Angiotensin-(1–7)-induced activation of ERK1/2 is cAMP/protein kinase A-dependent in glomerular mesangial cells

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Angiotensin-(1–7) infusion has been reported to attenuate experimental and vascular smooth muscle cells (13). In this regard, protective role for Ang-(1–7) because Ang-(1–7) prevented mechanism within the RAS (15, 20, 31). Studies support a suggested that Ang-(1–7) is an important counterbalancing proliferative effects (10, 11, 16, 30, 32, 34, 35). It has been II to Ang-(1–7) (33). Ang-(1–7) activates the mas receptor and Ang-(1–7) by ACE. In addition, ACE2 can also convert ANG ACE2 converts ANG I to Ang-(1–9) which can be converted to converting enzyme 2 (ACE2) has been shown to be protective of the RAS is angiotensin II (ANG II) (18), angiotensin- development and progression of chronic kidney disease includ- ing diabetic nephropathy. Although the main effector peptide of the RAS is angiotensin II (ANG II) (18), angiotensin-converting enzyme 2 (ACE2) has been shown to be protective under many pathological settings (2, 8, 25, 27, 28, 37, 40, 43). ACE2 converts ANG I to Ang-(1–9) which can be converted to Ang-(1–7) by ACE. In addition, ACE2 can also convert ANG II to Ang-(1–7) (33). Ang-(1–7) activates the mas receptor and is associated with vasodilating, natriuretic, diuretic, and anti-proliferative effects (10, 11, 16, 30, 32, 34, 35). It has been suggested that Ang-(1–7) is an important counterbalancing mechanism within the RAS (15, 20, 31). Studies support a protective role for Ang-(1–7) because Ang-(1–7) prevented ANG II-induced MAPK activation in proximal tubular cells (35) and vascular smooth muscle cells (13). In this regard, Ang-(1–7) infusion has been reported to attenuate experimental glomerulonephritis (44). However, other studies suggest that some of the cellular effects of Ang-(1–7) may be deleterious; for example, stimulation of growth factor expression and cell proliferation by Ang-(1–7) in MC have been reported (45). In addition, deletion of the gene for the mas receptor in mice is associated with decreased inflammation in kidneys subjected to unilateral ureteral obstruction (9).

The mas receptor is expressed by glomerular mesangial cells (MC) but the intracellular signal transduction pathways activated by Ang-(1–7) have not been fully elucidated. Accordingly, the goal of the current study was to elucidate the intracellular signal- ing pathways linking Ang-(1–7) to the activation of the canonical mitogen-activated protein kinase extracellular signal-related kinase (ERK1/2) in MC. We observed that the activation of ERK1/2 by Ang-(1–7) was dependent on generation of cAMP and the subsequent activation of protein kinase A (PKA).

MATERIALS AND METHODS

Cell culture. Harlan Sprague-Dawley rat MC (SD-MC) were cultured in DMEM supplemented with 20% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in 95% air-5% CO2. Before treatment, the cells were starved in serum-free medium overnight. Experiments were carried out using cells between passages 11 to 20.

Chemicals. Angiotensin-(1–7), ANG II, DB-cAMP, H-89 and pCPT-cAMP, DPI, NAC, catalase, and BAPTA-AM were obtained from Sigma (St. Louis, MO). SQ22536, KT5720, AG1478, PD158780, and apocynin were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). A-779 was purchased from Bachem California (Torrance, CA). A23187 was purchased from Alexis (Enzo Life Sciences, Ann Arbor, MI). Ang-(1–7) (10−11 M) was used for experiments unless mentioned otherwise.

Western blot analysis. After treatment, cells were washed twice with ice-cold PBS and transferred to microcentrifuge tubes and centrifuged at 18,000 g for 30 s. Cell pellets were resuspended and lysed on ice with lysis buffer (Cell Signaling, Beverly, MA) containing 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After 20 min of incubation in the lysis buffer, a 10-min centrifugation at 18,000 g at 4°C was performed. The total cell lysate was collected, and the protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples with equal concentrations of cellular protein (25 μg) were mixed with 6× SDS sample loading buffer containing 125 mM Tris·HCl (pH 6.8), 2% SDS, 20% glycerol, 0.2% bromophenol blue, and 5% β-mercaptoethanol and were boiled for 5 min, and separated by a 10% SDS-PAGE gel and then transferred onto Immobilon polyvinylidene fluoride membrane (Millipore, Bedford, MA). After being blocked with 5% skim milk, primary antibodies were applied (1:1,000 phospho-ERK1/2, 1:1,000 total-ERK1/2, purchased from Cell Signaling) and allowed to incubate overnight at 4°C. After extensive washing in TBS-Tween 20, the membranes were then
incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. After being washed, the membranes were incubated with enhanced chemiluminescence system detection kit (Millipore, Billerica, MA). Total ERK1/2 was used as loading control, and densitometry was measured using Scion Image software (Scion, Frederick, MD).

cAMP assay and PKA assay. MC were cultured in 60-mm culture dishes to confluence and subsequently serum deprived for 18 h. The cells were then treated with 10^{-11} M Ang-(1–7) for 15 min and lysed. The amount of cAMP extracted from each dish was quantified by cAMP assay according to the manufacturer’s instructions and normalized against total protein as determined by Bradford assay. For PKA assay, MC were cultured in 60-mm culture dishes to confluence and subsequently serum deprived for 18 h. The cells were pretreated with the potent PKA-selective inhibitor H89 (1 μM) for 30 min before Ang-(1–7) stimulation. The cells were lysed and adjusted to the same concentration of protein in each sample. An IMPAK-PAK Assay Kit (Molecular Devices, Sunnyvale, CA) was used to measure PKA enzyme activity according to the manufacturer’s instruction.

Fluorescence imaging. SD-MC were transferred to six-well plates containing autoclaved glass slips and grown to 80% confluence. Cells were serum-starved overnight. Cells were pretreated by the inhibitors and/or 10^{-11} M Ang-(1–7) for 15 min at 37°C. After treatment, cells were washed three times with ice-cold PBS. Cells were fixed with 4% paraformaldehyde for 30 min, and then washed again. Blocking with 5% goat serum was performed at room temperature over 1 h, and cells were then incubated overnight at 4°C with anti-phospho-ERK1/2 antibody (1:100; Molecular Probes, Eugene, OR) and detected using an FB12 luminometer (Huntsville, AL). The protein concentration was determined with the Bradford assay (Bradford Laboratories).

Lucigenin chemiluminescence for NADPH oxidase activity. SD-MC were plated on 60-mm dishes with growth medium. Cells were cultured for 72 h to 90% confluence, then exposed to experimental reagents for the indicated times and concentrations, washed with ice-cold PBS twice, collected, and sonicated. After 20 min of centrifugation at 1,000 rcf and 4°C, 100 μl of supernatant were assayed by adding lucigenin (5 μM, Sigma), NADPH (1 mM, Merck KGaA), and detected using an FB12 luminometer (Huntsville, AL). The protein concentration was determined with the Bradford assay (Bradford Laboratories).

Statistical analysis. Results are expressed as means ± SE, unless otherwise specified. Student’s t-test was used for comparison between two groups. Comparisons between multiple groups were performed by one-way ANOVA followed by Bonferroni correction using GraphPad software.

RESULTS

Ang-(1–7) induces phosphorylation of ERK1/2. We first examined the time- and concentration-dependent effect of
Fig. 3. Effect of DPI and apocynin on the activation of ERK1/2 in SD-MC on Ang-(1–7) (A) or ANG II (B)-induced ERK1/2 phosphorylation. A: cells were pretreated with 10 μM DPI or 100 μM apocynin for 1 h before exposure to 10^{-11} M Ang-(1–7) for 5 min. B: cells were pretreated with 10 μM DPI or 100 μM apocynin for 1 h before exposure to 10^{-7} M ANG II for 5 min. *P < 0.05 vs. untreated control cells. †P < 0.05 compared with cells treated with ANG II alone. ‡P < 0.001 compared with cells treated with ANG II alone. Each experiment was performed in triplicate.

Ang-(1–7) on the phosphorylation of ERK1/2 in our primary rat MC (Fig. 1, A and B). As expected, there was a time-dependent activation of ERK1/2 and we observed a peak effect at 5 min. Although we observed a stronger intensity for phospho-ERK2 (p42 MAPK) following treatment with Ang-(1–7), the responses of phospho-ERK1 and phospho-ERK2 paralleled one another. ERK1/2 was activated at a dose of 10^{-12} M Ang-(1–7) and there was only a modest dose-dependent effect on the phosphorylation of ERK1/2 at higher concentrations of the peptide. Ang-(1–7) (10^{-11} M) was used in subsequent experiments.

**Effect of A-779 on Ang-(1–7)-induced ERK1/2 phosphorylation.** To relate activation of ERK1/2 by Ang-(1–7) to the mas receptor, we studied the effect of the mas receptor antagonist A-779. A-779 inhibited Ang-(1–7)-induced ERK1/2 phosphorylation in a dose-dependent manner (Fig. 2A). Pretreatment with the AT₁ blocker losartan did not prevent Ang-(1–7)- induced ERK1/2 phosphorylation (Fig. 2B), although the dose of losartan effectively blocked ANG II-induced ERK1/2 phosphorylation (Fig. 2C). Pretreatment with the AT₂ blocker PD123319 did not attenuate Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 2B) or ANG II-induced ERK1/2 phosphorylation in the primary SD-MC (Fig. 2C).

**Effect of DPI and apocynin on Ang-(1–7)-induced ERK1/2 phosphorylation.** To better understand the transduction pathways linking Ang-(1–7)/mas receptor interaction(s) to the phosphorylation of ERK1/2, we first studied the effect of NADPH oxidase inhibition. Cells were pretreated with the NADPH oxidase inhibitors DPI (10 μM) or apocynin (100 μM) for 60 min before the administration of Ang-(1–7). Neither NADPH oxidase inhibitors reduced Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 3A), although pretreatment with the inhibitors significantly attenuated ANG II-induced phosphorylation of ERK1/2 (Fig. 3B).

Ang-(1–7)-induced ERK1/2 phosphorylation is independent of the epidermal growth factor receptor. Transactivation of the epidermal growth factor receptor (EGFR) has been linked to ERK1/2 signaling by ANG II (5). To assess the role of EGFR in Ang-(1–7)-induced ERK1/2 phosphorylation, we pretreated SD-MC with the EGFR kinase inhibitors AG1478 and PD158780. Neither EGFR kinase inhibitors attenuated Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 4A), although the equivalent doses prevented both EGF-induced ERK1/2 phosphorylation (Fig. 4B) and ANG II-induced ERK1/2 phosphorylation (Fig. 4C).

Ang-(1–7) increases cAMP levels in SD-MC and Ang-(1–7)-induced ERK1/2 phosphorylation is cAMP-dependent. In some cell types, mainly of neuronal origin, activation of ERK1/2 downstream of Gs-α-coupled receptors is cAMP-dependent. Ang-(1–7) increased cAMP levels in the SD-MC, an effect that was abrogated by pretreatment with the mas receptor antagonist A-779 (Fig. 5A). The selective adenylyl cyclase inhibitor DPI (10 μM) for 60 min before the administration of Ang-(1–7) and there was only a modest dose-dependent inhibition. Cells were pretreated with 10 μM DPI or 100 μM apocynin for 1 h before exposure to 10^{-11} M Ang-(1–7) for 5 min. *P < 0.05 compared with untreated control cells. †P < 0.05 compared with cells treated with ANG II alone. Each experiment was performed in triplicate.

Fig. 4. Effect of epidermal growth factor (EGF) receptor antagonists on activation of ERK1/2 in SD-MC. A: cells were preincubated with 0.2 μM AG-1478 for 20 min or 10 μM PD158780 for 2 h and then treated with 10^{-11} M Ang-(1–7). B: cells were preincubated with 0.2 μM AG-1478 for 20 min or 10 μM PD158780 for 2 h and then treated with 10^{-7} M ANG II for 5 min. Values are means ± SE. *P < 0.001 compared with untreated SD-MC. †P < 0.001 compared with SD-MC treated with ligand alone. ‡P < 0.05 compared with cells treated with ANG II alone. Each experiment was performed in triplicate.
SQ22536 also prevented Ang-(1–7)-induced increase in cAMP levels (Fig. 5A). Taken together, these findings suggest that the mas receptor is coupled to Gs and that the binding of Ang-(1–7) activates adenylyl cyclase in SD-MC. We then studied the effect of SQ22536 on Ang-(1–7)-induced activation of ERK1/2, and we found that pretreatment with the adenylyl cyclase inhibitor prevented phosphorylation of ERK1/2 (Fig. 5B).

Ang-(1–7) increases PKA activity in SD-MC and Ang-(1–7)-induced ERK1/2 phosphorylation is PKA-dependent. It has been proposed that there is a signal transduction pathway that includes cAMP, PKA, and MEK leading to the activation of ERK1/2 (6, 23, 24). We first found that Ang-(1–7) increased PKA activity, an effect that was inhibited by the reversible PKA inhibitor H89 (Fig. 6A). We then studied the effect of the reversible PKA inhibitors H89 and KT5720 on Ang-(1–7)-induced activation of ERK1/2 and found that pretreatment with the PKA inhibitors prevented phosphorylation of ERK1/2 (Fig. 6, B and C). To further relate cAMP and PKA to ERK1/2 phosphorylation in our primary SD-MC, we studied the effect of the cell-permeable cAMP analog pCPT-cAMP on ERK1/2 phosphorylation. Treatment with the pCPT-cAMP led to a time-dependent activation of ERK1/2 in the SD-MC (Fig. 7A). This analog leads to the activation of both PKA- and cAMP-activated guanine-nucleotide exchange factors (Epac) so we also examined the effect of inhibiting PKA on pCPT-cAMP-induced phosphorylation of ERK1/2. Inhibition of PKA by H89 attenuated pCPT-cAMP-induced phosphorylation of ERK1/2, as did pretreatment with the MEK inhibitor PD98059 (Fig. 7B). Taken together, these findings suggest that the signal transduction pathway linking Ang-(1–7) to the activation of ERK1/2 is dependent on both cAMP and PKA and does not proceed through Epac.

**DISCUSSION**

The RAS plays a key role in both renal physiology and the pathogenesis of chronic kidney disease. All components of the RAS are expressed in the kidney and ANG II is generated at higher levels in the kidney than in the systemic circulation. The recent discovery of ACE2 has refocused attention on angiotensin peptide processing and the generation of other biologically relevant angiotensin peptides. ACE2 is highly expressed in the kidney and generates Ang-(1–7). In vivo studies suggest that Ang-(1–7) may exert a protective effect in the kidney. For example, Giani et al. (15) showed that Ang-(1–7) reduced proteinuria and renal fibrosis in hypertensive rats. Similarly,
Zhang et al. (44) found that Ang-(1–7) infusion attenuated glomerulosclerosis in rats, and more recently attenuated kidney injury in mice with type 2 diabetes mellitus (26). However, the signal transduction pathways downstream of Ang-(1–7) in kidney cells have not been fully elucidated.

Our first major observation was that Ang-(1–7) activated ERK1/2 in SD-MC in a mas receptor/cAMP/PKA-dependent manner. Although MCs express the G protein-coupled receptor mas, it has been reported that Ang-(1–7) can interact with the AT1 receptor in rat heart and renal cortex (14, 17), and more recently that Ang-(1–7) exhibits modest affinity for the AT2 receptor (39). However, we found that activation of ERK1/2 was downstream of the mas receptor because the mas receptor antagonist A-779 prevented ERK1/2 phosphorylation while neither the AT1 receptor blocker losartan nor the AT2 receptor antagonist PD123319 had an effect on ERK1/2 phosphorylation, in accord with previous studies (45). Given the above reports on Ang-(1–7)/AT1 receptor interactions we also determined that the dose of losartan, we used inhibited ANG II-induced activation of ERK1/2.

We consistently observed a stronger intensity for phospho-ERK2 (p42 MAPK) immunostaining than for phospho-ERK1 receptor (39). However, we found that activation of ERK1/2 was downstream of the mas receptor because the mas receptor antagonist A-779 prevented ERK1/2 phosphorylation while neither the AT1 receptor blocker losartan nor the AT2 receptor antagonist PD123319 had an effect on ERK1/2 phosphorylation, in accord with previous studies (45). Given the above reports on Ang-(1–7)/AT1 receptor interactions we also determined that the dose of losartan, we used inhibited ANG II-induced activation of ERK1/2.

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We consistently observed a stronger intensity for phospho-ERK2 (p42 MAPK) immunostaining than for phospho-ERK1
(p44 MAPK) immunostaining following treatment with Ang-(1–7) and ANG II in the Western blot analyses. Although there is evidence suggesting different biologic roles for ERK1 and ERK2 (12, 29, 42), it is widely accepted that ERK1 and ERK2 are regulated in a similar manner. In addition, despite the difference in the intensity of the signals for phospho-ERK1 and phospho-ERK2, the responses to our experimental manoeuvres paralleled one another. We therefore did not distinguish between ERK1 and ERK2 in the presentation of our data.

A number of signal transduction pathways link G protein-coupled receptors to the activation of ERK1/2. One central pathway utilized by the AT1 receptor is NADPH oxidase activation and several studies have shown that inhibition of NADPH oxidase attenuates AT1-dependent activation ERK1/2 (18). In addition, transactivation of the EGFR by ANG II/AT1 is also an important determinant of ANG II-dependent activation of ERK1/2 (5). We therefore studied the effect of two inhibitors of NADPH oxidase and two inhibitors of EGFR kinase activity and found that while these reagents effectively inhibited ANG II-induced activation of ERK1/2, there was no effect on Ang-(1–7)-induced ERK1/2 activation. These findings suggest that neither NADPH oxidase nor transactivation of the EGFR plays a role in the signal transduction pathway linking Ang-(1–7)/mas receptor to ERK1/2, unlike ANG II/AT1 receptor cell signaling, and that there is a fundamental difference in the mechanisms linking these two peptide ligands and their cognate receptors to the activation of ERK1/2.

Initially, we did not expect that cAMP/PKA signaling would play a role in the activation of ERK1/2 because Gs-α-coupled receptors inhibit ERK1/2 activation in many cell types, due at least in part to a cAMP/PKA-dependent inhibition of the serine threonine kinase Raf-1 that is upstream of MEK and ERK1/2 (4). Moreover, there have been conflicting reports on the effect of Ang-(1–7) on cAMP levels in kidney cells in vitro (45). However, it has also been reported that ERK1/2 is activated in neuronal cells by Gs-α-coupled receptors (24). Although the signal transduction pathway linking Gs-coupled receptors to ERK1/2 is not well-defined, it may proceed through cAMP, PKA, and MEK because other studies have implicated a role for the GTP-binding Ras and Raf-1 (6, 21, 38). Our second major observation was that inhibition of adenyl cyclase attenuated Ang-(1–7)-induced activation of ERK1/2. We therefore measured cAMP levels in our SD-MC and we found a modest increase that was prevented by pretreatment of the cells with the mas receptor antagonist A-779 and the adenyl cyclase inhibitor SQ22536. These novel findings suggest that the mas receptor is coupled to Gs-α and that ligand binding activates Gs-α and its effector molecule adenyl cyclase.

The Ang-(1–7)-induced increase in cAMP levels was also associated with an increase in PKA activity in the SD-MC. However, cAMP can lead to an increase in ERK1/2 independent of PKA, an effect that may be mediated by cAMP-activated guanine-nucleotide exchange factors (cAMP-GEFs, also known as Epacs) (23). This pathway has been described in kidney cells (44). We next treated our SD-MC with two different PKA inhibitors before exposure to Ang-(1–7) to determine whether ERK1/2 activation was dependent on PKA. Both compounds inhibited the Ang-(1–7)-induced ERK1/2 phosphorylation. Taken together with our finding that a stable cAMP analog activated ERK1/2 in the SD-MC in a PKA- and MEK-dependent manner, these findings support the conclusion that Ang-(1–7) signals to ERK1/2 via cAMP/PKA and not via cAMP-GEFs. Finally, we compared the effect of PKA inhibition and EGFR inhibition on the activation and nuclear translocation of ERK1/2 by confocal microscopy and fluorescence imaging. In accord with the Western blot analyses, PKA inhibition but not EGFR kinase inhibition prevented nuclear translocation of ERK1/2.

We believe that these findings have some relevance to experimental models of kidney injury, especially with respect to a protective role of Ang-(1–7). Infusion of Ang-(1–7) attenuates experimental glomerulonephritis and diabetic nephropathy and the inhibition of NADPH oxidase and ERK1/2 may contribute to these beneficial effects. On the other hand, it is difficult to reconcile these findings with reports that Ang-(1–7) promotes TGF-β1 and extracellular matrix protein production in MC in vitro (45) and contributed to epithelial-to-mesenchymal transformation (3). It may be that these findings are linked with angiotensin-converting enzyme 2 attenuates angiotensin II-induced collagen production via AT1 receptor-phosphoinositide 3-kinase-Akt pathway. Endocrine Res 39: 139–147, 2011. 3. Burns WC, Velkoska E, Dean R, Burrell LM, Thomas MC. Angiotensin II mediates epithelial-to-mesenchymal transformation in tubular

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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