Angiotensin-(1–7) inhibits growth of cardiac myocytes through activation of the mas receptor

E. Ann Tallant, Carlos M. Ferrario, and Patricia E. Gallagher

Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Winston-Salem, North Carolina

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Address for reprint requests and other correspondence: E. Ann Tallant, Hypertension and Vascular Disease Center, Wake Forest Univ. School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157-1032 (e-mail: atallant@wfubmc.edu).

Peptide hormones such as ANG II and endothelin contribute to cardiac remodeling after myocardial infarction by stimulating myocyte hypertrophy and myofibroblast proliferation. In contrast, angiotensin-(1–7) [ANG-(1–7)] infusion after myocardial infarction reduced myocyte size and attenuated ventricular dysfunction and remodeling. We measured the effect of ANG-(1–7) on protein and DNA synthesis in cultured neonatal rat myocytes to assess the role of the heptapeptide in cell growth. ANG-(1–7) significantly attenuated either fetal bovine serum- or endothelin-1-stimulated [3H]leucine incorporation into myocytes with no effect on [3H]thymidine incorporation. [D-Ala7]-ANG-(1–7), the selective ANG type 1–7 (AT1–7) receptor antagonist, blocked the ANG-(1–7)-mediated reduction in protein synthesis in cardiac myocytes, whereas the AT1- and AT2-angiotensin peptide receptors were ineffective. Serum-stimulated ERK1/ERK2 mitogen-activated protein kinase activity was significantly decreased by ANG-(1–7) in myocytes, a response that was also blocked by [D-Ala7]-ANG-(1–7). Both rat heart and cardiac myocytes express the mRNA for the mas receptor, and a 59-kDa immunoreactive protein was identified in both extracts of rat heart and cultured myocytes by Western blot hybridization with the use of an antibody to mas, an ANG-(1–7) receptor. Transfection of cultured myocytes with an antisense oligonucleotide to the mas receptor blocked the ANG-(1–7)-mediated inhibition of serum-stimulated MAPK activation, whereas a sense oligonucleotide was ineffective. These results suggest that ANG-(1–7) reduces the growth of cardiomyocytes through activation of the mas receptor. Because ANG-(1–7) is elevated after treatment with angiotensin-converting enzyme inhibitors or AT1 receptor blockers, ANG-(1–7) may contribute to their beneficial effects on cardiac dysfunction and ventricular remodeling after myocardial infarction.

Cardiac hypertrophy; mitogen-activated protein kinases

Angiotensin-(1–7) [ANG-(1–7)] is an endogenous peptide hormone that produces unique physiological responses that are often opposite to those of the well-characterized angiotensin peptide ANG II (15). The ability of ANG II to increase blood pressure is documented; it is a potent vasoconstrictor, it stimulates thirst and aldosterone release, and inhibition of its production or effect with the use of angiotensin-converting enzyme (ACE) inhibitors or ANG type 1 (AT1) receptor antagonists reduces mean arterial pressure (45). In addition, ANG II stimulates vascular growth as well as hypertrophy in terminally differentiated cells. In contrast, ANG-(1–7) reduces the blood pressure of hypertensive dogs and rats (4, 33), alters renal fluid absorption (11, 19, 22), causes vasodilation (6, 34, 35), and participates in the antihypertensive responses to ACE inhibition or AT1 receptor blockade in hypertensive rats (24, 25). In addition, we showed that ANG-(1–7) reduces vascular growth in vitro and in vivo (17, 42, 44), suggesting that ANG-(1–7) may act as an endogenous regulator of cell growth. Thus ANG-(1–7) opposes both the pressor and proliferative effects of ANG II.

Several recent studies also suggest that ANG-(1–7) participates in the regulation of cardiac function. Averill et al. (2) first reported the presence of intense ANG-(1–7) immunoreactivity within the myocytes in the rat heart as well as a significant increase in ANG-(1–7) immunoreactivity in the myocytes surrounding an ischemic zone. These findings correlate with studies showing the presence of ANG-(1–7) in the venous effluent collected from the canine coronary sinus (38) and the generation of ANG-(1–7) from ANG I and ANG II in the interstitial fluid collected from microdialysis probes placed in the canine left ventricle (52). Recent studies identified a novel enzyme that converts ANG II to ANG-(1–7), ACE2, which plays a critical role in cardiac function (12). We showed an upregulation of ACE2 mRNA by losartan treatment of rats after myocardial infarction (23). The increased ACE2 mRNA correlated with elevated plasma ANG-(1–7) and reversal of cardiac remodeling. In addition, Zisman et al. (54) showed that ANG-(1–7) is made in the intact human heart, and its production was suppressed by an ACE inhibitor, suggesting that a major pathway for the formation of ANG-(1–7) was directly dependent on the availability of ANG II as a substrate. Collectively, these results provide evidence of a role for ACE2 and ANG-(1–7) in the heart as well as in improved cardiac function after treatment with AT1 receptor antagonists.

ANG-(1–7) is a poor competitor of the AT1 or AT2 angiotensin receptor (26, 27, 49), and the majority of responses to ANG-(1–7) are not blocked by AT1 or AT2 receptor antagonists (3, 6, 27, 35). However, many of these responses are inhibited by [D-Ala7]-ANG-(1–7), a modified form of ANG-(1–7) in which prolinate at position 7 is replaced by d-Ala (39, 48), [d-Ala7]-ANG-(1–7) selectively blocks responses to ANG-(1–7), is a poor competitor of the AT1 or AT2 receptor, and does not block pressor or contractile responses to ANG II (5, 16, 39). Santos et al. (40) recently identified the orphan G protein-coupled receptor mas as an ANG-(1–7) receptor. Chinese hamster ovary cells transfected with mas have a high-affinity binding site for 125I-labeled ANG-(1–7), which is competed for by [D-Ala7]-ANG-(1–7), and vasodilatory and renal responses to ANG-(1–7) are attenuated in mas-depleted mice.

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Loot et al. (30) reported that ANG-(1–7) infusion after coronary artery ligation was associated with the preservation of left ventricular function, reversal of cardiac remodeling, and reduction in myocyte size. These results suggest a role for ANG-(1–7) in both the regulation of cardiac dynamics and myocyte growth. We evaluated the effect of ANG-(1–7) on neonatal rat myocyte cell growth and investigated whether the mas receptor mediated this response to gain further insight into the cellular mechanisms responsible for the actions of ANG-(1–7) in cardiac function and remodeling.

**METHODS**

**Materials.** ANG-(1–7), endothelin-1 (ET-1), and [d-Ala²]–ANG-(1–7) were obtained from Bachem California (Torrance, CA). DMEM-F12, FBS, penicillin, streptomycin, and Oligofectamine were obtained from Gibco Invitrogen (Gaithersburg, MD). Phospho-specific antibodies against ERK1/ERK2 were obtained from Cell Signaling (Cambridge, MA). Antisense and sense oligonucleotides to mas were synthesized by the Wake Forest University Cancer Center DNA Synthesis Core. All other reagents were purchased from Sigma Chemical (St. Louis, MO).

**Isolation of neonatal rat cardiac myocytes.** Neonatal rat cardiomyocytes were isolated from the ventricles of Sprague-Dawley rats by proteolytic digestion and differential plating, as previously described (1, 37). Myocytes were collected and maintained in DMEM-F12 containing 10% FBS, 10 μg/ml insulin, 10 μg/ml holo-transferrin, 100 μM bromodeoxyuridine (to prevent proliferation of nonmyocytes), and the antibiotics ampicillin and streptomycin. Before their use, myocytes were incubated for 48 h in the same media in the absence of serum and bromodeoxyuridine (serum-free media). Cells isolated by this protocol routinely showed positive immunoreactive staining for antisarcosmic myosin (1:100 dilutions, Sigma) and little immunoreactivity labeling with antibodies to α-smooth muscle-specific actin, fibronectin, or vimentin (1:100 dilutions; Sigma). All experimental procedures were performed in accordance with guidelines set forth by the Institutional Animal Care and Use Committee.

**Measurement of thymidine and leucine incorporation.** Tritiated thymidine and leucine incorporation into cardiac myocytes was measured in cells in 24-well culture plates. Cells were treated for 48 h in the presence and absence of 1% FBS, 10 mM ET-1, and ANG receptor antagonists as indicated in individual experiments. One microcurie of either [³H]thymidine or [³H]leucine per milliliter of culture medium was added to each well. The incorporation of [³H]thymidine into newly synthesized DNA or [³H]leucine into newly synthesized protein was determined after precipitation of acid-insoluble material with ice-cold 10% trichloroacetic acid. The acid-insoluble material was dissolved in 0.25 N NaOH-0.1% sodium dodecyl sulfate and quantified by liquid scintillation spectrometry.

**Measurement of ERK1/ERK2 activities and Western blot hybridization.** Cells were incubated with 1% FBS for 10 min or preincubated with 100 nM ANG-(1–7) for 6 h followed by a 10-min treatment with 1% FBS. Cell lysates were prepared by washing the cells with PBS (50 mM NaHPO₄ and 0.15 mM NaCl, pH 7.2) containing 0.01 mM NaVO₄ to prevent the dephosphorylation of activated, phosphorylated proteins. Cellular protein was solubilized in lysis buffer (100 mM NaCl, 50 mM l NaF, and 5 mM l EDTA, 1% Triton X-100, and 50 mM l Tris-HCl, pH 7.4) containing 0.01 mM l NaVO₄, 0.1 mM l PMSF, and 0.6 μmol/l leupeptin for 30 min on ice. The supernatant was clarified by centrifugation (12,000 g for 10 min at 4°C), and the protein concentration was measured by the Lowry method (31).

For Western blot hybridization, solubilized proteins (20 μg/well) were separated on 10% polyacrylamide gels with the use of the buffer system of Laemmli and then transferred to polyvinylidene difluoride membranes (Amersham Pharmacia, Piscataway, NJ) by electrophoresis. Nonspecific binding to the membranes was blocked by incubation in 5% Blotto (5% evaporated milk; 0.1% Tween 20 in 50 mmol/l Tris-HCl, pH 7.4; and 50 mmol/l NaCl). Membranes were subsequently probed with a specific antibody to the activated phosphorylated form of the ERK1/ERK2 MAPKs (1:1,000 dilution; Cell Signaling Technology, Beverly, MA) to measure the level of the phosphorylated ERK or with a specific antibody to mas (1:2,000 dilution) to identify the mas receptor, followed by incubation with goat anti-rabbit antibody (1:1,000 dilution) coupled to horseradish peroxidase. Actin (β-actin; Abcam) immunostaining was used as a loading control. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagents and quantified by densitometry.

**Immunocytochemistry.** Myocytes were grown in eight-well chamber slides for immunocytochemical analysis. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated overnight at 4°C with mas antibody purified on a peptide-affinity column. Cells were subsequently incubated with an FITC-coupled goat anti-rabbit antibody (1:200 dilution) and visualized by fluorescence microscopy.

**Measurement of ERK1/ERK2 activities and Western blot hybridization.** RNA was isolated from rat hearts or cultured neonatal myocytes with the use of the TRIzol reagent (GIBCO Invitrogen, Carlsbad, CA) as directed by the manufacturer. The RNA was incubated with RQ1 DNase (Promega, Madison, WI) to eliminate any residual DNA that would amplify during the PCR. The RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Approximately 1 μg of total RNA was reverse transcribed with the use of avian myeloblastosis virus RT in a 20-μl reaction mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in RT buffer. The RT reaction product was heated at 95°C to terminate the reaction. For real-time PCR, 2 μl of the resultant cDNA were added to TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with the mas-specific primer/probe set, and amplification was performed on an ABI 7000 Sequence Detection System. The primer/probe set consisted of forward primer 5’-TTCAATGCATCTCAGCTTTG-3’, reverse primer 5’-GTICTTCGATTTCTCCACCAAA-3’, and probe 5’-FAM-TTCACTCCGCTCATGTTAG-NFQ-3’. The mixtures were heated at 50°C for 2 min and at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate, and 18S ribosomal RNA, amplified with the use of the TaqMan Ribosomal RNA control kit (Applied Biosystems), served as an internal control. The results were quantitated as Cᵢ values, where Cᵢ is defined as the threshold cycle of PCR at which the amplified product is first detected and the values are expressed as the ratio of target to control.

**Transfection of myocytes with antisense oligonucleotides.** Myocytes were transfected with mas sense and antisense oligonucleotides for 4 h at 37°C with the use of Oligofectamine according to the manufacturer’s directions. The sense oligonucleotide was 5’-ATGGACCAATCACAATAATGAC-3’, and the antisense oligonucleotide was 5’-GTATATTGGATATTGCCAT-3’. Cells were incubated before their use for an additional 48 h in OptiMem containing 10% FBS. Transfected cells were treated with 1 μmol/l ANG-(1–7) for 6 h and stimulated with 1% FBS for 10 min. ERK1/ERK2 activity was determined with the use of a phospho-specific antibody as described in Measurement of ERK1/ERK2 activities and Western blot hybridization.

**Statistics.** All data are presented as means ± SE. Statistical differences were evaluated by ANOVA followed by Dunnett’s post hoc test. The criterion for statistical significance was set at P < 0.05.

**RESULTS**

Inhibition of cardiomyocyte cell growth by ANG-(1–7). Cardiac myocytes were isolated from neonatal rat hearts to test the hypothesis that ANG-(1–7) regulates the growth of cardiac cells. Myocytes were incubated in media depleted of serum for

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*References cited in the text are available in the original document.*
48 h and then treated with 1% FBS or 10 nM ET-1 in the presence and absence of ANG-(1–7). Either [3H]thymidine, to measure incorporation into DNA as an indicator of DNA synthesis, or [3H]leucine, as an indicator of protein synthesis, was added to separate myocyte cultures. ANG-(1–7) had no effect on [3H]thymidine incorporation into cardiac myocytes. Both FBS and ET-1 stimulated [3H]leucine incorporation into protein; FBS increased [3H]leucine incorporation by 186.1 ± 37.1% above control (n = cells isolated from 5 litters of neonatal rat pups, in triplicate). ET-1 caused a 48.6 ± 17.2% increase above control (n = cells isolated from 3 litters of neonatal rat pups, in triplicate). Treatment with ANG-(1–7) caused a significant reduction in both serum- and ET-1-stimulated [3H]leucine incorporation into cardiomyocytes, as shown in Fig. 1. Figure 2 shows that [D-Ala7]-ANG-(1–7), the selective AT1–7 receptor antagonist, blocked the inhibitory effects of ANG-(1–7) in serum-stimulated cardiac myocytes. In contrast, blockade of AT1 and AT2 receptors with losartan and PD-123319, respectively, had no effect on the inhibitory actions of ANG-(1–7) on cardiac protein synthesis. These results suggest that ANG-(1–7) inhibits protein synthesis in cardiomyocytes with no effect on DNA production through activation of a [D-Ala7]-ANG-(1–7)-sensitive receptor.

**Reduction in MAPK activity by ANG-(1–7).** Mitogens such as FBS and ET-1 stimulate the MAPKs ERK1 and ERK2 in neonatal rat cardiac myocytes (1, 37). We stimulated ERK1 and ERK2 activities in myocytes by the addition of 1% FBS in the presence and absence of 100 nmol/l ANG-(1–7) to determine whether ANG-(1–7) reduced serum-stimulated MAPK activity. No phospho-ERK1 or -ERK2 activity was detected in unstimulated myocytes, indicating an absence of active MAPKs before stimulation. Immunoreactive phospho-ERK1 and -ERK2 were visualized when 1% FBS was added to myocytes for 10 min, demonstrating that serum stimulates ERK1/ERK2 activities (as shown in Fig. 3 A); preincubation with 100 nM ANG-(1–7) reduced both phospho-ERK1 and -ERK2. Serum-stimulated ERK1/ERK2 MAPK activities were reduced by 10.220.33.1 on May 7, 2017 http://ajpheart.physiology.org/ Downloaded from
significantly reduced by ANG-(1–7) in myocytes, as shown graphically in Fig. 3B. The ANG-(1–7)-mediated reduction in serum-stimulated ERK1/ERK2 activities was blocked by the ANG-(1–7) receptor antagonist [d-Ala7]-ANG-(1–7), indicating that the ANG-(1–7)-inhibition of serum-stimulated MAPK activities was mediated by a [d-Ala7]-ANG-(1–7)-sensitive receptor.

Identification of receptor-mediating effect of ANG-(1–7) in cardiac myocytes. The mRNA for mas, a reported ANG-(1–7) receptor, and a 59-kDa mas immunoreactive protein were detected in both rat hearts and neonatal rat cardiomyocytes, as shown in Fig. 4. Relative quantification of mas mRNA by RT real-time PCR suggests that the adult rat heart contains approximately five times more mas mRNA than do cultured myocytes. An affinity-purified mas antibody was also used to localize the protein in cultured neonatal rat myocytes. As shown in Fig. 5, punctuate staining of the mas receptor was visualized over the cell surface of cultured myocytes, with an absence of any immunoreactivity in the cell nuclei (Fig. 5). In contrast, little immunoreactivity was observed when the affinity-purified antiserum was preabsorbed with 10 μmol/l of the peptide used to generate the antibody. Thus mas mRNA and protein are present in cardiac myocytes.

Antisense oligonucleotides against mas were transfected into neonatal rat myocytes to determine whether mas mediates the inhibition of MAPK activity by ANG-(1–7). After a 48-h incubation, myocytes were treated with ANG-(1–7) in the presence of losartan and ERK1/ERK2 activation by FBS was measured with the use of phospho-specific antibodies. As shown in Fig. 6, ANG-(1–7) caused a 45% reduction in both FBS-stimulated ERK1 and ERK2 activities in cells transfected with a sense oligonucleotide, in agreement with studies in transfected cells as described in Reduction in MAPK activity by ANG-(1–7). In contrast, transfection with a mas antisense oligonucleotide completely blocked the ANG-(1–7)-mediated inhibition of serum-stimulated MAPK activity.

DISCUSSION

Peptide hormones, such as ANG II and ET-1, contribute to cardiac remodeling after myocardial infarction by stimulating...
myocyte growth and myofibroblast proliferation. In these studies, we found that the heptapeptide ANG-(1–7) caused a significant reduction in either serum or ET-1 [3H]leucine incorporation into neonatal rat cardiac myocytes. In contrast, neither serum nor ANG-(1–7) had any effect on [3H]thymidine incorporation into myocytes. These results indicate that ANG-(1–7) reduces the growth of cultured neonatal myocytes. In a previous study by Loot et al. (30), ANG-(1–7) was infused into rats for 8 wk after coronary artery ligation. The infusion with ANG-(1–7) improved cardiac function and was associated with a significant decrease in myocyte size. Our in vitro studies showing a reduction in myocyte cell growth by ANG-(1–7) were conducted in myocytes from neonatal rat pups. Although myocytes from neonatal rats are phenotypically different from myocytes in an adult rat, our studies in neonatal rat myocytes show a similar reduction in myocyte growth.

ANG-(1–7) also caused a significant reduction in serum-stimulated ERK1/ERK2 activities. Tallant and Clark (47) previously showed that ANG-(1–7) inhibits ANG II or PDGF stimulation of ERK1 and ERK2 in vascular smooth muscle cells (VSMCs). Furthermore, Tallant and Gallagher (50) recently reported that the ANG-(1–7)-mediated inhibition of MAPK signaling in VSMCs was prevented by pretreatment with either a serine/threonine or tyrosine phosphatase inhibitor, suggesting that ANG-(1–7) upregulates a protein phosphatase(s) to inhibit ERK1/ERK2 activities. ANG-(1–7) may reduce ERK1/ERK2 activity in cardiac myocytes by stimulating or inducing the expression of an MAPK phosphatase. Transfection of MAPK phosphatase-1 into neonatal cardiac myocytes inhibited the expression of atrial natriuretic factor, ventricular myosin light chain 2, and β-myosin heavy chain in response to the hypertrophic agonists phenylephrine or ET-1 (18). In addition, constitutive expression of MAPK phosphatase-1 in cultured myocytes with the use of adenovirus-mediated gene transfer blocked the activation of ERK1/ERK2 and prevented agonist-induced hypertrophy (7). Alternatively, ANG-(1–7) could inhibit MAPK activity in cardiomyocytes by a reduction in MAPK-ERK1/2 (MEK1/2) activity.

The MAPKs ERK1 and ERK2 are activated by hypertrophic agonists in cultured cardiac myocytes (1, 9, 43). The transfection of constitutively active MEK1 augmented atrial natriuretic factor promoter activity in cultured myocytes, whereas a dominant-negative MEK1 construct attenuated its activity (20). Furthermore, the MEK1 inhibitor PD-98059 reduced agonist-induced natriuretic promoter activity in cardiac cells (29). Transgenic mice containing an activated MEK1 cDNA exhibited a phenotype of compensated cardiac hypertrophy (8). This is consistent with the hypertrophic response observed in cardiomyocytes infected with an activated MEK1-encoding adenovirus (8, 51). Glennon et al. (21) used antisense oligonucleotides to reduce p42 and p44 (ERK2 and ERK1) by 44% and 60% in cardiac myocytes; this resulted in significant reductions in cardiac myocyte morphology and gene expression that did not require total depletion of MAPK. Collectively, these results provide strong evidence that activation of the MEK1/ERK1/ERK2 pathway plays a critical role in myocyte growth. Because ANG-(1–7) reduced mitogen-stimulated ERK1/ERK2 activities in cultured myocytes, our results are consistent with a role for the heptapeptide in regulating myocyte growth.

Both the ANG-(1–7)-mediated reduction in [3H]leucine incorporation and the ERK1/ERK2 signaling were blocked by the ANG-(1–7)-selective receptor antagonist [n-Ala2]-ANG-(1–7). These results suggest that the antigurowth response to ANG-(1–7) is mediated by an AT1–7 receptor. Santos et al. (40) demonstrated that the orphan G protein-coupled receptor mas is an ANG-(1–7) receptor. We recently reported that antisense oligonucleotides and small interfering RNAs to mas inhibited the antiproliferative response to ANG-(1–7) in VSMCs (46). We showed that mas mRNA and protein are present in the rat heart as well as in cardiac myocytes isolated from the neonatal rat heart. The antibody to mas labeled a 59-kDa protein, suggesting the presence of a glycosylated form of the mas receptor in the rat heart and cultured cardiac myocytes. Furthermore, we show that an antisense oligonucleotide to the mas receptor blocks the stimulation of cardiomycocyte serum-stimulated ERK1/ERK2 activities. This same antisense oligonucleotide also prevented the ANG-(1–7)-mediated inhibition of MAPK activity in VSMCs and reduced mas immunoreactivity to 69.9 ± 8.8% (unpublished observation). These results suggest that mas mediates the antigurowth response to ANG-(1–7) in cardiac myocytes.
Several lines of evidence suggest that ANG II plays a critical role in mediating cardiac hypertrophy through direct effects on contractility, induction of growth-promoting genes, increased protein synthesis, and cell growth (41). In the mature heart, ANG II causes cardiac hypertrophy independent of its effect on blood pressure, whereas blockade of the renin-angiotensin system attenuates or reverses the molecular and cellular adaptations to pressure overload (13, 36). In contrast, ANG-(1–7) attenuates the development of heart failure postmyocardial infarction (30) as well as acts as an antihypertrophic factor during myocardial ischemia-reperfusion (38). Because ANG-(1–7) reduces the agonist-mediated increase in protein synthesis and MAPK signaling in cultured myocytes and because plasma ANG-(1–7) levels are increased after treatment with ACE inhibitors or AT1 receptor blockers (10, 28, 32), ANG-(1–7) may participate in the improvement in cardiac function.

In conclusion, we showed that ANG-(1–7) inhibits protein synthesis in cardiac myocytes through activation of the cardiac mas receptor and inhibition of the MAPK pathway. ANG-(1–7) immunoreactivity is present in the heart (2) along with a novel enzyme that generates ANG-(1–7) from ANG II, ACE2 (14, 53, 54). Because we showed an increase in ACE2 mRNA and ANG-(1–7) in the heart after blockade of AT1 receptors that correlates with improved cardiac function after coronary artery ligation (2, 23), our results suggest that ANG-(1–7) may contribute to the beneficial effects of AT1 receptor blockers on cardiac dysfunction and ventricular remodeling after myocardial infarction.

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