, vol/vol), and the chloroform/methanol extract was transferred to a test tube. Cardiac homogenate was further homogenized with a Polytron in 0.5 Mmol/L HCl, which was added to the chloroform/methanol extract, shaken, and then centrifuged. The upper phase was aspirated, dried, and stored at ~30°C. The water-soluble inositol phosphates were eluted with formic acid and ammonium formate by using Dowex columns, according to the method established by Berridge.22

**Preparation of Cell Fraction, Measurement for MAPK Activity, and Immunoblotting**

Isolation of cells from human LV tissue was performed as previously reported with a slight modification.13 Briefly, pieces of LV free walls kept in oxygenated Tyrode’s solution were minced finely with scissors, incubated with Joklik medium containing 0.3% collagenase for 10 minutes at 37°C, then mechanically pipetted; the obtained supernatant was collected into ice-cold DMEM with 20% FCS. This sample fraction was considered to contain the dissociated intact cells, mostly cardiac fibroblasts, smooth muscle α-actin for vascular smooth muscle cells, desmin for cardiomyocytes, and a polyclonal antibody against von Willebrand factor for detection of endothelial cells. Immunocytochemistry was performed by Biotin/Avidin system (Elite ABC kit, Vector Lab), using dianminobenzidine tetrahydrochloride as a substrate.

**Reagents and Statistical Methods**

All reagents were purchased from Sigma Chemical Co, unless otherwise indicated. Losartan was provided by DuPont Merck Pharmaceutical (Wilmington, Del). PD123177 and PD123319 were provided by Parke-Davis and Warner-Lambert Co (Ann Arbor, Mich), and CGP42112A was purchased from NEOSYSTEM (Strasbourg, France). Results are expressed as mean±SE. Data analyses were performed using a commercially available statistical program (SAS, Statistical Analysis System, SAS Institute Inc) on an IBM PC. Statistical analyses in Figures 2 through 7 were performed with 1-way ANOVA, and actual P values from the ANOVAs are shown in the respective figure legends. We proceeded to pairwise contrasts (control versus conditions) using Dunnett’s multiple-comparison test only if the outcome of 1-way ANOVA was significant in Figures 2 through 5 and Figure 7. In Figure 6B and 6C, the pairwise comparisons (untreated control versus Ang II treatment, Ang II versus Ang II+losartan, or Ang II+PD) were performed with Holm’s stepdown procedure. Data were considered statistically significant when P was <0.05.

**Results**

Both AT1-R and AT2-R Were Expressed in Human LV

Membrane fractions from all autopsy samples had high-affinity binding sites, with a Ki of 0.89±0.15 nM (Figure 1A and 1B). Competition experiments suggested the presence of 2 classes of binding sites (Figure 1C), and nonlinear least-squares regression analysis indicated the existence of 2 populations of Ang II binding sites, one with high (K1 = 7.2±0.2 nM) and the other with low (K2 = 0.14±0.03 μM) affinity for losartan. When CGP42112A was used for inhibition, K1 values of high- and low-affinity sites were 0.15±0.03 mM and 0.64±0.28 μM, respectively. The relative ratio of high- and low-affinity sites for CGP42112A was calculated as 59% and 41%, respectively, in the nonfailing heart group (n = 4, Table). The ratio of AT1-R/AT2-R by losartan inhibition was calculated as 57/43%.

To further confirm the accuracy of the assay method, we determined AT2-R numbers in LVs from 5 DCM patients (Table) using AT2-R selective ligand [125I]-CGP42112A and compared them with AT2-R numbers determined using [125I]-Sar1,Ile8 Ang II. AT2-R numbers determined using [125I]-CGP42112A were 9.7±1.4 fmol/mg protein (patient No. 9: 7.9, No. 10: 7.1, No. 11: 8.0, No. 12: 14.9, and No. 13: 10.7), similar to AT2-R numbers calculated by CGP42112A inhibition (Table), and the interassay variation in AT2-R numbers determined with the 2 different methods was <10% (8.6±1.4%).

Next we examined the purity of membrane fractions. The activity of the membrane marker ouabain-sensitive Na+,K+-ATPase was 15±0.86, 16±1.2, and 16±1.3 μmol/mg protein
per hour in autopsy samples from the nonfailing, OMI, and DCM hearts (patient No. 9 to No. 13), respectively. The activity in autopsy OMI samples was comparable with that in operation samples (176.6 ± 0.64). These values were similar to those reported in animal hearts, suggesting that the membranes contained an enriched cardiac membrane fraction.

Ventricular AT1-R Numbers Were Decreased in Both OMI and DCM, Whereas Ventricular AT2-R Numbers Were Upregulated in DCM

As shown in Figure 2 (top) and the Table, AT1-R numbers were significantly decreased about 63% in the OMI and DCM groups, respectively, compared with those in the nonfailing heart group (4.1 ± 0.2 fmol/mg protein), whereas AT2-R numbers in the AMI group were significantly increased about 54% relative to those in the nonfailing heart group. On the other hand, AT1-R numbers were markedly (293%) increased in the DCM group (11.4 ± 1.3 fmol/mg protein) relative to those in the nonfailing heart group (2.9 ± 0.2). There were no significant differences in AT2-R numbers between AMI (3.0 ± 0.3), OMI (3.9 ± 0.5), and nonfailing heart groups.

AT1-R and AT2-R Were Detected in Atrial Tissues, and Atrial AT1-R but not AT2-R Was Downregulated in Failing Hearts

We also measured AT1-R and AT2-R numbers in membrane fractions from right atria. As shown in Figure 3 (top), AT1-R numbers were significantly decreased about 43% and 51% in the OMI and DCM groups, respectively, compared with those in the nonfailing heart group (6.9 ± 0.4 fmol/mg protein), whereas AT1-R numbers in the AMI group were not significantly different from those in the nonfailing group. On the other hand, AT2-R numbers in the AMI, OMI, and DCM groups did not significantly differ from those in the nonfailing heart group (Figure 3, bottom).

Change in AT1-R Numbers Was due to Regulation in AT1-R mRNA Level

AT1-R mRNA levels in the AMI group were significantly increased by 113% compared with those in the control group (Figure 4). In the OMI and DCM groups, AT1-R mRNA levels were downregulated by 46% and 57% (P < 0.01), respectively, compatible with changes at protein levels. Similar regulation of AT1-R was also observed in operation and biopsy samples. GAPDH mRNA levels were similar between all study groups including nonfailing and ischemic hearts (Figure 4A).

Upregulation in AT2-R Numbers in DCM Hearts Was due to Increased AT2-R mRNA Accumulation

AT2-R mRNA levels in both autopsy and biopsy samples from DCM were increased by 213% and 177%, respectively, compared with the nonfailing heart group (Figure 5). This increase agreed with the change at the protein level (Figure 2, top). There was no significant difference in AT2-R mRNA levels between AMI, OMI, and nonfailing heart groups.

AT1-R–Mediated Inositol Phosphate Production Was Decreased in Cardiac Homogenate From Failing Heart Samples

AT1-R–mediated response of inositol phosphate production was measured using cardiac homogenates from LV tissue samples including both myocyte and nonmyocyte compartments in the presence of AT1-R antagonist PD123319 (Figure 6A). AT1-R–mediated response was markedly inhibited in OMI and DCM hearts, and this inhibition (P < 0.01) at 1 μmol/L Ang II was about 54% and 72% in autopsy samples from OMI and DCM, respectively, and 59% in operation samples from OMI. Although these experiments were performed in the presence of PD123319, the reduced response was observed to a similar extent even in the absence of...
PD123319, and pretreatment with losartan (10 μmol/L) completely blocked the inositol phosphate production by 1 μmol/L Ang II (data not shown). Thus, AT1-R–mediated inositol phosphate response of failing hearts was decreased in the tissue homogenate, including both myocyte and nonmyocyte compartments, in good agreement with the result of AT1-R downregulation in mRNA levels and membrane fractions prepared from tissue samples of failing hearts.

Ang II–induced MAPK Activity Was Attenuated in Cell Fraction Isolated From DCM Heart

Exposure of cell fraction isolated from nonfailing hearts to Ang II (1 μmol/L) induced a significant increase (94%, P<0.001) in MAPK activities compared with those in the untreated samples (Figure 6B). Next we tested the effects of pretreatment with losartan and PD123319 on the increase in MAPK activities induced by Ang II. Ang II–induced MAPK activities were almost completely abolished by pretreatment with 5 μmol/L of losartan (P<0.001), whereas pretreatment with PD123319 did not cause a significant change (Figure 6B). On the other hand, exposure of cell fraction from DCM hearts to Ang II (1 μmol/L) did not result in a significant increase in MAPK activities relative to those in the untreated samples (Figure 6C). We also tested the effects of pretreatment with losartan and PD123319 on the increase in MAPK activities induced by Ang II. Ang II–induced MAPK activities (274±10 pmol·min⁻¹·mg⁻¹) were further (36%, P<0.001) increased by PD123319 pretreatment (371±27 pmol·min⁻¹·mg⁻¹) whereas pretreatment with losartan significantly (24%, P<0.05) inhibited Ang II–induced MAPK activities (Figure 6C).

AT1-R and AT2-R Numbers and mRNA Levels in Autopsy Samples Were Similar to Those in Samples Obtained at Biopsy or Operation

The above-mentioned studies were mainly examined using autopsy samples within 1 hour after death. To test whether the length of time transpired before hearts were removed affected Ang II receptor mRNA and numbers, we measured its expression level using LV samples obtained during operation of aneurysmectomy from OMI patients (n=4) and biopsy specimens from DCM patients (n=5). As shown in Figure 2, total Ang II receptor numbers and the relative ratio of AT1-R/AT2-R in operation samples from OMI hearts were very similar to those in autopsy samples. AT1-R mRNA levels in operation and biopsy samples from OMI or DCM were comparably downregulated compared with those in the nonfailing heart group (Figure 4B), whereas AT2-R mRNA levels were markedly increased in biopsy samples from DCM (Figure 5B). AT1-R–mediated inositol phosphate production response was reduced to a similar extent between operation and autopsy OMI samples (Figure 6A). Taken together with
the findings that GAPDH mRNA levels were comparable between all study groups (Figure 4) and that RNA quality, judging from 28S/18S ratio of rRNA, was high in autopsy samples (data not shown), it was likely that the length of time transpired before autopsy hearts were removed had no significant influence on the distribution pattern of Ang II receptor subtypes.

Expression of Hypertrophy Marker and Extracellular Matrix Genes Was Markedly Increased in DCM Hearts

To examine the development of cardiac hypertrophy and interstitial fibrosis, ANP and BNP mRNA levels, as hypertrophy markers, and collagen type 1 and fibronectin mRNA levels, known as major extracellular matrix components, were quantified. ANP and BNP mRNA levels in OMI hearts were markedly increased compared with those in nonfailing hearts, and this level was comparable with the levels in DCM hearts (Figure 7). Collagen type 1 and fibronectin mRNA levels were also significantly increased in both OMI and DCM hearts, whereas the increase in DCM was more prominent than in OMI (Figure 7). Although in the AMI hearts ANP and BNP mRNA levels were moderately augmented compared with those in OMI and DCM, collagen type 1 and fibronectin mRNA levels did not significantly differ from those in nonfailing hearts. These findings suggest that the development of ventricular hypertrophy was similar between OMI and DCM hearts, whereas the degree of cardiac fibrosis estimated by deposition of extracellular matrix components was more remarkable in DCM hearts than in OMI hearts.

AT2-R Was Highly Localized in Fibrous Regions in DCM Hearts

To identify the cells expressing AT2-R in DCM hearts, cryostat LV sections were incubated with [125I]-[Sar1,Ile8] Ang II, followed by exposure to photographic emulsion. Regions with interstitial fibrosis were distinguished from the surrounding myocardium (indicated by arrows in Figure 8 No. 1) by staining with hematoxylin and eosin. Tissue distributions of Ang II receptors were evaluated by the numbers of silver grains overlying the myocardium. As shown in Figure 8 No. 2, Ang II binding sites were abundant in the regions with interstitial fibrosis with fewer binding sites in the surrounding myocardium. Competition with PD123319 strongly inhibited Ang II binding in fibrous regions (Figure 8 No. 3), whereas losartan did not appreciably affect these binding sites (Figure 8 No. 4), indicating that fibrous regions are the major site for AT2-R expression. Nonspecific
bindings in both fibrous regions and surrounding myocardium (Figure 8 No. 5) were fewer than the binding sites in the presence of PD123319 (Figure 8 No. 3), suggesting that AT1-R is present in both fibroblasts and myocytes at a very low expression level. In contrast, there was no obvious inhibition in Ang II binding by pretreatment with losartan (Figure 8 No. 4) compared with the binding in the absence of competitors (Figure 8 No. 2). As the binding affinity suggested by $K_i$ value is about 10-fold higher in PD123319 than in losartan,23 such difference in Ang II binding might be involved in a lack of obvious inhibition by losartan. Although AT2-R sites were highly localized in fibrous regions and its expression level in myocardium was much lower (Figure 8 No. 4), nonspecific binding sites in myocardium (Figure 8 No. 5) were obviously fewer than those

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**Figure 4.** RNase protection analyses of AT1-R mRNA levels in LV from autopsy, biopsy, and operation samples. A, Total RNA (40 μg) was hybridized with AT1-R RNA probes followed by digestion with RNases, resulting in a 210-bp protected signal for AT1-R mRNA. GAPDH mRNA as an internal RNA control quantified by Northern blotting (10 μg of total RNA) was also shown. Exposure times of AT1-R and GAPDH mRNA signals were 20 days and 2 days, respectively. B, Densities of AT1-R mRNA signals measured by densitometry were normalized relative to those of GAPDH mRNA signals. The relative value of a non-failing heart patient was arbitrarily normalized to 1, and the relative values in other groups were shown as arbitrary units. OMI hearts obtained at autopsy ($n=5$) or during operation of aneurysmectomy ($n=4$) and DCM hearts obtained from autopsy ($n=5$, patient No. 9 to No. 13 in Table) or biopsy ($n=5$) were examined. Nonfailing ($n=4$) and AMI ($n=3$) hearts were obtained from autopsy samples (Table). Actual P value resulting from 1-way ANOVA test was $P<0.0001$. *$P<0.001$ vs nonfailing heart group by Dunnett’s multiple-comparison test.

**Figure 5.** RNase protection analyses of AT2-R mRNA levels in LV from autopsy, biopsy, and operation samples. A, Total RNA (40 μg) was hybridized with AT2-R RNA probes followed by digestion with RNases, resulting in a 90-bp protected signal for AT2-R mRNA. Exposure time was 20 days. B, AT2-R mRNA levels were quantified as described in Figure 4. LV sample profiles and numbers were same as in Figure 4. Actual P value resulting from the 1-way ANOVA test was $P<0.0001$. *$P<0.001$ vs nonfailing heart group by Dunnett’s multiple-comparison test.
in the presence of losartan (Figure 8 No. 4), suggesting that AT2-R is also expressed in cardiac myocytes at a much lower level.

We also examined the distribution of Ang II receptor subtypes in the LV tissue from the nonfailing heart (Figure 9 No. 1). Ang II binding sites were present more numerously in the interstitial region than in the myocardium (Figure 9 No. 2). Interestingly, Ang II binding in the interstitial region was nearly abolished by pretreatment with PD123319 (Figure 9 No. 3), and losartan pretreatment inhibited the Ang II binding to a lesser extent (Figure 9 No. 4), suggesting that AT2-R is the predominant subtype present in fibroblasts in the normal human heart. On the other hand, Ang II binding in the myocardium was predominantly inhibited by losartan pretreatment (Figure 9 No. 3). Considering that the Ang II binding in the myocardium in the failing state was less affected by pretreatment with losartan (Figure 8 No. 4), these findings suggest that the AT2-R is chiefly expressed in myocardium rather than in interstitial regions, and in the heart failure state, its expression level is downregulated.

The presence of fibrous regions in myocardium was clearly shown by hematoxylin-eosin staining (indicated by arrows in Figure 8 No. 1). To further identify the cell types, we stained LV sections using antibodies against vimentin (for fibroblasts), desmin (for myocytes), smooth muscle α-actin (for vascular smooth muscle cells), or von Willebrand factor (for endothelial cells). Cells present in fibrous regions were positive for vimentin but were negative for other cell markers, whereas cells surrounding this region were positive for desmin (data not shown), demonstrating that the majority of the cells present in fibrous regions are fibroblasts.
The new findings of the present human study were that (1) AT₁-R expression was increased in human hearts with prominent deposition of extracellular matrix components, and fibroblasts present in interstitial regions were the major cell type responsible for AT₁-R expression. (2) AT₁-R reexpressed in the fibroblasts exerted an inhibitory effect on Ang II–induced mitogen signals, (3) the expression of AT₁-R in atrial and LV tissues was downregulated during chronic heart failure, and biochemical responsiveness assessed by Ang II–induced inositol phosphate production was significantly attenuated in the failing hearts. We also showed upregulation of ventricular AT₁-R in hearts with AMI, consistent with the data previously demonstrated in animal studies.\textsuperscript{33–35}

The present human study was mainly carried out using autopsy samples obtained immediately after death. Ang II receptor levels and Na⁺,K⁺-ATPase activities in samples obtained at operation or biopsy were very similar to those in autopsy samples, and also RNA quality, judging from 28S/18S ratio of rRNA and GAPDH mRNA, was high in autopsy samples. Ang II–induced inositol phosphate production was decreased to a similar extent between autopsy and operation samples. This evidence established that the length of time transpired before hearts were removed from autopsy samples had no significant influence on the purity of plasma membrane or AT₁-R and AT₂-R numbers. Measurement of the plasma membrane marker ouabain-sensitive Na⁺,K⁺-ATPase activity indicated that the membrane was an enriched cardiac membrane fraction. The expression pattern of AT₁-R and AT₂-R observed using plasma membranes was compatible with that in combined membrane/light vesicle fractions, suggesting that the change in the expression of Ang II receptors cannot be explained by internalization of receptors. The result of autoradiography using samples that did not undergo the process of membrane preparation was also consistent with the data of binding assays. These findings demonstrate that the low level of AT₁-R and upregulation of AT₂-R in DCM hearts did not result from an inappropriate method for membrane preparation or the process of receptor internalization. AT₁-R and AT₂-R numbers did not differ between the patients treated or untreated with ACE inhibitors (Table). Given that production of Ang II is inhibited in the patients treated with ACE inhibitors and that there is no relationship between plasma Ang II levels and ventricular Ang II receptor numbers in cardiomyopathic hamsters,\textsuperscript{36} it is unlikely that plasma Ang II levels influence the expression pattern of cardiac Ang II receptors.

Total Ang II receptor numbers in LV were in the range of 3.8 to 17.3 fmol/mg protein (Table), similar to the results reported by the previous studies,\textsuperscript{13–16} but were lower than the receptor numbers (118, n=5) determined by de Gasparo et al.\textsuperscript{17,37,38} de Gasparo et al used combined fractions including plasma membranes and internalized receptors, whereas we and other studies\textsuperscript{13–16} measured Ang II receptor numbers using only membrane fractions. In fact, Regitz-Zagrosek et al.\textsuperscript{14} found that the B\textsubscript{max} values determined with combined fractions were increased compared with those using only membranes. Uraña et al\textsuperscript{13} reported that Ang II receptor numbers were not significantly different between normal and DCM hearts. They prepared membranes using frozen tissue samples from the middle portion of LVs. In contrast, we used the base of LVs, in which Ang II receptor numbers were higher than in the middle portion\textsuperscript{13} and prepared membranes without the freezing process of tissue samples that caused decreases in AT₁-R and AT₂-R numbers (see Materials and Methods). Thus, the discrepancy may have been due to methodological differences, such as dissimilarities in the purity of the membrane fractions, the portion of ventricles examined, or membrane preparation methods.

We found that cardiac fibroblasts present in the interstitial region are the major cell type expressing AT₁-R in LV tissues from either normal or failing heart, in good agreement with
localization of AT2-R in human atrial tissues or in LV of cardiomyopathic hamsters. Because of limited resolution of the autoradiographic technique and the homogeneous distribution of relatively low-density myocardial Ang II binding sites, the cellular resolution of AT1-R and AT2-R in the normal or failing myocardium remained unclear. Although the presence of the interstitial region might suggest that the control nonfailing heart examined in the present study is not perfectly the normal heart, the interstitial region was observed in all control hearts with normal heart function. Thus, it remains to be determined whether the dominant expression of AT2-R in the interstitial region in the control heart reflects the normal distribution pattern of Ang II receptor subtypes in fibroblasts present in the normal human myocardium or is partially modified by pathological changes associated with interstitial fibrosis. However, these findings imply that the expression level of cardiac AT2-R is likely determined by the extent of the region with interstitial fibrosis and also have the interesting pharmacological implications for the predicted actions of AT2-R. We reported using cardiac fibroblasts from myopathic hamster hearts that AT2-R had an inhibitory effect on AT1-R–mediated DNA and collagenous protein synthesis. In the present study, we also found that AT2-R significantly inhibited Ang II–induced MAPK activation in fibroblast compartment from the failing hearts. However, considering that pretreatment with PD123319 does not completely normalize the Ang II–induced MAPK activity, the downregulation of AT2-R in the fibroblast compartment from the failing hearts might be also partially involved in the decrease in Ang II–induced MAPK activity. Taken together with our previous data that AT2-R has an inhibitory effect on AT1-R–mediated DNA and collagenous protein synthesis in fibroblasts from myopathic hamster hearts, it is likely that AT2-R in cardiac fibroblasts at least partially has the ability to inhibit the progression of interstitial fibrosis in failing hearts by decreasing Ang II–induced proliferation of fibroblasts as well as extracellular matrix protein accumulation.

We reported that in rats after AMI, AT1-R expression is increased, in good agreement with the result in the present human study. Related studies using rats after AMI showed that upregulation of AT1-R is mainly due to myocyte hypertrophy. In the present study, ANP and BNP mRNA levels were increased in AMI hearts, supporting a positive relationship between hypertrophy and AT1-R expression. However, ANP and BNP mRNA levels were increased more in OMI and DCM hearts, which contrasted with downregulation of AT1-R in failing hearts. Thus, it appears that the expression of AT1-R is differentially regulated between acute and chronic heart failure, and additional studies will be required to identify this differential mechanism.

It was not defined in the present study whether downregulation of AT1-R in failing hearts occurred at the myocyte level or was due to relative dilution of the myocyte compartment by nonmuscle cells. Using hematoxylin and eosin staining, we found that in nonfailing hearts, interstitial fibrosis in myocardium was reduced and in OMI hearts, its extent tended to be slightly increased. However, in DCM hearts (although the
presence of replacement fibrosis was already confirmed by biopsy), greater fibrous regions were scattered in myocardium. Collagen type 1 and fibronectin mRNA levels, known as major extracellular matrix components and as a specific marker for fibroblast proliferation,31,32 were more markedly increased in DCM hearts than in OMI hearts (Figure 7). Although these data indicated that the extent of interstitial fibrosis was much greater in DCM hearts, AT1-R numbers were similarly downregulated in both OMI and DCM hearts (Figure 2). We used ANP and BNP as a specific marker for myocyte hypertrophy and found that both mRNA levels were markedly increased in OMI and DCM groups to a similar extent compared with those in nonfailing hearts, suggesting comparable hypertrophy of remaining viable myocytes in both groups. Thus, although in the present study we could not quantitatively define the ratio between myocytes and nonmyocytes by the histological data, these data using specific markers for fibroblast and myocyte strongly suggested that AT1-R downregulation was unlikely due to relative dilution of myocyte compartment by nonmuscle cells. Although previous studies reported downregulation of AT1-R in human failing hearts,15–18 the exact mechanism responsible for its regulation has not been clarified yet. Additional studies will be needed to determine whether AT1-R downregulation reflects a decrease in numbers of viable myocytes in failing hearts or is due to specific regulation of gene transcription, transcript stability, or a currently unidentified mechanism at myocyte level.

The present study demonstrated that AT1-R expression was decreased in failing hearts as observed in β-adrenergic receptor,40 and that Ang II–induced inositol phosphate response was also decreased in the compartment including both myocytes and nonmyocytes. These findings suggest that AT1-R–mediated effects leading to elevation of intracellular free calcium might conceivably result in sluggish calcium transients and hence reduced contractile force in patients with heart failure. Recently, we have reported that cardiac myocyte–specific overexpression mice of AT2-R shows attenuated response to Ang II–induced chronotropic action.5 Taken together with the inhibitory effect of AT2-R reexpressed in cardiac fibroblasts on cell proliferation and production of collagenous matrix proteins,22 an induction of AT2-R in failing human hearts likely has desirable effects on cardiac function or the remodeling process, and hence selective stimulation of AT2-R by treatment with AT1-R antagonists might result in cardiac protection as explained by an unexpected lower risk of mortality than the ACE inhibitor in older patients with heart failure.41

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Angiotensin II Type 2 Receptor Is Upregulated in Human Heart With Interstitial Fibrosis, and Cardiac Fibroblasts Are the Major Cell Type for Its Expression

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