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Key Role of P38 Mitogen-Activated Protein Kinase and the Lipoxygenase Pathway in Angiotensin II Actions in H295R Adrenocortical Cells

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Angiotensin II (ANG II) can activate the mitogen-activated protein kinases (MAPKs) and stress-activated protein kinases in several cell types. We have previously shown that the 12-lipoxygenase (12-LO) pathway of arachidonic acid metabolism is a mediator of ANG II–induced aldosterone synthesis in adrenal glomerulosa cells. To evaluate the role of MAPK activation in ANG II and the effects of LO on aldosterone synthesis, experiments were performed using the human adrenocortical cell line H295R, which secretes aldosterone in response to ANG II. MAPK activities were determined by Western immunoblotting using specific antibodies to their activated phosphorylated forms. ANG II led to a dose-dependent increase in extracellular signal-regulated kinase (ERK1/2) activity in these cells, with a peak at 5 min and lasting up to 3 h. The effects of ANG II were blocked by the ANG-II Type 1 receptor antagonist losartan. A specific 12-LO product, 12(*S*)-hydroxyeicosatetraenoic acid (12-HETE), had no direct effect on ERK activity. However, both ANG II and 12-HETE led to significant dose-dependent increases in p38 MAPK activity with peak effects at 5 min. By contrast, the 15-LO product, 15-HETE, had no effect on p38 MAPK activity. Furthermore, two dissimilar 12-LO inhibitors, CDC and baicalein, blocked ANG II–induced p38 MAPK activation. ANG II significantly increased aldosterone release, and this effect was inhibited by the LO inhibitor baicalein, as well as a specific p38 MAPK inhibitor, SB202190, but not by PD098059, a specific inhibitor of the ERK activator MEK. In summary, in H295R cells, ANG II activated ERK and p38 MAPKs, ANG II–induced p38 MAPK was mediated by 12-LO activation, and ANG II–induced aldosterone synthesis was prevented by 12-LO- and p38 MAPK–specific inhibitors. These results suggest, for the first time, that activation of p38 MAPK, either directly or via LO activation, participates in aldosterone's stimulatory effects of ANG II in adrenal cells.

Key Words: Angiotensin II; lipoxygenase; aldosterone; mitogen-activated protein kinase; aldosterone synthase.

Introduction

Angiotesin II (ANG II) has important actions in several target tissues including heart, vascular smooth muscle, brain, kidney, and adrenal gland *(1)*. It is a major regulator of the steroid aldosterone in the zona glomerulosa of the adrenal gland *(2)*. ANG II exerts many of its cellular effects by binding to ANG II Type l (AT1) receptor coupled to G protein *(3,4)*. Its actions on steroidogenesis of adrenal glomerulosa have been linked to subsequent phospholipase C (PLC) and PLD activation *(5,6)*. PLC activation leads to the hydrolysis of phosphatidylinositol 4,5-biphosphate to 1,4,5-IP_3 and diacylglycerol, which, in turn, leads to intracellular calcium mobilization and the stimulation of protein kinase C (PKC) *(7,8)*. PLD hydrolyzes phosphatidylcholine into phosphatidic acid and choline *(6)*. Then DAG and phosphatidic acid can be converted into arachidonic acid, which may be subsequently hydrolyzed by multiple pathways including the 12-lipoxygenase (12-LO) pathway, which forms products such as 12(*S*)-hydroxyeicosatetraenoic acid (12-HETE) *(9)*. Our earlier studies demonstrated that the 12-LO pathway of arachidonate metabolism participates in ANG II–induced aldosterone synthesis in rat and human adrenal glomerulosa cells *(10–12)*. However, the mechanisms by which 12-LO products mediate Ang II actions in the adrenal are not clear.

One of the main impediments to the understanding of mechanisms underlying the regulation of aldosterone by ANG II has been the unavailability of suitable cell lines that would retain the typical responses of ANG II for extended time periods. It was recently demonstrated that the NCI-H295R cell line, cultured from a human adrenocortical tumor, is a good model for these purposes *(13,14)*. This cell line has since been extensively used to examine various cellular

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and molecular aspects of adrenal steroidogenesis and growth. In the present study, we used this H295R adrenal cell line as a model for ANG II–responsive aldosterone secretion to define the signaling mechanisms that regulate ANG II–induced aldosterone release in human glomerulosa cells, as well as the role of 12-LO in this process.

ANG II is a potent mitogen for adrenal cortical and glomerulosa cells *(15–18)*. These responses, similar to the steroidogenic effects, are mediated by AT1 receptor. It has also been shown that ANG II–induced aldosterone in H295R cells is mediated by the AT1 receptor *(19)*. While the steroidogenic responses of ANG II in the adrenal have been associated with changes in intracellular calcium and activation of PLC, much less is known regarding the potential involvement of the mitogen-activated protein kinases (MAPKs) and stress-activated protein kinases in this process. The family of MAPKs, including extracellular signal-regulated kinases (ERKs), p38, and c-Jun-aminoterminal kinase (JNK), is part of the extracellular signal-initiated protein cascades that mediate cellular growth, differentiation, and apoptotic responses *(20,21)*. ANG II has been shown to activate the MAPKs in cells such as vascular smooth muscle cells and cardiac cells *(22–24)*. Furthermore, these MAPKs seem to mediate the growth-promoting effects of ANG II. Tian et al. *(25)* showed that ANG II activates the ERK1/2 MAPK in bovine adrenal glomerulosa cells via PKC. However, the role of ERK and p38 MAPKs in ANG II–induced aldosterone synthesis is not clear. In the present study, we have evaluated the role of MAPK activation in ANG II and the effects of 12-LO on aldosterone synthesis in H295R cells.

Results

To evaluate the signaling mechanisms of ANG II action in H295 cells, we first tested the effects of ANG II on the activity of ERK1/2 MAPK. Serum-starved H295R cells were treated with ANG II for various time periods, and cell lysates were electrophoresed and immunoblotted with an antibody specific to phospho-ERK1/2. The blots were then stripped and reprobed with an antibody to nonphospho-ERK to determine equal protein loading. Ratios of intensity in phospho- to nonphospho-bands were used to quantitate activity at each point. Similar to other ANG II—responsive cell types, ANG II led to a potent dose-dependent increase in ERK1/2 activity in H295R cells, as seen in Fig. 1 by the increase in intensity of the phospho-ERK bands (upper bands). This peaked at 5 min (20- ± 2-fold over control; $p < 0.001$), was clearly evident at 30 min, and lasted at least 3 h (Fig. 2). There were no significant differences in the levels of nonphospho-ERK under these conditions (Figs. 1 and 2, lower bands).

To determine whether this effect of ANG II was mediated by the AT1 receptor, we examined the effects of the AT1 receptor antagonist losartan. Figure 3 shows that a 15-min pretreatment of the cells with losartan led to a clear attenuation of ANG II–induced ERK1/2 activation at 5 min as well as at 3 h. These results suggest that the effects of ANG II on ERK1/2 activation in H295R cells are also mediated by AT1 receptor.

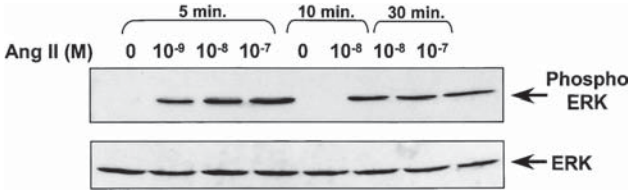

Fig. 1. Effect of short-term ANG II treatment on ERK activity in H295R cells. H295R cells in 100-mm dishes were grown to 80% confluency and then serum starved for 24 h. Fresh medium containing 0.2% bovine serum albumin (BSA) was replaced, and the cells were preincubated for 1 h prior to ANG II treatment for the time periods shown. Cells lysates were then processed for electrophoresis and immunoblotting to detect phospho-ERK (upper bands) or ERK (lower bands). Results shown are representative of three to four experiments.

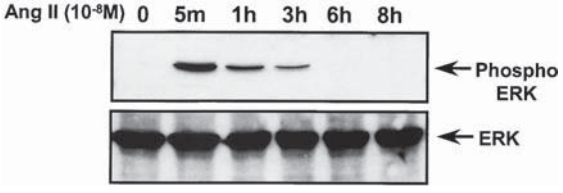

Fig. 2. Effect of long-term ANG II treatment on ERK activity in H295R cells. Experiments were performed as described in Fig. 1 except that ANG II treatment was up to 8 h. Results shown are representative of three experiments.

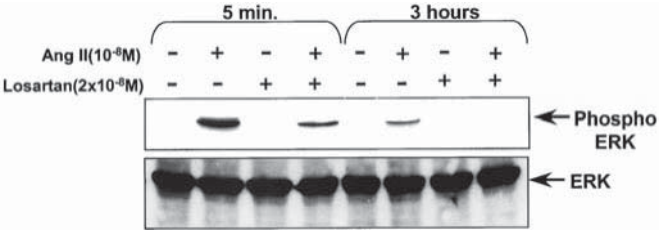

Fig. 3. Effect of AT1 receptor blocker losartan on ANG II–induced ERK activity in H295R cells. Serum-depleted H295R cells were preincubated alone for 1 h in fresh medium with no additions followed by an additional 15 min with or without losartan. They were then incubated for 5 min or 3 h with or without ANG II. Results shown are representative of three experiments.

Effect of 12-HETE on ERK1/2 Activation

Since our earlier studies had indicated that the 12-LO pathway could mediate some of the effects of ANG II, we examined whether the 12-LO product 12-HETE could directly activate ERK1/2 in these cells. We found that 12-HETE had no effect on ERK1/2 activity when tested over a wide range of concentrations and at two time points, 10 min and 30 min (Fig. 4).

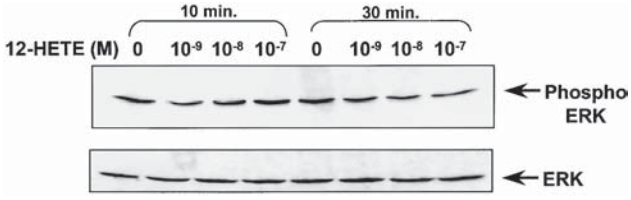

Fig. 4. Effect of 12-HETE on ERK activity in H295R cells. Experiments were performed as described in Fig. 1 except that cells were treated with various concentrations of 12-HETE for 10 or 30 min. Controls received only the vehicle ethanol. Results shown are representative of three experiments.

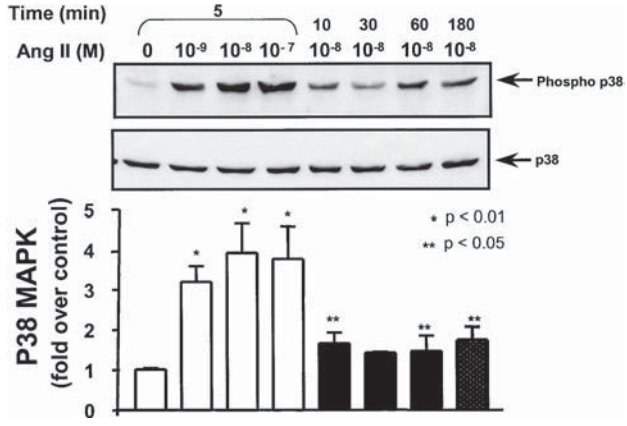

Fig. 5. Effect of ANG II on p38 MAPK activity. Experiments were performed as described in Fig. 1 except that cell lysates were electrophoresed and probed with an antibody to phospho-p38 MAPK (upper bands), and stripped and probed with an antibody to p38 MAPK (lower bands). The bar graph shows cumulative data from three to four experiments (densitometric data normalized for non-phospho-p38 MAPK levels).

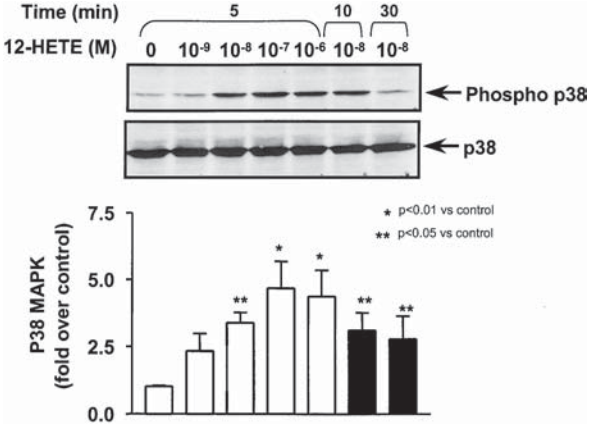

Fig. 6. Effect of 12-HETE on p38 MAPK activity. Experiments were performed as described in Fig. 5 except that treatments were with 12-HETE or the vehicle ethanol. The bar graph represents the data analyses from three to four experiments.

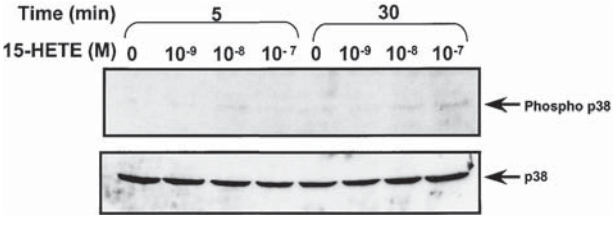

Fig. 7. 15-HETE has no effect on p38 MAPK activity. Experiments were performed as described in Fig. 6 except that cells were stimulated with various concentrations of 15-HETE for 5 or 30 min. Results shown are representative of two experiments.

Effect of ANG II on p38 MAPK Activity

Owing to recent observations that the p38 MAPK activity may mediate some of the effects of ANG II in vascular smooth muscle cells *(26,27)*, we examined whether ANG II could activate p38 MAPK in the H295R cells. Figure 5 depicts the dose response as well as time course of ANG II on p38 MAPK activity as assessed by an increase in levels of phospho-p38 MAPK levels (upper band). The same blot was stripped and reprobed with an antibody to nonphospho-p38 (lower band). Results show that ANG II led to a potent dose-dependent increase in p38 MAPK activity at 5 min with a peak dose effect around 10^{-8} *M* (upper bands). Furthermore, the stimulatory effect of ANG II was evident even up to 3 h. By contrast, there was no change in the levels of the nonphospo-p38 MAPK (lower bands). The bar graph showing cumulative data from multiple experiments (normalized for nonphospho-p38 levels) indicates that the effects of ANG II on p38 MAPK are highly significant. The peak time effect of ANG II was at 5 min at 10^{-8} *M* (4.5 ± 1.0-fold over control; $p < 0.001$). These results show, for the first time, that ANG II is a potent inducer of the p38 MAPK activity in H295R adrenal cells.

Effect of 12-HETE on p38 MAPK Activity

We next evaluated whether 12(*S*)-HETE has direct effects on p38 MAPK activation. Figure 6 shows a time course and dose response effect of 12-HETE. Similar to ANG II, 12-HETE had a potent, significant stimulatory effect on p38 MAPK activation (upper bands), and this remained sustained up to 30 min. The bar graph shows the densitometric quantitation. These results show, for the first time, that 12-HETE is a potent inducer of p38 MAPK in these adrenal cells. By contrast, the 15-LO product, 15(*S*)-HETE, had no effect on p38 MAPK activity (Fig. 7).

Effect of 12-LO Inhibitors on ANG II–Induced p38 MAPK Activity

To test the hypothesis that 12-HETE can mediate the effects of ANG II via key signaling pathways, we tested the effects of two structurally dissimilar specific 12-LO inhibitors, baicalein and cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), on ANG II–induced p38 MAPK activation. Neither inhibitor alone altered kinase activity (Fig. 8). How-

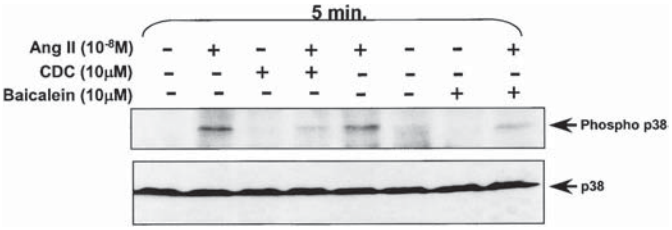

Fig. 8. Effect of 12-LO inhibitors (CDC and baicalein) on ANG II–induced p38 MAPK activity in H295R cells. Serum-depleted cells were preincubated for 1 h without any additions followed by an additional 15 min with each of the two LO inhibitors CDC or baicalein, or with the vehicle dimethylsulfoxide. They were then treated with ANG II for 5 min, and the cells were worked up for Western immunoblotting to detect phospho-p38 or p38. Results shown are representative of two experiments.

ever, Fig. 8 shows that each of the two inhibitors (10 μ*M*) could attenuate ANG II–induced p38 MAPK activity. These results implicate a role for 12-LO activation in ANG II–induced p38 MAPK activity.

Effect of ANG II and 12-HETE on JNK Activity

We also examined the effects of ANG II and 12-HETE on a third member of the MAPK family—JNK. JNK activity was determined by an immune-complex kinase assay *(28)*. We noted that both ANG II and 12-HETE, at doses and time intervals that elicited a potent increase in p38 MAPK activation, failed to induce JNK activation (results not shown).

Effect of a MEK (ERK) and a p38 MAPK Inhibitor on ANG II–Induced Aldosterone Release by H295R Cells

Evidence shows that the MAPKs, particularly ERKs, can mediate the growth-promoting effects of ANG II in several cell types. Our next objective was to determine whether either ERK1/2 or p38 MAPK activation played any role in the steroidogenic effects of ANG II. Therefore, we examined whether inhibitors of these pathways could attenuate ANG II–induced aldosterone secretion in the H295R cells. Table 1 indicates that the ERK1/2 pathway inhibitor (PD98059) at 1.0 and 10.0 μ*M* had no significant effect on ANG II–induced aldosterone synthesis. However, Table 2 shows that the specific p38 MAPK inhibitor SB202190 (0.5 μ*M*) significantly, but not completely, blocked the stimulatory effect of ANG II on aldosterone. SB202190 alone did not alter aldosterone synthesis. These results suggest that activation of p38 MAPK by ANG II may, at least in part, signal the steroidogenic machinery.

12-LO Inhibitors Attenuate ANG II–Induced Aldosterone, and 12-HETE Can Restore Effects of ANG II During LO Inhibition

To determine the functional role of 12-LO activation in ANG II–induced aldosterone, we evaluated whether the 12-LO inhibitor baicalein could block the effects of ANG II.

Table 1
Effect of MEK Inhibitor PD098059 on ANG II–Induced Aldosterone Synthesis in H295R Cells[a]

Agent	Aldosterone (fold over control)
ANG II (0.1 μ*M*)	1.70 ± 0.11[b]
ANG II + PD098059 (10 μ*M*)	1.66 ± 0.04[b]
ANG II + PD098059 (1 μ*M*)	1.53 ± 0.11[b]
PD098059 (10 μ*M*)	0.70 ± 0.02
PD098059 (1 μ*M*)	1.13 ± 0.10

[a] Results are expressed as mean ± SE from three to four separate experiments.

[b] $p < 0.01$ vs control cells incubated in medium alone.

Table 2
Effect of p38 MAPK Inhibitor SB202190 on ANG II–Induced Synthesis in H295R Cells[a]

Agent	Aldosterone (fold over control)
ANG II (0.1 μ*M*)	1.99 ± 0 0.12
ANG II + SB202190 (0.5 μ*M*)	1.04 ± 0.07[b]
SB202190 (0.5 μ*M*)	0.86 ± 0.13[b]

[a] Results are expressed as mean ± SE from six separate experiments.

[b] $p < 0.01$ vs ANG II, but no change vs control cells incubated in medium alone.

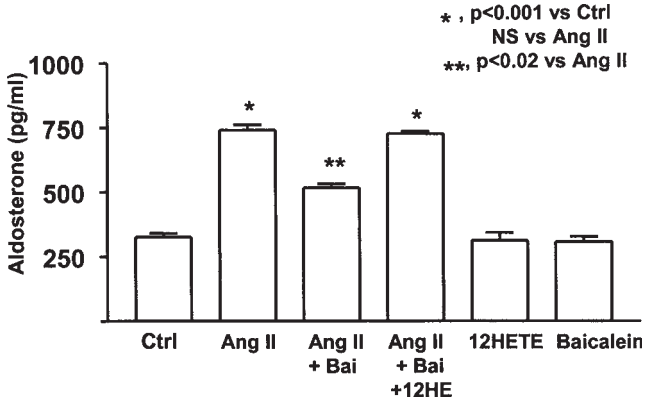

Fig. 9. 12-HETE restores ANG II–induced aldosterone synthesis in the presence of the LO inhibitor baicalein in H295R cells. H295R cells were serum depleted and preincubated for 1 h with fresh serum-depleted medium without any additions. They were then treated for 15 min with baicalein alone (10 μ*M*) or along with 12-HETE (0.1 μ*M*). Then ANG II (0.1 μ*M*) was added and the cells were incubated for 6 h. Aldosterone levels in the supernates were quantitated by specific radioimmunoassay (RIA). Results shown are the mean ± SEM from three experiments. NS, not significantly different from ANG II.

Figure 9 shows that ANG II–induced aldosterone was significantly attenuated by baicalein (10 μ*M*). Baicalein or 12-HETE alone had no direct effect on aldosterone synthesis. However, coaddition of 12-HETE (0.1 μ*M*) with baicalein

could restore the stimulatory effects of ANG II during inhibition by baicalein. These results support the involvement of the 12-LO pathway in the aldosterone-stimulatory effect of ANG II in H295 cells.

Discussion

We used the H295R adrenocortical cell line to evaluate the MAPKs involved in ANG II–induced aldosterone secretion, as well as the potential role of 12-LO activation in this process. We showed that ANG II is a potent inducer of ERK1/2 and p38 MAPK, but not JNK activation, in these cells. Furthermore, two dissimilar 12-LO inhibitors blocked ANG II–induced p38 MAPK, but not ERK1/2 activation. The 12-LO product, 12-HETE, could lead to the activation of p38 MAPK, but not ERK1/2 or JNK in these cells. By contrast, the 15-LO product, 15-HETE, did not stimulate p38 MAPK activity, indicating the specificity of 12-HETE actions. A 12-LO inhibitor blocked ANG II–induced aldosterone and addition of 12-HETE could restore the stimulatory effect of ANG II during 12-LO inhibition. Furthermore, a p38 MAPK inhibitor, but not an ERK1/2 inhibitor, attenuated ANG II–induced aldosterone synthesis. Overall, our results demonstrate, for the first time, that ANG II–induced aldosterone synthesis may be mediated, at least in part, via p38 MAPK activation. Furthermore, the results suggest that 12-LO activation plays a role in ANG II activation of p38 MAPK and aldosterone synthesis in H295R cells.

We have previously shown that 12-LO inhibitors could block ANG II, but not adrenocorticotropic hormone or potassium-induced aldosterone secretion, by freshly isolated rat and primary cultures of human adrenal glomerulosa cells *(10)*. In that study, we examined the potential signal transduction pathways involved. While it is now well known that the family of MAPKs plays a key role in the growth-promoting effects of ANG II, much less is known about their role in the steroidogenic effects of ANG II. The H295R cell line provides a reliable source of cells that retain ANG II responses in culture for studies such as ours *(13,14,29)*. ANG II induces not only aldosterone production but also aldosterone synthase mRNA in these cells *(29)*. Recent studies have used subtraction hybridization to identify novel ANG II–responsive genes in these H295R cells *(30)*. We have demonstrated ANG II activation of two members of the MAPK pathway in these cells and also shown that the p38 MAPK pathway may mediate the steroidogenic effects of ANG II. These current studies do not provide data on the upstream activators or the downstream transcription factor targets of p38 that may mediate the steroidogenic effect or induction of aldosterone synthase by ANG II. These will be the focus of future studies. Interestingly, a very recent report indicates that src tyrosine kinase may mediate ANG II–induced aldosterone in these cells *(31)*.

p38 MAPK is being actively studied owing to observations that it has multiple cellular functions and that specific isoforms can mediate inflammatory, hypertrophic, and apoptotic functions *(32–36)*. p38 MAPK has been implicated in cellular hypertrophic effects and gene expression *(32–36)*. There may be an important link between the 12-LO and p38 MAPK cascades. Recent observations showed that cardiac fibroblasts overexpressing the mouse 12-LO cDNA had increased cellular hypertrophy as well as p38 MAPK expression *(37)*, further underscoring a novel interaction between p38 MAPK and 12-LO activation. Our studies evaluated, for the first time, the role of p38 MAPK in adrenal aldosterone synthesis, and a potential relationship with 12-LO. Four isoforms of p38 have been identified recently (α, β, γ, δ) *(35,38–41)* that are dual phosphorylated and activated by MKK6 and MKK3. The antibody used in our Western blots crossreacts with all isoforms. Thus, our present studies do not reveal the relative contribution of individual p38 isoforms to the effects of ANG II.

Adrenal glomerulosa cells express the LO enzyme, and ANG II treatment could induce the expression of LO mRNA in these cells *(42)*. Furthermore, ANG II could increase formation of the 12-LO product, 12-HETE, in glomerulosa cells, and the LO pathway plays a key role in ANG II–induced aldosterone synthesis *(10,11)*. In vascular smooth muscle cells, evidence shows that LO products have growth and chemotactic effects, and also mediate ANG II–induced hypertrophic responses *(43,44)*. We also observed that the LO pathway could mediate the mitogenic effects of ANG II in Chinese hamster ovary cells overexpressing the ANG II AT1 receptor *(45)*. These cells also appeared to have a putative 12(*S*)-HETE receptor (unpublished observations). In the present study, it is possible that 12-HETE effects are receptor mediated and that there could be a novel crosstalk between the 12-HETE receptor and the ANG II receptor.

In summary, our results suggest, for the first time, that activation of p38 MAPK, either directly or via 12-LO pathway activation, plays a role, at least in part, in the aldosterone-stimulatory effects of ANG II in human adrenal cells.

Materials and Methods

Cell Culture

H295R cells were obtained from American Type Culture Collection; cultured in Dulbecco's modified Eagle's medium/F12 (1:1) with 5% fetal calf serum (FCS), 0.2% BSA, 100 U/mL of penicillin G, and 100 μU/mL of streptomycin; and incubated at 37ºC under 5% CO_2. Some experiments were performed with H295 cells obtained as a generous gift from Dr. W. E. Rainey (University of Texas Southwestern Medical Center at Dallas). Medium was changed every 3 d.

MAPK Activities

Cells (80% confluent) were starved with FCS-free medium for 24 h prior to treatment with desired concentrations of agents. The cells were immediately cooled and lysed with lysis buffer containing 20 m*M* Tris (pH 7.5), 150 m*M* NaCl,

1 m*M* EDTA, 1 m*M* EGTA, 1% Triton X-100, 25 m*M* $Na_4P_{2O}O_7$, 1 m*M* β-glycerol-phosphate, 1 m*M* Na_3VO_4, 1 µg/mL of leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride. The lysates were sonicated for 10 s on ice before centrifuging for 20 min at full speed in an Eppendorf microcentrifuge, and the supernatants were saved for evaluation of MAPK activities.

The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were washed 30 min with Tris-buffered saline (TBS) plus 0.1% Tween-20 at room temperature and then incubated in blocking buffer containing TBS with 0.1% Tween-20, 5% nonfat dry milk overnight. The membranes were incubated with primary antibody (anti-ERKs, anti-p38 MAPK, or the corresponding phosphospecific antibodies obtained from New England BioLabs) for 2 h at room temperature, washed completely with TBS, and incubated with secondary antibody (horseradish peroxidase–conjugated anti–rabbit antibody) in blocking buffer with gentle agitation for 1 h at room temperature. After washing with TBS, the phospho-MAPKs were detected by enhanced chemiluminescence method using an ECL kit from Amersham.

JNK-MAPK activity was assayed by a specific immune-complex kinase assay as described previously *(28)*.

Measurement of Aldosterone

H295R cells were plated in 12-well dishes. Eighty percent confluent cells were serum starved overnight and placed in fresh medium containing only 0.2% BSA. Cells were preincubated for 1 h and then treated with ANG II for 6 h at 37ºC. In some experiments, the cells were pretreated for 15 min with agents (12-HETE, p38 MAPK inhibitor [SB202190] Upstate Biotechnology, or MEK inhibitor [PD98059] New England BioLabs) prior to ANG II treatment. At the end of incubation, a 0.2-mL aliquot of supernatant was removed for measurement of aldosterone. Aldosterone was extracted from the cell incubates with methylene chloride and measured by a specific RIA using a kit from ICN as described previously *(10,11)*.

Data Analyses

Data are expressed as the mean ± SEM of multiple experiments. Paired student's *t*-tests were used to compare two groups, or analysis of variance with Dunnett posttest for multiple groups using PRISM software (Graph Pad, San Diego, CA). Bands from kinase assays were quantitated on a laser densitometer. Statistical significance was detected at the 0.05 level.

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