The Effects of Angiotensin II and Specific Angiotensin Receptor Blockers on Embryonic Cardiac Development and Looping Patterns


Department of Developmental Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina 29208

The role of angiotensin II (Ang II) in the early embryonic development of the heart has not been examined. We have used RT-PCR to identify mRNA for angiotensinogen, angiotensin-converting enzyme, and the Ang II AT1 and AT2 receptors in embryonic day 10.25 Sprague-Dawley rats, and have used confocal microscopy to localize the AT1 receptor to the greater curvature of the developing ventricle in these animals at embryonic days (ED) 9.25 and 10.25. The antibodies used in immunolocalization studies did not distinguish between the AT1a and AT1b receptor subtypes. In whole embryo culture, Ang II added to the culture media resulted in increased ventricular growth and myocyte hypertrophy when treated embryos were compared to cultured littermate controls. Use of Losartan and PD123,319 to block the Ang II AT1 and AT2 receptors resulted in reduced ventricular development and cardiac dilation when compared to control and Ang II-treated embryos. Addition of Ang II and PD123,319 to the culture media also resulted in cardiac loop inversions which may be associated with disruption of normal myofibrillar development. These results clearly indicate an important role for Ang II in the early embryonic development of the heart.

INTRODUCTION

The components of the renin-angiotensin system and their roles in adult cardiac hypertrophy have been well documented and reviewed (Baker et al., 1992; Dzau, 1995; Holtz, 1993; Katz, 1994; Lorell, 1995). In adult hearts, increased hemodynamic load results in increased levels of angiotensin II (Ang II) that stimulates significant hypertrophy and remodeling of cardiac structure via increases in intracellular protein levels and interstitial collagens. These remodeling effects of Ang II are receptor mediated and two primary types of receptors, AT1 and AT2, have been identified (Sechi et al., 1992, 1993; Shanmugam et al., 1994a,b). Currently, the majority of known effects of Ang II in the heart are mediated via subtypes of the AT1 receptor (Guthrie, 1995; Kohout and Rogers, 1995).

Recent evidence from in vivo studies indicates that Ang II also acts as a growth factor and has a potential role in late fetal and neonatal development of the heart. Beinlich et al. (1993, 1995) found that Ang II is required for the normal neonatal cardiac development of pigs by blocking the effects of angiotensin-converting enzyme and the Ang II AT1 receptor. In these studies, neonatal cardiac growth was inhibited by blocking the function of Ang II. Other in vivo evidence for the role of Ang II as a growth factor in intact fetal and neonatal hearts is primarily circumstantial. These studies have shown changes in the temporal and spatial localization of Ang II receptors or receptor mRNA in various regions of the developing rat heart as embryos increase in age from ED 11 (Shanmugam et al., 1994a) through ED 19 (Sechi et al., 1992, 1993; Shanmugam et al., 1994a,b) and up to 10 days after birth (Sechi et al., 1992, 1993; Shanmugam et al., 1994a; Suzuki et al., 1993). No information is available concerning the role of Ang II as a growth factor in the early embryonic development of the rat heart prior to ED 11.

Data from experimentation with isolated myocytes and fibroblasts from late fetal and early neonatal hearts also support the role of Ang II as a growth factor. Results from studies pertinent to this report indicate that: (1) Ang II stimulates the hypertrophy of myocytes (Aceto and Baker, 1990; Baker and Aceto, 1990; Sadoshima et al., 1993); (2) mechanical stress causes the release of Ang II directly from cardiac myocytes which acts as an initial mediator of the stretch induced hypertrophic response of myocytes (Sadoshima et al., 1993); (3) both the AT1 and AT2 AngII receptor...
subtypes are present in the heart and are developmentally regulated (Sechi et al., 1992, 1993; Sadoshima and Izumo, 1993a; Suzuki et al., 1993; Kojima et al., 1994; Yamazaki et al., 1995); and (4) activation of the AT1 Ang II receptor subtype mediates hypertrophy of cardiac myocytes via activation of the immediate-early genes c-fos and c-myc, and the late response genes for actin and myosin heavy chains (Yamazaki et al., 1993; Sadoshima and Izumo, 1993b; Sadoshima et al., 1995).

Given the above evidence that Ang II is a potential growth factor that may affect cardiac development, we initiated studies to determine the effects of Ang II on early heart development in the rat embryo. Specifically, we have: (1) examined the in vivo expression and distribution of the Ang II AT1 receptor subtype from embryonic days (ED) 9.25 and 10.25; (2) utilized the whole-embryo culture system to determine the effects of exogenous Ang II on the rate of myocyte differentiation and heart development; and (3) used Losartan and PD123,319, which are specific blockers of the AT1 and AT2 receptor subtypes, respectively, to determine their effects on cardiac development in whole-embryo culture.

MATERIALS AND METHODS

Reagents

Unless noted, reagents for whole-embryo culture, immunocytochemistry, and RT–PCR analyses were purchased from Sigma Chemical Co. (St. Louis, MO) and those for electron microscopy were from Polysciences, Inc., (Warrington, PA).

Animals

Time-pregnant Sprague-Dawley rats were obtained from Harlan Laboratory (Indianapolis, IN). Day 0 of pregnancy was considered midnight of the overnight breeding period. Animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care with food and water ad libitum. All experimental protocols were approved by the University Institutional Animal Care and Use Committee.

RT–PCR

To determine at what developmental stage Ang II may be expressed and play a role in cardiac myocyte development, we performed RT–PCR analyses for angiotensinogen, angiotensin-converting enzyme, and the AT1 and AT2 receptors using isolated hearts from embryos that had developed for 10.25 days in vivo. Angiotensinogen, the precursor molecule for Ang II, was used to determine the potential for the presence of Ang II. Since Ang II is a product of the renin–angiotensinogen cascade, mRNA for Ang II does not exist. Attempts to isolate cardiac tissue for RT–PCR at earlier time points in development resulted in the inclusion of some noncardiac tissue.

After the appropriate gestational period, hearts were carefully removed from 10 to 15 embryos and pooled. Total RNA was isolated from the pooled hearts by the one-step guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RT–PCR was performed with the Genamp RNA PCR kit (Perkin-Elmer, Norwalk, CT) using 2 μg of total RNA per reaction. Briefly, Moloney murine leukemia virus RT and random primers were used for cDNA synthesis from RNA. The cDNA was amplified with AmpliTaq DNA polymerase and primers for the rat aortic vascular smooth muscle Ang II type 1 receptor. Specific cDNA sequences used are shown in Table 1. Primer Detective software (Clontech, La Jolla, CA) was used to select the Ang II primers. These primers were synthesized by the Oligonucleotide Synthesis Facility at the University of South Carolina (Institute for Biological Research Technology, Columbia, SC). Primers for β-actin (Clontech) were used to test the integrity of the RNA. Thermal cycling was carried out for 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 30 s. Amplification of the appropriate DNA was verified by separation of RT–PCR products on 2% agarose gels along with PCR DNA markers (Promega Corp., Madison, WI) for size reference. Gels were stained with 10 μg/ml ethidium bromide and photographed against UV light with a Polaroid camera. Controls were included that omitted the reverse transcriptase from the RT reactions to ensure that RNA and not contaminating DNA was amplified. RT–PCR in the absence of reverse transcriptase resulted in no amplification of contaminating DNA with any of the primers used in these studies. Treatment of RNA samples with RNase-free DNase followed by RT–PCR resulted in the amplification of the appropriate products.

Whole Embryo Culture

Methods for whole embryo culture have been published previously (Price et al., 1992; Nakagawa et al., 1997) and are similar to those originally described by New (1978) and modified by Sadler et al. (1982). Age and developmentally matched embryos selected for culture were transferred with intact visceral yolk sacs into culture bottles containing 4.0 ml of rat serum and 1.0 ml of Tyrode’s solution (0.137 M NaCl, 2.7 mM KCl, 3.6 mM NaH2PO4H2O, 27 mM d-glucose, 3.6 mM NaHCO3, 1.36 M CaCl2, 0.5 mM MgCl2, and 0.13 mM streptomycin). For each mother, 2–3 embryos were designated for a treatment group. Ang II was added to the culture media at concentrations of 500 pg/ml, 10 ng/ml, or 100 μg/ml, and PD123,319 was added at concentrations of 1, 10, or 100 μg/ml, and PD123,319 was added at 3, 30, or 300 nM. Control cultures contained no Ang II, Losartan, or PD123,319. Following 24 h in culture, the culture medium was replaced with fresh medium containing Ang II, Losartan, or PD123,319 as described above. After designated times in culture, embryos were removed, scored by Brown and Fabro’s (1981) morphological scoring system, and prepared for confocal and transmission electron microscopy as described below.

Confocal Scanning Laser and Transmission Electron Microscopy

Embryos allowed to develop in vivo and in culture were rinsed in Tyrode’s solution with 50 mM KCl to relax the heart, preserved in 2% paraformaldehyde and stained for confocal microscopy (Price et al., 1996). To visualize general heart morphology, selected embryos (ED 9.25–10) were stained in a 1:20 dilution of rhodamine phalloidin (Molecular Probes, Eugene, OR) in phosphate-buffered saline plus 0.02% sodium azide (PBS–A) overnight at 4°C. Rhodamine phalloidin is a specific stain for F-actin, a primary component of the developing cardiac contractile apparatus. To facilitate pene-
tation of rhodamine phalloidin and imaging of larger hearts, embryos older than ED 10 were encased in 13% polyacrylamide gel using a modification of the procedure described by Hansen and Dryer (1981). After fixation, embryos were rinsed in PBS-A and placed in polyacrylamide gel that was immediately polymerized with 2% ammonium persulfate. Polyacrylamide gel encased embryos were sectioned at 200 μm with an Oxford vibratome and sections were stained with rhodamine phalloidin.

For labeling of Ang II AT1 receptors, either whole embryos or acrylamide sections of embryos were incubated in a 1:20 dilution of a polyclonal antibody to the AT, receptors (Chemicon International Inc., Temecula, CA) for 24 h at 4°C, rinsed in PBS-A, and stained overnight at 4°C in a 1:50 dilution of FITC. For double labeling, embryos were first labeled for the AT1 receptors followed by rhodamine phalloidin. Controls consisted of either nonimmune IgG or PBS buffer substituted for the primary antibody. Antibodies for the detection of AT2 receptors are not available. All embryos and sections were examined using a Bio-Rad MRC 1000 CSLM.

For TEM, embryos were rinsed in Tyrode's solution with 50 mM KCl and processed as previously described (Price et al., 1996).

**Protein Accumulation**

To determine the effects of angiotensin, Losartan, and PD123,319 on embryos older than ED 10 were cultured for 48 h in the presence of 10 μCi/ml of [35S]methionine. Following culture, embryos were rinsed in ice-cold Moscona's saline (136.8 mM NaCl, 28.6 mM KCl, 11.9 mM NaHCO3, 9.4 mM glucose, 0.08 mM NaH2PO4, pH 7.4) and the hearts were removed and transferred to an Eppendorf tube containing ice-cold 10% trichloracetic acid (TCA). Hearts were centrifuged at 4°C until 15,000 rpm, rinsed in 10% TCA, and stained overnight in 4°C in a 1:50 dilution of FITC. For double labeling, embryos were first labeled for the AT1 receptors followed by rhodamine phalloidin. Controls consisted of either nonimmune IgG or PBS buffer substituted for the primary antibody. Antibodies for the detection of AT2 receptors are not available. All embryos and sections were examined using a Bio-Rad MRC 1000 CSLM.

For TEM, embryos were rinsed in Tyrode's solution with 50 mM KCl and processed as previously described (Price et al., 1996).

**TABLE 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
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<tbody>
<tr>
<td>Angiotensinogen</td>
<td>5'-TCTCTGGACTTATCCACTGACC-3'</td>
<td>nt 875-896</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-TCACGAGAAAGTTGTTCTGGC-3'</td>
<td>nt 1215-1236</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>5'-AACCCATCTCTGCTAGACATG-3'</td>
<td>nt 66-87</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-AGATGTTGAGCAAGTCTGTG-3'</td>
<td>nt 498-519</td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>5'-TTCCGAGATGTCAAGAACC-3'</td>
<td>nt 680-697</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GCTAGGCTGATTACATGC-3'</td>
<td>nt 1247-1264</td>
</tr>
<tr>
<td>Actin</td>
<td>5'-CATTTTCAATCTGGCTGTG-3'</td>
<td>nt 3296-3313</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GGTGGACTCTTTATACG-3'</td>
<td>nt 3647-3666</td>
</tr>
</tbody>
</table>

on the SDS gels in relation to molecular weight standards. The relative amounts of MHC and actin in the various treatment groups are expressed as a percentage of that present in untreated control embryos.

**Morphometry and Statistical Analyses**

For comparative measurements of treated and control animals, embryos were photographed at 15× on an Olympus dissecting microscope. Photographic negatives were projected via a Dage MTI CCD72 camera to an Image 1 system (Universal Imaging, West Chester, PA) and measurements of ventricular length and width were obtained from the projected image. Ventricular length was determined as the distance from the apex to the base of the ventricle and ventricular width was measured as the distance across the plane of the ventricle with the greatest diameter (Nakagawa et al., 1997).

To determine changes in cardiac growth associated with a specific treatment, measurements of ventricular length and width for the control embryos of a litter were made and an average value for each parameter was obtained. The average value from the control embryos was then used to compare variations in cardiac growth of the treated embryos from the same litter. Each treatment group consisted of embryos from at least three mothers and a minimum sample size of six embryos per group. Changes in cardiac growth, expressed as a percentage of the control, are presented both as raw, or actual increases in growth, and as normalized data against the overall increase in embryo crown–rump length (Nakagawa et al., 1997). Data from all whole embryo culture runs were then pooled and t tests were performed to determine differences in the means of the control and treated populations.

To compare the proportions of the control and treated populations in which cardiac inversions occurred, the method of Sokal and Rohlf (1969) to test the equality of population percentages was used.

**RESULTS**

**In Vivo Expression and Localization of the AT1 Receptor and Angiotensinogen mRNA**

Using RT-PCR, mRNA for angiotensinogen, angiotensin-converting enzyme, and the AT1 and AT2 receptor sub-
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FIG. 1. RT-PCR products showing the expression of mRNA for β-actin used as a control and the AT1 and AT2 receptors, angiotensinogen, and angiotensin-converting enzyme at in vivo embryonic day 10.25. The expression of these mRNAs from isolated hearts identifies the potential for an active angiotensin system in the early embryonic heart.

Effects of Ang II, Losartan, and PD 123,319 on Ventricular Length and Width in Whole Embryo Culture

The addition of Ang II to the culture media of embryos isolated at ED 9.25 and cultured for 48 h significantly increased both the ventricular length and width of treated hearts at the three concentrations of Ang II used. At ED 10.25 plus 48 h in culture, the actual increases in cardiac growth were not as pronounced and were significant only at the higher concentrations of Ang II that were added to the culture media (Figs. 3A and 3B). The decrease in the observed effect of Ang II on cardiac growth as the embryos increased in age and size may be a result of reduced quantities of Ang II reaching the heart via diffusion through the body wall, and the potential for an increased number of available Ang II receptors in the body wall and other organs binding Ang II before it diffuses to the cardiac region.

To test if increases in heart growth were simply in response to increases in overall body size and development, cardiac growth was normalized against an overall increase in body size (crown–rump length) in response to Ang II treatment. The majority of data points tested remained significant at the P < 0.05 test level (Figs. 3A and 3B). This indicates that cardiac growth in response to the addition of Ang II to the culture media increases at a greater rate than overall growth of the embryo.

Since the effects of Ang II on cardiac growth in whole embryo culture were not as pronounced in the cultures of ED 10.25 animals, receptor blocking experiments were carried out only on ED 9.25 animals maintained in culture for 48 h. Experiments with Losartan, a specific blocker for the AT1 receptor, again resulted in significantly larger hearts at the three concentrations tested (Figs. 4A and 4B). Blocking of the AT2 receptors with PD 123,319 also resulted in larger hearts at the highest concentration (300 nM) used (Figs. 4A and 4B). Combining the highest concentrations of Losartan (100 μg/ml) and PD 123,319 (300 nM) also resulted in a significant increase heart size.

Morphological Assessment of the Effects of Ang II, Losartan, and PD 123,319 on Cardiac Development in Whole Embryo Culture

Myocardial development provides a potential explanation for the apparent paradox of increased heart size in response to both the increasing concentrations of available Ang II and the blocking of the Ang II receptors. Figures 5A–5D show rhodamine phalloidin-stained confocal images taken from a control embryo (A) and embryos treated with 10 μg/ml of Ang II (B), 100 μM Losartan (C), or 300 nM PD 123,319 (D). All images are single optical sections taken from comparable areas near the caudal portion of the ventricular loop. The myocardium and developing trabeculae of the Ang II-treated embryo showed the greatest intensity of staining for f-actin, which indicates that the increased ventricular length and width may be due to cardiac hypertrophy. Transmission electron micrographs (Figs. 6A and 6B) comparing myofibrillar development in similar areas of the ventricular walls of control and Ang II-treated embryos also indicated that myofibrillar development was more advanced in Ang II-treated embryos than in controls. Definitive sarcomeres and well-developed Z-bands were frequently found in the Ang II-treated embryos, but were rarely observed in control embryos.

Conversely, rhodamine-phalloidin staining of the myocardium of the Losartan-treated embryo showed that the myocardial wall in many areas was a single myocyte layer thick.
FIG. 2. (A–D) Colocalization of the Ang II AT1 receptor antibody (green) and f-actin (red) in embryonic hearts. Images are stacked confocal Z-series projected dorsal to ventral which results in the right side of the heart being viewed as the left side of the image. In the ED 9.25 heart (A), the Ang II AT1 receptor is localized primarily on the right side (arrow) in the region of the presumptive ventricle. This is also the region in which myofibrillogenesis is first initiated. At ED 10.25 (B), AT1 receptor antibody localization continues to be greatest along the greater curvature of the developing ventricle (arrow). Receptor localization is also heavy in the developing membranes of the body wall at both embryonic stages (*). In the area of the greater curvature of the ventricle, the AT1 receptor antibody was localized on the membranes of a majority of the cells in the developing epi- and myocardium (C), and was closely associated with the Z-bands of the developing myofibrils (arrows, D). Scale bars, 100 μm for A and B; 25 μm for C and D.
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appeared to be less extensive than in the control and Ang II-treated embryos (Figs. 5A–5D). This indicates that the observed increase in ventricular length and width associated with Losartan and PD123,319 treatment may be a result of cardiac dilation in response to reduced myofibrillar tension in the developing heart wall.

Biochemical Assessment of Cardiac Actin and Myosin Heavy Chain (MHC) Accumulation in response to Ang II, Losartan, and PD123,319

As expected from the morphological observations, both actin and MHC levels significantly increased when ED...
FIG. 5. (A–D) Single optical sections collected with the confocal microscope from the caudal region of the ventricular loop in embryos allowed to develop 9.25 days in vivo and an additional 48 h in culture and stained for f-actin with rhodamine phalloidin. Developing myofibrils were found in control embryos (A), and embryos were treated with 100 μg/ml of Ang II (B), 100 μM Losartan (C), and 300 nM PD123,319 (D). However, actin development appeared to be more extensive in the control (A) and Ang II (B)-treated embryos than in the embryos treated with Losartan (C) and PD123,319 (D). In addition, the wall of the developing myocardium (arrows) in control (A) and Ang II (B)-treated embryos consisted of multiple layers of cells, whereas areas of the myocardial wall of the Losartan-treated embryo (C) were frequently a single myocyte thick (arrow). Scale bar, 25 μm.

10.25 embryos were treated with 10 μg/ml of Ang II for 48 h in culture (Fig. 7). However, Ang II did not significantly increase the levels of these contractile proteins in the ED 9.25 embryos cultured for 48 h. This may be a result of the limited distribution of the AT1 receptor in the heart during the initial period of culture (Fig. 2A). The biochemical assay used included the entire heart and may not have been sensitive enough to determine an increase in contractile proteins in the limited cardiac region expressing the AT1 receptor during the initial culture period of ED 9.25 embryos. Treatment of embryos with either 100 μM Losartan or 30 nM PD123,319 resulted in a large degree of variability between culture runs and levels of actin and MHC that were not significantly different from those found in control embryos. This may again be related to the biochemical assessment utilizing the entire heart, whereas the morphological assessment of development focused on the specific areas in which the AT1 receptor was found.

Cardiac Inversions Associated with Angiotensin II, Losartan, and PD123,319 Treatment in Whole Embryo Culture

When treated with Ang II and PD123,319, embryos that had been allowed to develop for 9.25 days in vivo and 48 h in culture also exhibited an increased frequency of cardiac inversions in which the ventricular loop developed to the left rather than to the right (Fig. 8). No other characteristics of disrupted
FIG. 6. (A, B) Transmission electron micrographs from similar areas of the developing ventricle of control (A) and Ang II (10 μg/ml)-treated embryos (B). Embryos were allowed to develop for 9.25 days in vivo followed by 48 h in culture. Myofibrillar development in the Ang II-treated embryo was more extensive and sarcomere and Z-band organization were more advanced than in control embryos. Scale bar, 10 μM.
The Incidence of Cardiac Inversions in Embryos Allowed to Develop in Vivo for 9.25 Days Followed by 48 h of Development in Whole Embryo Culture Increased with the Addition of 10 ng and 10 μg/ml of Ang II, 100 μM Losartan, and PD123,319

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sample size</th>
<th>Number of cardiac/tail inversions</th>
<th>Percentage of cardiac inversions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>55</td>
<td>3/1</td>
<td>5.5</td>
</tr>
<tr>
<td>500 pg/ml Ang II</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 ng/ml Ang II</td>
<td>27</td>
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<td>0</td>
</tr>
<tr>
<td>10 μg/ml Ang II</td>
<td>36</td>
<td>8/0</td>
<td>22.2*</td>
</tr>
<tr>
<td>1 μM Losartan</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 μM Losartan</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100 μM Losartan</td>
<td>14</td>
<td>2/0</td>
<td>14.3</td>
</tr>
<tr>
<td>3 nM PD123,319</td>
<td>13</td>
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<td>23.1</td>
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<tr>
<td>30 nM PD123,319</td>
<td>8</td>
<td>3/0</td>
<td>37.5*</td>
</tr>
<tr>
<td>300 nM PD123,319</td>
<td>8</td>
<td>1/0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Note. Treatment with Ang II, Losartan, and PD123,319 did not affect other aspects of left–right body symmetry as indicated by normal development of the tail curvature in embryos that exhibit cardiac loop inversions. *The frequency of cardiac inversions is significantly higher than that of the control population at the P < 0.05 level.

DISCUSSION

In Vivo Expression of the Angiotensinogen System Components

RT-PCR of isolated hearts indicates that message for the necessary components of a local cardiac angiotensin system is present as early as ED 10.25 in the rat, and localization of the AT1 receptor was observed by confocal microscopy at ED 9.25. Hunt et al. (1995) have previously used autoradiography to localize Ang II receptors in the myocardium of ED 14 rats and to identify temporal variations in the density of the receptors through fetal and neonatal development. Others (Sechi et al., 1992, 1993; Matsubara et al., 1994; Shanmugam et al., 1994a,b) have also shown temporal and spatial variations in the expression of both the AT1 and AT2 receptor subtypes during late fetal, early neonatal, and adult development, but did not examine early embryonic cardiac expression of the receptors. During myocardial development, the total density of Ang II receptors peaks during neonatal development and subsequently decreases in the adult (Sechi et al., 1992), although subsequent changes in receptor density have been noted in cases of ventricular hypertrophy (Lopez et al., 1994).

Effects of Exogenous Angiotensin II and Specific Receptor Blockers during Cardiac Development

Identification of mRNA for the various components of the angiotensinogen system and immunolocalization of the AT1 receptor in the embryonic heart does not indicate that the system is functional at this early stage in development. However, modification of cardiac growth patterns, myofibrillogenesis, and accumulation of actin and MHC in response to the addition of Ang II, Losartan, or PD123,319 in whole embryo culture does imply that the AT1 and AT2 receptors are responsive, and that Ang II has the potential...
to play a significant role in the early growth and development of the embryonic heart. The increase in cardiac inversions in response to either increased levels of Ang II or the blocking of the AT2 receptor with PD123,319 also indicates that a proper balance between Ang II levels and receptor function is required for normal cardiac morphogenesis.

Studies with isolated cardiac myocytes have also indicated that the addition of Ang II to the culture media results in myocyte hypertrophy (Aceto and Baker, 1990; Baker and Aceto, 1990; Miyata and Haneda, 1994; Sadoshima and Izumo, 1993a) and an increase in the rate and force of myocyte contraction (Abdelatif et al., 1991; Rogers et al., 1989; Kohout and Rogers, 1995). In isolated cardiac myocytes, the binding of Ang II to the AT1 receptors initiates a phosphotyrosine cascade that activates protein kinase C and the expression of the immediate early genes c-fos and c-myc, and the late response genes for actin and myosin (Sadoshima and Izumo, 1993b; Sadoshima et al., 1995; Yamazaki et al., 1993, 1995a). These studies provide additional evidence that Ang II has the potential to increase the rate of myofibrillogenesis as we observed in whole embryo culture, and to induce cardiac hypertrophy that would result in the increases in the ventricular length and width we observed in these experiments.

Blocking the function of the AT1 receptor by the addition of Losartan blocks the hypertrophic response of isolated neonatal cardiac myocytes to Ang II (Sadoshima and Izumo, 1993a; Sadoshima et al., 1995), normal hypertrophy of the neonatal left ventricle (Beilich et al., 1993, 1995), and pathological hypertrophy of the left ventricle in response to increases in cardiovascular pressure (Rockman et al., 1994; de Gasparo et al., 1994). Similarly, the addition of Losartan to the whole embryo culture media resulted in decreased ventricular development as indicated by the relatively thin myocardial walls and an apparent reduction in the development of distinct myofibrils in the ventricular myocardium and trabeculae. A similar, but less pronounced effect on ventricular development was observed when the AT2 receptors were blocked by the addition of PD123,319.

Angiotensin and Cardiac Looping

Cardiac looping is a complex process that is dependent on a number of genetic and physical factors that may be affected by Ang II. It has previously been reported that the addition of Ang II to the culture media of isolated cardiac fibroblasts increases the autocrine production of transforming growth factor-β1 (TGF-β1) in isolated neonatal cardiac fibroblasts (Lee et al., 1995; Sharma et al., 1994). Initial expression of two members of the TGF-β1 subfamily, nodal and lefty, is also asymmetric in the early embryo (Collignon et al., 1996; Meno et al., 1996). Both are transiently found on the left side of the embryo and disruption of nodal expression results in cardiac positioning defects. The asymmetric distribution and possible interactions of these growth factors with Ang II indicate that subsequent asymmetric changes in protein accumulation levels may be critical in the normal development of cardiac looping (Brown and Wolpert, 1990; Beddington, 1996).

Normal myofibrillar differentiation has previously been identified as a requirement for the development of dextral looping patterns in the embryonic heart. Most hypotheses that attempt to explain cardiac looping involve phenomena such as right side dominance in development of myocytes at the base of the heart tube (Itasaki et al., 1989, 1991; Price et al., 1996), specific pattern formation of myofibrils and components of the extracellular matrix in different layers and regions of the myocardium (Manasek, 1981; Manasek et al., 1984; Shiraiishi et al., 1992, 1995; Itasaki et al., 1989, 1991; Price et al., 1996), and mechanical tension on myocytes in the greater curvature of the heart tube as it loops (Taber et al., 1995). During loop formation, cell number does not change significantly (Manasek, 1981), but cells along the greater curvature of the loop flatten and increase their apical surface area, while cells in the lesser curvature do not undergo this shape change (Manasek et al., 1984). Concurrent with changes in phenotype, myofibrils in the concave area of the heart loop develop more rapidly than in the convex portions of the myocardium (Itasaki et al., 1991; Price et al., 1996).

The right side, or convex area, of the developing heart loop is the region in which the Ang II AT1 receptor is predominantly found in ED 9.25 and 10.25 hearts. In vitro studies with isolated cardiac myocytes have indicated a direct relationship between mechanical stress and the role of Ang II in cardiac myocyte hypertrophy (Sadoshima and Izumo, 1993c; Sadoshima et al., 1993; Yamazaki et al., 1995a,b). These studies indicate that mechanical stress stimulates the secretion of Ang II from cardiac myocytes and that Ang II mediates mechanical load-induced cardiac myocyte hypertrophy. This may, in part, explain the more advanced cellular and myofibrillar development in the greater curvature of the ventricular loop (Manasek et al., 1981, 1984; Itasaki et al., 1991; Shiraiishi et al., 1992, 1995; Price et al., 1996).

However, in previous studies in which myofibrillogenesis was disrupted by the addition of cytochalasin D crystals at the right base of the heart, polymerization of actin filaments in this area was prevented (Itasaki et al., 1991). This resulted in myofibrils on the left side of the heart being more advanced than those on the right side, and a 100% occurrence of cardiac inversions. The concentration of AT1 receptors on the right side of the heart, and the addition of Ang II to the culture media, should result in an increased rate of myofibrillogenesis in this area which is essentially the opposite of the results from the studies by Itasaki et al. (1991). This apparent inconsistency is difficult to explain, but the disruption of normal myofibrillary patterns in the developing heart is a contributing factor to abnormal cardiac looping.

Whole embryo culture has previously been used to identify other factors, including receptor-mediated adrenergic stimulation, that induces situs inversus and an associated high incidence of cardiac inversions (Fujinaga and Baden, 1991a,b). Of particular interest is that both adrenergic and
angiotensin receptors are coupled to G-proteins that, when activated, induce an increase in intracellular calcium levels (Miyata and Haneda, 1994; Fujinaga et al., 1994; Delièse et al., 1993). In the studies by Fujinaga et al. (1994), use of nifedipine, an L-type Ca\(^{2+}\) channel blocker, decreased the occurrence of adrenergic-induced situs inversus associated with the intracellular Ca\(^{2+}\) increases. Both the adrenergic \(\alpha1B\) and angiotensin AT1 receptors are linked to the phospholipase C pathway (Fujinaga and Baden, 1994; Lokuta et al., 1994) which may indicate that shared pathways exist in systems that control development of body symmetry.

In the studies by Fujinaga and Baden (1991b), a high percentage (20–30\%) of the control embryos cultured prior to the early neural plate stage also developed cardiac inversions. However, in our cultures and those by Fujinaga and Baden in which culture was started during the early neural plate stage (approximately ED 9.0–9.25), inversions occurred in only 5–6\% of the control embryos and 20–40\% of the treated embryos. Initiation of culture following the early neural plate stage did not result in a significant number of cardiac inversions in either study.

The use of rhodamine phallolidin in this study focuses the morphological assessment of the effects of Ang II, Losartan, and PD123,319 primarily on myocyte development. Ang II also affects the development and function of cardiac fibroblasts (Dostal et al., 1992; Schorb et al., 1993; Sadoshima and Izumo, 1993a; Sadoshima et al., 1995) and extracellular matrix components (Burgess et al., 1994; Brilla et al., 1993), and interacts with other growth factors such as PDGF (Bobik et al., 1990; Nafililan et al., 1989), which have important roles in normal cardiac differentiation (Levene et al., 1994; Schattenman et al., 1996). We have recently initiated whole embryo culture studies to further examine these interactions and their potential role in cardiac differentiation.

### REFERENCES


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