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Phospholipase A_2-mediated activation of mitogen-activated protein kinase by angiotensin II

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ABSTRACT **In renal proximal tubule epithelial cells, a membrane-associated phospholipase A_2 (PLA_2) is a major signaling pathway linked to angiotensin II (Ang II) type 2 receptor (AT_2). The current studies were designed to test the hypothesis that membrane-associated PLA_2-induced release of arachidonic acid (AA) and/or its metabolites may serve as an upstream mediator of Ang II-induced mitogen-activated protein kinase (MAPK) activation. Ang II stimulated transient dose-dependent phosphorylation of MAPK with a maximum at 1 μM (10 min). Inhibition of PLA_2 by mepacrine diminished both AA release and MAPK phosphorylation, induced by Ang II. Furthermore, AA itself induced time- and dose-dependent phosphorylation of MAPK, supporting the importance of PLA_2 as a mediator of Ang II signaling. The effects of both Ang II and AA on MAPK phosphorylation were protein kinase C independent and abolished by the inhibitor of cytochrome P450 isoenzyme, ketoconazole. Moreover, 5,6-epoxyeicosatrienoic acid and 14,15-epoxyeicosatrienoic acid, the cytochrome P450-dependent metabolites of AA, significantly stimulated MAPK activity in renal proximal tubule epithelial cells. These observations document a mechanism of Ang II-induced MAPK phosphorylation, mediated by PLA_2-dependent release of AA and cytochrome P450-dependent production of epoxy derivatives of AA.**

Angiotensin II (Ang II), a potent vasoconstrictor and modulator of ion transport, also is known to induce mitogenesis associated with mitogen-activated protein kinase (MAPK) in a variety of cells (1–6). Earlier studies described a protein kinase C (PKC)-dependent mechanism of Ang II-induced activation of MAPK in adrenocortical cells and cardiac fibroblasts (1, 5), which also involved phospholipase C (PLC)-mediated production of inositol trisphosphates and diacyl glycerol (1, 5). More recently, an alternative $p21^{ras}$-dependent mechanism was described in cardiac myocytes, which involved phosphorylation of adaptor protein Shc by intracellular tyrosine kinases and association of Shc with Grb2/Sos complex, followed by activation of $p21^{ras}$ (6). In renal proximal tubular epithelial (RTE) cells, the Ang II signaling is not linked to PLC (7, 8). Our laboratory (7) and others (9, 10) have demonstrated that a major second messenger of Ang II in proximal tubular epithelium is phospholipase A_2 (PLA_2) and its product, arachidonic acid (AA). Therefore, we reasoned that AA and/or its metabolites could mediate the effect of Ang II on MAPK. This hypothesis also was based on observations demonstrating that AA and its metabolites elicited mitogenic activity in other cells (11–13).

In the present study we propose a PLA_2-dependent mechanism of activation of MAPK in proximal tubule epithelium, mediated by AA and/or its cytochrome P450-dependent metabolites, which may provide a linkage between Ang II receptor and intracellular tyrosine kinases in the $p21^{ras}$-dependent pathway of activation of MAPK cascade.

EXPERIMENTAL PROCEDURES

Materials. Sar^1-Ang II, a stable analog of Ang II, used in experiments, was obtained from Peninsula Laboratories. AA was obtained from Biomol (Plymouth Meeting, PA). 5,6-Epoxyeicosatrienoic acid (EET) and 14,15-EET were synthesized as previously described (14). The inhibitors of AA metabolism were from Sigma. [^{3}H]AA was from New England Research Products (Boston, MA). Protease inhibitors were from Boehringer Mannheim. Polyclonal antibodies against phosphorylated MAPK (PMAPK) were from New England Biolabs. Polyclonal antibodies against p42/p44 MAPK (αMAPK 63480) were kindly provided by M. Dunn (Medical College of Wisconsin, Milwaukee, WI).

Cell Culture. The rabbit RTE cells were isolated from male New Zealand White rabbits (Hazelton Research Products, Denver, PA) as previously described (15). The standard growth medium for RTE cells was a 50:50 mixture of DMEM and Ham's F-12 media supplemented with 15 mM Hepes (pH 7.4), 0.35 mg/ml of L-glutamine, 0.6 mg/ml of sodium bicarbonate, 100 units/ml of penicillin, 100 mg/ml of streptomycin, 5 μg/ml of bovine insulin, 5 μg/ml of human transferrin, 0.5 μM hydrocortisone, and 5% fetal bovine serum. The first-passaged RTE cells were serum-starved for 48 hr before the experiment.

Phosphorylation and Activation of MAPK. After stimulation with different agonists, the cells were washed twice with ice-cold PBS, lysed in a buffer containing 25 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml of pepstatin, 50 μg/ml of bestatin, 200 μM Na-orthovanadate, and 1 mM NaF. The lysed cells were scraped and centrifuged at 14,000 rpm for 20 min in the microcentrifuge. Phosphorylation of MAPK was determined by Western blot analysis of cell lysates with antibodies against αPMAPK, or as an electrophoretic mobility shift by immunoblotting with MAPK antibodies (αMAPK 63480).

MAPK activity was measured as described previously (16). Briefly, MAPK was immunoprecipitated from cell lysates with ERK1 antibodies followed by kinase assay with myelin basic protein (MBP) as a substrate. The phosphorylation of MBP

Abbreviations: Ang II, angiotensin II; AA, arachidonic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; MAPK, mitogen-activated protein kinase; PLA_2, phospholipase A2, PKC, protein kinase C; RTE, renal proximal tubular epithelial; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; $mPLA_2$, membrane-associated PLA_2; PMAPK, phosphorylated MAPK.

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was quantified by liquid scintillation spectrometry after separation by electrophoresis.

Measurement of AA Release. RTE cells, grown in 12-well plates, were labeled with [^{3}H]AA (1 μCi/ml) for 4 hr and washed three times with 1 ml of DMEM/F12 containing 0.1% BSA. After stimulation with agonists for 5 min, the medium was removed, and the radioactivity in the medium was quantified by liquid scintillation spectrometry.

RESULTS

Effect of Ang II and AA on MAPK Phosphorylation. Incubation of RTE cells with Ang II resulted in a dose-dependent phosphorylation of p42 and p44 isoforms of MAPK (Erk1 and Erk2, respectively) as determined by immunoblotting the cell lysates with αPMAPK, as well as by electrophoretic mobility shift of ERK1, detected by Western blotting with p42/p44 MAPK antibodies (αPMAPK, Fig. 1). Phosphorylation of MAPK was detectable at 10 nM Ang II and reached maximum at 1 μM Ang II. This is consistent with the involvement of the relatively low affinity Ang II binding site on the apical surface of previously described rabbit proximal tubule epithelium by our laboratory (17).

Previous studies have demonstrated that Ang II stimulates PLA_2 and rapid release of AA, using both intact RTE cells (18, 19) and renal brush border membrane vesicles (9). To evaluate the role of PLA_2 in the mechanism of activation of MAPK, we first examined the influence of PLA_2 inhibitor, mepacrine, on Ang II-induced release of AA and phosphorylation of MAPK. Ang II stimulated more than a 2-fold increase in AA release within 5 min of incubation with RTE cells, and this effect was significantly decreased by 76 ± 13% in the presence of mepacrine (Fig. 2*A*). Preincubation of RTE cells with mepacrine also diminished MAPK phosphorylation induced by Ang II (Fig. 2*B*), suggesting the importance of PLA_2 in Ang II signaling. Selective inhibitors of $cPLA_2$ ($AACOCF_3$) and $sPLA_2$ (manoalide) did not alternate Ang II-induced MAPK phosphorylation (data not shown). Because AA can be produced in an alternative manner through PLC signaling pathways and diacylglycerol (DAG), we used the specific DAG lipase inhibitor RHC-80267 but found no effect on basal or Ang II-induced MAPK phosphorylation (data not shown).

Direct addition of AA resulted in dose-dependent phosphorylation of MAPK in RTE cells (Fig. 3*A*). The effect of AA was detectable at 1.5 μM and reached maximum at 15 μM. Direct assessment of MAPK activity confirmed that phosphorylation of MAPK was associated with an increase in its activity (data not shown). The time course of MAPK phosphorylation demonstrated that the effect of AA on MAPK phosphorylation was more rapid (maximum at 3–5 min) than the effect of Ang II (maximum at 10–15 min), consistent with the hypothesis that AA mediates MAPK phosphorylation, induced by Ang II (Fig. 3 *B* and *C*).

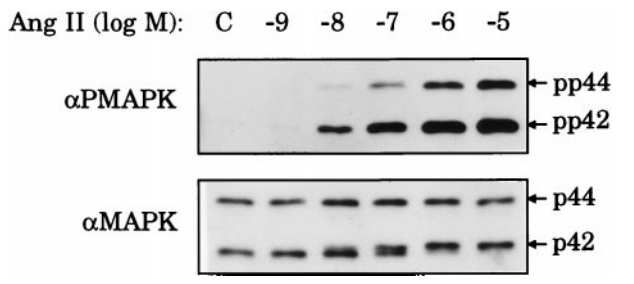

FIG. 1. Dose-dependent effect of Ang II on MAPK phosphorylation. Serum-starved RTE cells were treated with various concentrations of Ang II for 10 min, and cell lysates were analyzed by Western blotting with antibodies against PMAPK or against MAPK as indicated. Shown is a representation of two independent experiments with similar results.

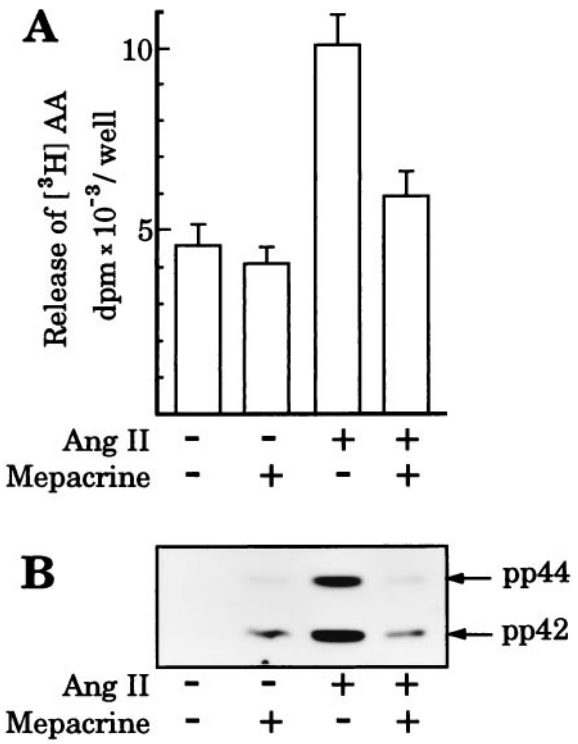

FIG. 2. Effect of mepacrine on Ang II-induced AA release (*A*) and MAPK phosphorylation (*B*). (*A*) Serum-starved RTE cells were prelabeled with [^{3}H]AA for 4 hr as described in *Experimental Procedures*, incubated with mepacrine (500 μM, 5 min), followed by stimulation with Ang II (1 μM, 5 min). The radioactivity in the extracellular medium was counted by liquid scintillation spectrometry. Data represent mean ± SEM from a single experiment performed in triplicate in parallel with measurement of MAPK phosphorylation. Two experiments had identical results. (*B*) RTE cells were preincubated with mepacrine as in *A* followed by stimulation with Ang II for 10 min. Cell lysates were analyzed by Western blotting with αPMAPK antibodies. Shown is a representation of three experiments with similar results.

Activation of MAPK by Ang II and AA Is PKC Independent. To evaluate the role of PKC in the mechanism of MAPK activation, we examined the effect of the PKC inhibitor staurosporine on MAPK phosphorylation, induced by Ang II

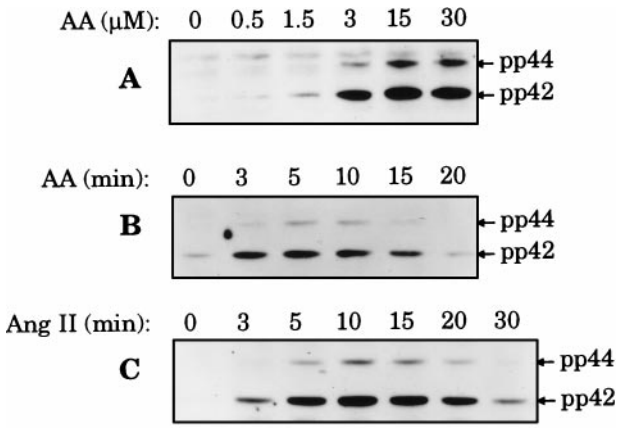

FIG. 3. Effect of AA and Ang II on MAPK phosphorylation. Serum-starved RTE cells were incubated with various concentrations of AA for 5 min (*A*), with 15 μM AA (*B*), or with 1 μM Ang II (*C*) for various times. Cell lysates were immunoblotted with αPMAPK antibodies.

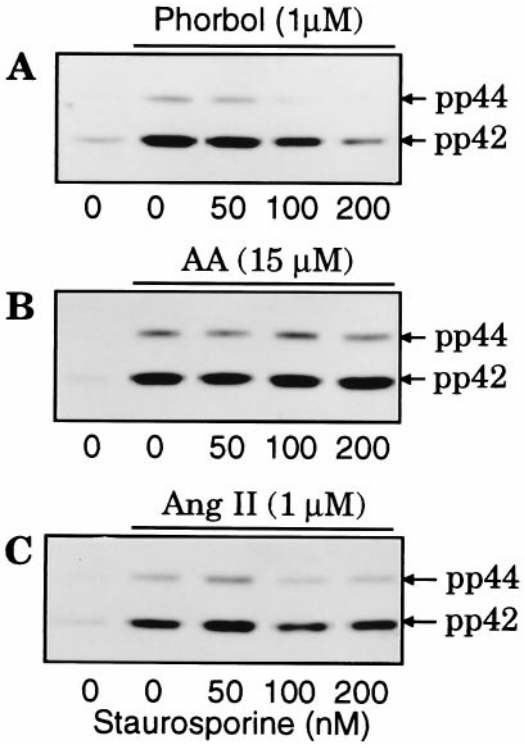

FIG. 4. Effect of staurosporine on MAPK phosphorylation induced by PMA, AA, and Ang II. Serum-starved RTE cells were preincubated with different concentrations of staurosporine for 5 min, followed by stimulation with 1 μM PMA for 10 min (*A*), 15 μM AA for 5 min (*B*), or 1 μM Ang II for 10 min (*C*). Cell lysates were analyzed by Western blotting with αPMAPK antibodies. Shown is a representation of two independent experiments with similar results.

and AA, as compared with the PKC activator phorbol 12-myristate 13-acetate (PMA). The choice of staurosporine was based on the fact that it is one of the most potent and less selective inhibitors of PKC, having the broadest range of

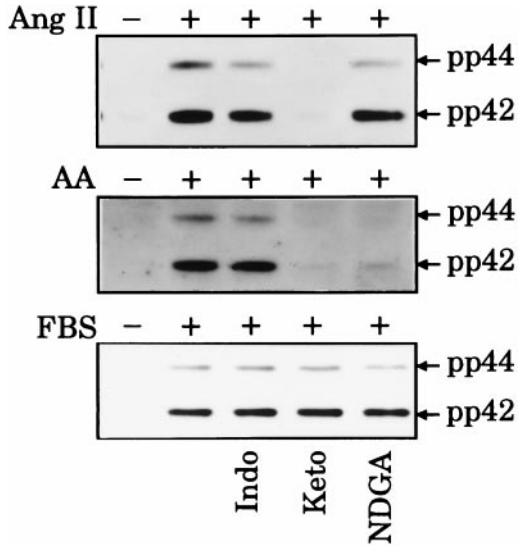

FIG. 5. Effect of inhibitors of AA metabolism on phosphorylation of MAPK induced by Ang II, AA, or fetal bovine serum (FBS). Serum-starved RTE cells were preincubated with indomethacin (100 μM), ketoconazole (30 μM), or nordihydroguaiaretic acid (NDGA) (10 μM) for 5 min, followed by stimulation with 1 μM Ang II for 10 min, 15 μM AA for 5 min, or 10% FBS as indicated. Cell lysates were immunoblotted with αPMAPK antibodies. Shown is a representation of three independent experiments with identical results.

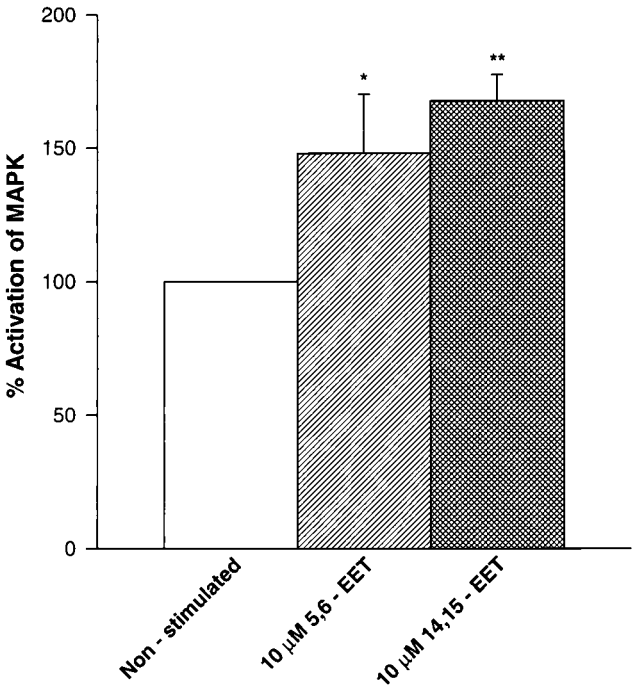

FIG. 6. Effect of AA, 5,6-EET, and 14,15-EET on MAPK activity. Serum-starved RTE cells were stimulated with 5,6-EET (10 μM) or 14,15-EET (10 μM) for 5 min, and MAPK activity was measured in MAPK immunoprecipitates as described in *Experimental Procedures*. Data represent the results of three experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.001$.

activity toward different specific isoforms of PKC (19). Thus, when the involvement of a specific isoform of PKC is not known, staurosporine can be a useful tool for defining the role of PKC in general.

As shown in Fig. 4, PMA stimulated phosphorylation of MAPK in RTE cells to the same extent as Ang II and AA. Preincubation of cells with staurosporine resulted in a dose-dependent inhibition of MAPK phosphorylation induced by PMA with a maximal effect at 200 nM (Fig. 4*A*). In contrast, 200 nM of staurosporine did not inhibit AA-induced MAPK phosphorylation (Fig. 4*B*) and had a negligible effect on Ang II-induced MAPK phosphorylation (Fig. 4*C*). These data suggest that in RTE cells the mechanism of MAPK activation by Ang II and AA is PKC independent in contrast to earlier reports in other cell types (20, 21). This supports our additional studies that document tyrosine phosphorylation of the adapter protein Shc, association with Grb2/Sos, and activation of $p21^{ras}$ (22).

Cytochrome P450 Products Mediate the Effect of Ang II and AA on MAPK Phosphorylation. To investigate whether MAPK phosphorylation is mediated by metabolites of AA, we first examined the effect of several specific inhibitors of AA metabolism on phosphorylation of MAPK. Previous studies have documented that in renal proximal tubular epithelium, AA is metabolized via cytochrome P450 system, rather than through the cyclooxygenase or lipoxygenase pathways of eicosanoid biosynthesis (7). In our experiments, the cyclooxygenase inhibitor indomethacin (100 μM) had no effect on phosphorylation of MAPK induced by Ang II or AA (Fig. 5). In contrast, the inhibitor of cytochrome P450 isozymes ketoconazole (100 μM) completely abolished phosphorylation of MAPK induced by both Ang II and AA (Fig. 5). Interestingly, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 10 μM) abolished MAPK phosphorylation induced by AA, but not by Ang II (Fig. 5). All three inhibitors had no effect on serum-induced MAPK phosphorylation (Fig. 5). These results

suggest that the cytochrome P450-dependent metabolites of AA may mediate the effect of Ang II on activation of MAPK.

To validate the role of cytochrome P450 in the mechanism of MAPK phosphorylation, we examined a direct effect of epoxy derivatives of AA, implicated in proximal tubular epithelial signaling linked to Ang II (7), on the activity of MAPK. As shown in Fig. 6, addition of 5,6-EET and 14,15-EET significantly increased MAPK activity in RTE cells by 48% and 67%, respectively, as compared with control. The other AA metabolites [5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, 15-HETE, 20-HETE, leukotrienes B_4 and C_4], some of which have been previously reported to be implicated in mitogenesis and MAPK activation (11–13, 20, 23), had no effect on MAPK in RTE cells (data not shown).

DISCUSSION

Phosphorylation and activation of MAPK by Ang II has been previously demonstrated in adrenocortical (1), renal mesangial (2), vascular smooth muscle cells (3), and cardiac myocytes and fibroblasts (4, 5). In these cells, the mechanism of MAPK activation by Ang II primarily involves signaling linked to inositol-specific PLC. Our present work documents a critical role of PLA_2 as an alternative mechanism of Ang II-induced activation of MAPK in RTE cells. Thus, inhibition of AA release by mepacrine attenuated Ang II-induced phosphorylation of MAPK in RTE cells (Fig. 2) and AA at low (1.5–3 μM) concentrations mimicked the effect of Ang II on MAPK with a striking parallel in time course (Fig. 3). Additional studies from this laboratory have documented that this PLA_2-mediated signaling is linked to the relatively low affinity type 2 (AT_2) Ang II receptor (18, 22) that is pharmacologically distinct from the AT_2 receptor cloned previously (24, 25). Moreover, this apical AT_2 receptor mediates Ang II-induced AA release and downstream events such as Shc/Grb2/Sos and $p21^{ras}$ association (18, 22). Thus, this represents an additional signaling model for AT_2 receptor subtypes and G protein-coupled receptors as well.

The question about specific isoform of PLA_2, involved in the mechanism of Ang-II induced MAPK activation in RTE cells, is of great importance. The evidence obtained from our laboratory (19) and others (9) indicates a membrane-associated PLA_2 ($mPLA_2$) as the prime second messenger of Ang II in renal proximal tubule epithelium. This $mPLA_2$ is distinct from the cytosolic PLA_2 ($cPLA_2$), or the secretory PLA_2 ($sPLA_2$) by several criteria (19). In RTE cells, a rapid activation of $mPLA_2$ (19) and AA release (18) is initiated within the first minutes of stimulation with Ang II and precedes the activation of MAPK, which in our experiments was significantly activated only after 5 min of incubation with Ang II (Fig. 3*C*). In contrast, phosphorylation and activation of $cPLA_2$ induced by Ang II occurs later (after 15 min) and most likely is MAPK dependent as reported by others (19, 26). The role of an alternate PLC/diacylglycerol (DAG) lipase-dependent source for AA in Ang II-induced MAPK activation also can be ruled out because (*i*) PLC is not activated by Ang II in RTE cells (7, 8), and (*ii*) the specific inhibitor of DAG lipase RHC-80267 had no effect on the basal or Ang II-induced MAPK phosphorylation in RTE cells. Taken together, these data suggest the importance of a mPLA2 in the mechanism of Ang II-induced activation of MAPK in RTE cells. In support of this hypothesis are our observations that selective $cPLA_2$ inhibitor $AACOCF_3$ and selective $sPLA_2$ inhibitor manoalide did not attenuate MAPK phosphorylation induced by Ang II.

Our experiments suggest that the mechanism of MAPK activation by both Ang II and AA is PKC independent because a nonselective inhibitor of PKC staurosporine did not affect Ang II/AA-induced but blocked PMA-induced MAPK phosphorylation (Fig. 4). These results are in contrast to two previous reports using rat liver epithelial cells and vascular smooth muscle cells where PKC was documented to mediate AA-induced activation of MAPK (20, 21). Using the current model, this laboratory has documented that both Ang II and AA stimulated the $p21^{ras}$-dependent pathway upstream of MAPK activation in RTE cells (22), a PKC-independent signaling pathway (6). This pathway involves tyrosine phosphorylation of adaptor protein Shc, its association with Grb2/Sos complex followed by activation of $p21^{ras}$. In G protein-coupled receptor signaling, the adaptor protein Shc has been reported to be phosphorylated by Src-related tyrosine protein kinases (6) or by receptor tyrosine kinases (27). The present studies provide linkage between the tyrosine kinase and this AT_2 receptor, placing $mPLA_2$ and AA upstream of Shc phosphorylation in the mechanism of MAPK activation by Ang II. More studies are in progress evaluating the mechanism whereby AA activates tyrosine kinase signaling.

In many cells, AA is metabolized by different enzymes into a variety of derivatives, which mediate its biological actions. In proximal tubule epithelium, cytochrome P450 enzymes represent a major Ang II-induced pathway of AA metabolism (28, 29). Epoxy derivatives of AA (EETs) can mimic the effect of Ang II and AA on ion transport (7, 29). In our experiments, the inhibitor of cytochrome P450 ketoconazole abolished MAPK phosphorylation induced by both Ang II and AA (Fig. 5). Moreover, the relevant metabolites of AA in RTE cells 5,6-EET and 14,15-EET activated MAPK (Fig. 6). These data strongly suggest that cytochrome P450-dependent epoxy derivatives of AA may mediate the effect of Ang II and AA on MAPK phosphorylation. Of interest is the fact that the inhibitor of lipoxygenase nordihydroguaiaretic acid (NDGA) also abolished AA-induced MAPK phosphorylation. Some lipoxygenase products of AA (12-HETE, 15-HETE, leukotriene B_4) have been reported to activate MAPK in other cell types (11, 20, 23). However, direct addition of these products to RTE cells had no effect on MAPK phosphorylation (data not shown). Moreover, because NDGA failed to inhibit Ang II-induced phosphorylation of MAPK (Fig. 5), the involvement of lipoxygenase in the mechanism of MAPK activation by Ang II seems to be unlikely.

In conclusion, this study documents the essential role of a $mPLA_2$, AA, and its cytochrome P450-dependent epoxy derivatives in PKC-independent phosphorylation of MAPK induced by Ang II. This represents an alternative mechanism of MAPK activation and may provide a linkage between this Ang II AT_2 receptor and the $p21^{ras}$-dependent pathway of activation of the MAPK cascade.

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