Molecular Characterization of Angiotensin II–Induced Hypertrophy of Cardiac Myocytes and Hyperplasia of Cardiac Fibroblasts

Critical Role of the AT₁ Receptor Subtype

Jun-ichi Sadoshima, Seigo Izumo

Increasing evidence suggests that angiotensin II (Ang II) may act as a growth factor for the heart. However, direct effects of Ang II on mammalian cardiac cells (myocytes and nonmyocytes), independent of secondary hemodynamic and neurohumoral effects, have not been well characterized. Therefore, we analyzed the molecular phenotype of cultured cardiac cells from neonatal rats in response to Ang II. In addition, we examined the effects of selective Ang II receptor subtype antagonists in mediating the biological effects of Ang II. In myocyte culture, Ang II caused an increase in protein synthesis without changing the rate of DNA synthesis. In contrast, Ang II induced increases in protein synthesis, DNA synthesis, and cell number in nonmyocyte cultures (mostly cardiac fibroblasts). The Ang II–induced hypertrophic response of myocytes and mitogenic response of fibroblasts were mediated primarily by the AT₁ receptor. Ang II caused a rapid induction of many immediate-early genes (c-fos, c-jun, jun B, Egr-1, and c-myc) in myocyte and nonmyocyte cultures. Ang II induced “late” markers for cardiac hypertrophy, skeletal α-actin and atrial natriuretic factor expression, within 6 hours in myocytes. Ang II also caused upregulation of the angiotensinogen gene and transforming growth factor-β1 gene within 6 hours. Induction of immediate-early genes, late genes, and growth factor genes by Ang II was fully blocked by an AT₁ receptor antagonist but not by an AT₂ receptor antagonist. These results indicate that (1) Ang II causes hypertrophy of cardiac myocytes and mitogenesis of cardiac fibroblasts, (2) the phenotypic changes of cardiac cells in response to Ang II in vitro closely mimic those of growth factor response in vivo and of load-induced hypertrophy in vivo, (3) all biological effects of Ang II examined here are mediated primarily by the AT₁ receptor subtype, and (4) Ang II may initiate a positive-feedback regulation of cardiac hypertrophic response by inducing the angiotensinogen gene and transforming growth factor-β1 gene. (Circulation Research 1993;73:413-423)

Key words • angiotensin II • AT₁ receptor • immediate-early genes • mitogenesis • hypertrophy

The renin-angiotensin system plays a critically important role in the control of cardiovascular and renal homeostasis.1-3 Previously, this system has been considered to be an endocrine system, in which angiotensinogen is produced in the liver and secreted into the systemic circulation, where the successive proteolytic cleavages by renin and angiotensin converting enzyme (ACE) occur to produce the biologically active peptide angiotensin II (Ang II).1,2

Recently, however, there is accumulating evidence for the existence of an independent tissue (local) renin-angiotensin system in several organs. This concept is supported by evidence derived from biochemical, immunohistochemical, and molecular biological demonstration of all components of the renin-angiotensin system, including renin, angiotensinogen, ACE, angiotensin I (Ang I), Ang II, and Ang II receptors in local tissues, including the heart and blood vessels. Besides its potent vasoconstrictive effect, Ang II has been suggested to work as an autocrine/paracrine factor regulating growth of local tissues such as blood vessel, kidney, and heart.3-7

The direct growth effect of Ang II has been extensively characterized in vascular smooth muscle cells, where Ang II has been shown to promote hypertrophy in vitro.6-10 Interestingly, Ang II also causes hyperplasia in some smooth muscle cells in culture, such as the aorta of spontaneously hypertensive rats or the renal arteri-oles of normal rats.11,12 Thus, Ang II directly or in combination with other growth factors may play an important role in the development of vascular hypertrophy and elevated arterial resistance in hypertension.

Several studies in vivo have suggested that Ang II may also be a critical factor in mediating cardiac hypertrophy. First, chronic infusion of subpressor doses of Ang II to rats caused ventricular hypertrophy without changes in blood pressure.13 Second, in a genetic model of hypertension, normalization of blood pressure by sympatholytic agents or by direct vasodilators did not cause regression of cardiac hypertrophy, whereas treatment with an ACE inhibitor did.14 Third, treating the rats having abdominal aortic coarctation with an ACE...
inhibitor prevented left ventricular hypertrophy, even though carotid artery pressure in these rats was not different from that in the untreated animals. Fourth, treatment of newborn pigs with an ACE inhibitor interfered with the physiological hypertrophy of the left ventricle associated with normal growth. Finally, treatment of patients suffering from myocardial infarction with ACE inhibitors prevented cardiac dilatation. These observations are consistent with, though do not prove, the notion that Ang II may act as an endogenous growth factor for the myocardium. However, ACE inhibitors lower blood pressure and also inhibit kinin and bradykinin metabolisms. Therefore, some effects of ACE inhibitors might not have been due to inhibition of Ang II production.

Recently, Baker et al. have shown that Ang II increases protein synthesis in chick cardiac myocytes in vitro. However, molecular characterization of the phenotypic changes in cardiac myocytes in response to Ang II has not been reported. Furthermore, it is not known whether Ang II has any direct effects on cardiac nonmyocyte populations (fibroblasts, endothelial cells, smooth muscle cells, etc), which account for as many as 50% of the total cell number of the heart. Thus, the objectives of the present experiments are (1) to characterize the phenotypic changes induced by Ang II in cardiac myocytes and nonmyocytes (primarily fibroblasts) in vitro and (2) to examine which receptor subtype (AT1 or AT2) mediates biological effects of Ang II in myocytes and nonmyocytes.

**Materials and Methods**

**Materials**

All culture reagents were purchased from GIBCO, Gaithersburg, Md. All radiochemicals were obtained from Du Pont–New England Nuclear, Boston, Mass. Losartan and PD123319 were generous gifts from Du Pont Merck, Wilmington, Del. and Parke-Davis, Ann Arbor, Mich, respectively. All angiotensin-related peptides were purchased from Peninsula Laboratories, Belmont, Calif. All other chemicals were from Sigma Chemical Co, St Louis, Mo.

**Preparation of Myocyte-Rich Culture**

Primary cultures of the neonatal rat cardiac myocyte were prepared as described previously. To selectively enrich for myocytes, dissociated cells were preplated for 1 hour, during which period the nonmyocytes attached readily to the bottom of the culture dish. The resultant suspension of myocytes was plated onto gelatin-coated 35-mm or 60-mm culture dishes at a density of 1×10^5 cells/cm². Bromodeoxyuridine (BrdU, 100 μM) was added during the first 24 to 36 hours to prevent proliferation of nonmyocytes. All experiments were done in the serum-free condition 24 to 48 hours after changing to the serum-free medium. Using this method, we routinely obtained myocyte-rich cultures with 90% to 95% myocytes (hereafter referred to as myocyte cultures), as assessed by microscopic observation of cell beating and by immunofluorescence staining with a monoclonal antibody (MF20) against sarcomeric myosin heavy chain.

**Preparation of Nonmyocyte-Rich Culture**

Highly enriched cultures of cardiac nonmyocytes (hereafter referred to as nonmyocyte cultures) were prepared passing twice the cells adherent to the culture dish during the preplating procedure. Until the second passage, cells were maintained in the same culture medium as above, except that 10% calf serum was used and BrdU was not used. After the second passage, the same serum-free medium as above was used. In this nonmyocyte culture, less than 10% of the cells were sarcomeric myosin positive. The percentage of myosin-positive cells did not increase with time, arguing against the notion that myosin-negative cells are undifferentiated cardioblasts. Immunostaining with an antibody against smooth muscle, α-actin (IBL Research Products, Cambridge, Mass), revealed that less than 10% of cells were positive. Incubating nonmyocyte culture with the fluorescence-labeled acetylated low density lipopro-
tein (Biomedical Technologies Inc, Stoughton, Mass) revealed that less than 2% of the cells took up acetylated low density lipoprotein (a marker for endothelial cells). These preliminary characterizations of nonmyocyte suggest that the majority of the cells in nonmyocyte culture are likely to be fibroblasts, as defined by the lack of the markers for cardiac myocytes, smooth muscle, or endothelial cells.

Isolation and Northern Blot Analysis of RNA

Isolation of total cellular RNA and Northern blot analysis were performed as described previously. The probes c-fos, c-jun, Egr-1 (Zif/268), c-myc, skeletal α-actin, atrial natriuretic factor (ANF), and glyceraldehyde-3-phosphate dehydrogenase were used as described previously. The following probes were also used: (1) jun B, a 1.8-kb EcoRI fragment of the mouse jun B cDNA, (2) angiotensinogen, an EcoRI fragment of the rat angiotensinogen cDNA clone pGEM3, and (3) transforming growth factor-β (TGF-β), a Sac I/Pvu II fragment of the porcine TGF-β clone pTGFβ33. The relative amounts of a specific mRNA were quantified by laser densitometry of the corresponding autoradiograms in the linear response range of the x-ray films. The hybridization signals of specific mRNAs were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA to correct for differences in loading and/or transfer. The levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were not affected by Ang II (see “Results”).

Incorporation of [3H]Phenylalanine

As an index of protein synthesis, [3H]phenylalanine incorporation was measured as described previously. After incubation in serum-free medium for 24 hours, the cells were stimulated with Ang II (10 nM) for 48 hours in the presence of [3H]phenylalanine (10 μCi/mL) and unlabeled phenylalanine (0.36 mM) in the medium. The cells were washed with phosphate-buffered saline (PBS), and 10% trichloroacetic acid (TCA) was added at 4°C for 60 minutes to precipitate protein. The control condition, parallel cultured cells were harvested at the same time course without Ang II stimulation. The precipitate was washed three times with 95% ethanol and then resuspended in 0.15N NaOH. Aliquots were counted in a scintillation counter. The results were expressed as counts per minute per dish.

Incorporation of [3H]Thymidine and Cell Counts

[3H]Thymidine uptake measurement and cell counts were performed as described. For this experiment, BrdU was omitted from the culture medium. Cells were grown in a serum-free medium for 24 hours and then stimulated with 10 nM Ang II. After 18 hours, [3H]thymidine (5 μCi/mL) was added for 6 hours. Cells were then washed with PBS and harvested with 10% TCA. TCA-precipitable counts were measured as above.

BrdU Incorporation

A mixed culture of cardiac myocytes and nonmyocytes was prepared by omitting the preplating procedure. Cells were kept in a serum-free medium for 48 hours and then stimulated with Ang II (100 nM) for 24 hours. Control cultures were prepared without stimulation with Ang II. In both preparations, BrdU (10 μM) was added for the last 5 hours. Cells were fixed in methanol for 10 minutes at −20°C, rehydrated in PBS,
and incubated in 2N HCl for 1 hour at 37°C. After neutralization in 0.1 M borate buffer (pH 8.5), cells were washed in PBS and processed for the immunofluorescent staining. Fluorescein isothiocyanate (FITC)— conjugated mouse monoclonal antibody against BrdU (Bu 5.1 FITC, IBL Research Products) and MF20 were used.21 For the detection of MF20, Texas red—coupled goat antibodies to mouse immunoglobulins (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) were used. For double-label experiments, FITC-conjugated anti-BrdU antibody was applied after completion of the staining with MF20.

Immunohistochemistry

Immunofluorescence cell staining was performed as described previously.20 For primary antibodies, rabbit serum 456 against c-fos (Medac, Hamburg, Germany) and MF20 were used. Secondary antibodies were FITC-conjugated or Texas red—coupled goat antibodies to immunoglobulins of rabbit or mouse (Jackson ImmunoResearch Laboratories). For double-label experiments, both primary and secondary antibodies were applied simultaneously.

Statistics

Data are given as mean±SEM. Statistical analysis was performed using analysis of variance and unpaired Student's t test as appropriate. Significance was accepted at P<.05.

Results

Ang II Causes Hypertrophy of Cardiac Myocytes

We examined the effects of Ang II on protein synthesis and the rate of DNA synthesis in the myocyte and nonmyocyte cell fractions (see "Materials and Methods") of primary cultured neonatal rat heart cells. In myocytes, Ang II (10 nM) caused a significant increase in protein synthesis as measured by [3H]phenylalanine incorporation over 48 hours (Fig 1, A). The magnitude of increase in [3H]phenylalanine incorporation induced
by Ang II (10 nM) was comparable to that induced by norepinephrine (20 μM) and was smaller than that induced by phorbol 12-myristate 13-acetate (1 μM) (Fig 2, A), two well-characterized hypertrophic stimuli for neonatal cardiac myocytes.26-28 In contrast, Ang II (10 nM) did not increase DNA synthesis as measured by [3H]thymidine uptake over 24 hours (Fig 1, B). Lack of DNA synthesis in response to Ang II in myocytes was also confirmed by double immunostaining with an anti-myosin antibody and an anti-BrdU antibody (Fig 3, see below). These results suggest that Ang II has a hypertrophic effect (increase in protein synthesis without DNA synthesis) on neonatal rat cardiac myocytes.

**AT1 Receptor Mediates Ang II–Induced Hypertrophy of Myocytes**

Recently, the presence of two Ang II receptor subtypes (AT1 and AT2) has been reported on the basis of binding site analyses using nonpeptide Ang II receptor antagonists. The prototypical antagonist of the AT1 receptor is losartan (DuP 753) and that of the AT2 receptor is PD123319.29 We examined which Ang II receptor subtype was linked to protein synthesis in the neonatal rat cardiac myocyte. Neither losartan (1 μM) nor PD123319 (1 μM) significantly affected the basal level of protein synthesis in nonstimulated myocytes (Fig 2, B). Losartan completely suppressed the Ang II–induced increase in protein synthesis, whereas PD123319 did not suppress it significantly. The results suggest that the Ang II–induced increase in protein synthesis in cardiac myocytes is mediated by the AT1 receptor.

**Ang II Causes Hyperplasia of Nonmyocytes via AT1 Receptors**

In cardiac nonmyocyte culture (mostly fibroblasts, see “Materials and Methods”), Ang II (10 nM) treatment also caused a significant increase in [3H]phenylalanine incorporation over 48 hours (Fig 1, A). Interestingly, Ang II caused a significant increase in [3H]thymidine uptake over 24 hours in these cells (Fig 1, B), although the magnitude of increase caused by Ang II was 8% to 10% of that caused by 20% fetal calf serum (data not shown).

To confirm Ang II–induced DNA synthesis in nonmyocytes, the cells were labeled with the thymidine analogue BrdU, and a double immunofluorescent analysis was performed using anti-BrdU antibody and anti-sarcomeric myosin antibody (Fig 3). We deliberately used a mixed culture of myocytes and nonmyocytes for this analysis to examine both cell types in the same microscopic field. In control cells cultured in the serum-free medium for 48 hours, no staining was observed by anti-BrdU antibody in any of the myosin-positive cells (Fig 3, a and c; arrowheads). In myosin-negative cells (thick arrows), 6.5% of the cells were BrdU-positive (26 of 400 cells counted). When the cells were treated with Ang II for 24 hours, a clear nuclear staining pattern by BrdU antibody was observed in 32% of the myosin-negative cells (Fig 3, b and d; thick arrows and 128 of 400 cells counted) but never in myosin-positive cells (Fig 3, b and d; arrowheads) in the multiple fields examined. The mitogenic effect of Ang II on nonmyocytes was also confirmed by counting the number of cells (see below). These results suggest that Ang II has a mitogenic effect on nonmyocytes but not on myocytes.

We next examined which receptor subtype mediates the Ang II–induced mitogenic effect on nonmyocytes. As shown in Fig 4, A, losartan prevented Ang II–induced increase in [3H]thymidine uptake, whereas PD123319 did not. Similar results were observed when cell numbers were counted (Fig 4, B). Ang II caused a 30% increase in cell number over 24 hours, and this increase was com-

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**FIG 4.** Bar graphs showing the effect of nonpeptide angiotensin II (Ang II) receptor antagonists on Ang II–induced [3H]thymidine incorporation (A) and cell counts (B) in nonmyocytes. Cells were pretreated with each antagonist for 30 minutes and then stimulated with Ang II (10 nM) for 24 hours in the presence of the antagonist. Ang II (10 nM) was supplemented at 12 hours to compensate for decrease due to degradation by an endogenous angiotensinase. The concentrations of the antagonists used are as follows: losartan, 1 μM; and PD123319, 1 μM. A, [3H]Thymidine (5 μCi/mL) was added from 18 to 24 hours after addition of Ang II. Data are normalized to the mean counts per minute of the control value without drugs, which was set as 1. Data are mean±SEM obtained from five samples in each group. *P<.01 vs control without antagonists. B, Cell count was performed at 24 hours. Data are expressed as number of cells per dish and are mean±SEM obtained from five samples in each group. *P<.01 vs control without antagonist. Minus sign indicates Ang II alone.
completely prevented by losartan but not by PD123319 (Fig 4, B). Thus, the AT₁ receptor mediates Ang II–induced mitogenesis of cardiac nonmyocytes.

Induction of Immediate-Early Genes

A variety of stimuli that induce cardiac hypertrophic response, such as mechanical load, α₁ and β-adrenergic receptor agonists, endothelin-1, fibroblast growth factors, and TGF-β₁, have been shown to induce immediate-early (IE) genes as one of the earliest nuclear events (see References 20, 27, and 30-33; reviewed in Reference 34). It has been shown that Ang II causes the induction of IE genes such as c-fos and c-myc in vascular smooth muscle cells. Therefore, we examined the effect of Ang II on the IE gene expression in myocytes and nonmyocytes. Since the expression pattern of the IE genes has been reported to be stimulus specific, we examined the expression of three different classes of transcription factors: (1) c-fos, c-jun, and jun B (members of “leucine zipper” class genes), (2) Egr-1 (a “zinc finger” class gene), and (3) c-myc (a “helix-loop-helix”–containing gene). Representative Northern blots are shown in Fig 5. In both myocytes and nonmyocytes, Ang II induced c-fos, c-jun, jun B, Egr-1, and c-myc. IE genes c-fos, jun B, and Egr-1 showed a peak induction at approximately 30 minutes, whereas c-jun and c-myc showed a later peak at approximately 30 minutes to 1 hour. The duration of augmented expression of c-fos was shorter than that of the others, reverting to the control level at 2 hours, whereas that of the others showed moderately elevated expression even at 2 hours.

Recently Roux et al reported that, in the absence of serum, the Fos protein could not be translocated into nucleus and stayed in cytoplasm in rat embryonic fibroblasts and mouse fibroblast cell lines. Therefore, we examined whether growing the cardiac myocytes and nonmyocytes in serum-free conditions actually leads to translocation of Fos protein to the nucleus after its synthesis in the cytoplasm in response to Ang II stimulation. Double immunofluorescence staining was performed on a mixed cell culture using anti-Fos and anti-sarcomeric myosin antibodies. Both myosin-positive cells (Fig 6, a through c; arrowheads) and myosin-negative cells (arrows) expressed Fos protein 1 hour after treatment with Ang II (Fig 6, a). No specific Fos signals were observed in nontreated control culture (Fig 6, d). Thus, in cardiac myocytes and nonmyocytes, Ang II induces translocation of Fos to the nucleus in the absence of serum. Induction of Fos protein was a transient response because little Fos signal was detectable 3 hours after Ang II treatment (data not shown).

Induction of c-fos by Ang II Is Mediated Primarily by AT₁ Receptors

Ang II induced c-fos expression in a dose-dependent manner in cardiac myocytes (Fig 7, A). The induction of c-fos was detected at 10 pM, and maximum induction was observed at approximately 100 nM. The dose-response relation of the c-fos induction as quantitated

![Fig 5. Angiotensin II (Ang II)–induced expression of immediate-early genes in myocytes and nonmyocytes in the neonatal rat heart. Representative Northern blots of myocytes (left) and nonmyocytes (right) are shown. The same blots were hybridized serially by different probes to demonstrate the different kinetics of each immediate-early gene. Myocyte and nonmyocyte fractions were prepared as described in the text. Cells were stimulated with Ang II (100 nM) for the times indicated on the top. Ethidium bromide staining of 18S and 28S RNA shown below showed that an equal amount of RNA was loaded in each lane. The serial hybridization resulted in higher background and lower signal intensity as seen in c-jun and c-myc probes that were hybridized after c-fos and EGR-1 probes. However, in other blots, significant induction of c-jun and c-myc by Ang II was observed. Similar results were obtained from two additional experiments.](http://circres.ahajournals.org/)

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by laser densitometry showed a half-maximum concentration (EC50) of 1 to 2 nM. This is consistent with a known $K_a$ of Ang II to its receptor in cardiac myocytes.

Figure 7, B, is a representative Northern blot showing the effects of nonpeptide Ang II receptor antagonists on Ang II (1 nM)–induced c-fos expression. The AT1 receptor antagonist losartan (100 nM) almost completely suppressed c-fos expression, but the same concentration (100 nM) of the AT2 antagonist PD123319 did not show significant inhibition. At 100-fold higher concentration (10 μM), PD123319 partially suppressed Ang II–induced c-fos expression. This effect may be due to a partial block of the AT1 receptor at this concentration of PD123319, although we cannot formally rule out the possibility that a minor component of Ang II–induced c-fos expression may be mediated by the AT2 receptor.

Effects of Angiotensin Metabolites on c-fos Induction

Recently, the existence of the local renin-angiotensin system has been reported in various tissues, including the rat heart. To identify the biological activity of several metabolites of the renin-angiotensin system, we examined c-fos inducibility by exogenously applying angiotensin-related peptides to the cardiac myocyte. Fig 8 shows a representative Northern blot. Ang I (100 nM) and its aminopeptidase-cleavage product [des-Asp1]Ang I (100 nM) induced c-fos expression (lanes 8 and 10). However, when Ang I and [des-Asp1]Ang I were applied with the ACE inhibitor captopril (10 μM) to prevent their conversion into Ang II and angiotensin III (Ang III), respectively, they did not induce c-fos (lane 9 and data not shown). This suggests that not only is there an endogenous ACE-like activity in cultured cardiac cells but that Ang I and [des-Asp1]Ang I require this ACE-like activity to induce c-fos in cardiac myocytes.

Among the Ang II degradation products, Ang III (10 nM) induced c-fos expression almost as potently as did Ang II (Fig 8, lane 4). However, the aminopeptidase cleavage products Ang II-(3-8) (100 nM) and Ang II-(4-8) (100 nM) did not induce c-fos (lanes 5 and 6). [Sar1, Ile8]Ang II (100 nM), a nonselective competitive inhibitor of Ang II, did not induce c-fos by itself (lane 3) and completely prevented the c-fos induction by Ang II (10 nM) (lanes 1 and 2). As expected, captopril did not prevent c-fos induction by Ang II or Ang III (data not shown).

Ang II Causes Induction of Fetal Genes, Angiotensinogen Gene, and TGF-β1 Gene

It is known that cardiac hypertrophy in vivo and in vitro is accompanied by changes in the muscle phenotype characterized by the expression of “fetal”-type genes, such as skeletal α-actin and the ventricular expression of ANF. Therefore, we examined the expression of these fetal genes in response to Ang II (Fig 9, A). Although ANF and skeletal α-actin mRNAs were detectable under control conditions in these neo-
natal ventricular myocytes, 6 to 24 hours of treatment with Ang II significantly increased the expression of ANF and skeletal α-actin genes in a time-dependent fashion.

Some growth factors stimulate or repress transcription of their own gene and other growth factor genes, which provides a positive- or negative-feedback regulation of cell growth. It has been shown that Ang II increases the amount of angiotensinogen mRNA in the rat liver. Therefore, we examined whether Ang II affects the accumulation of angiotensinogen mRNA in cardiac myocytes. We also examined levels of TGF-β, mRNA in response to Ang II stimulation because TGF-β is known to be a potent inducer of the fetal genes in neonatal rat cardiac myocytes. As shown in Fig 9, a significant induction of angiotensinogen mRNA and TGF-β mRNA was observed 6 hours after Ang II treatment, and this effect appeared more pronounced at 24 hours.

Increased Expression of “Late” Genes Is Mediated by AT₁ Receptors

We next examined whether increase in “late” genes by Ang II stimulation was mediated by AT₁ or AT₂ receptors. Ang II–induced increases in mRNAs encoding skeletal α-actin, ANF, TGF-β₁, and angiotensinogen were significantly suppressed by losartan but not by PD123319 (Fig 10 and data not shown). These results suggest that induction of late genes is primarily mediated by the AT₁ receptor subtype.

Discussion

We have analyzed the effects of Ang II in primary cultured cardiac myocytes and nonmyocytes (mostly fibroblasts). The major findings are as follows: First, Ang II causes hypertrophy of cardiac myocytes and hyperplasia of cardiac nonmyocytes. Second, both hypertrophy of myocytes and hyperplasia of nonmyocytes are mediated by the AT₁ receptor. Third, Ang II induces
the expression of a number of IE genes, such as c-fos, c-jun, jun B, Egr-1, and c-myc, in both myocytes and nonmyocytes. Fourth, Ang II induces the “fetal program” (induction of skeletal α-actin and ANF) and induces expression of the angiotensinogen gene and TGF-β1 gene. Fifth, Ang II–induced changes in IE genes and late genes are primarily mediated by the AT1 receptor.

The increase in the rate of protein synthesis by Ang II in the neonatal rat cardiac myocytes seems to be compatible with that in chick heart cells reported by Baker and Aceto13 (40% above the control value over 120 hours). However, the Ang II–induced increase in the protein synthesis in cardiac myocytes was smaller than that in the vascular smooth muscle cells reported by Berk et al.9 It is possible that Ang II has a greater growth effect on the smooth muscle cells than on cardiac myocytes. However, the experiment of Berk et al was carried out in the presence of 0.4% calf serum; thus, a potential synergistic effect with residual serum–derived growth factors cannot be excluded. Moreover, Geisterfer et al18 have reported that in rat aortic smooth muscle cells Ang II (1 μM) increases the rate of protein synthesis by approximately 25% over 48 hours, which is compatible with our results.

It is of interest that Ang II was mitogenic to cardiac nonmyocytes, whereas it was strictly hypertrophic to cardiac myocytes. The latter fact may not be solely due to the terminally differentiated state of neonatal cardiac myocytes, because at this developmental stage some myocytes are reported to still retain their ability to synthesize DNA in response to serum stimulation.33 It would also be interesting to determine whether the cell cycle regulatory genes, such as cyclins and cyclin-dependent kinases, are regulated differently in response to Ang II in myocytes and nonmyocytes. However, it should be emphasized that Ang II is not as strong a mitogen as fetal calf serum, because the Ang II–induced increase in thymidine incorporation is only 8% to 10% of that seen with fetal calf serum. Ang II has been shown to be mitogenic to Swiss 3T3 cells44 and some types of vascular smooth muscle cells.11,12

van Krimpen et al45 reported that increased DNA synthesis in interstitial cells after myocardial infarction was inhibited by an ACE inhibitor independent of its effect on afterload changes. Weber and Brilla46 found that Ang II causes fibrosis and increased collagen deposition in the cardiac interstitium. This effect was prevented by an ACE inhibitor and by an aldosterone inhibitor. In our in vitro system, the mitogenic effect of Ang II on nonmyocytes is most likely a direct effect of Ang II, rather than being mediated by aldosterone, because the latter hormone is not known to be produced in cardiac cells. Therefore, the beneficial effects of ACE inhibitors in myocardial remodeling after myocardial infarction may be due to a decreased production of Ang II, although it is not possible to rule out other nonspecific effects of ACE inhibitors in vivo.

It has been shown that both AT1 and AT2 receptors are expressed in rat neonatal cardiac myocytes and possibly in nonmyocytes.47 Our pharmacological studies indicate that all aspects of Ang II–induced myocyte hypertrophy examined (increase in protein synthesis and induction of IE genes and late genes) are primarily mediated by the AT1 receptor subtype. Our results also demonstrate that the mitogenic response of nonmyocytes is mediated by the AT1 receptor. The importance of the AT1 receptor in normal growth of newborn pig hearts in vivo has recently been demonstrated.10 At present, physiological roles of the AT1 receptor are not known in cardiac cells.

Recently, cDNAs encoding the AT1 receptors have been cloned.48,49 The deduced amino acid sequence predicts seven membrane spanning regions, typical of G protein–coupled receptors. The receptors for other hypertrophic stimuli, endothelin-I and α-adrenergic ag-
Hybridization significantly by two additional antagonists, also have similar structure. These receptors are believed to couple to Gq-type G proteins (reviewed in Reference 50). The phenotypic resemblance of Ang II–induced hypertrophy to that induced by endothelin-1 or α-adrenergic agonists may be due to the shared intracellular signaling in response to these agonists. On the other hand, TGF-β1 and basic fibroblast growth factor also cause similar phenotypic changes in cardiac myocytes. However, the initial signal transduction pathways by these agonists are different from those of G protein–coupled receptors. It remains to be determined at what point the convergence of the signaling occurs in response to various hypertrophic stimuli.

In the present study, we have used neonatal cardiac myocytes and nonmyocytes. There remains a possibility that the response of neonatal cardiac myocytes and nonmyocytes may differ from that of adult cells. It has been reported that the responses to α-agonists are different between neonatal and adult myocytes. Although several in vivo studies using ACE inhibitors suggest that Ang II may act as a growth factor for adult heart, a direct proof for this hypothesis awaits a study using cultured adult myocytes. Interestingly, Moalic et al have reported that infusion of Ang II did not induce c-fos or c-myc in an isolated perfused adult rat heart, presumably because of the absence of Ang II receptors in the adult rat heart. Very recently, however, Dostal and Baker have reported that chronic infusion of Ang II causes cardiac hypertrophy and that this is prevented by losartan in adult rat hearts. It remains to be determined whether our in vitro results using neonatal cells may apply for the adult heart in vivo.

It is of interest that Ang II increases the expression of the angiotensinogen gene. This raises the possibility that Ang II may initiate a positive-feedback regulation of cardiac growth. Ang II also increases expression of the TGF-β1 gene. TGF-β1 is known to induce “fetal” genes in cardiac myocytes and enhance the synthesis of the extracellular matrix by fibroblasts. Ang II has been shown to induce platelet-derived growth factor and TGF-β1 in smooth muscle cells. It is likely that Ang II may induce other growth factor genes in cardiac myocytes as well. It is possible that the mitogenic effect of Ang II on nonmyocytes may be due to an enhanced autocrine/paracrine production of growth factors by Ang II stimulation.

In summary, we have demonstrated that Ang II causes hypertrophy of cardiac myocytes and mitogenesis of nonmyocytes (primarily fibroblasts) via AT1 receptor stimulation. Elucidation of the mechanisms of Ang II–induced cardiac growth deserves further investigation because Ang II is clearly emerging as one of the most important mediators of cardiac hypertrophy in vivo.

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References


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