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Angiotensin-(1–7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells

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Angiotensin-converting enzyme 2 (ACE2) is a homolog of ACE, which is not blocked by ACE inhibitors. High amounts of ACE2 are present in the proximal tubule, and ACE2 catalyzes generation of angiotensin 1–7 (Ang-(1–7)) by this segment. Ang-(1–7) binds to a receptor distinct from the AT_1 or AT_2 Ang II receptor, identified as the *mas* receptor. We studied the effects of Ang-(1–7) on Ang II-mediated cell signaling pathways in proximal tubule. In primary cultures of rat proximal tubular cells, activation of mitogen-activated protein kinases (MAPK) was detected by immunoblotting, in the presence or absence of agonists/antagonists. Transforming growth factor-β_1 (TGF-β_1) was measured by enzyme-linked immunosorbent assay. Ang II (5 min, 10^{-7} M) stimulated phosphorylation of the three MAPK (p38, extracellular signal-related kinase (ERK 1/2), and c-Jun N-terminal kinase (JNK)). While incubation of proximal tubular cells with Ang-(1–7) alone did not significantly affect MAPK phosphorylation, Ang-(1–7) (10^{-7} M) completely inhibited Ang II-stimulated phosphorylation of p38, ERK 1/2, and JNK. This inhibitory effect was reversed by the Ang-(1–7) receptor antagonist, D-Ala^7-Ang-(1–7). Ang II significantly increased production of TGF-β_1 in proximal tubular cells, an effect that was partly inhibited by Ang-(1–7). Ang-(1–7) had no significant effect on cyclic 3′,5′-adenosine monophosphate production in these cells. In summary, Ang-(1–7) inhibits Ang II-stimulated MAPK phosphorylation in proximal tubular cells. Generation of Ang-(1–7) by proximal tubular ACE2 could thereby serve a protective role by counteracting the effects of locally generated Ang II.

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The renin–angiotensin system plays a crucial role in the regulation of renal and cardiovascular function. Although angiotensin II (Ang II) is the best characterized product of the renin–angiotensin system, Ang-(1–7), a heptapeptide that lacks phenylalanine at position 8 of the Ang II peptide is thought to be an important biologically active component of this system.[1] In 2000, a new pathway of Ang-(1–7) formation was described, involving catalysis by an angiotensin-converting enzyme (ACE)-related carboxypeptidase, ACE2.[2,3] ACE2 differs in its specificity and physiological role from classical ACE, and is not blocked by ACE inhibitors. Thus, ACE2 is responsible for the conversion of Ang-(1–10) to Ang-(1–9), which is directly transformed to Ang-(1–7) by ACE. ACE2 also directly cleaves Ang II to form Ang-(1–7).[2]

ACE2 is found in the kidney, with a relative abundance in the proximal straight tubule.[4–6] Recently, we determined that ACE2 is widely expressed in rat nephron segments, with relatively high concentrations in the proximal tubule, and we showed that ACE2 leads to generation of Ang-(1–7) in this segment.[6] In several tissues, the effects of Ang-(1–7) appear to oppose those caused by Ang II. Thus, in contrast to Ang II, Ang-(1–7) displays vasodilator, natriuretic, diuretic, and antiproliferative actions.[7–10] The vasodilatory effects of Ang-(1–7) are associated with lowering of blood pressure, prevention of cardiac pathophysiology, and attenuation of the renal abnormalities associated with hypertension.[11–15] Ang-(1–7) increases the release of nitric oxide[16,17] and the vasodilator prostacyclin[18] and potentiates the hypotensive effects of bradykinin.[19] These findings suggest that Ang-(1–7) may limit the pressor and proliferative effects of Ang II.

The mitogen-activated protein kinase (MAPK) cascade is an important intracellular mediator of responses related to cell growth and differentiation, survival, and cell death. Ang II is a well-known activator of this signaling pathway, and Ang II is also capable of inducing the synthesis of the profibrotic cytokine transforming growth factor-β_1 (TGF-β_1) in proximal tubule, associated with subsequent alterations in cell growth and matrix production.[20,21] Although it is now evident that Ang-(1–7) can be produced by proximal tubular cells, the effect of Ang-(1–7) on these Ang II-stimulated

signaling events is unknown. Accordingly, in the current study, we examined whether Ang-(1–7) affects Ang II-mediated MAPK signaling pathways in primary cultures of rat proximal tubular cells. Our hypothesis was that Ang-(1–7) might inhibit these pathways, suggesting that it acts as a counterregulatory factor to Ang II in the proximal tubule.

RESULTS

Ang II phosphorylates three distinct MAPK (p38, ERK 1/2 and JNK) in rat proximal tubular cells

Ang II induces phosphorylation of p38, extracellular signal-related kinase (ERK) 1/2, and JNK MAPK in several cell types.[22–24] In primary cultures of rat proximal tubular cells incubated with Ang II for 5–60 min, significant concentration-dependent phosphorylation of p38 was detected, with peak stimulation at 5-min incubation with Ang II 10^{-7} M (Figure 1: p38: 1.95 ± 0.22-fold stimulation, $P < 0.005$ vs control, $n = 3$–10). Ang II (10^{-7} M) also stimulated the phosphorylation of ERK 1/2 and JNK, with a peak effect at 5-min incubation (ERK1/2: 1.70 ± 0.19-fold stimulation, $P < 0.001$ vs control, $n = 4$; JNK: 2.73 ± 0.73-fold stimulation, $P < 0.05$ vs control, $n = 4$). Similar stimulatory effects of Ang II were observed on both ERK1 and 2, and on the p54 and p46 JNK proteins. Ang II had no effect on the amount of nonphosphorylated MAPK proteins.

Rat proximal tubular cells express mRNA for the *mas* receptor

To determine if primary cultures of rat proximal tubular cells express mRNA for the putative receptor for Ang-(1–7), *mas*, reverse transcription-polymerase chain reaction (RT-PCR) was performed on RNA isolated from these cells. In three separate experiments, RT-PCR generated a single band corresponding to the expected 313 bp product for the *mas* cDNA in rat proximal tubular cells (results not shown). RT-PCR also confirmed the presence of *mas* mRNA in freshly isolated rat renal cortex.

Effect of Ang-(1–7) on Ang II-induced MAPK phosphorylation

Incubation of cells with Ang-(1–7) (10^{-7} M) alone caused a small, nonsignificant increase in p38 MAPK phosphorylation (1.19 ± 0.11-fold of control, $P = $ NS, $n = 7$). However, pretreatment with 10^{-7} M Ang-(1–7) for 20 min significantly inhibited the Ang II-stimulated phosphorylation of p38 MAPK, and the other two MAPK (p38: 0.73 ± 0.11-fold of control, $P < 0.001$ vs Ang II, $n = 7$; ERK 1/2: 0.95 ± 0.12-fold of control, $P < 0.05$ vs Ang II, $n = 5$–6; JNK: 1.15 ± 0.13-fold of control, $P < 0.01$ vs Ang II, $n = 5$) (Figures 2–4). The stimulatory effects of Ang II on MAPK phosphorylation were also significantly inhibited in the presence of the AT_1 receptor antagonist losartan (10^{-6} M) (p38: 1.00 ± 0.24-fold of control, $P < 0.001$ vs Ang II alone; ERK 1/2: 0.84 ± 0.16-fold of control, $P < 0.05$ vs Ang II alone; JNK: 0.91 ± 0.09-fold of control, $P < 0.001$ vs Ang II alone) (Figures 2–4). Interestingly, the AT_2 receptor antagonist PD123319 (10^{-6} M) also caused an inhibitory effect on Ang II-stimulated p38 phosphorylation (Figure 2).

The selective Ang-(1–7) receptor antagonist, D-Ala^7-Ang-(1–7) (10^{-5} M), reversed the inhibitory effect of Ang-(1–7) on Ang II-stimulated MAPK phosphorylation (Ang II + Ang-(1–7) + D-Ala^7-Ang-(1–7): p38: 2.07 ± 0.32-fold of control, $P = $ NS vs Ang II, $n = 6$; ERK 1/2: 1.32 ± 0.07-fold of control, $P = $ NS vs Ang II, $n = 5$; JNK: 1.76 ± 0.16-fold of control, $P = $ NS vs Ang II, $n = 5$) (Figures 5–7).

Effect of Ang-(1–7) on Ang II-stimulated synthesis of TGF-β_1

Incubation of rat proximal tubular cells with Ang II (10^{-7} M) for 48 h produced a significant increase in the levels of TGF-β_1 in the cell supernatant (Ang II: 27.3 ± 3.3 pg/ml vs control:

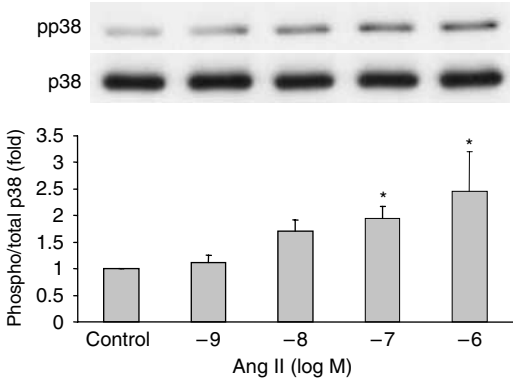

Figure 1 | Ang II induces concentration-dependent phosphorylation of p38 MAPK in rat proximal tubular cells. The effect of Ang II (10^{-9}–10^{-6} M) on p38 MAPK phosphorylation (pp38 MAPK) is shown, with a representative Western blot depicted above. Cells were treated with Ang II at the indicated concentrations for 5 min, and Western analysis was performed for phosphorylated p38 (pp38) and total p38 protein. Ang II had no significant effect on the amount of total p38. Values are means ± s.e. *$P < 0.05$ compared with control ($n = 3$–10).

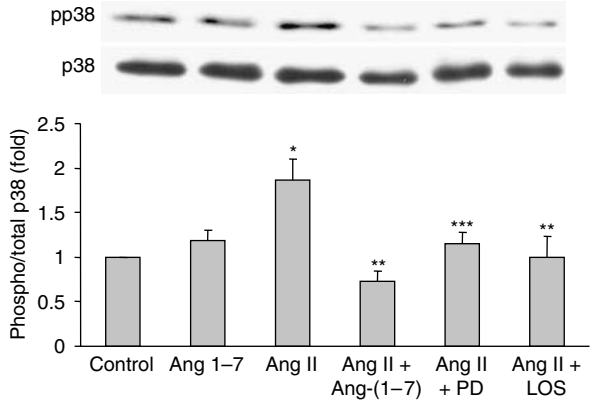

Figure 2 | Ang-(1–7) inhibits Ang II-stimulated p38 MAPK phosphorylation. Shown is the effect of pretreatment of rat proximal tubular cells for 20 min with Ang-(1–7) (10^{-7} M), the AT_1 receptor antagonist losartan (LOS, 10^{-6} M), or the AT_2 receptor antagonist PD123319 (PD, 10^{-6} M), followed by administration of Ang II (10^{-7} M) for 5 min, on phosphorylation of p38 MAPK (pp38). Representative Western blot is shown above the graph. Values are means ± s.e. *$P < 0.001$ vs control, **$P < 0.001$ vs Ang II, and ***$P < 0.05$ vs Ang II ($n = 7$).

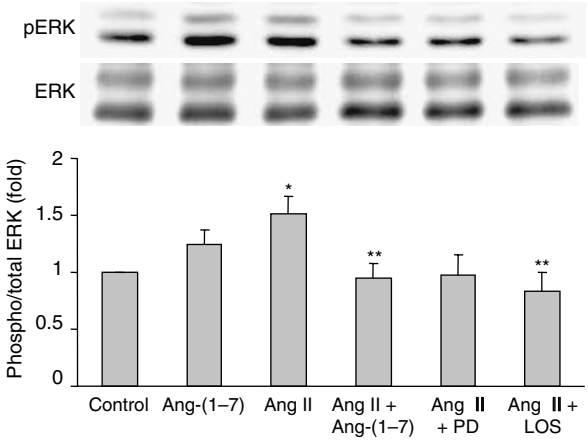

Figure 3 | Ang-(1–7) inhibits Ang II-stimulated phosphorylation of ERK 1/2. Shown is the effect of pretreatment of rat proximal tubular cells for 20 min with Ang-(1–7) (10^{-7} M), the AT_1 receptor antagonist losartan (LOS, 10^{-6} M), or the AT_2 receptor antagonist PD123319 (PD, 10^{-6} M), followed by administration of Ang II (10^{-7} M) for 5 min, on phosphorylation of ERK 1/2 (pERK). Representative Western blot is shown above the graph. Values are means ± s.e. *$P<0.05$ vs control, **$P<0.05$ vs Ang II ($n=5$–6).

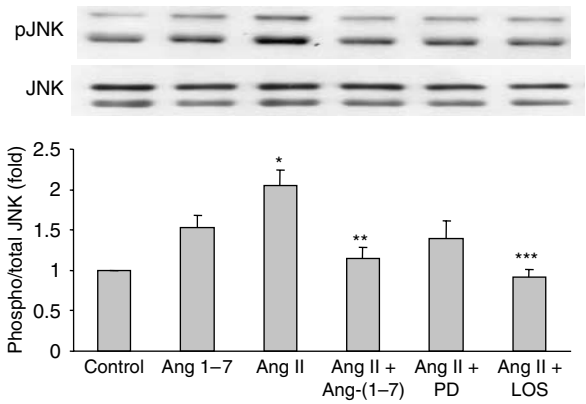

Figure 4 | Ang-(1–7) inhibits Ang II-stimulated phosphorylation of JNK. Shown is the effect of pretreatment of rat proximal tubular cells for 20 min with Ang-(1–7) (10^{-7} M), the AT_1 receptor antagonist losartan (LOS, 10^{-6} M), or the AT_2 receptor antagonist PD123319 (PD, 10^{-6} M), followed by administration of Ang II (10^{-7} M) for 5 min, on phosphorylation of JNK (pJNK). Representative Western blot is shown above the graph. Values are means ± s.e. *$P<0.001$ vs control, **$P<0.01$ vs Ang II, ***$P<0.001$ vs Ang II ($n=5$).

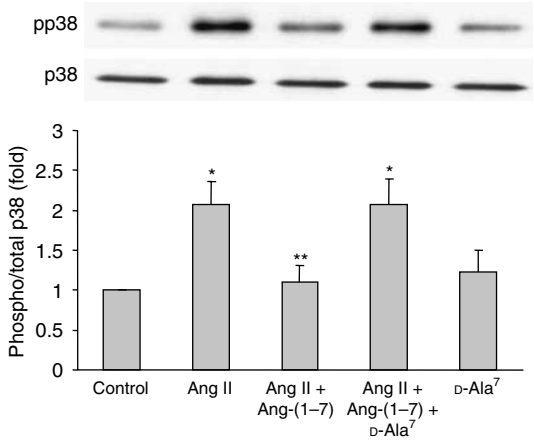

Figure 5 | D-Ala^7-Ang-(1–7) reverses the inhibitory effect of Ang-(1–7) on Ang II-stimulated p38 phosphorylation. Rat proximal tubular cells were pretreated with D-Ala^7-Ang-(1–7) (D-Ala^7, 10^{-5} M) for 10 min prior to administration of Ang II, with or without Ang-(1–7) (10^{-7} M). Shown is effect on phosphorylation of p38. *$P<0.01$ vs control, **$P<0.05$ vs Ang II or vs Ang II + Ang-(1–7) + D-Ala^7, $n=6$.

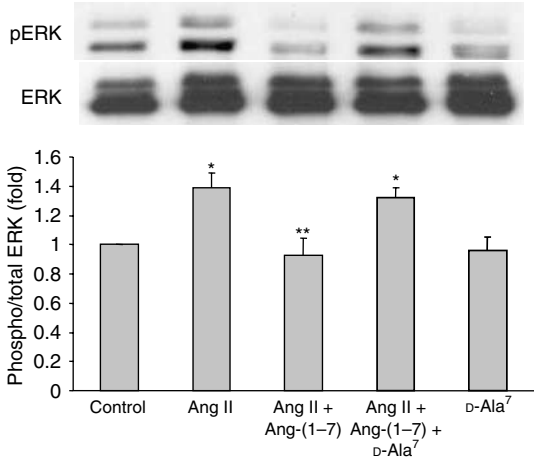

Figure 6 | D-Ala^7-Ang-(1–7) reverses the inhibitory effect of Ang-(1–7) on Ang II-stimulated ERK phosphorylation. Rat proximal tubular cells were pretreated with D-Ala^7-Ang-(1–7) (D-Ala^7, 10^{-5} M) for 10 min prior to administration of Ang II, with or without Ang-(1–7) (10^{-7} M). Shown is effect on phosphorylation of ERK. *$P<0.05$ vs control, **$P<0.005$ vs Ang II, and $P<0.05$ vs Ang II + Ang-(1–7) + D-Ala^7, $n=5$.

3.7 ± 2.3 pg/ml, $P<0.001$, $n=7$). Ang-(1–7) (10^{-7} M) alone had no effect on TGF-β_1 secretion by proximal tubular cells (Ang-(1–7): 5.9 ± 2.9 pg/ml, $n=7$). However, Ang-(1–7) caused a partial reduction in the levels of TGF-β_1 induced by stimulation with Ang II (Ang II + Ang-(1–7): 17.3 ± 3.5 pg/ml, $P<0.05$ vs control, $n=7$) (Figure 8).

Effect of Ang-(1–7) on cAMP production in rat proximal tubular cells

Ang-(1–7) stimulates cyclic 3′,5′-adenosine monophosphate (cAMP) synthesis in freshly isolated rat inner medullary collecting duct suspensions and in rat vascular smooth muscle cells.[25,26] To determine if Ang-(1–7) activates adenylate cyclase in rat proximal tubular cells, cAMP was measured after exposing cells to Ang-(1–7) for 10 or 30 min. Neither Ang-(1–7) (10^{-7} M) nor Ang II (10^{-7} M) had any effect on cAMP levels at either time point, whereas parathyroid hormone (PTH) (10^{-7} M) and forskolin (10^{-4} M) stimulated cAMP (30 min: control: 12.14 ± 1.56 pmol/mg; Ang-(1–7): 13.33 ± 1.07 pmol/mg; Ang II: 11.63 ± 1.07 pmol/mg; PTH: 24.45 ± 4.56 pmol/mg; $P<0.01$ vs control; forskolin: 190.58 ± 21.78 pmol/mg; $P<0.001$ vs control; $n=3$–5).

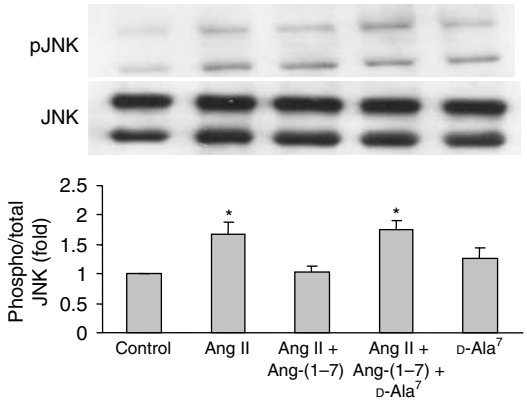

Figure 7 | D-Ala^7-Ang-(1–7) reverses the inhibitory effect of Ang-(1–7) on Ang II-stimulated JNK phosphorylation. Rat proximal tubular cells were pretreated with D-Ala^7-Ang-(1–7) (D-Ala^7, 10^{-5} M) for 10 min prior to administration of Ang II, with or without Ang-(1–7) (10^{-7} M). Shown is effect on phosphorylation of JNK. *$P<0.05$ vs control or vs Ang II + Ang-(1–7), $n=5$.

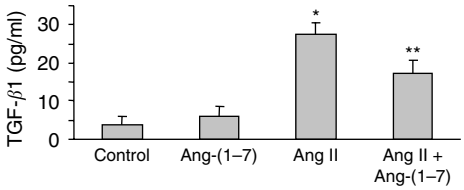

Figure 8 | Effect of Ang-(1–7) on Ang II-stimulated production of TGF-β1 in rat proximal tubular cells. Cells were treated with Ang II (10^{-7} M) for 48 h, with or without Ang-(1–7) (10^{-7} M). Values are means ± s.e.m. *$P<0.001$ vs control, **$P<0.05$ vs control ($n=7$).

DISCUSSION

The MAPK family of serine/threonine kinases consists of at least three broad families (p38 kinase, extracellular signal-regulated kinase (ERK 1/2), and c-Jun N-terminal kinase (JNK)) that regulate several processes, including cellular growth, differentiation, and survival. The MAPK family transduces signals from the cell surface to the nucleus in response to growth factors and cell stress.[27] Ang II stimulates a number of cellular responses via p38, ERK 1/2, and JNK in several cell types, including vascular smooth muscle cells and glomerular mesangial cells.[22–24,28,29] In proximal tubular cells derived from Wistar-Kyoto rats, and in porcine proximal tubular cells stably expressing the rabbit AT_1 receptor, Ang II increases ERK 1/2 and JNK activity.[30,31] Similarly, Ang II activates ERK 1/2 in opossum kidney proximal tubular cells,[32] and induces phosphorylation of p38 MAPK and ERK 1/2 in cultured mouse proximal tubular cells.[33]

The present studies demonstrate that Ang II rapidly induces phosphorylation of p38, ERK 1/2, and JNK MAPK in rat proximal tubular cells. This effect is prevented by incubation with losartan, suggesting a major role for the AT_1 receptor in mediating the response. However, the data also reveal that preincubation of cells with PD123319 caused a partial inhibition of the stimulatory effects of Ang II on p38 phosphorylation. This suggests that AT_2 receptor activation may participate in the MAPK response to Ang II, even though cells were derived from adult rats, where intrarenal AT_2 expression is relatively low. Since losartan completely blocked the MAPK phosphorylation induced by Ang II, at micromolar concentration, we cannot rule out the possibility that some nonspecificity exists, associated with use of these antagonists. Previous studies have linked AT_1 receptor activation to MAPK stimulation in proximal tubular cells,[31] and since Ang II reduces ERK 1/2 phosphorylation in LLC-PK$_1$ cells expressing the AT_2 receptor (and not the AT_1 receptor),[34] we suggest that the major pathway for MAPK activation in rat proximal tubular cells is via the AT_1 receptor.

The major finding of the present study is that Ang-(1–7) significantly reduced Ang II-induced phosphorylation of p38, ERK 1/2, and JNK MAPK in rat proximal tubular cells. Ang-(1–7) is a heptapeptide component of the renin-angiotensin system, and studies indicate that Ang-(1–7) may inhibit processes that are stimulated by Ang II, such as vasoconstriction, growth of vascular smooth muscle cells, cell proliferation, and stimulation of fibrogenic responses.[7,8,35] However, the effects of Ang-(1–7) on MAPK signaling in proximal tubule have not been studied. In this regard, in rat vascular smooth muscle cells, Ang-(1–7) inhibits Ang II-stimulated ERK 1/2 phosphorylation,[26,36] while having no effect alone on ERK 1/2, consistent with our results in proximal tubular cells. Ang-(1–7) has also been shown to inhibit serum-stimulated cell proliferation in vascular smooth muscle cells, an effect dependent on release of prostacyclin, subsequent production of cAMP, and activation of cAMP-dependent protein kinase A.[26] Similarly, in rat inner medullary collecting duct, Ang-(1–7) stimulates cAMP production and water transport, the latter effect abolished by prostaglandin E2 or protein kinase A inhibition.[25] In the current studies in rat proximal tubular cells, in contrast, we observed no effect of Ang-(1–7) on cAMP production. This suggests that other signaling pathways mediate the inhibitory effects of Ang-(1–7) on Ang II responses in the proximal tubule. Ang-(1–7) has been linked to the production of arachidonic acid in proximal tubular cells,[37] and it is also conceivable that Ang-(1–7) directly activates cytoplasmic phosphatases, leading to inhibition of agonist-stimulated MAPK activation.

We have recently demonstrated that the rat proximal tubule expresses relatively high levels of ACE2,[6] an enzyme that catalyzes the formation of Ang-(1–7), either directly from Ang II or via conversion of Ang-(1–10) to Ang-(1–9), followed by ACE-dependent cleavage to Ang-(1–7).[2,3] Synthesis of Ang-(1–7) via an ACE2-dependent pathway has been demonstrated in proximal tubule,[6] and indeed urinary levels of Ang-(1–7) are higher than those found in the circulation, suggesting substantial intrarenal synthesis.[38] Furthermore, Ang-(1–7), ACE2, and *mas* protein have been immunohistochemically localized to mouse proximal tubule, suggesting the existence of a local autocrine/paracrine system for production and action of this peptide.[39] The proximal tubule also expresses the enzymes necessary for synthesis of Ang II,

and concentrations of Ang II in the vicinity of this segment have been reported to be in the nanomolar range, or at least 100-fold higher than those in plasma.[40] The current studies suggest, therefore, that the synthesis of Ang-(1–7) in proximal tubule could represent an endogenous pathway that limits the ability of local Ang II to stimulate cell growth responses, via inhibition of MAPK activation.

Although Ang-(1–7) has limited affinity for Ang II AT_1 or AT_2 receptors, its actions in the kidney and other sites appear to be mediated by distinct cell surface receptors.[41] In 2003, Santos *et al.*[42] identified the G protein-coupled *mas* protein as a putative receptor for Ang-(1–7). In the current studies, we demonstrated the presence of *mas* mRNA in rat proximal tubular cells. The existence of a distinct receptor is also supported by pharmacologic studies. Thus, in water-loaded rats, the antidiuretic action of Ang-(1–7) is blocked by the selective Ang-(1–7) receptor antagonist D-Ala^7-Ang-(1–7), which has a very low affinity for classical Ang II receptor subtypes (AT_1 and AT_2).[43] Moreover, in bovine aortic endothelial cells, neither AT_1- nor AT_2-selective receptor antagonists significantly competed for ^{125}I-Ang-(1–7) binding.[44] In our studies, we observed a small but nonsignificant stimulatory effect of Ang-(1–7) alone on p38 MAPK phosphorylation. Studies have reported that the effects of Ang-(1–7) may be blocked by AT_1 receptor antagonists under certain conditions, suggesting partial agonist activity of Ang-(1–7) on the AT_1 receptor.[45,46] Our data strongly support the existence of a distinct receptor for Ang-(1–7) in rat proximal tubular cells, since the inhibitory effect of Ang-(1–7) on stimulation of all three MAPK by Ang II was completely reversed by D-Ala^7-Ang-(1–7). Moreover, in rat cardiac myocytes, Ang-(1–7) inhibits phosphorylation of ERK 1/2, an effect that is completely blocked by transfection of cells with antisense oligonucleotide to the *mas* receptor, providing further support for the role of *mas* as a distinct binding site for Ang-(1–7).[47]

Ang II stimulates the synthesis of TGF-β_1 in proximal tubular cells.[20,21] In pathophysiologic states such as diabetic nephropathy or urinary tract obstruction, Ang II-stimulated production of TGF-β_1 represents a final common pathway that is thought to accelerate the progression of tubulointerstitial fibrosis.[48,49] Although the precise mechanisms of Ang II-induced TGF-β_1 expression remain unclear, in mouse mesangial cells Ang II regulates TGF-β_1 production via a pathway involving MAPK.[50] Conversely, TGF-β_1 can directly activate all three MAPK signaling pathways.[51] Our data confirm that Ang II significantly stimulates the secretion of active TGF-β_1 from rat proximal tubular cells. Ang-(1–7) caused a partial decrease in Ang II-stimulated levels of TGF-β_1, compared to those associated with Ang II alone. Our results are in agreement with studies by Wolf *et al.*,[21] who showed that transfection of proximal tubular cells with the *c-mas* oncogene (encoding the putative receptor for Ang-(1–7)) caused an inhibition of Ang II-stimulated TGF-β_1 production. In the present studies, the stimulation of TGF-β_1 secretion remained significantly elevated, compared to control cells. The data suggest, therefore, that the production of TGF-β_1 mediated by Ang II in rat proximal tubular cells involves additional pathways besides any of the three MAPKs. Thus, Ang-(1–7)-mediated production of arachidonic acid,[37] or other signals, might be involved in regulating the secretion of TGF-β_1 in proximal tubule.

In summary, our data indicate that in rat proximal tubular cells, Ang-(1–7) inhibits Ang II-mediated MAPK phosphorylation, and partly suppresses Ang II-stimulated increases in TGF-β_1. ACE2-mediated production of Ang-(1–7) may counteract the effects of locally generated Ang II in the proximal tubule, thereby protecting against the development of progressive tubulointerstitial fibrosis, a hallmark of chronic kidney disease.

MATERIALS AND METHODS

Isolation and culture of rat proximal tubular cells

Suspensions of rat proximal tubules were isolated by collagenase digestion of renal cortices, followed by Percoll gradient centrifugation, essentially as described.[52] Briefly, kidneys were removed aseptically from anesthetized 200–250 g male Sprague–Dawley rats, and renal cortices were dissected, gently minced, and suspended in a solution consisting of (in mmol/l) 105 NaCl, 24 $NaHCO_3$, 5 KCL, 1.5 $CaCL_2$, 1.0 $MgSO_4$, 2.0 Na_2HPO_4, 5 D-glucose, 1.0 alanine, 4.0 Na lactate, 10 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.4), 0.2% bovine serum albumin, 0.03% collagenase (type IV; Sigma, St Louis, MO, USA), and 0.01% soybean trypsin inhibitor (Sigma) (buffer A). The suspension was gassed with 5% CO_2–95% O_2 for 30 min at 37°C. After digestion, the cortical suspension was strained through a 250 μm brass sieve and then centrifuged for 1 min at 100 *g*. The pellet was resuspended in buffer A without collagenase or trypsin inhibitor and recentrifuged three times at 100 *g*. The pellet was then applied to a 42% Percoll solution of identical ionic composition as buffer A, which had been previously chilled to 4°C. The Percoll solution was centrifuged at 26 000 *g* for 30 min at 4°C, and the digested tissue separated into four distinct bands. The F_4 layer, enriched in proximal tubular segments, was removed and suspended in buffer A without collagenase or trypsin inhibitor and centrifuged. The final pellet was suspended in culture medium and tubules were seeded onto culture plates. The procedure yields a highly purified preparation of proximal tubules (97% by microscopy), and viability of segments was >95%, as determined by exclusion of trypan blue (10 mg/dl).

Cells plated for culture were initially grown in a defined medium consisting of Dulbecco's modified Eagle's medium-F12, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (50 nM), and 3,3′,5-triiodo-L-thyronine (2.5 nM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. At 24 h after plating, cells were switched to defined medium without serum. The cells were maintained at 37°C, in a humidified environment of 5% CO_2 in Dulbecco's modified Eagle's medium/F-12 medium. Experiments were performed on subconfluent cells (~75% confluence) that had been rendered quiescent by 24 h incubation in serum- and hormone-free culture medium.

RT-PCR

RT-PCR was performed to demonstrate the presence of mRNA for the putative receptor for Ang-(1–7), the *mas* protein, in rat proximal tubular cells. Briefly, RNA (1 μg) was isolated from primary cultures

of rat proximal tubular cells, and rat kidney cortex (RNeasy, Qiagen, Mississauga, ON, Canada). The RNA was reverse transcribed, denatured at 94°C for 3 min, and then subject to 40 cycles of PCR at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, followed by extension at 72°C for 10 min, using a commercial kit (Applied Biosystems, Foster City, CA, USA). The selected primers were designed to generate a 313 bp product, corresponding to bp 267–579 of the rat *mas* cDNA (GenBank). The upstream primer was 5′-ctatgctttagactat gaactctct-3′, and the downstream primer was 5′-gaggatggctatgaagat gatgac-3′. PCR products were identified by running samples on agarose gels stained with ethidium bromide.

Western blotting

After 24 h in hormone- and serum-free medium, subconfluent proximal tubular cells were incubated for various times (up to 60 min) with Ang II (10^{-6}–10^{-9} M) (Bachem Bioscience Inc., King of Prussia, PA, USA), followed by immunoblot assays for phosphorylated MAPK. In certain experiments, cells were pretreated with either 10^{-7} M Ang-(1–7) (Bachem Bioscience Inc.), 10^{-6} M losartan (Merck Research Laboratories, Rahway, NJ, USA), 10^{-6} M PD123319 (Sigma) or 10^{-5} M D-Ala^7-Ang-(1–7) (Bachem Bioscience Inc.) for 10–20 min prior to application of Ang II. The phosphorylation state of the MAPK p38, ERK 1/2 and JNK was measured by Western blotting, as described.[34] Briefly, cells were lysed in a buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% w/v sodium dodecyl sulfate, 10% glycerol, 50 mM DTT, and 0.1% w/v 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% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Ltd, 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 1:1000 dilution of antiphosphospecific antibodies to p38 MAPK (Cell Signaling Technology, New England Biolabs Ltd, Pickering, ON, Canada), ERK 1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and JNK (Santa Cruz Biotechnology, Inc.). Membranes were then incubated with 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ, USA), and then washed. Phosphorylated proteins were detected by enhanced chemiluminescence (Amersham) on Hyperfilm (Amersham). Prestained standards were used as molecular weight markers (Bio-Rad). To control for protein loading, all membranes were stripped and re-probed with antibodies to unphosphorylated p38 MAPK (Cell Signaling Technology), ERK 1/2 (Santa Cruz Biotechnology, Inc.), and JNK (Santa Cruz Biotechnology, Inc.). Signals for phosphorylated MAPK proteins on Western blots were quantified by densitometry and corrected for unphosphorylated protein levels, using an image-analysis software program (Kodak Densitometer 1S440CF). For ERK, graphic data show the p42 signal, while for JNK, graphic data indicate the p54 signal. Similar effects were observed on p44 ERK and p46 JNK.

Enzyme-linked immunosorbent assay assay for TGF-β_1

The secretion of active TGF-β_1 from cultured rat proximal tubular cells was quantified using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. For each experiment, a TGF-β_1 standard curve was constructed using varying concentrations of recombinant human TGF-β_1 protein, and a curve-fitting software program was used to quantify TGF-β_1 protein concentration in the culture media. All data were corrected for cell protein content, measured by Bradford assay.

cAMP assay

Quiescent primary cultures of rat proximal tubular cells on plastic dishes were incubated in Dulbecco's modified Eagle's medium/F12 medium supplemented with isobutyl-methylxanthine (10^{-4} M) and 0.5% bovine serum albumin, along with either Ang-(1–7) (10^{-7} M), Ang II (10^{-7} M), PTH (10^{-7} M), or forskolin (10^{-4} M), for 10 or 30 min. The culture medium was then aspirated and replaced with ice-cold 10% trichloroacetic acid (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 (Intermedico, Markham, ON, Canada). Experiments were performed in duplicate, and results are expressed as pmoles cAMP/mg protein.

Statistical analysis

Results are presented as means $\pm$ s.e. Significance, considered as $P<0.05$, was determined by analysis of variance, with Bonferroni correction, in all cases involving multiple comparisons.

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