Angiotensin (1–7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects

Michikado Iwata, Randy T. Cowling, Devorah Gurantz, Cristina Moore, Shen Zhang, Jason X.-J. Yuan, and Barry H. Greenberg

Angiotensin (1–7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. Am J Physiol Heart Circ Physiol 289: H2356–H2363, 2005. First published July 15, 2005; doi:10.1152/ajpheart.00317.2005.—Angiotensin (1–7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. Angiotensin (1–7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. Angiotensin (1–7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects.

Recently, an alternative pathway of the RAS involving a homolog of angiotensin-converting enzyme (ACE), termed ACE2, was discovered (8, 25). One of the properties of this carboxypeptidase is its ability to hydrolyze ANG II with high catalytic efficiency (26). This reaction results in the generation of ANG-(1–7) (8, 26). In the vascular system, ANG-(1–7) induces vasodilation, attenuates ANG II-stimulated vasoconstriction, and inhibits cell growth (20, 23). In vascular smooth muscle cells (VSMCs), specific receptors for ANG-(1–7), AT1–7 receptors, which are distinct from the AT1 and AT2 receptors, are considered to mediate these effects (20, 23). Recent studies demonstrated that the Mas protooncogene could function as a receptor for ANG-(1–7) in vitro and in vivo (18, 22).

Most previous studies of ANG-(1–7) have focused on its vascular actions (20, 23). There is evidence, however, that ACE2 is expressed in the heart (30) and that ANG-(1–7) can be generated directly within the myocardium (1). Targeted disruption of ACE2 in mice produces severe defects in cardiac function (5). Infusion of ANG-(1–7) in rats after a MI improves cardiac function and attenuates the development of heart failure (14). Thus ANG-(1–7) may act in the heart as an autocrine/paracrine hormone to affect cardiac structure and function.

Evidence that exposure of cardiac fibroblasts to ANG II stimulates a variety of profibrotic functions as well as induction of growth factors that can act in an autocrine or paracrine fashion within the myocardium indicates that cardiac fibroblasts play a crucial role in RAS-induced cardiac remodeling (11, 13, 15, 19, 27). Little is known, however, about the effects of ANG-(1–7) on these cells. We hypothesized that cardiac fibroblasts possess specific receptors for ANG-(1–7) and that ANG-(1–7) and ANG II have distinct and independent effects on cardiac fibroblast functions involved in cardiac remodeling. To test these hypotheses, we investigated characteristics of ANG-(1–7) binding to adult rat cardiac fibroblasts (ARCFs) and the effects of ANG-(1–7) on cellular functions associated with cardiac remodeling in these cells.

MATERIALS AND METHODS

Cell Cultures

Cardiac fibroblasts were isolated from ventricles of adult male Sprague-Dawley rats (~8 wk old, 200–250 g) as described previously (27). Cells were cultured in DMEM (GIBCO, Carlsbad, CA) supplemented with penicillin-streptomycin-fungizone (GIBCO) plus 10% FCS.
FBS in a humidified incubator containing 10% CO₂ at 37°C until confluence and subsequently passaged. Cells at passage 1 or greater were cardiac fibroblasts free of contaminating cells (≥99% purity) (27). Cells from passages 1 through 2 were grown to confluence and then serum deprived for 24 h before treatments, unless otherwise specified.

Cardiomyocytes were isolated from ventricles of 1- to 2-day-old Sprague-Dawley rats as described previously (13) with minor modification. The myocyte-rich layer on a discontinuous Percoll gradient was plated onto uncoated cell culture dishes in DMEM with 10% FBS for 45 min. Myocytes in the nonadherent fraction were resuspended in plating medium (13) and seeded onto gelatin-coated dishes at a density of 1 × 10⁵ cells/cm². After overnight incubation, plating medium was replaced with maintenance medium (13) containing 0.1 mM bromodeoxyuridine (Sigma, St. Louis, MO) for 24 h. After an additional 24-h incubation with serum-free maintenance medium, myocytes were treated as described below. These protocols were approved by the University of California, San Diego Animal Subject Committee for the preparation of cells.

Receptor Binding Assays

Binding assays were performed in duplicate on intact adherent ARCFs grown to confluence in multiwell plates as described previously (10). On the basis of our previous experience (10), cells were incubated with a radioligand, [125I]labeled ANG-(1–7) ([125I]ANG-(1–7)) or [3H]ANG II (Amersham Biosciences), for that 4°C. Specific binding was determined in the presence of an unlabeled ANG (10⁻⁵ M) corresponding to the radiolabeled ligand. After incubation, cells were harvested in ice-cold 10% TCA at 4°C overnight. Cells were washed with cold PBS at 4°C and lysed with 0.2 N NaOH for counting radioactivities. Binding parameters were derived by analyzing the data with GraphPad Prism software (version 3.00, GraphPad Software, San Diego, CA).

Measurement of Cytosolic Ca²⁺ Concentration

Cytosolic Ca²⁺ concentration ([Ca²⁺]cyt) in single ARCFs was measured with the Ca²⁺-sensitive fluorescent indicator fura-2 as previously described (29).

Collagenase-Sensitive [³H]Proline Incorporation Assay

Effects of ANGs on collagen synthesis in ARCFs were assessed by measuring cellular [³H]proline uptake. Cells were treated in serum-free medium with ANGs in the presence of 1 µCi/ml [³H]proline (PerkinElmer, Boston, MA) for 24 h in triplicate. After washing three times with ice-cold PBS, cellular protein was precipitated with ice-cold 10% TCA at 4°C overnight. Cells were washed with 10% TCA twice and dissolved in 0.5 N NaOH at 37°C for 1 h. After neutralization with 0.5 N HCl, collagenase was added at the final concentration of 1 mg/ml in Tris-CaCl₂ buffer (in mM: 50 Tris, 15 CaCl₂, pH 7.6) and incubated 37°C for 1 h. The reaction was terminated by adding ice-cold 10% TCA and centrifuging at 14,500 g for 10 min. The resultant supernatant was used for measurement of incorporated radioactivity with a scintillation counter.

Fibroblast Conditioned Medium

After 24-h serum starvation, ARCFs were washed twice with fresh serum-free medium and stimulated for 6 h in 0.1 ml/cm² of serum-free medium with ANG II (10⁻⁷ M), ANG-(1–7) (10⁻⁵ M), or both after 1-h pretreatment with ANG-(1–7) (10⁻⁷ M). Control and mock fibroblast conditioned medium (CM) were also prepared from unstimulated cells and from dishes without cells, respectively.

[³H]Leucine Incorporation Assay

Effects of ANGs and fibroblast CM on protein synthesis in cardiomyocytes were assessed by measurement of cellular [³H]leucine uptake. Cardiomyocytes were treated on 24-well plates with ANG II (10⁻⁷ M) or ANG-(1–7) (10⁻⁷ M), both after 1-h pretreatment with ANG-(1–7) (10⁻⁷ M) or the fibroblast CM in the presence of [³H]-angiotensin II (10⁻⁶ M) to block effects of carried-over ANGs for 24 h. [³H]leucine (PerkinElmer) was added to each well to a final concentration of 1 µCi/ml for the last 6 h. Incorporated radioactivity was measured as described previously (17).

Northern Blotting

Total RNA was isolated from cultured ARCFs with an RNeasy Mini kit (Qiagen, Valencia, CA). Probes for rat atrial natriuretic factor (ANF), endothelin-1 (ET-1), leukemia inhibitory factor (LIF), and transforming growth factor-β1 (TGF-β1) mRNA were generated by RT-PCR (Tian one tube RT-PCR system; Roche) as described previously (10) and purified with a gel extraction kit (Qiagen). Primers used for RT-PCR were ANF: 5'-CAGCATGCGGCCTCTTTCCTCATC-3' (sense), 5'-GCCCGGCTCTCATGATCATC-3' (antisense); ET-1: 5'-ATCTTCTGGCCTGGACATC-3' (sense), 5'-GCTGATCTGCACA-3' (antisense); LIF: 5'-ATGAAAACTGGCCTCAAC-3' (sense), 5'-CTGGACCACCGCACTAATGACT-3' (antisense); and TGF-β1: 5'-ACTGCTACCACTGGACCATC-3' (sense), 5'-ACTGAGGCGAAGCTGTGA-3' (antisense). Equal amounts of RNA were separated on a 1% MOPS-formaldehyde-agarose gel and transferred to a nylon membrane. RNA was fixed to the membrane by heating at 80°C for 2 h. Prehybridization, preparation of ³²P-labeled probes, hybridization, washing, and visualization of the membrane were performed as described previously (3). The membranes were stripped and reprobed with a cDNA representing nucleotides 2837–4436 of human 28S rRNA to standardize RNA loading.

Statistics

Data are expressed as means ± SE. Comparisons between a standardized group and control were performed by a one-sample t-test. Comparisons between three or more groups were performed by one-way analysis of variance, accompanied by post hoc tests as described in Figs. 1 and 5–7. Statistical significance was defined as P ≤ 0.05.

RESULTS

Characteristics of ANG-(1–7) Binding to ARCFs

Saturation binding assays using ARCFs and [¹²⁵I]ANG-(1–7) demonstrated a dose-dependent increase in specific binding. Receptor density was estimated to be 131 fmol/mg protein, with an affinity of 11.3 nM (Fig. 1A), which was derived by
analyzing the ANG-(1–7) competition curve shown in Fig. 1B. The number of ANG-(1–7) binding sites, estimated on the basis of the number of cells per well, was $10.4 \times 10^3$ per cell. As shown in Fig. 1B, competition assays for $[^{125}\text{I}]$ANG-(1–7) binding revealed dose-dependent inhibition by ANG-(1–7) that fit a model of two-site competition, with the high-affinity site comprising 49 ± 10% of the total specific binding. Analysis of the competition curve showed that ANG-(1–7) competed for the high-affinity sites with an IC$_{50}$ of 1.2 nM (log IC$_{50}$ = −0.7), whereas ANG II competed weakly at the single site with an IC$_{50}$ of 13.5 μM (log IC$_{50}$ = −4.9 ± 1.2), indicating that the affinity of ANG-(1–7) for at least the high-affinity site is much greater than that of ANG II. Binding assays using angiotensin receptor blockers (10$^{-6}$ M) shown in Fig. 1C revealed that ANG-(1–7) binding was significantly inhibited by A-779 and [Sar$^1$,Thr$^8$]-ANG II but not by valsartan or PD-123319. These results demonstrate that specific ANG-(1–7) receptors are present on ARCFs. To determine the effects of ANG-(1–7) on ANG II binding to ARCFs, competition assays for $[^{125}\text{I}]$ANG II binding were performed. Most $[^{3}\text{H}]$ANG II binding to ARCFs was mediated by the AT$_1$ receptors, because binding was almost completely inhibited by valsartan but not by PD-123319 (both 10$^{-6}$ M) (data not shown). Whereas ANG II prevented $[^{3}\text{H}]$ANG II binding in a dose-dependent manner, with an IC$_{50}$ of 9.5 nM (log IC$_{50}$ = −8.0 ± 0.1), ANG-(1–7) competed poorly for the binding, with an IC$_{50}$ of 21 μM (log IC$_{50}$ = −4.7 ± 0.7) (Fig. 1D). These results indicate that the affinity of ANG-(1–7) for AT$_1$ receptors is very much lower than that of ANG II. The ANG-(1–7) competition curves also demonstrate that nanomolar concentrations of ANG-(1–7) compete little with ANG II for the AT$_1$ receptors. Because previous work reported that pretreatment with high concentrations of ANG-(1–7) reduced ANG II binding in VSMCs (2), dose-dependent effects on $[^{3}\text{H}]$ANG II binding of pretreatment for 1 h at 37°C with ANG-(1–7) as well as ANG II were examined in ARCFs. Pretreatment with ANG-(1–7) shifted the competition curve to the left, with an IC$_{50}$ of 2.3 nM (log IC$_{50}$ = −6.6 ± 0.1). Although pretreatment with ANG-(1–7) shifted the competition curve slightly to the left, with an IC$_{50}$ of 8.5 μM (log IC$_{50}$ = −5.1 ± 0.3), nanomolar concentrations of ANG-(1–7) had no significant effects on $[^{3}\text{H}]$ANG II binding (Fig. 1D). These results demonstrate that nanomolar concentrations of ANG-(1–7) interact with specific receptors that are distinct from the AT$_1$ and AT$_2$ receptors on ARCFs and that ANG-(1–7) pretreatment has minimal effect on ANG II binding.

**Effects of ANG-(1–7) on Cellular Functions of ARCFs**

**Effects on $[\text{Ca}^{2+}]_{cyt}$**. To discern differences in function of the specific receptors, effects of ANGs (10$^{-7}$ M) and pretreatment with ANG-(1–7) (10$^{-7}$ M) on $[\text{Ca}^{2+}]_{cyt}$ were examined in ARCFs. As shown in Fig. 2, extracellular application of ANG II induced a transient increase in $[\text{Ca}^{2+}]_{cyt}$, whereas ANG-(1–7) had no effect on $[\text{Ca}^{2+}]_{cyt}$ in ARCFs superfused with 1.8 mM Ca$^{2+}$-containing solution. Neither costimulation nor 1-h pretreatment with ANG-(1–7) altered ANG II-induced increases in $[\text{Ca}^{2+}]_{cyt}$. These results show that ANG II and ANG-(1–7) act on distinct receptors that have different effects on cytosolic Ca$^{2+}$ transients in ARCFs.

**Effects on collagen synthesis**. To determine whether ANG II and ANG-(1–7) have different effects on ARCF functions, changes in collagen synthesis in response to the peptides were
assessed by a collagenase-sensitive [3H]proline incorporation assay. ANG-(1–7) inhibited [3H]proline incorporation in a dose-dependent manner, and the maximal effect was obtained by 10^{-7} M. This inhibitory effect of ANG-(1–7) (10^{-7} M) was not significantly affected by the presence of valsartan (Fig. 3A).

As shown in Fig. 3B, although ANG II (10^{-7} M) increased [3H]proline incorporation by 17 ± 6%, pretreatment with ANG-(1–7) (10^{-7} M) completely reversed the ANG II effects, resulting in a decrease in [3H]proline incorporation by 12 ± 4% compared with nonstimulated control. These results suggest that ANG-(1–7) and ANG II act on distinct receptors to exert opposing effects on collagen synthesis of ARCFs and that pretreatment with ANG-(1–7) could completely inhibit collagen synthesis stimulated by ANG II.

Effects on induction of growth factors. The effects of ANGs (10^{-7} M) on mRNA expression of three growth factors, TGF-β1, ET-1, and LIF, were examined in ARCFs. ANG II induced a maximal 2.2 ± 0.2-fold increase in TGF-β1 mRNA expression at 12 h after stimulation, whereas ANG-(1–7) slightly decreased the mRNA level by 8 ± 1% at 3 h (Fig. 4). However, pretreatment with ANG-(1–7) did not have significant inhibitory effects on ANG II-induced increases in TGF-β1 mRNA expression (data not shown). ANG II induced 3.5 ± 0.2-fold and 14.3 ± 0.7-fold increases in ET-1 and LIF mRNA expression, respectively, whereas ANG-(1–7) induced 60 ± 8% and 47 ± 13% decreases in ET-1 and LIF mRNA expression, respectively (Fig. 5A). These effects of ANG-(1–7) were not significantly affected by the presence of valsartan (Fig. 5B). As shown in Fig. 6, pretreatment with ANG-(1–7) significantly inhibited the ANG II-induced increase in ET-1 and LIF mRNA expression in a time-dependent manner, with maximal reductions of 104 ± 8% and 40 ± 3%, respectively. These results show that ANG II and ANG-(1–7) have different and sometimes opposing effects and that pretreatment with ANG-(1–7) inhibits ANG II-induced upregulation of some growth factors in ARCFs.

Effects of fibroblast CM on cardiomyocyte hypertrophy. Because ANG II has been reported to induce cardiomyocyte hypertrophy through paracrine release of growth factors from cardiac fibroblasts (9, 11, 13, 19), effects of CM from ARCFs treated with ANGs (10^{-7} M) on myocyte hypertrophy were assessed. Figure 7A shows that CM from unstimulated ARCFs (control CM) increased [3H]leucine incorporation and that this effect was significantly increased by ANG II stimulation of fibroblasts. The effect of ANG II-treated CM was significantly reduced by pretreating ARCFs with ANG-(1–7) (10^{-7} M) for 1 h. As shown in Fig. 7B, ANG II-treated CM also induced a 3.5 ± 0.3-fold increase in ANF mRNA expression in cardiomyocytes, whereas this effect was reduced by 39 ± 16% by pretreating ARCFs with ANG-(1–7). Direct treatment of cardiomyocytes with ANG II, ANG-(1–7), or ANG-(1–7) before ANG II did not alter [3H]leucine incorporation or ANF mRNA expression. These results demonstrate that ANG-(1–7) pretreatment of ARCFs inhibits ANG II-induced hypertrophic responses in cardiomyocytes that are mediated by paracrine factors.

DISCUSSION

The local cardiac RAS plays an important role in regulating remodeling of the heart (7). Recent studies demonstrate that ANG-(1–7), a peptide with effects on vascular cells that differ
ANG-(1–7) indicate that ARCFs specifically bind ANG-(1–7) fibroblasts.

mediated by ANG II-stimulated release of factors from cardiac and II-induced stimulatory effects on these functions of ARCFs; collagen synthesis, induction of growth factors, and ANG nase-sensitive \[ 3H\]proline incorporation assay.

Compared with estimates of the AT1 receptors on \[3H\]proline to assess the effects on collagen synthesis by a collage-\[3H\]proline incorporation assay.

However, our inability to perform binding assays with higher concentrations of [125I]ANG-(1–7) because of interference of [3H\]proline binding (\% of total binding), may have affected the accuracy of estimation of the receptor density. The results of competition assays on [125I]ANG-(1–7) binding indicating the existence of high-affinity binding sites for ANG-(1–7) on ARCFs are compatible with previous reports showing the presence of high-affinity binding sites on cultured cells as well as intact tissues from organs such as brain and kidney (20, 23, 24).

Our findings that ANG II competes poorly with [125I]ANG-(1–7) for binding sites on ARCFs whereas ANG-(1–7) is a very weak competitor for [3H]ANG II binding sites strongly suggest that ANG-(1–7) and ANG II interact with separate and distinct receptors on these cells. Recent studies have demonstrated that the Mas protooncogene, originally considered to be an “orphan” G protein-coupled receptor, functions as a receptor for ANG-(1–7) (18, 22). Our preliminary study indicates that ARCFs as well as cardiomyocytes express Mas mRNA (unpublished data). Our findings in the present study are consistent with the possibility that Mas is a receptor for ANG-(1–7) in ARCFs.

Effects of ANG-(1–7) on Cellular Functions of ARCFs

The results of experiments evaluating the effects of the ANG peptides on Ca2+ flux in ARCFs support the concept that ANG II and ANG-(1–7) receptors have distinct signaling. The lack of an effect of ANG-(1–7) pretreatment on ANG II-induced increases in [Ca2+]cyr is also consistent with and reinforces the binding studies, which demonstrated the absence of competition between ANG II and ANG-(1–7) at nanomolar concentrations.

ANG-(1–7) and ANG II had opposite effects on collagen synthesis as assessed by a collagenase-sensitive \[3H\]proline incorporation assay, and ANG II-induced increase in incorporation was significantly inhibited by ANG-(1–7) pretreatment. These results imply that activation of receptors for ANG II and from those of ANG II (20, 23), is generated directly in the heart (1, 30) through an alternative pathway of the RAS involving ACE2 (30). Because cardiac fibroblasts are extensively involved in cardiac remodeling (11, 13, 15, 19, 27), we sought to determine whether ANG-(1–7) interacts with cardiac fibroblasts and affects cellular functions in ways that might influence the remodeling process. Our results provide evidence that 1) ANG-(1–7) binds to receptors on ARCFs that are distinct from the AT1 and AT2 receptors; 2) ANG-(1–7) inhibits collagen synthesis, induction of growth factors, and ANG II-induced stimulatory effects on these functions of ARCFs; and 3) ANG-(1–7) inhibits cardiomyocyte hypertrophy that is mediated by ANG II-stimulated release of factors from cardiac fibroblasts.

**ANG-(1–7) Receptors in ARCFs**

The results of the saturation binding assays using [125I]ANG-(1–7) indicate that ARCFs specifically bind ANG-(1–7). Compared with estimates of the AT1 receptors on ARCFs from a previous report, the number of ANG-(1–7) receptors corresponds to 33% of the AT1 receptor density (4). However, our inability to perform binding assays with higher concentrations of [125I]ANG-(1–7) because of interference of radioligand solution, as well as relatively high nonspecific binding (~50% of total binding), may have affected the accuracy of estimation of the receptor density. The results of competition assays on [125I]ANG-(1–7) binding indicating the existence of high-affinity binding sites for ANG-(1–7) on ARCFs are compatible with previous reports showing the presence of high-affinity binding sites on cultured cells as well as intact tissues from organs such as brain and kidney (20, 23, 24).

Our findings that ANG II competes poorly with [125I]ANG-(1–7) for binding sites on ARCFs whereas ANG-(1–7) is a very weak competitor for [3H]ANG II binding sites strongly suggest that ANG-(1–7) and ANG II interact with separate and distinct receptors on these cells. Recent studies have demonstrated that the Mas protooncogene, originally considered to be an “orphan” G protein-coupled receptor, functions as a receptor for ANG-(1–7) (18, 22). Our preliminary study indicates that ARCFs as well as cardiomyocytes express Mas mRNA (unpublished data). Our findings in the present study are consistent with the possibility that Mas is a receptor for ANG-(1–7) in ARCFs.

**Effects of ANG-(1–7) on Cellular Functions of ARCFs**

The results of experiments evaluating the effects of the ANG peptides on Ca2+ flux in ARCFs support the concept that ANG II and ANG-(1–7) receptors have distinct signaling. The lack of an effect of ANG-(1–7) pretreatment on ANG II-induced increases in [Ca2+]cyr is also consistent with and reinforces the binding studies, which demonstrated the absence of competition between ANG II and ANG-(1–7) at nanomolar concentrations.

ANG-(1–7) and ANG II had opposite effects on collagen synthesis as assessed by a collagenase-sensitive \[3H\]proline incorporation assay, and ANG II-induced increase in incorporation was significantly inhibited by ANG-(1–7) pretreatment. These results imply that activation of receptors for ANG II and

**From those of ANG II (20, 23), is generated directly in the heart (1, 30) through an alternative pathway of the RAS involving ACE2 (30). Because cardiac fibroblasts are extensively involved in cardiac remodeling (11, 13, 15, 19, 27), we sought to determine whether ANG-(1–7) interacts with cardiac fibroblasts and affects cellular functions in ways that might influence the remodeling process. Our results provide evidence that 1) ANG-(1–7) binds to receptors on ARCFs that are distinct from the AT1 and AT2 receptors; 2) ANG-(1–7) inhibits collagen synthesis, induction of growth factors, and ANG II-induced stimulatory effects on these functions of ARCFs; and 3) ANG-(1–7) inhibits cardiomyocyte hypertrophy that is mediated by ANG II-stimulated release of factors from cardiac fibroblasts.

**ANG-(1–7) Receptors in ARCFs**

The results of the saturation binding assays using [125I]ANG-(1–7) indicate that ARCFs specifically bind ANG-(1–7). Compared with estimates of the AT1 receptors on ARCFs from a previous report, the number of ANG-(1–7) receptors corresponds to 33% of the AT1 receptor density (4). However, our inability to perform binding assays with higher concentrations of [125I]ANG-(1–7) because of interference of radioligand solution, as well as relatively high nonspecific binding (~50% of total binding), may have affected the accuracy of estimation of the receptor density. The results of competition assays on [125I]ANG-(1–7) binding indicating the existence of high-affinity binding sites for ANG-(1–7) on ARCFs are compatible with previous reports showing the presence of high-affinity binding sites on cultured cells as well as intact tissues from organs such as brain and kidney (20, 23, 24). Our findings that ANG II competes poorly with [125I]ANG-(1–7) for binding sites on ARCFs whereas ANG-(1–7) is a very weak competitor for [3H]ANG II binding sites strongly suggest that ANG-(1–7) and ANG II interact with separate and distinct receptors on these cells. Recent studies have demonstrated that the Mas protooncogene, originally considered to be an “orphan” G protein-coupled receptor, functions as a receptor for ANG-(1–7) (18, 22). Our preliminary study indicates that ARCFs as well as cardiomyocytes express Mas mRNA (unpublished data). Our findings in the present study are consistent with the possibility that Mas is a receptor for ANG-(1–7) in ARCFs.

**Effects of ANG-(1–7) on Cellular Functions of ARCFs**

The results of experiments evaluating the effects of the ANG peptides on Ca2+ flux in ARCFs support the concept that ANG II and ANG-(1–7) receptors have distinct signaling. The lack of an effect of ANG-(1–7) pretreatment on ANG II-induced increases in [Ca2+]cyr is also consistent with and reinforces the binding studies, which demonstrated the absence of competition between ANG II and ANG-(1–7) at nanomolar concentrations.

ANG-(1–7) and ANG II had opposite effects on collagen synthesis as assessed by a collagenase-sensitive \[3H\]proline incorporation assay, and ANG II-induced increase in incorporation was significantly inhibited by ANG-(1–7) pretreatment. These results imply that activation of receptors for ANG II and
ANG-(1–7) results in induction of opposite effects on some cellular functions of cardiac fibroblasts and that ANG-(1–7) can inhibit some ANG II effects in these cells. In addition, these results suggest that ANG II and ANG-(1–7) may have opposite effects on collagen deposition and cardiac fibrosis during cardiac remodeling.

Cardiac fibroblasts produce a variety of growth factors that are believed to influence remodeling of the heart (11, 13, 15, 19). Generation of factors such as TGF-β1, ET-1, and LIF from cardiac fibroblasts has been shown to be stimulated by ANG II (9, 11, 19). In the present study, ANG-(1–7) was shown to decrease gene expression of these growth factors. In addition, pretreatment with ANG-(1–7) significantly inhibited ANG II-induced increases in gene expression of ET-1 and LIF but not TGF-β1. These results suggest that ANG-(1–7) does not reverse gene expression of all the growth factors stimulated by ANG II but does significantly inhibit induction of some growth factors. Although the mechanisms responsible for the inhibitory effects of ANG-(1–7) were not clarified in the present study, the effects may be mediated by release of secondary factors such as prostanoids or nitric oxide as has been reported in VSMCs (16, 20). The absence of a significant effect of ANG-(1–7) pretreatment on ANG II binding argues against this effect being related to competition for the receptor site or receptor internalization (2).

Our observation that ANG-(1–7) significantly inhibited growth factor gene expression suggests that this peptide might also attenuate ANG II-stimulated hypertrophy of cardiomyocytes that is mediated by release of growth factors from fibroblasts. In this study, CM from ANG II-stimulated fibroblasts increased [3H]leucine incorporation and ANF mRNA expression, both of which are indicators of myocyte hypertrophy. This result is consistent with previous studies (11, 13, 19). Although ANG-(1–7) did not directly affect [3H]leucine incorporation or ANF mRNA expression in myocytes, pretreatment of fibroblasts with this peptide significantly attenuated the hypertrophic response that was induced by CM from ANG II-stimulated fibroblasts. Thus, our findings indicate that ANG II induction of myocyte hypertrophy depends at least partly on the release of substances by cardiac fibroblasts and that this effect is attenuated by ANG-(1–7) pretreatment of fibroblasts.

These results may help provide an explanation for the observations of Loot et al. (14), who found that infusion of ANG-(1–7) in rats in the midst of post-MI cardiac remodeling resulted in a reduction in myocyte cross-sectional area and preservation of cardiac function. Our findings are also compatible with those from a recent study showing that chronic generation of ANG-(1–7) by using an ANG-(1–7)-producing fusion protein attenuates myocyte hypertrophy and induces cardioprotective effects in rats (21).

### Study Limitations and Clinical Implications

Neonatal cardiomyocytes were used in these studies to examine the effects of fibroblast CM on myocyte hypertrophy. There could be differences in the response to fibroblast CM between neonatal and adult myocytes. However, our findings of the inhibitory effects of ANG-(1–7) on myocyte hypertrophy are consistent with the observations of Loot et al. (14) and others (21) who showed an antihypertrophic effect of ANG-(1–7) in the adult rat heart.

Most of our experiments were performed with nanomolar concentrations of ANG peptides. Although plasma levels of these peptides are generally in the picomolar range, interstitial levels in the heart are >100-fold higher (i.e., in the nanomolar range) (6, 7). ANG I and ANG II infusion into the interstitial compartment of the left ventricle of dogs increases ANG-(1–7) levels >400- and 60-fold, respectively, and this corresponds to...
final concentrations of ~10–100 nM (28). In contrast, in vivo evidence that endogenous concentrations of ANG-(1–7) are increased to higher (e.g., micromolar levels) is lacking (2). Because ANG-(1–7) is actually generated in rat myocardium (1) and in the intact and failing human heart (30), the concentrations of ANG-(1–7) used in these experiments appear to be relevant.

Because ANG-(1–7) is enzymatically generated by the degradation of ANG II (26, 30), AT1 receptor antagonists might be able to not only block AT1 receptor-mediated ANG II effects but also intensify ANG-(1–7) effects by increasing ANG-(1–7) levels within the myocardium, as they increase plasma ANG-(1–7) levels (12). In this regard, the results of the present study might provide an additional mechanism to help explain how AT1 antagonists inhibit cardiac remodeling. Our observations also raise the possibility that novel interventions designed to increase ANG-(1–7) levels in the remodeling heart might be effective as a means of limiting remodeling and preventing the future development of heart failure.

In conclusion, our results demonstrate that ARCFs possess specific receptors for ANG-(1–7) that are distinct from the AT1 and AT2 receptors. Evidence that ANG-(1–7) and ANG II have separate effects on ARCF cellular functions suggests that the effects of these peptides on cardiac remodeling might differ. Specifically, our findings raise the possibility that ANG-(1–7) might limit the profibrotic and hypertrophic effects of ANG II.
on cardiac cells. Thus this study suggests that ANG-(1–7) may help regulate cardiac remodeling, and it provides new insights into the complex functions of the cardiac RAS.

ACKNOWLEDGMENTS

We thank Drs. Joan Heller Brown, Wolfgang Dillmann, and Paul A. Insel for their comments.

GRANTS

This study was supported by National Heart, Lung, and Blood Institute Grants RO1-HL-63909 (B. Greenberger) and HL-66012 (J.-X. Yuan), an award from the American Heart Association, Western States Affiliate (0425095Y, M. Iwata), and a charitable contribution from the Joyce and Jesse Spencer Fund.

REFERENCES


