Brief Definitive Communication

Rat Angiotensin II (Type 1A) Receptor mRNA Regulation and Subtype Expression in Myocardial Growth and Hypertrophy

Junichi Suzuki, Hiroaki Matsubara, Masaya Urakami, Mitsuo Inada

Two subtypes of angiotensin II (Ang II) receptors (AT₁ and AT₂) are distinguished by using the respective specific antagonists. In the present study, we report the regulation of cardiac AT₁, type A (AT₁A) receptor mRNA levels and the expression pattern of AT₁ and AT₂ receptors in the growth of the heart and the development and regression of cardiac hypertrophy. The ventricular AT₁A mRNA level and the density of Ang II receptors at the neonatal period were significantly increased (3.5-fold and 2.5-fold, respectively) and then downregulated with maturation. The cardiac hypertrophy established in spontaneously hypertensive rats or two-kidney one-clip renovascular hypertensive rats resulted in substantial increases in ventricular AT₁A mRNA levels (threefold) and Ang II receptor densities (twofold) as compared with those in respective control rats, whereas the receptor affinity was similar. The proportion of AT₁ and AT₂ subtypes in the specific Ang II binding in ventricular membranes prepared from normal adult rats was nearly equal. This proportion did not change significantly in the development of myocardial hypertrophy. The regression of cardiac hypertrophy by the normalization of elevated blood pressure completely reversed the increased levels of AT₁A mRNA and the receptor density to the control level. Thus, AT₁ and AT₂ receptors are present in rat ventricular myocardium, and their expression is developmentally regulated and upregulated in response to hypertrophic change. Ang II action exerted through the increased number of Ang II receptors may contribute to the growth of the heart and thus to the maintenance of established hypertrophy as one of the hormones involved in hypertrophy development. (Circulation Research 1993;73:439-447)

KEY WORDS • angiotensin II • hypertrophy • AT₁ receptors • AT₂ receptors

The hypertrophic action of angiotensin II (Ang II) could be mediated by circulating or locally produced hormone. Evidence demonstrating the presence of an endogenous renin-angiotensin system in the heart includes the demonstration of mRNAs for angiotensinogen and renin, angiotensin I (Ang I) converting enzyme, and Ang II receptors and the detection of Ang I and Ang II radioimmunoreactivities. Upregulation of left ventricular angiotensinogen and Ang I converting enzyme mRNAs has been described in association with pressure-overload cardiac hypertrophy, suggesting that a cardiac renin-angiotensin system may be activated in cardiac hypertrophy.

Recently, two benzylimidazole derivatives have been used to distinguish Ang II receptor subtypes in several tissues. DuP 753 and TCV-116 inhibit the Ang II type 1 (AT₁) receptor, which is the predominant subtype in the adrenal cortex, vasculature, kidney, and liver, whereas PD123319 and CGP42112A inhibit the Ang II type 2 (AT₂) receptor, which is present in the adrenal medulla or brain. The previous study has reported that both AT₁ and AT₂ subtypes are equally distributed over the myocardium. Ang II is one of a growing number of peptide hormones that have been implicated in the regulation of cellular growth and cardiocyte hypertrophy. Beilich et al have demonstrated that Ang II is required for growth of the newborn pig heart by the use of the AT₁ receptor antagonist. Although this accumulated evidence suggests an activated local renin-angiotensin system in the hypertrophied heart, little is known in terms of the expression pattern of rat cardiac AT₁ and AT₂ receptors, partly because of the paucity of expression in the rat heart. The successful cloning of rat, bovine, human, and mouse AT₁ receptors has made it possible to study the mechanism of gene expression in the heart at the molecular level. Unexpectedly, rat and mouse hearts have two AT₁ receptor subtypes, whereas human and bovine hearts seem to have only one subtype. These two AT₁ receptor subtypes in the rat have high homologous sequences and similar binding and functional characteristics. The AT₁ receptor that is predominantly expressed in the kidney is termed AT₁A and that which is predominantly expressed in the adrenal gland is termed AT₁B. The purpose of the present study was to investigate the regulation of cardiac AT₁A receptor mRNA levels and the expression pattern of AT₁ and AT₂ receptors in the growth of the heart as well as the development and regression of cardiac hypertrophy.

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Materials and Methods

Quantitative Reverse Transcription and Polymerase Chain Reaction Assay

First-strand cDNA was synthesized from total RNA isolated by guanidium isothiocyanate–cesium chloride centrifugation with a Gene Amp RNA polymerase chain reaction (PCR) kit from Perkin-Elmer Cetus Instruments, Norwalk, Conn, with random hexamers (2.5 μmol/L) as primers. The resultant single-strand cDNA was assayed for levels of specific cDNA by quantitative PCR. The following PCR oligonucleotide primers (0.5 μmol/L) were chosen to amplify the AT1\textsubscript{A} cDNA: sense from the 5′ noncoding region (5′-GAGTCCCTGGTCCACCGAT- CACCGATCAC) and antisense from the coding region (5′-GGATGACGCCCCACGTGAATCGACATC). To obtain deletion-mutated cRNA (AT1\textsubscript{A}\textsubscript{-}), the 1046-bp PCR product was blunt-ended to attach phosphorylated HindIII linkers (New England Biolabs, Beverly, Mass) and subcloned into the HindIII site of pbUescript II KS(−) (Stratagene Inc, La Jolla, Calif), which was designated pKS. The plasmid was cut by Msc I and self-ligated in order to obtain the insert that lacked Msc I–Msc I (288 bp). The deletion-mutated cRNAs were synthesized from T\textsubscript{4} RNA polymerase (Takara Shuzo, Kyoto, Japan) after being linearized with BamHI. Total RNA (1 μg) and the deletion-mutated cRNA (10 pg) were simultaneously mixed and assayed by the reverse transcription (RT)–PCR method. Denaturing, annealing, and polymerase reactions were performed 30 times at 94°C for 45 seconds, 60°C for 1 minute, and 72°C for 1 minute, respectively. The sizes of RT-PCR products for AT1\textsubscript{A} mRNA and ΔAT1\textsubscript{A} RNA were 1046 and 758 bp, respectively. Specificity of gene amplification was confirmed by correspondence of the size of PCR products to that predicted from the cDNA sequence and by the restriction digestion pattern. As an internal control for input RNA, the rat α-tubulin probe was used for Northern blot analysis, because it has been reported that rat α-tubulin mRNA levels in rat ventricles are unaffected in response to developmental or hypertrophic changes. RNA samples were run on 3% formaldehyde and 1% agarose gels in MOPS buffer (20 mmol/L MOPS and 1 mmol/L EDTA, pH 7.4). The RNA was transferred to nylon filters in 10× standard saline citrate (1× standard saline citrate contains 0.15 mol/L NaCl and 1.5 mmol/L sodium citrate, pH 7.0). The filters were baked at 80°C for 2 hours and prehybridized and hybridized to the 5′-end-labeled rat α-tubulin (Onogene Science, Inc, Manhasset, NY) with [γ-32P]ATP using T\textsubscript{4} polynucleotide kinase under high stringency.\textsuperscript{22,25} The autoradiographic signals were measured by a scanning densitometer.

To quantify the AT1\textsubscript{A} mRNAs, a trace amount (5 μCi) of [35P]dCTP was included in the PCR reaction mixture. The bands of interest were excised from the agarose gel and analyzed in a scintillation counter to measure 35P incorporation and thus the quantity of the PCR product. To control for the efficiency of PCR amplification against tube-to-tube variation and the variability in the amount of input cDNA, AT1\textsubscript{A} signals were normalized to both deletion-mutated cRNA signals (for PCR efficiency) and rat α-tubulin signals (as internal control) measured by the scanning densitometer; the 32P amounts incorporated in AT1\textsubscript{A} signals were normalized with those in RT-PCR bands of deletion-mutated cRNA and autoradiographic α-tubulin signals. In the experiments examining AT1\textsubscript{A} mRNA levels in the developmental stages and in cardiac hypertrophy, the value in an appropriate control was normalized to 1 arbitrary unit for quantitative comparison.

Analysis of Ventricular Atrial Natriuretic Factor mRNA

For quantitative analyses of atrial natriuretic factor (ANF) mRNA, RNA samples that had been diluted serially five times were dotted on nitrocellulose filters; 0.625 to 10 μg total ventricular RNA samples were applied to the filters. The filters were air-dried, baked at 80°C for 2 hours, and prehybridized and hybridized to the radiolabeled rat ANF complementary DNA probe (782 bp), as described previously.\textsuperscript{22} The autoradiographic signals were measured by a scanning densitometer and plotted as a function of the amount of dotted RNA. Dot blot hybridization was performed for three separate RNA samples obtained from each experimental group. The mean of the slopes in the linear regression of these plots was taken as a quantitative index of relative ANF mRNA.\textsuperscript{22}

Animals

Male spontaneously hypertensive rats (SHR) at ages of 17, 20, and 24 weeks (n=4 at each age) and age-matched male Wistar Kyoto (WKY) rats serving as genetically normotensive control strains (n=4 at each age) were used in the study. Two-kidney one-clipping (2K1C) renovascular hypertensive rats (RHR) were produced from male Wistar rats (195 to 200 g) by placing a silver clip (0.2 mm) on the left renal artery and leaving the contralateral kidney untouched. Sham-operated rats served as controls. Animals were housed in a temperature-, humidity-, and light-controlled room, and a standard rat diet plus water was provided ad libitum. Systolic blood pressure and heart rate were measured once a week using a tail-cuff method, and body weight was checked. When systolic blood pressure reached a level greater than 160 mm Hg within 2 weeks after clipping, the rats were regarded as RHR.

Reversal of Renovascular Hypertension

Six weeks after clipping, RHR were divided into four groups. We reduced the high blood pressure by two procedures. The two groups (n=4 each) were treated with the AT\textsubscript{1} antagonist TCV-116 (Takeda Chemical Industries, Ltd, Osaka, Japan) or the AT\textsubscript{1} antagonist PD132319 (Parke-Davis, Warner-Lambert Co, Ann Arbor, Mich). The drugs were given in distilled water by oral gavage (1 mg/kg for TCV-116, 2 mg/kg for PD132319) twice daily for 4 weeks. In the third group (n=4), the clipped kidney was removed under ether anesthesia (nephrectomy). The remaining group of RHR received neither drug treatment nor nephrectomy, and henceforth, this group is referred to as untreated RHR. They were examined at 6 weeks (n=4) and 10 weeks (n=4) after clipping. In addition, the sham-operated control rats were also examined 6 weeks (n=4) and 10 weeks (n=4) after the sham operation.

Binding Assay

Ventricular membranes from rats were prepared using a slight modification of a published method by Baker.
et al.26 The rats were killed by decapitation, the hearts removed immediately, and the ventricles were dissected free from atria, rinsed in ice-cold saline, frozen in liquid nitrogen, and stored at -80°C. A homogenate (20% [wt/vol]) was prepared in 0.25 mol/L sucrose and 25 mmol/L Tris, pH 7.5, containing 0.5 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 mg/mL bacitracin, 4 μg/mL leupeptin, 4 μg/mL pepstatin, and 40 U/mL trasylol with a Polytron (twice for 30 seconds each) at half-maximal speed. The homogenate was sedimented at 10 000g for 20 minutes (twice), and the supernatant was centrifuged at 45 000g for 30 minutes. The pellet was resuspended in 0.6 mol/L KCl and 30 mmol/L histidine at pH 7.0 containing 0.5 mmol/L EDTA, 0.5 mmol/L PMSF, 10 mg/mL bacitracin, 4 μg/mL leupeptin, 4 μg/mL pepstatin, and 40 U/mL trasylol and resedimented at 45 000g for 30 minutes. The pellets obtained from the final centrifugation were washed three times and resuspended in 25 mmol/L Tris, pH 7.5, containing 10 mmol/L MgCl₂, 0.5 mmol/L PMSF, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 40 U/mL trasylol, and 10 mg/L bacitracin using a hand-driver glass/glass homogenizer. All centrifugations were performed at 4°C. The membrane preparations were immediately frozen in liquid nitrogen and held in aliquots at -80°C until used. The yield was approximately 1 mg protein per gram of heart. The assay buffer was 25 mmol/L Tris, pH 7.5, containing 10 mmol/L MgCl₂, 2 g/L bovine serum albumin (BSA), 10 mg/L bacitracin, and the peptidase inhibitors antipain, phosphoramidon, leupeptin, pepstatin, bestatin, and amastatin, each at 1 μg/mL, and 0.5 mmol/L PMSF.27 The labeled ligand was [125I]Ang II (final concentration, 200 to 2000 pmol/mL) purchased from Amersham, England. The mixtures were incubated at 22°C for 60 minutes in assay buffer (150 μL) with 50 μg membrane protein determined by the Bio-Rad assay using BSA as a standard. The reaction was stopped by the addition of 5 mL of ice-cold 25 mmol/L Tris, pH 7.5, and the mixtures were immediately filtered through glass fiber filters presoaked with 1 g/L BSA by vacuum filtration. The filters were washed twice with 5 mL of 25 mmol/L Tris, pH 7.5, and the trapped radioactive measurement was measured in a gamma scintillation counter. Nonspecific binding (determined in the presence of 1 μmol/L Ang II) was subtracted from total binding. Binding to AT1 and AT2 receptors was estimated by subtracting the nonspecific binding from the maximum saturation binding with 5 nmol/L [125I]Ang II after preincubation with 10 μmol/L PD123319 or 10 μmol/L TCV-116 for 30 minutes at 22°C, respectively.27 The Scatchard equation bound/free=(Bmax/Kd)-(bound/Kd), where Bmax and Kd are maximal binding site density and affinity, respectively, was used to calculate Kd and Bmax.

Na⁺,K⁺-ATPase Activity and Cathepsin D Activity

Approximately 15 μg of protein from the membrane fraction was preincubated in 0.1 mL of the standard medium containing 50 mmol/L imidazole-HCl (pH 7.0), 100 mmol/L NaCl, 16 mmol/L KCl, 4 mmol/L MgCl₂, 1 mmol/L EDTA, and 100 μg/mL saponin 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. The incubation continued for 10 minutes, and the reaction was terminated by cooling and addition of 0.05 mL cold 35% trichloroacetic acid. The inorganic phosphate (P_i) released in the incubation medium was immediately measured according to the modification of the method of Parvin and Smith.28 The Na⁺,K⁺-ATPase activity corresponds to the difference between P_i released in the absence and presence of ouabain into the incubation medium. Cathepsin D activity was determined spectrophotometrically using the method of Anson.29

Reagents and Statistical Methods

All reagents were purchased from Sigma Chemical Co, St Louis, Mo, unless otherwise indicated below. Results are expressed as mean±SEM. Analysis of variance and the Newman-Keuls test were used for multigroup comparisons. Values of P<.05 were considered statistically significant.

Results

Developmental Changes in Ventricular AT₁₅ mRNA Levels

We have used a method for quantification of mRNA levels based on randomly primed RT-PCR as previously described,23 because of the low levels of AT₁₅ mRNA accumulations in the rat heart. To optimize the quantitative RT-PCR, the range of concentrations of sample RNA and internal control cRNA as well as the number of amplification cycles were chosen within the exponential phase (Fig 1, A), where the quantity of amplified product would be directly proportional to the quantity of starting target sequence.23 The linear relation in the ratio of sample RNA to internal control cRNA was maintained throughout the range we observed (Fig 1, B). To further prove the validity of this method, we have two kinds of RNA samples: one (sample A) is the total RNA isolated from adult rat ventricles, and the other (sample B) is composed of 50% Cos-7 cell total RNA that has no detectable AT₁₅ signal as assessed by our RT-PCR assay and 50% adult rat ventricle total RNA (same as that used in sample A). One microgram of sample A or sample B combined with 2.5, 5, 10, 20, or 50 pg of the deletion-mutated cRNA (ΔAT₁₅) was reverse-transcribed, and the resultant cDNA mixtures were amplified by PCR. The ratios of radioactivities of AT₁₅ signals to those of ΔAT₁₅ signals were plotted against the amount of ΔAT₁₅ combined with total RNA samples. The ratio of slope obtained from sample A to that from sample B is 1.9, which agrees well with the expected value of 2.0 (Fig 1, C). This indicates that twofold differences in the RNA concentrations can be discernible by our RT-PCR assay and that the concentration of the deletion-mutated cRNA in this range (2.5 to 50 pg) is optimized for the assay.

The previous study has shown that AT₁₅ mRNA levels are developmentally regulated in a tissue-specific manner; the steady-state level of AT₁₅ mRNA in the neonatal stage is repressed in the liver but augmented in the kidney compared with the level in adult rats.17 Our present study demonstrates that AT₁₅ mRNA levels in the heart are also regulated developmentally. As shown in Fig 2, A, the AT₁₅ mRNA levels in the ventricles are increased by up to 3.5-fold in the neonatal stage relative to levels in 4-week-old rats. The increased levels declined to the steady-state levels at 4 weeks and thereafter remained at the same level for up to 17 weeks. Over
Changes of AT1A mRNA Levels in Development and Regression of Ventricular Hypertrophy

The upregulation of the mRNA levels for angiotensinogen and Ang I converting enzyme (ACE) in cardiac hypertrophy suggests that the cardiac renin-angiotensin system is activated in such a pathological state. Since little is known about the alteration of Ang II receptors in hypertrophy, we attempted to study AT1A expression at the mRNA levels in the two distinct experimental hypertrophy models of SHR and 2K1C RHR. The Table shows the hemodynamic data of SHR and its genetic normotensive control, WKY rats. SHR are genetically hypertensive rat strains that have been studied widely and intensively in hypertension research. An apparent hypertrophy shown by the ratio of left ventricular and body weight developed at 20 weeks, which was proven by the increased amounts of ANF mRNA, generally accepted as one of the markers of ventricular hypertrophy.

As shown in Fig 3, A, an apparent increase in AT1A mRNA levels was seen in 20-week-old SHR, and the levels increased up to threefold in 24-week-old SHR, whereas the α-tubulin signals used as internal controls appeared to be unchanged in these hypertensive rats. Similar results were seen consistently in separate experimental rats (n=4 at each time point), and representative data normalized by α-tubulin and ΔAT1A were shown. To verify further the normalized arbitrary values, the RNA samples were diluted serially five times and reassayed with RT-PCR in the presence of [32P]dCTP. The AT1A signals were plotted as a function of the amount of diluted RNA, and the mean of the slopes (n=4 at each time point) in the linear regression of these plots was determined. The results showed that the increases in the slopes of SHR ventricular AT1A mRNAs were compatible with increases in normalized arbitrary slope values.

To establish the upregulation of AT1A expression in cardiac hypertrophy, we examined 2K1C RHR with more prominent ventricular hypertrophy. The clipped artery produces higher levels of plasma renin, aldosterone, and Ang II, resulting in severe renovascular hypertension–induced hypertrophy. The elevated level of blood pressure can be completely normalized by the removal of the clipped kidney or treatment by Ang I converting enzyme inhibitor, accompanied by the regression of cardiac hypertrophy.

The Table shows the hemodynamic data and cardiac ANF mRNA levels in sham-operated control rats, 2K1C RHR untreated for 6 or 10 weeks after clipping, and 2K1C RHR treated with nephrectomy, AT1 receptor antagonist TCV-116, or AT2 receptor antagonist PD123319 for 4 weeks after the 6-week clipping. The presence of cardiac hypertrophy is evident by the remarkably increased values of the ratio of left ventricular and body weight (twofold) and left ventricular ANF mRNA levels (eightfold). Nephrectomy and treatment with AT1 antagonist TCV-116 for 4 weeks completely normalized the ventricular/body weight ratio and the elevated ventricular ANF mRNA levels, although AT2 antagonist PD123319 did not affect them as well as the elevated blood pressure levels, suggesting that the hemodynamic effects of Ang II on renovascular hypertension are mediated exclusively by the AT1 receptor.

The Table, B, shows the expression patterns of AT1A mRNA in the development (6 and 10 weeks after clipping) and regression of cardiac hypertrophy. Similar to the results of SHR, AT1A mRNA levels were augmented by more than threefold compared with control levels. Either removal of the clipped kidney or treatment by the AT1 antagonist TCV-116 reversed these changes to the normal steady-state levels.
together with the regression of hypertrophy. Treatment with AT2 receptor antagonist PD123319 did not affect the magnitude of hypertrophy nor the increased levels of AT1A mRNA. To verify further the normalized arbitrary values, the RNA samples were diluted serially five times and reassayed with RT-PCR in the presence of [3H]dCTP. The AT1A signal was plotted as a function of the amount of diluted RNA, and the mean of the slopes (n=4 in each experiment) in the linear regression of these plots was determined. The results showed that the increases in the slopes of 2KIC RHR ventricular AT1A mRNAs were compatible with those for the normalized arbitrary values.

**Ang II Binding Assay**

Saturation binding of [125I]Ang II to the ventricular membranes prepared from adult (12-week-old) rats is depicted in Fig 4, A. The data demonstrate the presence of specific sites with high affinity but limited density for Ang II in the presence of protease inhibitors. Analysis of the data as shown in the Scatchard plot suggests the presence of a single class of receptors with Kd of 0.9±0.2 nmol/L and Bmax of 16±0.7 fmol/mg protein (n=4). The activity of the plasma membrane marker, ouabain-inhibited Na+,K+-ATPase, was 15±0.8 μmol/mg protein per hour in ventricular membrane preparations from adult (12-week-old) rats. The value was comparable to that reported by Baker et al29 or Jones et al30 and indicated that the membrane preparation was an enriched fraction of cardiac membrane. Cathepsin D activity showed that there was no protease activity against hemoglobin in the final 45,000g supernatant or pellet, suggesting that lysosomal disruption was not a problem in membrane isolation procedures.

To characterize two subtypes of Ang II receptors in ventricular membranes of adult (12-week-old) rats, competition binding experiments were carried out by using their respective antagonists (Fig 4, B). In the presence of TCV-116 (10 μmol/L), ie, with the putative AT1 receptor unblocked, the remaining binding sites have a high affinity for PD123319 (IC50, 6±0.2 nmol/L). In the presence of PD123319 (10 μmol/L), with the putative AT1 unmasked, TCV-116 has a relatively high affinity (IC50, 18±0.3 nmol/L). The two receptor populations have an almost identical high affinity for Ang II (1.5±0.2 and 1.2±0.2 nmol/L), in the presence of TCV-116 and PD123319, respectively; see Fig 4, B). Therefore, from inhibition experiments with these antagonists, the proportion of the receptor subtypes can be estimated. By use of TCV-116, 49±2% (n=4) of the Ang II binding sites in 12-week-old rat ventricles were not blocked and can therefore be classified as AT1 receptors, whereas 51±2% (n=4) were sensitive to this compound and can be assigned to the AT2 receptor (Fig 2, B). The similar proportion of receptor subtypes was obtained in the reverse experiments using PD123319 as a competitor. These receptor studies were used to examine the developmental and hypertrophic changes in Ang II receptor subtype expression.

As shown in Fig 2, B, the Ang II receptor densities estimated by Bmax (femtomoles per milligram protein) were higher (more than twofold, P<.01) in neonatal rat ventricles as compared with those in mature rat ventricle...
Changes of Blood Pressure and Left Ventricular Atrial Natriuretic Factor mRNA Levels in Cardiac Hypertrophy of Spontaneously Hypertensive Rats and Renovascular Hypertensive Rats

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<th>SHR Study</th>
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<td>17 weeks</td>
<td>20 weeks</td>
<td>24 weeks</td>
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<td>(n=4)</td>
<td>(n=4)</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>184±4*</td>
<td>140±2</td>
<td>185±3*</td>
<td>136±3</td>
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<td></td>
<td>140±2</td>
<td>185±3*</td>
<td>136±3</td>
<td>185±5*</td>
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<tr>
<td>LV wt/body wt (mg/g)</td>
<td>2.4±0.05</td>
<td>2.3±0.03</td>
<td>2.7±0.04*</td>
<td>2.3±0.03</td>
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<td>2.3±0.03</td>
<td>2.7±0.04*</td>
<td>2.3±0.03</td>
<td>2.8±0.03*</td>
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<tr>
<td>LV ANF mRNA (OD/µg total RNA)</td>
<td>580±13*</td>
<td>141±12</td>
<td>663±16*</td>
<td>138±11</td>
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SHR, spontaneously hypertensive rats; n, number of rats; WKY, normotensive Wistar-Kyoto rats; RHR, two-kidney one-clip renovascular hypertensive rats; SBP, systolic blood pressure; ANF, atrial natriuretic factor; OD, optical density. Values are mean±SEM.

*P<.01 vs age-matched control rats.
†P<.05 vs age-matched control rats.

Discussion

The hypertrophic action of Ang II could be mediated by circulating or locally produced hormone. Although the constituents for an intracardiac renin-angiotensin system, including the mRNAs for angiotensinogen,2,6 renin,2 Ang I converting enzyme,3 and Ang II receptors,4 are present in the heart, the physiologically relevant autocrine or paracrine functions in the heart have not been established yet. In the present study, we showed for the first time that AT₁A mRNA accumulations are induced more than threefold in hypertrophied ventricles relative to control ventricles. This induction was seen consistently in SHR as well as 2K1C RHR, and the regression of hypertrophy with the AT₁ antagonist normalized the increase at mRNA levels, indicating that these interesting phenomena are a specific and consistent feature of hypertrophied myocardium. In the initial part of the experiment examining the AT₁A mRNA changes in the 2K1C RHR heart, in which plasma Ang II levels are elevated for long durations, we were interested in Ang II receptor downregulation because AT₁A mRNA levels are transiently downregulated by Ang II in cultured rat glomerular mesangial cells31 or cultured neonatal rat cardiocytes (H. Matsubara, unpublished observation). The present results indicate that hypertrophy-dependent induction of AT₁A mRNA could occur even in such a condition and suggest that the increased number of the receptor may contribute to the development of prominent hypertrophy seen in renovascular hypertension.

The AT₁ receptor has two subtypes (AT₁A and AT₁B) with highly homologous sequences and similar binding and functional characteristics.17,18 Very recently, Iwai et al19 have reported that the proportion of AT₁A mRNA levels to AT₁B mRNA levels in the normotensive 16-week-old WKY ventricles is 1.2 and that the AT₁B mRNA levels are increased in 16-week-old SHR ventricles as compared with 16-week-old WKY ventricles, whereas AT₁A mRNA levels were almost the same between the two strains at this age. Although the proportion of AT₁A mRNA and AT₁B mRNA levels was not determined in the present study, the AT₁A mRNA levels in 17-week-old SHR and AT₁B mRNA levels in 17-week-old WKY rats were similar to those in the 17-week-old WKY rats and thereafter significantly increased in the older SHR compared with age-matched WKY rats (Fig 3, A). Since the ventricular hypertrophy estimated by left ventricle/body weight ratio has not developed by 17 weeks in SHR (Table), the AT₁B mRNA accumulation may respond to the potential hypertrophic change more rapidly than does the AT₁A mRNA. It may be suggested that the hypertrophic change in ventricle regulates the ventricular AT₁A and AT₁B genes concordantly along with its development, which results in the stimulated expression of their translational products evaluated as increased AT₁ receptor densities.

The results of our binding assay demonstrate that the densities of high-affinity Ang II receptors are increased in the hypertrophied ventricle. Analysis of binding data using the inhibitors TCV-116 and PD123319 suggested that two classes (AT₁ and AT₂) of Ang II receptors with high affinity for the ligands are present in the ventricle in a nearly equal proportion, consistent with previous observations in the rabbit27 and rat.13 It has been shown that the AT₂ receptor is highly expressed in the mesenchymal tissue of the developing rat fetus and rapidly disappears after birth33 and that during embryonic and...
RHR

6 weeks

133±3
2.1±0.03
145±13
10 weeks

201±5*
3.3±0.3*
972±18*

untreated RHR

Control (n=4)

RHR (n=4)

Control (n=4)

RHR (n=4)

TCV-116 (n=4)

PD123319 (n=4)

Nephrectomy (n=4)

136±4
2.3±0.05
152±11
130±3
2.1±0.1
138±14
211±5*
3.5±0.4*
1143±24*
200±7*
3.3±0.5*
1056±20*

1056±20*

131±3
2.2±0.1
144±16

4 weeks

138±14

1056±20*

131±3
2.2±0.1
144±16

immediate postnatal development rat aortic smooth muscle cells express AT₂ receptors almost exclusively. These findings could indicate that Ang II and AT₂ receptors are involved in the regulation of tissue growth and development. In adult rabbit cardiomyocytes, Ang II stimulates hypertrophy in the cardiomyocytes and expression of early growth response gene 1. In chick heart cells, Ang II increases RNA and protein content. In addition to these studies in isolated tissues, a role for Ang II in the development of cardiac hypertro-

FIG 3. Northern blots (A and B, left) and bar graphs (A and B, right; C and D) show reverse transcription–polymerase chain reaction (RT-PCR) analyses of ventricular AT₁ receptor type A (AT₁A) mRNA levels in the growth of spontaneously hypertensive rats (SHR) and the development and regression of cardiac hypertrophy in two-kidney one-clip (2K1C) renovascular hypertensive rats (RHR). One microgram of ventricular total RNA and 10 μg of deletion-mutated AT₁A cRNA (ΔAT₁A) were assayed. The RT-PCR products were loaded onto a 1% agarose gel. The same RNA samples (A, left, 10 μg; B, left, 20 μg) were analyzed with Northern blots using α-tubulin probe as an internal control, and autoradiographic signals (1-day exposure) are shown at the bottom. Amplified cDNAs were normalized with RT-PCR products of ΔAT₁A and autoradiographic α-tubulin signals as described in “Materials and Methods.” A, right. The value of a ventricular RNA sample in Wistar-Kyoto (WKY) rats at 17 weeks was normalized to 1 arbitrary unit. Values given are the mean±SEM of four separate rat samples each. *P<.01 vs values in age-matched WKY rats. B, right. RHR were untreated or treated with 6 or 10 weeks after clipping, and the antihypertensive treatments by the nephrectomy of clipped kidney, TCV-116, or PD123319 continued for 4 weeks after the 6-week clipping. The value of a ventricular RNA sample in 6-week sham-operated control (CTR) was normalized to 1 arbitrary unit. Values given are the mean±SEM of four separate rat samples each. *P<.01 vs values in age-matched sham-operated CTR. C and D, Binding studies for specific angiotensin II (A II) receptor densities in ventricular membrane preparations from SHR and RHR are shown. The proportion of A II receptor subtypes AT1 and AT2 was analyzed as in Fig 2. B, Values given are the mean±SEM of four separate rat ventricular samples each. *P<.01 and †P<.05 vs age-matched respective CTR.
A number of cell types in the heart, such as sympathetic nerve terminals, cardiomyocytes, and fibroblasts, have Ang II–specific binding sites. Schorb et al have demonstrated that rat cardiac fibroblasts have a single class of Ang II high-affinity binding sites (IC50, 1.1 ± 0.2 nM/mL; Bmax, 784 ± 133 fmol/mg protein). Ang II receptors localized exclusively in the cultured cardiocytes could not be correctly characterized because the complete separation of fibroblasts from cardiocytes rich cultured cells was not possible. Therefore, our present data concerning Ang II receptors measured in the rat heart should be interpreted with caution, because various cells in which Ang II receptors localize may potentially contribute to the mRNA and binding results. In situ autoradiographic binding study indicates that Ang II receptors are widely and uniformly present throughout the rat heart. Thus, it appears very difficult to provide evidence for cellular localization of Ang II receptors through in vivo or in vitro analyses. Further studies would be required to determine which cell types have a substantial increase in Ang II receptor expression in the hypertrophied heart.

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