

## Angiotensin-(1–7) activates growth-stimulatory pathways in human mesangial cells

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**Zimpelmann J, Burns KD.** Angiotensin-(1–7) activates growth-stimulatory pathways in human mesangial cells. *Am J Physiol Renal Physiol* 296: F337–F346, 2009. First published December 3, 2008; doi:10.1152/ajprenal.90437.2008.—Angiotensin-(1–7) [Ang-(1–7)] is generated in part via ACE2-dependent degradation of angiotensin II (ANG II). In proximal tubular cells, Ang-(1–7) inhibits ANG II-stimulated phosphorylation of the mitogen-activated protein kinases (MAPKs) p38, extracellular signal-related kinase (ERK1/ERK2), and c-jun N-terminal kinase (JNK), suggesting that Ang-(1–7) protects against ANG II-mediated tubulointerstitial injury. We determined the effect of Ang-(1–7) on signaling and growth responses in cultured human mesangial cells. Ang-(1–7) increased phosphorylation of p38, ERK1/ERK2, and JNK MAPKs, which was blocked by the Ang-(1–7) antagonist A-779. Neither the AT<sub>1</sub> receptor antagonist losartan, nor the AT<sub>2</sub> antagonist PD123319 affected specific binding of [<sup>125</sup>I]Ang-(1–7) or Ang-(1–7)-stimulated p38 phosphorylation. Ang-(1–7) increased cell arachidonic acid release, an effect blocked by A-779. The p38 MAPK antagonist SB202190 completely prevented Ang-(1–7)-stimulated release of arachidonic acid, whereas inhibitors of ERK or JNK had no effect. Ang-(1–7) significantly enhanced DNA synthesis and increased production of transforming growth factor-β1 (TGF-β1), fibronectin, and collagen IV. Both A-779 and SB202190 blocked the Ang-(1–7)-stimulated increases in TGF-β1, fibronectin, and collagen IV. These data indicate that Ang-(1–7) activates MAPK phosphorylation via binding to a specific receptor in human mesangial cells. Stimulation of p38 MAPK phosphorylation by Ang-(1–7) leads to release of arachidonic acid and production of TGF-β1 and extracellular matrix proteins. We conclude that Ang-(1–7) exerts growth-stimulatory effects in human mesangial cells.

renin-angiotensin system; glomerulus; receptor Mas; extracellular matrix protein; transforming growth factor-β1

ACTIVATION OF THE RENIN-ANGIOTENSIN system (RAS) is associated with glomerular and tubular cell injury in chronic kidney diseases, including diabetic nephropathy. Within kidney cells, the octapeptide product of the RAS, angiotensin II (ANG II), stimulates a variety of signaling pathways linked to increased cell growth, activation of NADPH oxidase, and production of extracellular matrix proteins, via binding to its AT<sub>1</sub> receptor. In mesangial cells, binding of ANG II to AT<sub>1</sub> receptors stimulates protein synthesis and cell hypertrophy, via release of arachidonic acid and reactive oxygen species, and activation of Akt/PKB and the mitogen-activated protein kinase (MAPK) extracellular-signal-regulated kinases 1 and 2 (ERK1/ERK2) (10–12). In mesangial cells, ANG II also stimulates the production of the profibrotic cytokine transforming growth fac-

tor-β1 (TGF-β1), which is thought to be a critical mediator of progressive glomerulosclerosis (19).

Angiotensin-converting enzyme 2 (ACE2) was cloned in 2000 and found to be a homolog of ACE, which is not blocked by ACE inhibitors (6, 35). ACE2 is a monocarboxypeptidase that is highly expressed in the kidney and mediates the degradation of ANG II to the heptapeptide angiotensin-(1–7) [Ang-(1–7)]. ACE2 can also convert ANG I to angiotensin-(1–9), which can be metabolized to Ang-(1–7) by ACE (6). Male mice with gene deletion of ACE2 develop glomerulosclerosis at 12 mo of age, associated with glomerular MAPK activation (24). These effects are blocked by AT<sub>1</sub> receptor antagonism (24), suggesting that the renoprotective effects of ACE2 are mediated by a reduction in glomerular ANG II and decreased AT<sub>1</sub> receptor activation. Treatment of *db/db* diabetic mice with an inhibitor of ACE2 causes increased albuminuria and glomerular histological injury (38). Similarly, in ACE2 gene-knockout mice that are diabetic, accelerated glomerular injury occurs, and this is reversed by AT<sub>1</sub> receptor antagonism (37). Although these data suggest a prominent role for reduction in AT<sub>1</sub> receptor activity in mediating the protective effects of ACE2, the possible independent effects of ACE2-mediated production of Ang-(1–7) have not been studied.

In several tissues, Ang-(1–7) exerts vasodilatory and anti-proliferative properties, opposing the effects of ANG II (3, 27). Ang-(1–7) binds to a distinct plasma membrane G protein-coupled receptor, identified as the Mas protein (28). Impaired cardiac function and enhanced cardiac collagen production occur in receptor Mas-knockout mice, consistent with a cardioprotective role for Ang-(1–7) (29). However, the signaling pathways associated with Ang-(1–7) receptor activation are incompletely characterized. In proximal tubular cells, we have shown that Ang-(1–7) blocks ANG II-stimulated activation of the MAPKs p38, ERK1/ERK2, and c-jun N-terminal kinase (JNK), an effect reversed by a specific Ang-(1–7) antagonist (32).

The purpose of the present studies was to examine the effects of Ang-(1–7) on cell signaling and growth responses in mesangial cells. We predicted that, as in proximal tubule, Ang-(1–7) would inhibit MAPK activation associated with AT<sub>1</sub> receptor stimulation, via binding to its distinct receptor. Our results, however, indicate that Ang-(1–7) rapidly activates MAPKs in these cells, associated with stimulation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and release of arachidonic acid. Moreover, exposure of cells to Ang-(1–7) for 72 h stimulates DNA synthesis, as well as production of TGF-β1 and the extracellular matrix proteins fibronectin and collagen IV.

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## MATERIALS AND METHODS

**Cell culture.** A human mesangial cell line (ScienCell Research Laboratories, Carlsbad, CA) was used for all studies. The cells were grown on plastic tissue culture dishes at 37°C in a humidified environment of 5% CO<sub>2</sub>-95% air in mesangial cell medium supplied by the manufacturer. The mesangial cell medium contained 2% fetal bovine serum, 1% penicillin/streptomycin solution, and a cell growth supplement supplied by the manufacturer, consisting of essential and nonessential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, and trace minerals. The medium is bicarbonate-buffered and kept at pH 7.4. Cells were used for experiments at passages 2–7. Cells were rendered quiescent 24 h before experimentation by removal of serum and growth factor additives.

**Western blotting.** Human mesangial cells that were rendered quiescent were incubated for various times with agonists [Ang-(1-7), ANG II, obtained from Bachem Bioscience, King of Prussia, PA], followed by immunoblot assays for the MAPKs p38, ERK1/ERK2, and JNK. In some experiments, cells were preincubated for 15–30 min with the AT<sub>1</sub> receptor antagonist losartan (10<sup>-6</sup>–10<sup>-5</sup> M, Merck Research Laboratories, Rahway, NJ), the AT<sub>2</sub> receptor antagonist PD 123319 (10<sup>-6</sup>–10<sup>-5</sup> M, Sigma-Aldrich, St. Louis, MO), the Ang-(1-7) antagonist D-Ala<sup>7</sup>-Ang-(1-7) (A-779, 10<sup>-6</sup>–10<sup>-5</sup> M, Bachem Bioscience), the p38 MAP kinase antagonist SB202190 (Sigma-Aldrich), the ERK antagonist PD98059 (Sigma), or the JNK antagonist SP600125 (Toocris Bioscience, Ellisville, MO) before incubation with Ang-(1-7).

Western blotting was performed as we have previously described (32). Briefly, after stimulation cells were lysed in a buffer consisting of 62.5 mM Tris·HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, 50 mM DTT, and 0.01% wt/vol bromophenol blue. The lysate was then sonicated for 5 s and boiled for 5 min, followed by centrifugation at 12,000 g for 5 min to remove insoluble debris. After quantification of proteins, equal amounts of protein lysates (10 µg) were run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada). The membranes were blocked with 5% skim milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were then incubated for 16 h at 4°C with a 1:1,000 dilution of anti-phosphospecific antibodies to p38 MAPK (Cell Signaling Technology, New England Biolabs, Pickering, ON, Canada), ERK1/ERK2 (Cell Signaling Technology), and JNK (Cell Signaling Technology). The p38 antibody recognizes the α-, β-, γ-, and δ-isomers of p38, as per the manufacturer. In some experiments, the primary antibody was a rabbit polyclonal antibody to the receptor Mas, reactive to the human protein (Novus Biologicals, Littleton, CO). In other experiments, cells were incubated with Ang-(1-7) for up to 72 h, followed by immunoblotting for fibronectin on samples of the conditioned media. For these experiments, membranes were incubated for 16 h at 4°C with a 1:5,000 dilution of rabbit anti-human fibronectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with primary antibodies, membranes were incubated with 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibody (GE Health Care Bio-Sciences, Baie d'Urfe, Quebec, Canada) and then washed. Proteins were detected by enhanced chemiluminescence (ECL; GE Health Care Bio-Sciences) on Hyperfilm (GE Health Care Bio-Science). Prestained standards were used as molecular weight markers (Bio-Rad). To control for protein loading, all membranes were stripped and reprobed with antibodies to total p38, ERK1/ERK2, and JNK MAPKs (Cell Signaling Technology). Signals for phosphorylated MAPK proteins on Western blots were quantified by densitometry and corrected for total protein levels, using an image-analysis software program (Kodak Densitometer 1S440CF).

**[<sup>125</sup>I]Ang-(1-7) binding.** To determine specific binding of Ang-(1-7), human mesangial cells were grown to confluence in 24-well plastic dishes and incubated at 4°C in PBS buffer with 0.5% albumin (BSA,

Sigma-Aldrich), 1 mM EDTA, 0.25 mM phenanthroline, supplemented with 0.1 nM [<sup>125</sup>I]Ang-(1-7) (1,794 Ci/mmol, Phoenix Pharmaceuticals, Burlingame, CA), essentially as described (40). Cells in some wells were also incubated with either Ang-(1-7), A-779, losartan, PD123319, or ANG II, all at 10<sup>-6</sup> M. Experiments indicated that significant specific binding of [<sup>125</sup>I]Ang-(1-7) occurred in human mesangial cells even after 1-h incubation at 4°C (*n* = 4). The results are reported for cells incubated with the radiolabel for 4 or 12 h at 4°C. At the end of incubation, the buffer was removed and cells were washed four times with ice-cold PBS with 0.5% BSA, solubilized in 0.25 N NaOH-0.1% SDS, and cell-associated radioactivity was measured in a gamma counter. All binding experiments were performed in duplicate.

**Arachidonic acid release assay.** PLA<sub>2</sub> activity was measured by release of [<sup>3</sup>H]arachidonic acid as described (23). Briefly, cells were loaded with [<sup>3</sup>H]arachidonic acid (0.5 µCi/ml media, GE Health Care Bio-Sciences), added to the culture medium, for 24 h. The medium was then removed, and the cells were washed four times with isotope-free Hanks' buffer and then incubated in fresh medium with or without Ang-(1-7). Antagonists were added 30 min before the labeled medium was initially removed. Following addition of fresh medium, aliquots were removed at 15 and 30 min, to measure the release of [<sup>3</sup>H]arachidonic acid from the cells, by liquid scintillation spectrometry. After 30 min, the medium was discarded and the cells were lysed in 0.25 M NaOH with 0.1% SDS for measurement of cell-associated [<sup>3</sup>H]arachidonic acid. In some experiments, cells were preincubated with the PLA<sub>2</sub> antagonist methyl arachidonyl fluoro-

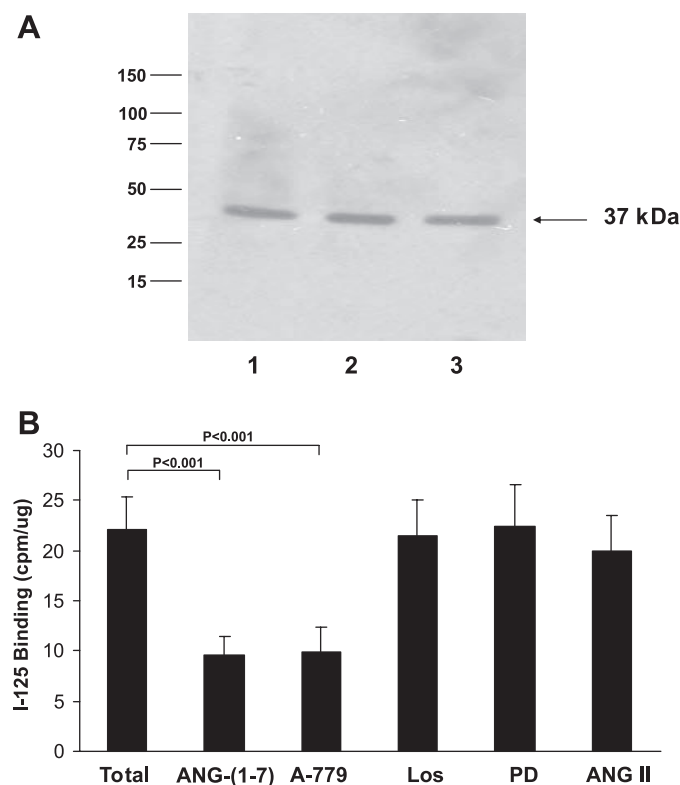


Fig. 1. Human mesangial cells express the receptor Mas and display specific binding for [<sup>125</sup>I]Ang-(1-7). A: representative immunoblot is shown (*n* = 3), showing expression of the 37-kDa Mas protein in primary cultures of rat proximal tubular cells (lane 1), human proximal tubular cells (lane 2), and human mesangial cells (lane 3). Each lane contains 10 µg protein. Molecular weight standards are indicated on the left. B: graph depicts binding of [<sup>125</sup>I]Ang-(1-7) (0.1 nM) in human mesangial cells, in the absence (Total) or presence of Ang-(1-7), A-779, losartan (Los), PD123319 (PD), or ANG II, all at 10<sup>-6</sup> M; *n* = 6 (4 experiments at 4-h incubation, 2 experiments at 12-h incubation).

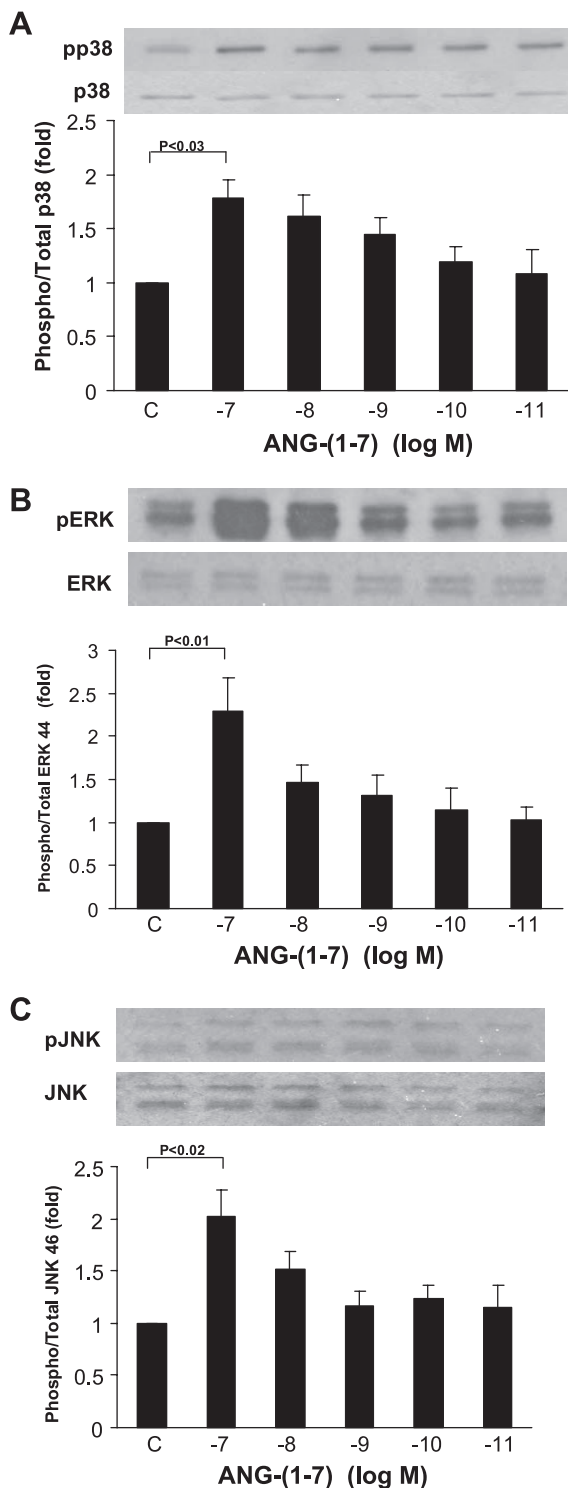


Fig. 2. Concentration-dependent stimulation of MAPK by Ang-(1-7) in human mesangial cells. Effect of Ang-(1-7) ( $10^{-11}$  to  $10^{-7}$  M) on phosphorylation of p38 (A;  $n = 6$ ), ERK (p44) (B;  $n = 5$ ), and JNK (p46; C;  $n = 4$ ). MAPK is shown compared with control (C). Similar results were obtained for p42 ERK and p54 JNK (not shown). Results are presented as the ratio of phosphorylated to nonphosphorylated MAPK protein. Above each graph is a representative immunoblot, with the phosphorylated MAPK at the *top* and the nonphosphorylated MAPK at the *bottom*, serving as a control for protein loading.

phosphonate (MAFP; Cedarlane Laboratories, Burlington, ON, Canada). Results are expressed as the percentage of the total cell-associated [ $^3$ H]arachidonic acid released into the medium. All experiments were performed in duplicate.

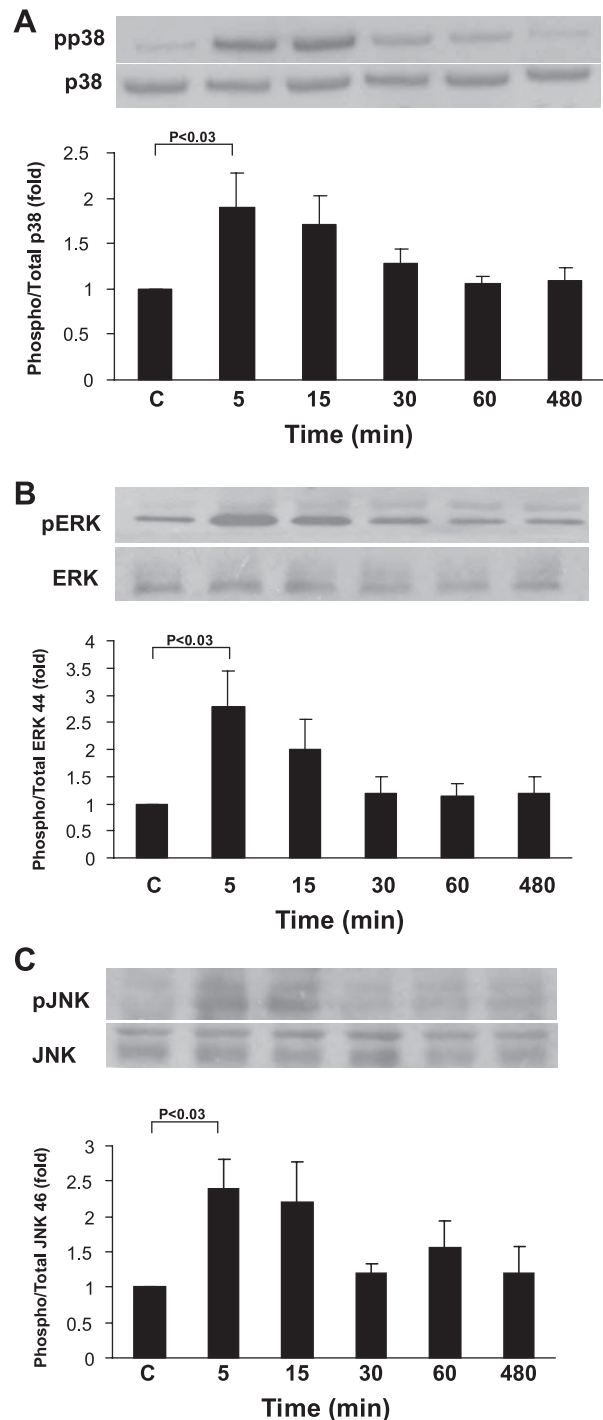


Fig. 3. Time course for stimulation of MAPK by Ang-(1-7) in human mesangial cells. Effect of incubation of cells with Ang-(1-7) ( $10^{-7}$  M, 5–480 min) on phosphorylation of p38 (A;  $n = 5-7$ ), ERK (p44; B;  $n = 5$ ), and JNK (p46; C;  $n = 4$ ) is shown, compared with control (C). Similar results were obtained for p42 ERK and p54 JNK (not shown). Results are presented as the ratio of phosphorylated to nonphosphorylated MAPK protein. Above each graph is a representative immunoblot, with the phosphorylated MAPK at the *top* and the nonphosphorylated MAPK at the *bottom*, serving as a control for protein loading.



**TGF- $\beta$ 1 ELISA.** Cells were incubated for 72 h with agonists, and aliquots of the culture media were collected. TGF- $\beta$ 1 was activated by treating the media with 1 N HCl for 10 min at room temperature, followed by neutralization with 1.2 N NaOH/0.5 M HEPES. The levels of TGF- $\beta$ 1 were determined by a quantitative sandwich enzyme immunoassay using a commercial TGF- $\beta$ 1 ELISA kit, according to the manufacturer's instructions (R&D Systems, TWC Biosearch International, Hong Kong, China). Results are reported as picograms TGF- $\beta$ 1 per milliliter per microgram protein.

**Collagen IV ELISA.** After incubation of mesangial cells for 72 h in the presence or absence of Ang-(1-7) or other agonists, collagen IV was measured in the cell culture supernatant, using a commercial ELISA kit (Chemicon, Temecula, CA). Results are reported as picograms collagen IV per milliliter per microgram protein.

**Measurement of DNA synthesis.** DNA synthesis was measured by [ $^3$ H]thymidine incorporation, as we have previously performed (40). Human mesangial cells were seeded onto 24-well culture plates and at 50–60% confluence were changed to media containing no serum, with or without Ang-(1-7) ( $10^{-7}$  M), A-779 ( $10^{-6}$  M), losartan ( $10^{-6}$  M), or PD123319 ( $10^{-6}$  M) for 70 h, followed by labeling for 2 h with 0.5  $\mu$ Ci/ml of [ $^3$ H]thymidine (Amersham). The cells were then washed four times with ice-cold PBS plus 0.5% BSA and lysed in 0.25 N NaOH with 0.1% SDS. DNA-incorporated thymidine was counted by scintillation spectrometry. All experiments were performed in duplicate.

**Measurement of cAMP.** Quiescent mesangial cells were incubated in medium supplemented with isobutyl-methylxanthine (IBMX;  $10^{-4}$  M) and 0.5% BSA, along with agonists for 30 min. The culture medium was then aspirated and replaced with ice-cold 10% TCA (vol/vol). After 30 min, the samples were extracted four times with four volumes of water-saturated ether. The pH of the samples was adjusted to 7.0 with Tris·HCl. Sample aliquots were assayed for cAMP, using a radioligand competitive binding assay kit (GE Health Care Bio-Sciences). Experiments were performed in duplicate.

**Measurement of cytosolic calcium.** For measurement of cytosolic calcium concentration, cells were grown on glass coverslips and incubated with 5  $\mu$ M fura 2-acetoxymethyl ester (fura 2-AM) in the presence of 0.005% Pluronic-F127 for 60 min at 37°C, as per our previous studies (40). Cells were then mounted in a Stallion high-speed digital workstation microscope (Carl Zeiss Canada, Toronto, ON, Canada) and continuously perfused at 37°C with a solution consisting of (in mM) 105 NaCl, 24 NaHCO $_3$ , 2 Na $_2$ HPO $_4$ , 5 KCl, 1.0 MgSO $_4$ , 1.5 CaCl $_2$ , 4 lactic acid, 5 glucose, 1 alanine, and 10 HEPES (pH 7.3) as well as 0.2% BSA, with or without agonists. Fluorescence was measured from dual monochromators, set at 340 and 380 nm, and cytosolic calcium was recorded continuously by photon counting, as the fluorescence emission intensity ratio from 340- and 380-nm excitation (Slidebook Software for Stallion, version 4.1, Intelligent Imaging Innovations, Denver, CO).

**Statistical analysis.** Results are presented as means  $\pm$  SE. Significance, considered as  $P < 0.05$ , was determined by ANOVA, with Bonferroni correction, in all cases involving multiple comparisons.

## RESULTS

**Human mesangial cells express the receptor Mas and specific binding for Ang-(1-7).** Initial experiments were performed to determine whether human mesangial cells express protein for Mas, identified as the receptor for Ang-(1-7) (28). Immunoblot assay revealed a single protein band of  $\sim$ 37 kDa, confirming expression of the receptor Mas in these cells (Fig. 1A). As shown in Fig. 1B, binding of [ $^{125}$ I]Ang-(1-7) to human mesangial cells was significantly inhibited in the presence of unlabeled Ang-(1-7) or A-779 but was not affected by either the AT $_1$  antagonist losartan, the AT $_2$  antagonist PD123319, or ANG II, all at  $10^{-6}$  M. A similar degree of inhibition of [ $^{125}$ I]Ang-(1-7)

specific binding was observed with Ang-(1-7) ( $10^{-7}$  M), compared with  $10^{-6}$  M Ang-(1-7) (65 vs. 70%, respectively,  $n = 2$ ). These studies confirm the presence of a specific binding site for Ang-(1-7) on these cells, which is distinct from AT $_1$  or AT $_2$  ANG II receptors.

**Effect of Ang-(1-7) on cAMP and cytosolic calcium.** Ang-(1-7) increases cAMP production in inner medullary collecting duct cells (17). In human mesangial cells, Ang-(1-7) ( $10^{-7}$  M) had no effect on cAMP levels, whereas the activators of adenylate cyclase forskolin and cicaprost, used as positive controls, caused significant increases (not shown). In rat mesangial cells, Ang-(1-7) has been reported to inhibit intracellu-

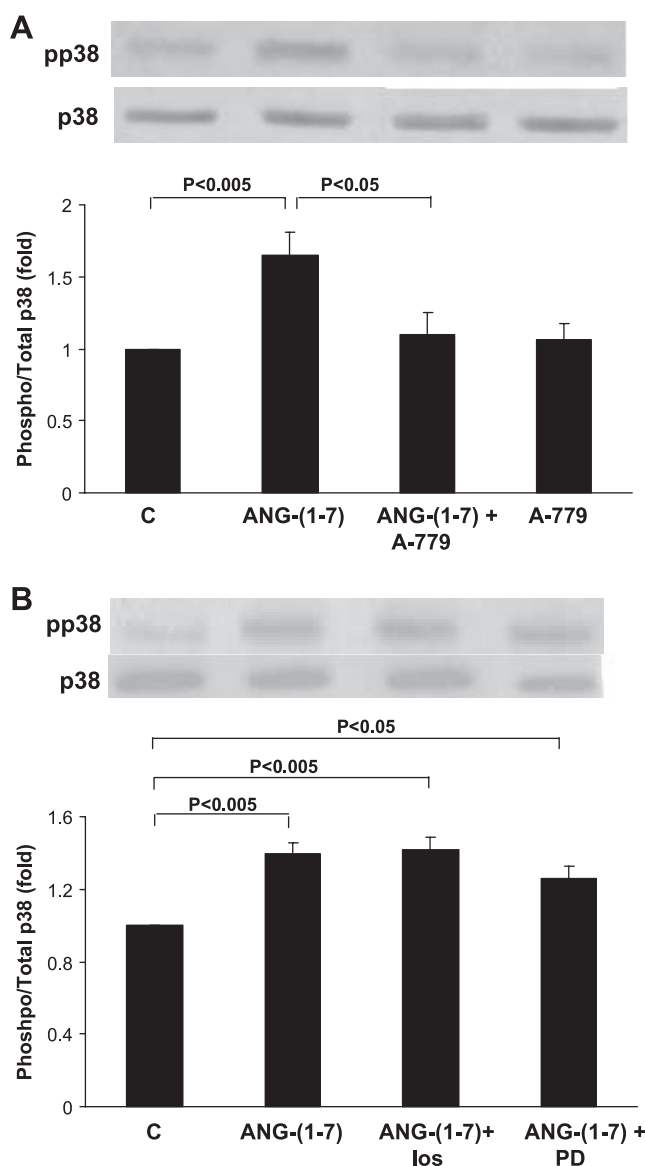


Fig. 4. Effect of A-779, AT $_1$ , and AT $_2$  receptor antagonists on Ang-(1-7)-stimulated phosphorylation of p38 MAPK in human mesangial cells. **A:** graph depicting effect of Ang-(1-7) ( $10^{-7}$  M) with or without A-779 ( $10^{-5}$  M) on p38 MAPK phosphorylation.  $P$  values are indicated;  $n = 4-7$ . **B:** graph depicting the effect of losartan (los;  $10^{-5}$  M) or PD123319 (PD;  $10^{-5}$  M) on Ang-(1-7)-stimulated p38 MAPK phosphorylation ( $10^{-7}$  M).  $P$  values are indicated;  $n = 5$ . For **A** and **B**, results are presented as the ratio of phosphorylated p38 to total p38 in arbitrary units. Representative blots are depicted above each graph, showing phosphorylated p38 (pp38) and total p38 (p38).

lar calcium increases induced by ANG II (5). In human mesangial cells, ANG II ( $10^{-7}$  M) caused a significant increase in cytosolic calcium, which was inhibited by losartan, but not by PD123319 (not shown). In contrast, Ang-(1-7) ( $10^{-7}$  M) had no effect on basal calcium levels, nor did it affect increases in intracellular calcium caused by ANG II (not shown).

**Effect of Ang-(1-7) on MAPK phosphorylation.** Immunoblot analysis was performed to determine the effect of Ang-(1-7) on phosphorylation of p38, ERK1/ERK2 (p44, p42), and JNK (p54, p46) MAPK. Ang-(1-7) caused a concentration-dependent stimulation of phosphorylation of all MAPKs, with maximal stimulation at  $10^{-7}$  M (Fig. 2). Stimulation of MAPK phosphorylation occurred early, with the maximal effect after 5-min incubation with Ang-(1-7) (Fig. 3).

To determine whether the stimulatory effects of Ang-(1-7) on MAPK phosphorylation involved binding to the receptor Mas, cells were preincubated with the Ang-(1-7) antagonist A-779 ( $10^{-5}$  M) before 5-min exposure to Ang-(1-7) ( $10^{-7}$

M). As shown in Fig. 4A, A-779 significantly inhibited Ang-(1-7)-stimulated p38 MAPK phosphorylation but had no effect alone on p38 phosphorylation. Furthermore, neither the AT<sub>1</sub> receptor antagonist losartan nor the AT<sub>2</sub> antagonist PD123319 inhibited p38 phosphorylation induced by Ang-(1-7) (Fig. 4B). Neither losartan ( $10^{-5}$  M) nor PD123319 ( $10^{-5}$  M) had any inhibitory effect on stimulation of phosphorylation of ERK1/ERK2 or JNK by Ang-(1-7) (not shown). Ang-(1-7)-stimulated phosphorylation of ERK1/ERK2 and JNK was also blocked by preincubation with A-779 (Fig. 5).

In proximal tubular cells, Ang-(1-7) blocks ANG II-stimulated p38 MAPK phosphorylation (32). In human mesangial cells, administration of ANG II ( $10^{-7}$  M) for 5 min stimulated p38 MAPK, an effect blocked by losartan, but not by PD123319 (Fig. 6A). The degree of stimulation of p38 MAPK phosphorylation by ANG II was similar to that observed with Ang-(1-7) at the equivalent concentration. However, Ang-(1-7) had no inhibitory effect on stimulation of p38 MAPK by ANG II,

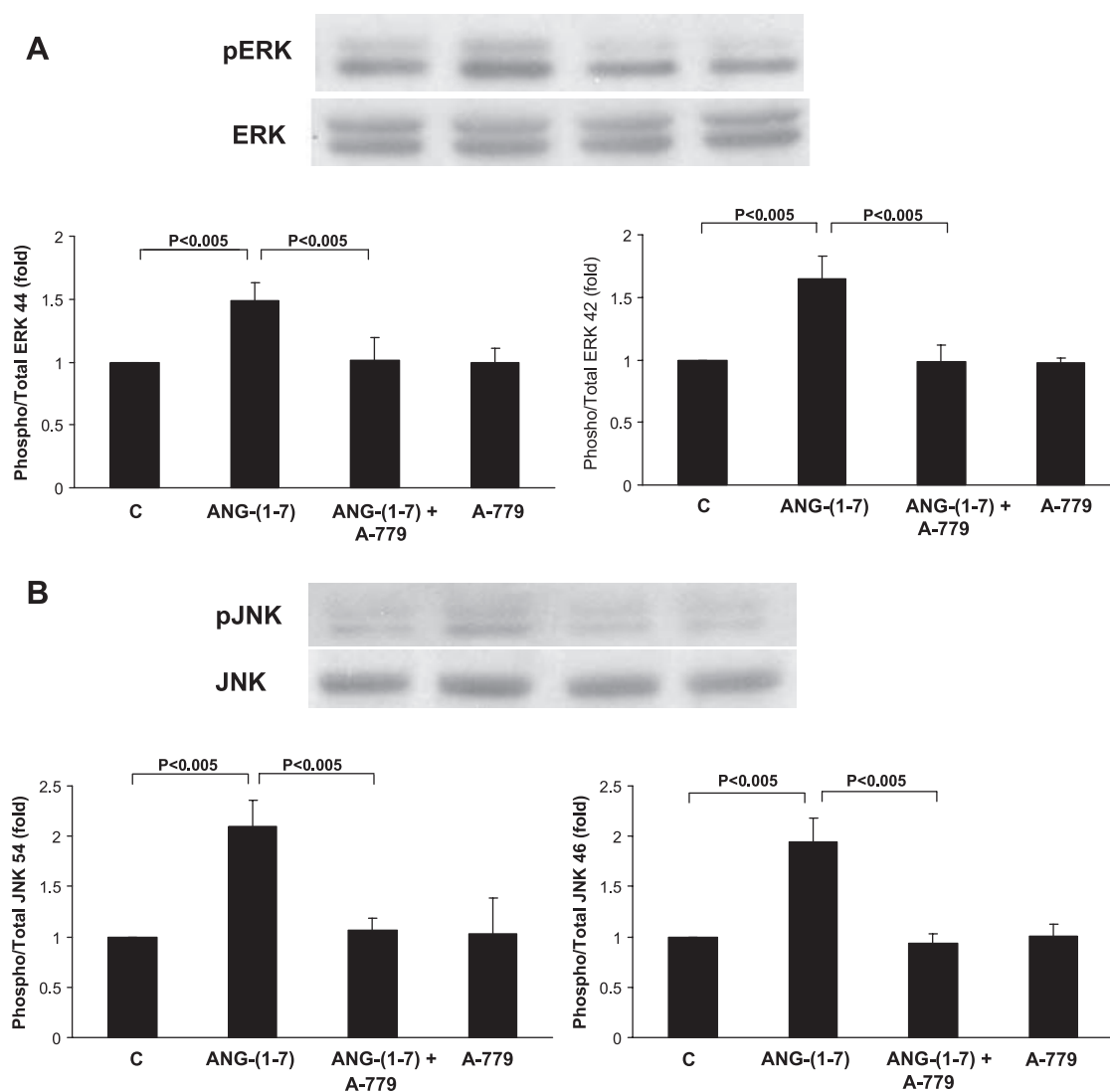


Fig. 5. Effect of A-779 on Ang-(1-7)-stimulated phosphorylation of ERK1/ERK2 and JNK in human mesangial cells. *A*: graphs depict effect of A-779 ( $10^{-5}$  M) on Ang-(1-7) ( $10^{-7}$  M)-stimulated phosphorylation of p44 (left) and p42 (right) MAPK, compared with control (C). Representative immunoblot is shown above, with phosphorylated ERK (pERK) and unphosphorylated ERK (ERK). *P* values are indicated; *n* = 4. *B*: graphs depict effect of A-779 ( $10^{-5}$  M) on Ang-(1-7) ( $10^{-7}$  M)-stimulated phosphorylation of p54 JNK (left) and p46 JNK (right) compared with control (C). Representative immunoblot is shown above, with phosphorylated JNK (pJNK) and unphosphorylated JNK (JNK). *P* values are indicated; *n* = 4–6.

indeed causing a small increase in phosphorylation in the presence of ANG II (Fig. 6B). In separate experiments, ANG II ( $10^{-7}$  M) stimulated ERK1/ERK2 phosphorylation in these cells by  $79 \pm 26\%$  ( $P < 0.02$  vs. control,  $n = 5$ ), an effect that was blocked by losartan ( $10^{-6}$  M), but not by PD123319 ( $10^{-6}$  M). The stimulatory effect of ANG II on ERK1/ERK2 was less

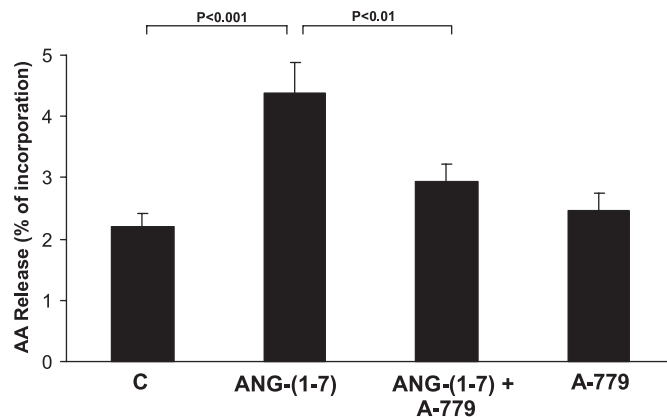
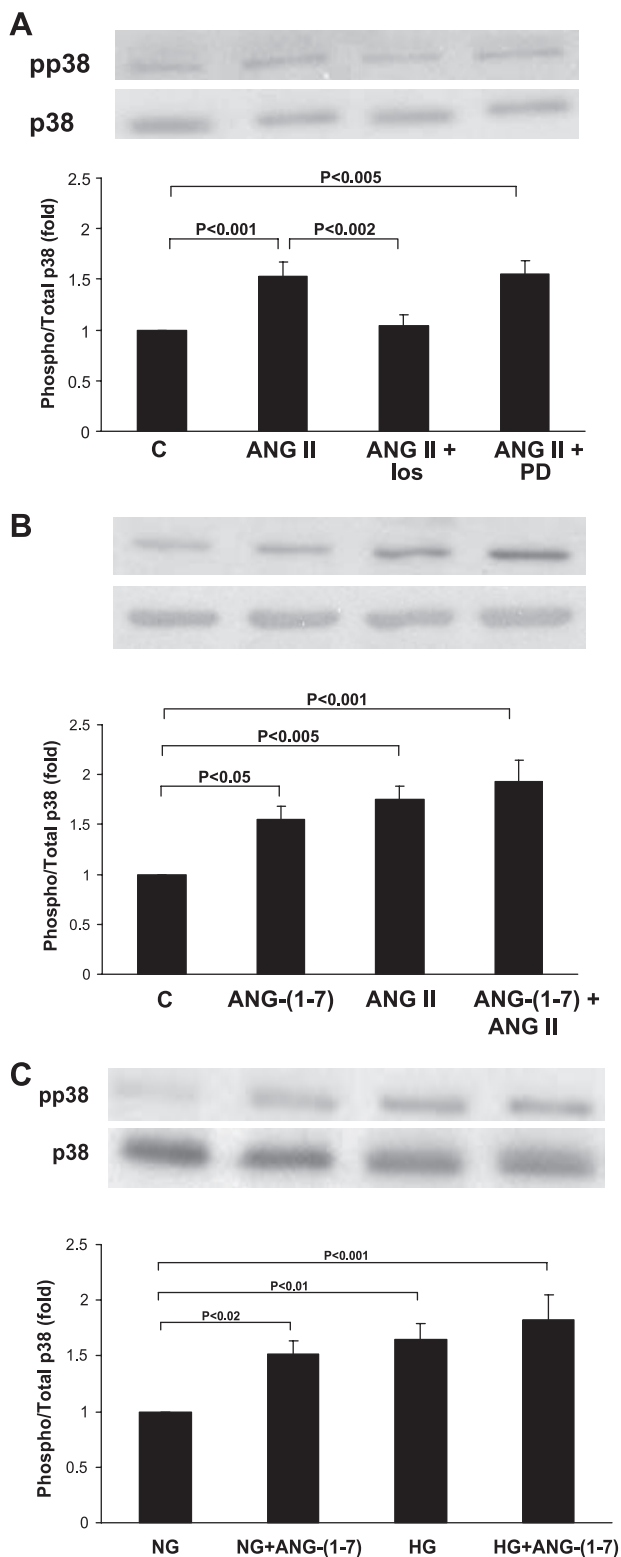


Fig. 7. Ang-(1-7) stimulates arachidonic acid release in human mesangial cells. Graph shows the effect of Ang-(1-7) ( $10^{-7}$  M) in the presence or absence of A-779 ( $10^{-6}$  M) on arachidonic acid (AA) release. Results are expressed as percentage of incorporated  $^3\text{H}$ arachidonic acid released from the cells.  $P$  values are indicated;  $n = 4-5$ .

than that observed with Ang-(1-7) at equivalent concentration (see Figs. 2 and 3).

High concentrations of extracellular glucose have been shown to activate p38 MAPK in renal cells (20, 36). Exposure of human mesangial cells for 60 min to media containing high glucose (25 mM) increased p38 MAPK phosphorylation compared with cells in normal glucose (5 mM), with L-glucose added to control for osmolality. Preincubation with Ang-(1-7) had no inhibitory effect on high glucose-stimulated phosphorylation of p38 MAPK (Fig. 6C).

**Arachidonic acid release.** In vascular smooth muscle cells, Ang-(1-7) stimulates the release of arachidonic acid (23). Similarly, in human mesangial cells loaded with  $^3\text{H}$ arachidonic acid, Ang-(1-7) ( $10^{-7}$  M) caused a significant release of arachidonic acid, an effect that was blocked by A-779 (Fig. 7). The inhibitor of PLA<sub>2</sub>, MAFP (50  $\mu\text{M}$ ), also completely blocked Ang-(1-7)-stimulated arachidonic acid release [control:  $1.77 \pm 0.26$  vs. Ang-(1-7) ( $10^{-7}$  M):  $2.49 \pm 0.18$  vs. Ang-(1-7)+MAFP:  $1.79 \pm 0.28\%$  release;  $P < 0.04$ , control or MAFP+Ang-(1-7) vs. Ang-(1-7);  $n = 4$ ]. In contrast, neither losartan nor PD123319 had any effect on Ang-(1-7)-stimulated release of arachidonic acid (not shown).

To determine whether activation of MAPKs by Ang-(1-7) in mesangial cells plays a role in stimulation of PLA<sub>2</sub>, cells were loaded with  $^3\text{H}$ arachidonic acid and then exposed to Ang-(1-7) in the presence or absence of inhibitors of p38, ERK1/ERK2, or JNK. As shown in Fig. 8, the p38 MAPK antagonist SB202190 ( $10^{-5}$  M) completely inhibited Ang-(1-7)-stimu-

Fig. 6. Effect of Ang-(1-7) on ANG II- and high glucose-stimulated phosphorylation of p38 MAPK. **A**: graph depicting the effect of ANG II ( $10^{-7}$  M) on p38 MAPK phosphorylation, in the presence or absence of losartan (los,  $10^{-5}$  M) or PD123319 (PD,  $10^{-5}$  M).  $P$  values are indicated;  $n = 4$ . **B**: graph showing the effect of Ang-(1-7) ( $10^{-7}$  M) on ANG II-stimulated p38 MAPK phosphorylation ( $10^{-7}$  M).  $P$  values are indicated;  $n = 6$ . **C**: graph depicts effects of Ang-(1-7) ( $10^{-7}$  M) in normal glucose (NG; 5 mM) or high glucose (HG; 25 mM for 60 min).  $P$  values are indicated;  $n = 6$ . To control for the possible effects of osmolality in these experiments, incubations in NG were supplemented with L-glucose (20 mM). For all graphs, results are presented as the ratio of phosphorylated p38 to total p38, in arbitrary units. Representative blot is depicted above each graph, showing phosphorylated p38 (pp38) and total p38 (p38).

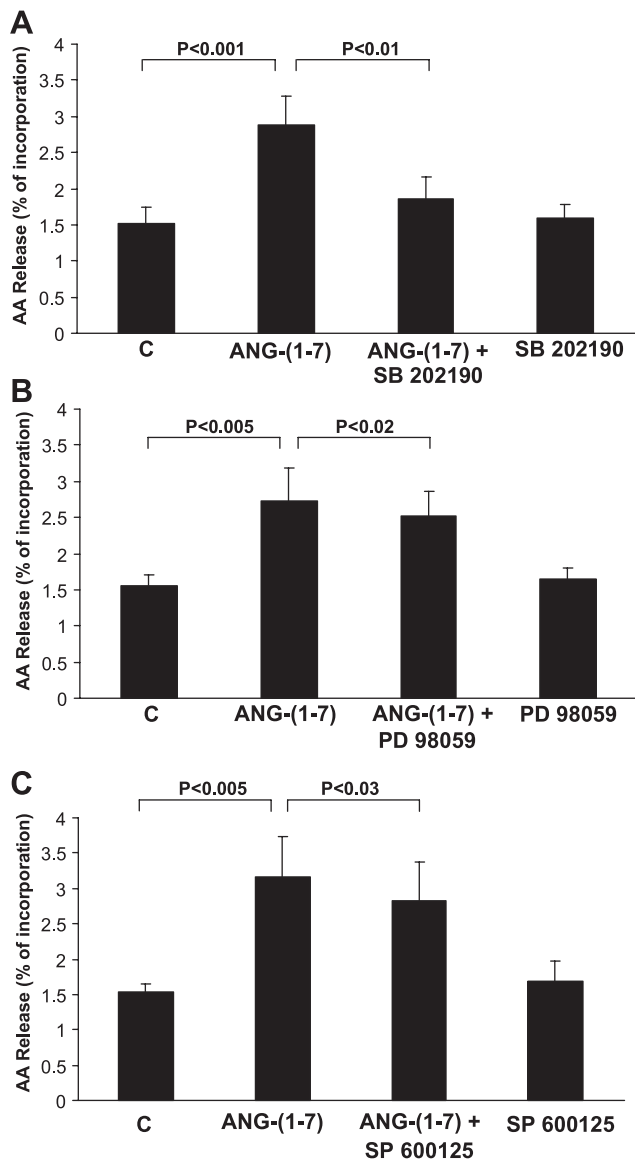


Fig. 8. Inhibition of p38 MAP kinase blocks Ang-(1-7)-stimulated release of AA. **A**: bar graph showing the effect of the p38 MAP kinase inhibitor SB 202190 ( $10^{-5}$  M) on Ang-(1-7) ( $10^{-7}$  M)-stimulated release of AA. *P* values are indicated; *n* = 8. **B**: bar graph depicting the effect of the ERK inhibitor PD 98059 ( $10^{-5}$  M) on Ang-(1-7) ( $10^{-7}$  M)-stimulated AA release; *n* = 5. **C**: bar graph depicting the effect of the JNK inhibitor SP 600125 ( $10^{-5}$  M) on Ang-(1-7) ( $10^{-7}$  M)-stimulated AA release. *P* values are indicated; *n* = 5.

lated arachidonic acid release, whereas inhibitors of ERK1/ERK2 (PD98059,  $10^{-5}$  M) or JNK (SP600125,  $10^{-5}$  M) had no effect. In separate experiments, the Ang-(1-7)-stimulated phosphorylation of p38, ERK1/ERK2, and JNK MAPK was blocked by preincubation of cells with their respective inhibitors SB202190 ( $10^{-5}$  M), PD98059 ( $10^{-5}$  M), and SP600125 ( $10^{-5}$  M) (*n* = 3–4, not shown).

**Effect of Ang-(1-7) on TGF- $\beta$ 1, fibronectin, and collagen IV production.** ANG II stimulates production of TGF- $\beta$ 1 and extracellular matrix proteins in mesangial cells (19). In human mesangial cells, exposure to Ang-(1-7) ( $10^{-7}$  M) for 72 h caused a significant increase in production of TGF- $\beta$ 1, which was blocked by preincubation with A-779 (Fig. 9A). Moreover, preincubation of cells with the p38 MAPK antagonist

SB202190 completely inhibited Ang-(1-7)-stimulated TGF- $\beta$ 1 production (Fig. 9B). In contrast, the ERK1/ERK2 antagonist PD98059 and the JNK antagonist SP600125 did not significantly affect Ang-(1-7)-stimulated TGF- $\beta$ 1 production (not shown).

Similarly, incubation of mesangial cells for 72 h with Ang-(1-7) ( $10^{-7}$  M) caused a significant stimulation of fibronectin and collagen IV production, and these effects were inhibited by A-779 and by preincubation with the p38 MAPK antagonist SB202190 (Figs. 10 and 11, respectively). However, the ERK1/ERK2 antagonist PD98059 and the JNK antagonist SP600125 did not significantly affect Ang-(1-7)-stimulated production of either fibronectin or collagen IV (not shown).

**Effect of Ang-(1-7) on DNA synthesis in mesangial cells.** Activation of MAPK has been associated with stimulation of cell growth responses, including cell mitosis. In cells incubated with Ang-(1-7) ( $10^{-7}$  M) for 72 h, a significant stimulation of [ $^3$ H]thymidine incorporation was observed, which was blocked by preincubation with A-779. In contrast, neither losartan nor PD123319 had any significant effect on Ang-(1-7)-stimulated DNA synthesis (Table 1). In these cells, ANG II ( $10^{-7}$  M) caused a significant increase in [ $^3$ H]thymidine incorporation ( $158.0 \pm 15.0\%$  of control) that was completely inhibited by losartan ( $10^{-6}$  M) ( $96.4 \pm 15.6\%$  of control, *n* = 3).

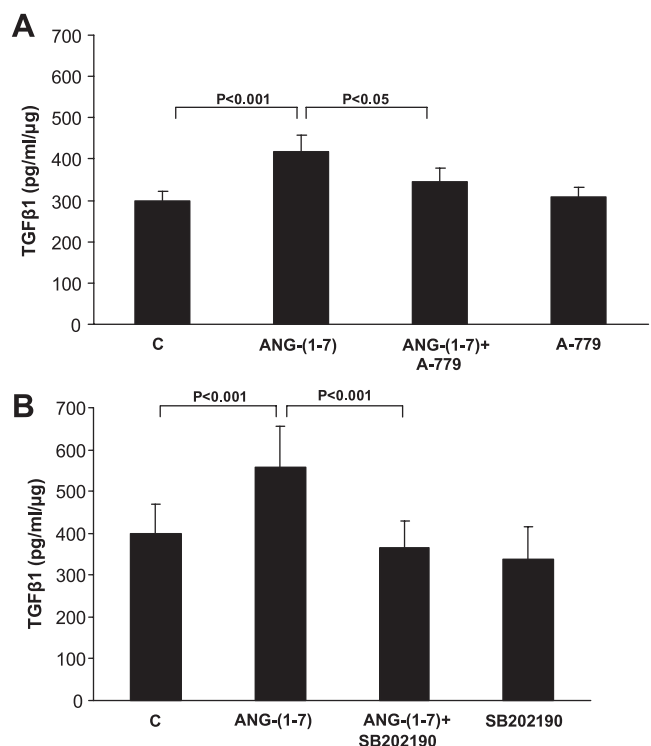


Fig. 9. Effect of Ang-(1-7) on production of TGF- $\beta$ 1 in human mesangial cells. Cells were incubated with Ang-(1-7) ( $10^{-7}$  M) for 72 h, followed by assay of TGF- $\beta$ 1 in the cell supernatant, as described in MATERIALS AND METHODS. **A**: graph depicts effect of Ang-(1-7), with or without A-779, on TGF- $\beta$ 1 production. *P* values are indicated. *P* < 0.001 for Ang-(1-7) vs. A-779; *n* = 10. **B**: graph depicts effect of Ang-(1-7), with or without the p38 MAPK inhibitor SB202190 ( $10^{-5}$  M), on TGF- $\beta$ 1 production. *P* values are indicated. *P* < 0.001 for Ang-(1-7) vs. SB 202190; *n* = 8.



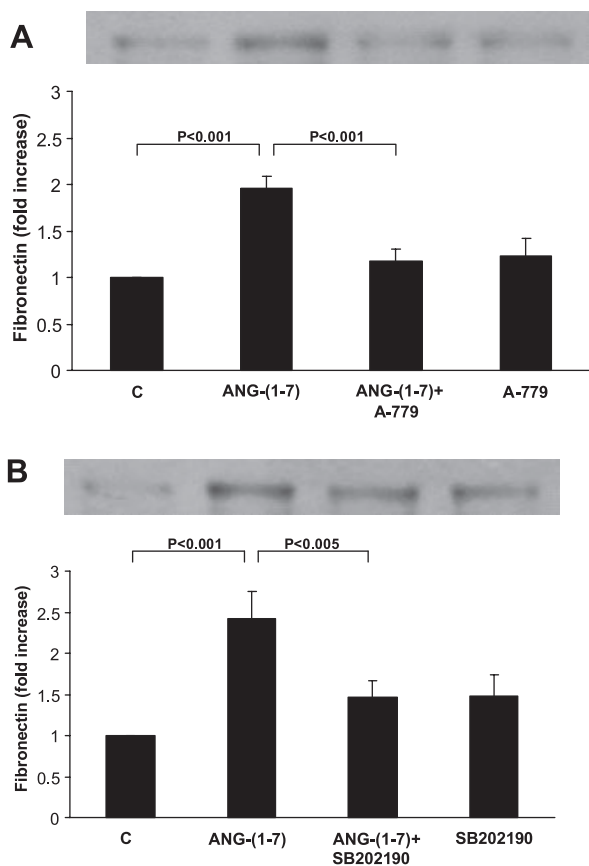


Fig. 10. Effect of Ang-(1-7) on production of fibronectin in human mesangial cells. Cells were incubated with Ang-(1-7) ( $10^{-7}$  M) for 72 h, followed by immunoblotting for fibronectin in the cell supernatant, as described in MATERIALS AND METHODS. *A*: graph depicts effect of Ang-(1-7), with or without A-779, on fibronectin production. *P* values are indicated.  $P < 0.003$  for Ang-(1-7) vs. A-779;  $n = 8$ . *B*: graph depicts effect of Ang-(1-7), with or without the p38 MAPK inhibitor SB202190 ( $10^{-5}$  M), on fibronectin production. *P* values are indicated.  $P < 0.003$  for Ang-(1-7) vs. SB 202190;  $n = 7-9$ . Representative immunoblots are depicted above each graph.

## DISCUSSION

The heptapeptide Ang-(1-7) is formed via cleavage of ANG II or ANG I and has been reported to be an endogenous vasodilator that counteracts many of the actions of ANG II. By contrast, the major new finding in the current study is that Ang-(1-7) stimulates cell growth responses classically associated with ANG II signaling in human mesangial cells, including phosphorylation of MAPK, activation of PLA<sub>2</sub>, enhanced DNA synthesis, and production of TGF- $\beta$ 1 and extracellular matrix proteins. These responses are mediated via binding of Ang-(1-7) to sites distinct from AT<sub>1</sub> or AT<sub>2</sub> receptors in these cells. Furthermore, our data indicate a signaling cascade that involves upstream activation of p38 MAPK, linked to downstream activation of PLA<sub>2</sub>, and production of TGF- $\beta$ 1, fibronectin, and collagen IV.

Our data support the existence of a specific G protein-coupled receptor for Ang-(1-7) in human mesangial cells. This is in agreement with the studies of Santos and colleagues, who have identified the Mas protooncogene as a likely candidate for the Ang-(1-7) receptor (28). In the current studies, we demonstrated the presence of the Mas protein, and we also showed that binding of [<sup>125</sup>I]Ang-(1-7) was inhibited by Ang-(1-7)

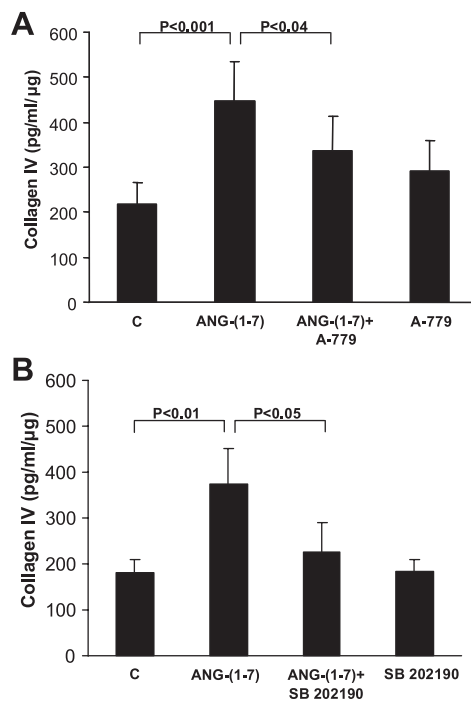


Fig. 11. Effect of Ang-(1-7) on production of collagen IV in human mesangial cells. Cells were incubated with Ang-(1-7) ( $10^{-7}$  M) for 72 h, followed by ELISA assay of collagen IV in the cell supernatant, as described in MATERIALS AND METHODS. *A*: graph depicts effect of Ang-(1-7), with or without A-779, on collagen IV production. *P* values are indicated.  $P < 0.003$  for Ang-(1-7) vs. A-779;  $n = 9$ . *B*: graph depicts effect of Ang-(1-7), with or without the p38 MAPK inhibitor SB202190 ( $10^{-5}$  M), on collagen IV production. *P* values are indicated.  $P < 0.010$  for Ang-(1-7) vs. SB 202190;  $n = 9$ .

and its antagonist A-779, but not by the AT<sub>1</sub> receptor antagonist losartan or the AT<sub>2</sub> antagonist PD123319. Similarly, stimulation of MAPK phosphorylation, PLA<sub>2</sub> activation, and DNA synthesis by Ang-(1-7) were completely blocked by A-779, but were not affected by losartan or PD123319. In this regard, in some tissues Ang-(1-7) has been shown to interact with the AT<sub>1</sub> receptor (4, 8, 9). In rat heart in vivo, for example, Ang-(1-7) stimulates the phosphorylation of Janus kinase 2 and insulin receptor substrate-1, an effect blocked by losartan (8). In rat glomeruli, specific binding of radiolabeled ANG II is displaced by both losartan and by nanomolar concentrations of Ang-(1-7), suggesting that Ang-(1-7) has affinity for ANG II AT<sub>1</sub> receptors (9). In the rat, Ang-(1-7) potentiates the decreases in blood pressure induced by bradykinin in vivo, an

Table 1. Effect of Ang-(1-7) on [<sup>3</sup>H]thymidine incorporation in human mesangial cells

Condition	[ <sup>3</sup> H]thymidine Incorporation, cpm/well
Control	1,502 ± 441
Ang-(1-7)	1,922 ± 508*
Ang-(1-7)+A-779	1,549 ± 473
A-779	1,490 ± 422
Ang-(1-7)+losartan	2,014 ± 572*
Losartan	1,467 ± 451
Ang-(1-7)+PD123319	1,881 ± 492†
PD123319	1,415 ± 501

Values are means ± SE. cpm, Counts per minute. Ang-(1-7) concentration was  $10^{-7}$  M, and concentrations of all other agents were  $10^{-6}$  M. \* $P < 0.001$  vs. control. † $P < 0.005$  vs. control;  $n = 5$  for each.



effect that is partially blocked by the bradykinin B2 receptor antagonist Hoe 140, but not by a receptor Mas antagonist (13), suggesting a possible interaction of Ang-(1-7) with B2 receptors. Recently, Silva et al. (31) demonstrated evidence for a subtype Ang-(1-7) receptor in rat aorta, since the Ang-(1-7) antagonist D-Pro<sup>7</sup>-Ang-(1-7) blocked the vasodilatory effects of Ang-(1-7), while A-779 had no inhibitory effect. In contrast, our data suggest that the stimulatory effects of Ang-(1-7) on MAPK phosphorylation, PLA<sub>2</sub> activation, and DNA synthesis in mesangial cells may be due to binding to the receptor Mas, and do not involve interaction with AT<sub>1</sub> or AT<sub>2</sub> receptors.

In glomerular mesangial cells, ANG II signaling pathways have been well characterized and include inhibition of adenylate cyclase and suppression of cAMP levels (7), stimulation of cytosolic calcium increases (5), activation of MAPKs, arachidonic acid release, and generation of reactive oxygen species (10-12). In the present studies in human mesangial cells, ANG II induced expected responses, including stimulation of calcium influx, phosphorylation of p38 and ERK1/ERK2 MAPK, and [<sup>3</sup>H]thymidine incorporation, all via the AT<sub>1</sub> receptor. Few studies have addressed the signaling of Ang-(1-7) in glomerular cells. In rat mesangial cells, Chansel et al. (5) demonstrated that Ang-(1-7) inhibited calcium transients induced by ANG II. We observed no effect of Ang-(1-7) on basal or ANG II-stimulated calcium increases in human mesangial cells, although we used concentrations of Ang-(1-7) that were up to 10-fold lower than in the studies in rat mesangial cells. Similarly, Ang-(1-7) had no effect on cAMP levels, suggesting that the mesangial cell receptor Mas is not linked to inhibitory G proteins. In contrast, in rat inner medullary collecting duct cell suspensions, Ang-(1-7) has been shown to stimulate cAMP levels and to consequently increase water permeability (22).

An unexpected finding in the present studies was the stimulation of all three MAPKs by Ang-(1-7) and the failure of Ang-(1-7) to inhibit ANG II- or high glucose-stimulated phosphorylation of p38. This contrasts to our previous studies in proximal tubular cells, where Ang-(1-7) potently inhibited ANG II-mediated phosphorylation of p38, ERK1/ERK2, and JNK and partly blocked ANG II-stimulated production of TGF-β1 (32). In vascular smooth muscle cells and cardiac myocytes, Ang-(1-7) inhibits ANG II-stimulated ERK1/ERK2 via the receptor Mas, associated with reduction in cell protein synthesis (33, 34, 39). Thus, the view has emerged that Ang-(1-7) may protect against ANG II-induced vascular injury, hypertension, or cardiac pathology via antagonism of AT<sub>1</sub>-mediated signaling events (3, 27). In this regard, it is of interest that in human aortic endothelial cells Ang-(1-7) stimulated Akt phosphorylation, associated with activation of endothelial nitric oxide (NO) synthase and production of NO (26). Since NO has been shown to inhibit MAPK activation induced by stretch in mesangial cells (17), further studies are required to determine whether Ang-(1-7)-stimulates Akt and NO release in these cells, which might modulate stimulatory effects on MAPK.

Ang-(1-7) stimulated the release of arachidonic acid in mesangial cells, an effect that was blocked by an inhibitor of PLA<sub>2</sub>. Moreover, inhibition of p38 MAPK reversed the stimulatory effect of Ang-(1-7) on arachidonic acid release, whereas inhibitors of the other two MAPKs had no effect. These data suggest that p38 MAPK activation is necessary for subsequent stimulation of PLA<sub>2</sub>. Release of arachidonic acid

by Ang-(1-7) has been demonstrated in other cell types, including vascular smooth muscle cells, and proximal tubular cells (1, 23). In rat mesangial cells, activation of cPLA<sub>2</sub> and arachidonic acid release induced by hydrogen peroxide and PDGF is partly blocked by inhibitors of ERK or p38 MAPK, suggesting that activation of MAPKs is upstream of PLA<sub>2</sub> activation, as observed in the current study (16). On the other hand, Gorin et al. (12) have shown that ANG II-induced ERK1/ERK2 activation is blocked by PLA<sub>2</sub> inhibition in rat mesangial cells, indicating that PLA<sub>2</sub> could be regulated downstream of MAPKs.

A novel finding in the current studies is the demonstration that in mesangial cells, Ang-(1-7) stimulates production of the cytokine TGF-β1, as well as synthesis of the extracellular matrix proteins fibronectin and collagen IV, mimicking the effects of ANG II. These effects are inhibited by the antagonist A-779 and are dependent on upstream activation of p38 MAPK, since they are blocked by the p38 inhibitor SB202190, but not by inhibitors of ERK1/ERK2 or JNK. Furthermore, Ang-(1-7) stimulates DNA synthesis in mesangial cells, independently of interaction with AT<sub>1</sub> or AT<sub>2</sub> receptors. These effects appear to contradict the growth-inhibitory properties of Ang-(1-7) reported in other tissues, particularly the vasculature and the heart. Thus Ang-(1-7) has been shown to reduce collagen synthesis in cardiac fibroblasts (18), and in a rat DOCA-salt model of hypertension, Ang-(1-7) prevented cardiac and perivascular fibrosis (14) and protected against cardiac myocyte hypertrophy and interstitial fibrosis in pressure overload (15). Vascular neointimal proliferation after balloon injury is also prevented by Ang-(1-7) (21). On the other hand, Ang-(1-7) has been reported to stimulate the proliferation of blood progenitor cells (25). The long-term effects of Ang-(1-7) on kidney cell growth and glomerular function remain unclear. In this regard, the glomerulus contains other cell types, including endothelial cells and podocytes, and these cells may respond differently to Ang-(1-7). In rats with streptozotocin-induced diabetes, Benter et al. (2) have reported that chronic Ang-(1-7) treatment attenuated proteinuria and restored vascular reactivity. In contrast, Shao et al. (30) recently showed that chronic administration of Ang-(1-7) caused an increase in kidney TGF-β1 mRNA and protein, associated with increased proteinuria and renal injury in streptozotocin-diabetic rats.

In summary, these studies demonstrate that in human mesangial cells, Ang-(1-7) stimulates phosphorylation of all three MAPKs, via binding to the specific Ang-(1-7) receptor. Phosphorylation of p38 MAPK leads to arachidonic acid release and downstream production of TGF-β1, fibronectin and collagen IV. Ang-(1-7) also stimulates mesangial cell DNA synthesis. To determine the impact of these responses on glomerular injury in pathophysiological states, further studies are required to investigate the effects of Ang-(1-7) on other cell types within the glomerulus.

#### GRANTS

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#### REFERENCES

1. Andreatta-van Leyen S, Romero MF, Khosia MC, Douglas JG. Modulation of phospholipase A2 activity and sodium transport by angiotensin (1-7). *Kidney Int* 44: 932-936, 1993.

2. **Benter IF, Yousif MH, Cojocel C, Al-Maghrebi M, Diz DI.** Angiotensin-(1-7) prevents diabetes-induced cardiovascular dysfunction. *Am J Physiol Heart Circ Physiol* 292: H666-H672, 2007.
3. **Burns KD.** The emerging role of angiotensin-converting enzyme 2 in the kidney. *Curr Opin Nephrol Hypertens* 16: 116-121, 2007.
4. **Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, Alemida AP.** Evidence for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. *Hypertension* 46: 937-942, 2005.
5. **Chansel D, Vandermeersch S, Oko A, Curat C, Ardaillou R.** Effects of angiotensin IV and angiotensin-(1-7) on basal and angiotensin II-stimulated cytosolic Ca<sup>2+</sup> in mesangial cells. *Eur J Pharmacol* 414: 165-175, 2001.
6. **Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S.** A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 87: E1-E9, 2000.
7. **Edwards RM, Stack EJ.** Angiotensin II inhibits glomerular adenylate cyclase via the angiotensin II receptor subtype 1 (AT1). *J Pharmacol Exp Ther* 266: 506-510, 1993.
8. **Giani JF, Gironacci MM, Munoz MC, Pena C, Turyn D, Dominici FP.** Angiotensin-(1-7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart in vivo: role of the AT1 and Mas receptors. *Am J Physiol Heart Circ Physiol* 293: H1154-H1163, 2007.
9. **Gironacci MM, Coba MP, Pena C.** Angiotensin-(1-7) binds at the type 1 angiotensin II receptors in rat renal cortex. *Regul Pept* 84: 51-54, 1999.
10. **Gorin Y, Kim NH, Fellers D, Bhandari B, Choudhury GG, Abboud HE.** Angiotensin II activates Akt/protein kinase B by an arachidonic acid/redox-dependent pathway and independent of phosphoinositide 3-kinase. *FASEB J* 15: 1909-1920, 2001.
11. **Gorin Y, Ricono JM, Kim NH, Bhandari B, Choudhury GG, Abboud HE.** Nox4 mediates angiotensin II-induced activation of Akt/protein kinase B in mesangial cells. *Am J Physiol Renal Physiol* 285: F219-F229, 2003.
12. **Gorin Y, Ricono JM, Wagner B, Kim NH, Bhandari B, Choudhury GG, Abboud HE.** Angiotensin II-induced ERK1/ERK2 activation and protein synthesis are redox-dependent in glomerular mesangial cells. *Biochem J* 381: 231-239, 2004.
13. **Greco AJ, Master RG, Fokin A Jr, Baber SR, Kadowitz PJ.** Angiotensin-(1-7) potentiates responses to bradykinin but does not change responses to angiotensin I. *Can J Physiol Pharmacol* 84: 1163-1175, 2006.
14. **Grobe JL, Mecca AP, Mao H, Katovich MJ.** Chronic angiotensin-(1-7) prevents cardiac fibrosis in DOCA-salt model of hypertension. *Am J Physiol Heart Circ Physiol* 290: H2417-H2423, 2006.
15. **Grobe JL, Mecca AP, Lingis M, Shenoy V, Bolton TA, Machado JM, Speth RC, Raizada MK, Katovich MJ.** Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). *Am J Physiol Heart Circ Physiol* 292: H736-H742, 2007.
16. **Hayama M, Inoue R, Akiba S, Sato T.** ERK and p38 MAP kinase are involved in arachidonic acid release induced by H<sub>2</sub>O<sub>2</sub> and PDGF in mesangial cells. *Am J Physiol Renal Physiol* 282: F485-F491, 2002.
17. **Ingram AJ, James L, Thai K, Ly H, Cai L, Scholey JW.** Nitric oxide modulates mechanical strain-induced activation of p38 MAPK in mesangial cells. *Am J Physiol Renal Physiol* 279: F243-F251, 2000.
18. **Iwata M, Cowling RT, Gurantz D, Moore C, Zhang S, Yuan JX, Greenberg BH.** 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.
19. **Kagami S, Border WA, Miller DE, Noble NA.** Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 93: 2431-2437, 1994.
20. **Kang MJ, Wu X, Ly H, Thai K, Scholey JW.** Effect of glucose on stress-activated protein kinase activity in mesangial cells and diabetic glomeruli. *Kidney Int* 55: 2203-2214, 1999.
21. **Langeveld B, van Gilst WH, Tio RA, Zijlstra F, Roks AJ.** Angiotensin-(1-7) attenuates neointimal formation after stent implantation in the rat. *Hypertension* 45: 138-141, 2005.
22. **Magaldi AJ, Cesar KR, de Araujo M, Simoes e Silva AC, Santos RA.** Angiotensin-(1-7) stimulates water transport in rat inner medullary collecting duct: evidence for involvement of vasopressin V2 receptors. *Pflügers Arch* 447: 223-230, 2003.
23. **Muthalif MM, Benter IF, Uddin MR, Harper JL, Malik KU.** Signal transduction mechanisms involved in angiotensin-(1-7)-stimulated arachidonic acid release and prostanoid synthesis in rabbit aortic smooth muscle cells. *J Pharmacol Exp Ther* 284: 388-394, 1998.
24. **Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R, Crackower MA, Backx PH, Penninger JM, Scholey JW.** Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. *Am J Pathol* 168: 1808-1820, 2006.
25. **Rodgers KE, Xiong S, diZerega GS.** Accelerated recovery from irradiation injury by angiotensin peptides. *Cancer Chemother Pharmacol* 49: 403-411, 2002.
26. **Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM.** Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49: 185-192, 2007.
27. **Santos RAS, Campagnole-Santos MJ, Andrade SP.** Angiotensin (1-7): an update. *Regul Pept* 91: 45-62, 2000.
28. **Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T.** Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 100: 8258-8263, 2003.
29. **Santos RA, Castro CH, Gava E, Pinheiro SV, Almeida AP, Paula RD, Cruz JS, Ramos AS, Rosa KT, Irigoyen MC, Bader M, Alenina N, Kitten GT, Ferreira AJ.** Impairment of in vitro and in vivo heart function in angiotensin-(1-7) receptor MAS knockout mice. *Hypertension* 47: 996-1002, 2006.
30. **Shao Y, He M, Zhou L, Yao T, Huang Y, Lu LM.** Chronic angiotensin-(1-7) injection accelerates STZ-induced diabetic renal injury. *Acta Pharmacol Sin* 29: 829-837, 2008.
31. **Silva DM, Vianna HR, Cortes SF, Campagnole-Santos MJ, Santos RA, Lemos VS.** Evidence for a new angiotensin-(1-7) receptor subtype in the aorta of Sprague-Dawley rats. *Peptides* 28: 702-707, 2007.
32. **Su Z, Zimpelmann J, Burns KD.** Angiotensin-(1-7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. *Kidney Int* 69: 2212-2218, 2006.
33. **Tallant EA, Clark MA.** Molecular mechanisms of inhibition of vascular growth by angiotensin-(1-7). *Hypertension* 42: 574-579, 2003.
34. **Tallant EA, Ferrario CM, Gallagher PE.** Angiotensin-(1-7) inhibits growth of cardiac myocytes through activation of the mas receptor. *Am J Physiol Heart Circ Physiol* 289: H1560-H1566, 2005.
35. **Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ.** A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 275: 33238-33243, 2000.
36. **Wilmer WA, Dixon CL, Hebert C.** Chronic exposure of human mesangial cells to high glucose environments activates the p38 MAPK pathway. *Kidney Int* 60: 858-871, 2001.
37. **Wong DW, Oudit GY, Reich H, Kassiri Z, Zhou J, Liu QC, Backx PH, Penninger JM, Herzenberg AM, Scholey JW.** Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. *Am J Pathol* 171: 438-451, 2007.
38. **Ye M, Wysocki J, William J, Soler MJ, Cokic I, Battle D.** Glomerular localization and expression of angiotensin-converting enzyme 2 and angiotensin converting enzyme: implications for albuminuria in diabetes. *J Am Soc Nephrol* 17: 3067-3075, 2006.
39. **Zhu Z, Zhong J, Zhu S, Liu D, Van Der Giet M, Tepel M.** Angiotensin-(1-7) inhibits angiotensin II-induced signal transduction. *J Cardiovasc Pharmacol* 40: 693-700, 2002.
40. **Zimpelmann J, Burns KD.** Angiotensin II AT2 receptors inhibit growth responses in proximal tubule cells. *Am J Physiol Renal Physiol* 281: F300-F308, 2001.