Immunolocalization of Subtype 2 Angiotensin II (AT$_2$) Receptor Protein in Rat Heart

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Abstract—Angiotensin II exerts its effects on cardiovascular function and water and sodium homeostasis by interacting with plasma membrane receptors on target organs. The existence of subtype 2 angiotensin II (AT$_2$) receptors in the rat heart has been demonstrated by ligand binding and reverse transcription–polymerase chain reaction. In the present study, the expression and localization of AT$_2$ receptor protein in the rat heart was investigated using an antipeptide polyclonal antibody against the native rat AT$_2$ receptor by light microscopic immunocytochemistry and Western blot analysis. In frozen tissue sections, positive immunostaining was observed in the myocardium and coronary vessels throughout the ventricle and atrium of neonatal and young rat hearts. Coronary vessels of the neonatal heart were more intensely stained compared with the surrounding myocardium. Positive immunoreactivity in the coronary vessels of young rats was localized to vascular endothelium but not in the smooth muscle cells. Preadsorption controls were all negative. Western blot analysis showed that the AT$_2$ receptor protein (≈44 kDa) was detectable from the AT$_2$ receptor–transfected COS-7 cells and neonatal rat cardiac myocytes but not from fibroblasts or young rat aortic smooth muscle cells. The neonatal rat heart expressed significantly more AT$_2$ receptors than young rat heart. These data provide the first direct evidence for the expression and localization of AT$_2$ receptor protein in the rat heart. (Hypertension. 1998;32:78-83.)

Key Words: receptors, angiotensin II ■ heart ■ immunocytochemistry ■ myocardium ■ rats

Angiotensin II (Ang II) plays an important role in the maintenance of cardiovascular homeostasis by controlling vascular tone, sodium excretion, hormone secretion, and neuronal activity. Ang II exerts major influences on the heart via its effects on systemic hemodynamics and blood volume. Two pharmacologically distinct subclasses of Ang II receptor, types 1 and 2 (AT$_1$ and AT$_2$), have been identified based on their inhibition by the nonpeptide antagonists losartan (AT$_1$) and PD 123319 (AT$_2$).$^{1,2}$ The cDNAs of both receptor subtypes have been cloned and sequenced. Although they both have a seven-transmembrane domain structure typical of G protein–coupled receptors, AT$_1$ and AT$_2$ receptors have only 34% homology at the protein level and display different functional properties and signal transduction mechanisms.$^3$ The AT$_1$ receptor is localized to the brain, heart, kidney, peripheral vasculature, and adrenal glands.$^4$ In contrast, the AT$_2$ receptor is abundantly and widely expressed in fetal tissues but is present only at low levels in limited organs in adults, including the brain, adrenal glands, uterine myometrium, and atretic ovarian follicles.$^5-8$ Almost all of the known physiological effects of Ang II are mediated through the AT$_1$ receptor. The biological role associated with the AT$_2$ receptor remains to be established. Recently, the AT$_2$ receptor has been shown to be reexpressed/upregulated in experimental cardiac hypertrophy, myocardial infarction, and neointimal lesions after vascular injury and skin wounds.$^9-12$

The expression of cardiac AT$_1$ receptor mRNA and binding sites have been localized to cardiac myocytes, fibroblasts, endothelial cells, presynaptic neurons, and conducting tissue in the heart.$^{13-18}$ In contrast, studies localizing the AT$_2$ receptor in the heart are largely limited to ligand binding and autoradiography studies, which have shown low levels of expression.$^{14-18}$ One recent study using an in situ hybridization technique reported that AT$_2$ receptor mRNA was observed only in the coronary artery but not in the myocardium in the developing rat heart.$^{19}$ The regional distribution of this subtype receptor in the heart has not been fully explored. In the present study, we used a specific polyclonal rabbit antipeptide antibody, directed against a synthetic peptide that corresponds to the NH-terminal extracellular tail of the native rat AT$_2$ receptor cDNA, to determine AT$_2$ receptor protein expression and its localization in the neonatal (1 day after birth) and young (4-week-old) rat heart.

Methods

All experiments were conducted with the approval of the Animal Research Committee of the University of Virginia School of Medicine.

Characterization of AT$_2$ Receptor Antiserum

AT$_2$ receptor–specific polyclonal antibody was raised in rabbits against a synthetic peptide sequence derived from the NH-terminal
extracellular tail (MKDNFSFAATSRNITSS, amino acids 1 to 17) of the native rat AT$_2$ receptor.\textsuperscript{20} Antibody specificity has been documented previously\textsuperscript{20} by its ability to identify AT$_2$ receptor protein expressed in COS-7 cells stably transfected with a 2.9-kb full-length rat AT$_2$ receptor cDNA from PC12W cells (a rat pheochromocytoma cell line; a generous gift from Dr Inagami, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tenn). This rat AT$_2$ receptor cDNA encodes a protein with 363 amino acid residues corresponding to a theoretical molecular weight of 41 303.\textsuperscript{21,22} These transfected cells have previously been shown to exhibit a high level of AT$_2$ receptor expression, specific receptor-ligand binding, and functional features characteristic of the AT$_2$ receptor.\textsuperscript{21,23}

Light Microscopic Immunocytochemistry

Timed pregnant and 4-week-old (70 to 100 g body weight) female Sprague-Dawley rats were obtained (Hilltop Laboratory Animals, Scottsdale, Pa). Neonatal rats were subjected to study at the age of 1 day. Rats were deeply anesthetized with pentobarbital sodium (80 mg/kg body wt IP). After a perfusion fix with 1% paraformaldehyde, or without perfusion in neonatal rats, the hearts were excised and fixed with 1% paraformaldehyde in PBS for 2 hours. Tissues were then cryoprotected overnight at 4°C in 30% sucrose in PBS, and frozen sections (10 to 12 $\mu$m) were cut. Immunoperoxidase immunocytochemistry was performed as previously described.\textsuperscript{20,22} After endogenous peroxidase was quenched by 0.3% H$_2$O$_2$ in methanol, the sections were blocked using 3% normal goat serum, or without perfusion in neonatal rats, the coronary vessels were more developed. Tissues were then incubated overnight at 4°C with one of the following, diluted (1:500) in 1.5% normal goat serum and 0.5% nonfat dry milk in PBS: (1) AT$_2$ receptor primary antiserum (3.1 protein mg/mL) and (2) AT$_2$ receptor primary antiserum preadsorbed against its synthetic peptide antigen. For preadsorption, antiserum was incubated overnight at 4°C with a 10-fold molar excess of the pure peptide immunogen. After washes in PBS, the immunostaining was detected with an avidin-biotin immunoperoxidase reaction (Vectastain ABC kit, Vector Laboratory) and visualized by diaminobenzidine staining. Tissue sections were lightly counterstained with hematoxylin, dehydrated, and placed under coverslips.

Western Blot Analysis of AT$_2$ Receptor Protein

Western blot analysis was performed as previously described.\textsuperscript{20,23} AT$_2$ receptor–transfected or –nontransfected COS-7 cells, primary cultures of neonatal rat ventricular myocytes and fibroblasts\textsuperscript{24,25} (kindly provided by Dr K.M. Baker, Weis Center for Research, Danville, Pa), and young rat aortic smooth muscle cells\textsuperscript{26} (kindly provided by Dr A. Hassid, Department of Physiology and Biophysics, University of Tennessee School of Medicine, Memphis) were extracted with lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.02% sodium azide, 100 $\mu$g/mL PMSF, 1 $\mu$g/mL aprotinin, 1% NP-40) and then centrifuged. The supernatant was stored at −70°C until the time of the experiment. After rats were deeply anesthetized, tissues (heart, adrenal gland, and whole brain) were dissected, minced, and immediately homogenized with Polytron in Buffer A (10% glycerol, 20 mmol/L Tris-HCl, 100 mmol/L NaCl, 2 mmol/L PMSF, 2 mmol/L EDTA, 2 mmol/L EGTA, 10 mmol/L sodium orthovanadate, 10 $\mu$g/mL leupeptin, 10 $\mu$g/mL aprotinin). The homogenate was centrifuged. The resultant pellet was resuspended in Buffer B (Buffer A with 1% NP-40), stirred, and centrifuged again. The supernatant was stored at −70°C until analysis.

Aliquots of solubilized samples were separated by SDS–polyacrylamide gel electrophoresis (5% acrylamide stacking gel and 8% running gel). Proteins were transferred onto a nitrocellulose membrane (0.2 $\mu$m, Schleicher & Schuell) by semidry electrophoretic blotting (Trans Blot SD DNA, Bio-Rad). The nitrocellulose membrane was soaked in Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, 150 mmol/L NaCl) containing 5% nonfat powdered milk and 0.1% Tween 20 to block nonspecific sites and then incubated with the AT$_2$ receptor antiserum (3.1 mg/mL, 1:1000 dilution in TBS with 5% nonfat milk and 0.1% Tween 20). Blots were washed, incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:5000 dilution, Amersham). Immunoreactivity was visualized with an ECL Western blotting detection kit (Amersham).

Protein concentrations were determined by micro-bicinchoninic acid protein assay (Pierce). Quantitative assessment of band densities was performed by scanning densitometry (ImageQuant, Molecular Dynamics). Data were expressed as mean±SE and analyzed by Student’s paired $t$ test.

Results

In frozen sections, positive immunostaining for the AT$_2$ receptor was observed in the myocardium and coronary vessels throughout the ventricle and atrium of the neonatal (Figure 1A) and young (Figure 2A, 2C, and 2E) rat heart. In the neonatal heart, the coronary vessels were more...
intensely stained compared with the surrounding myocardium (Figure 1A). The positive immunoreactive signal in the intracardiac vessels comes from vascular endothelium but not smooth muscle cells (Figure 2E). Consecutive sections processed with the antibody preadsorbed against its peptide antigen at the same dilution as the anti-AT2 receptor serum did not produce significant staining (Figure 1B, Figure 2B and 2D).

On the immunoblot, the AT2 receptor protein (≈44 kDa) was detected from the AT2 receptor–transfected COS-7 cells and neonatal cardiac myocytes but not from neonatal cardiac fibroblasts or young rat aortic smooth muscle cells (Figure 3). This band was observed in membranes from the whole hearts of neonatal and young rats. Moreover, in all seven comparisons, the density of this specific band in the neonatal heart was significantly greater than in the young rat heart by semiquantitative analysis (Figure 4).

**Discussion**

We characterized the site-specific distribution of the AT2 receptor protein in the neonatal and young rat heart using a specific polyclonal antipeptide antibody. The specificity of the antiserum used was validated by (1) positive immunostaining of AT2 receptor–transfected COS-7 cells and absence of staining in preadsorption control and nontransfected COS-7 cells in our previous study20; (2) the presence of a single band of the appropriate molecular mass (≈44 kDa) in COS-7 cells stably transfected with the native AT2 receptor and its absence in nontransfected COS-7 cells or adult rat aortic smooth muscle cells, which
have been shown to express Ang II receptors of AT1 but not AT2 subtype\(^{11,27}\); and (3) the presence of the same single band in rat adrenal gland and brain,\(^{20}\) tissues known to have abundant expression of the AT2 receptor.\(^{6,28,29}\)

Therefore, these results confirm that the antipeptide serum, directed toward a completely conserved region of the AT2 receptor, can recognize in an effective and selective manner its appropriate peptide antigen in both transfected cells and experimental tissues.

Our results in the present study are different from those of Reagan et al.,\(^{30}\) who described two bands (110 and 66 kDa) recognized by a protein-directed polyclonal AT2 receptor antiserum against a partially purified membrane protein from murine neuroblastoma N1E-115 cells. It is not clear to which amino acid sequence of the cell membrane protein their antiserum was directed, whereas our antibody was raised against a specific peptide sequence of the cloned rat AT2 receptor. The different species and immunogen used by Reagan et al could have accounted for the finding of higher molecular mass bands of mouse AT2 receptor protein than the presently observed molecular weight of the native rat AT2 receptor. Interestingly, no immunospecific proteins were detected by their antiserum in rat adrenal gland and PC12W cells, as well as in COS-1 cells transfected with the mouse AT2 receptor cDNA from N1E-115 cells.\(^{31,32}\) This lack of immunoreaction with the recently cloned rat AT2 receptor was interpreted as being due to the possible heterogeneity within the AT2 receptor subtype.\(^{31-33}\)

During the late embryonic and early postnatal periods, cells in the myocardium undergo a transition from growth by an increase in cell number to growth by an increase in cell size, and rapid vascular growth and capillary formation also occur in the developing rat heart. Previous studies using autoradiography and in situ hybridization techniques showed that high concentrations of AT2 receptor mRNA and binding sites are present in the vasculature of the newborn rat heart.\(^{19,34}\) While the existence of AT2 receptor mRNA and binding sites has been demonstrated in neonatal rat cardiomyocytes,\(^{17,35}\) Ang II receptor binding sites found on the neonatal cardiac fibroblasts are mainly, if not exclusively, of the AT1 subtype.\(^{18,25,35,36}\) Consistently, our present study demonstrated that the neonatal cardiac myocytes but not fibroblasts express AT2 receptor protein. The AT2 receptor protein exists not only in the myocardium but also in coronary vessels. Expression in the cardiac vasculature was significantly higher than in myocardium, which provides support for the hypothesis that Ang II may act as an angiogenic factor in the developing rat heart.

Studies using in situ hybridization and Northern blot analysis failed to generate detectable AT2 receptor transcripts from adult rat hearts.\(^{19,21,37}\) Competitive reverse transcription–polymerase chain reaction, using total RNA prepared from adult rat hearts, revealed a detectable transcript for the AT2 receptor gene.\(^{10}\) Quantitative autoradiography and radioligand binding studies both indicated that low levels of cardiac AT1 and AT2 receptor subtypes exist in essentially equal proportions in the adult rat heart,\(^{9,14,15}\) although 90% AT2 binding sites in the rat heart also were reported.\(^{16}\) Our results show that AT2 receptor protein in young rat heart was detectable in coronary vessels and myocardium but at a relatively lower level compared with neonatal heart. The positive immunoreactivity found in the coronary endothelial layer is in good agreement with results obtained from cultured coronary endothelial cells.\(^{38,39}\) The present immunoblotting results from vascular smooth muscle cells of the rat aorta confirmed that these cells do not normally express AT2 receptor, as shown by our preliminary immunocytochemical study.\(^{40}\) Further studies at the electron microscopic level would help to elucidate the exact cell type(s) with expression of AT2 receptor in the heart.

The role of cardiac AT2 receptors in control of heart function remains to be clarified. In isolated ischemic rat hearts, the cardioprotective effect of AT1 receptor blockade may be mediated in part by endogenously released Ang II via AT2 receptor stimulation.\(^{39}\) The regression of hyper-
tension-induced cardiac hypertrophy by AT₁ receptor antagonists may be due in part to an unopposed antigrrowth effect of Ang II mediated via the AT₂ receptor. Chronic AT₂ receptor antagonism blocked the improvement of cardiac function and regression of ventricular remodeling induced by AT₁ receptor antagonism in rats with chronic heart failure, whereas acute cardiac AT₂ receptor antagonism was shown to produce enhanced recovery from mechanical dysfunction after ischemia/reperfusion in isolated working rat hearts. Identification of the cardiac AT₂ receptor protein will help future exploration of the possible role of the AT₂ receptor in Ang II–mediated cardiovascular effects.

In summary, using a specific antipeptide polyclonal antibody directed toward the rat AT₂ receptor, we detected AT₂ receptor protein in the cardiac myocyte and coronary endothelium and provided evidence for the expression of AT₂ receptor protein in the coronary vessels and myocardium at a higher level in neonate than in young rats. These results support the hypothesis that expression of the cardiac AT₂ receptor protein is developmentally regulated and could therefore play a role in early cardiac growth and development.

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References


42. Ford WR, Clanachan AS, Jugdutt BI. Opposite effects of angiotensin AT\textsubscript{1} and AT\textsubscript{2} receptor antagonists on recovery of mechanical function after ischemia-reperfusion in isolated working rat hearts. Circulation. 1996;94:3087–3089.