Arachidonate-Induced Tyrosine Phosphorylation of Epidermal Growth Factor Receptor and Shc-Grb2-Sos Association

Nickolai O. Dulin, Andrey Sorokin, Janice G. Douglas

Abstract—Protein tyrosine phosphorylation induced by arachidonic acid (AA), an important lipid second messenger, was investigated in rabbit renal proximal tubule epithelial cells. AA stimulated tyrosine phosphorylation of a number of proteins with estimated molecular weights of 42, 44, 52, 56, 85, and 170/180 kDa. The phosphoproteins pp44 and pp42 were identified as 2 isoforms of mitogen-activated protein kinase (MAPK). Phosphorylation of MAPK in response to AA was transient, dose-dependent, and accompanied by an increase in its activity. The mechanism of AA-induced MAPK activation in RTE cells was protein kinase C–independent and involved tyrosine phosphorylation of adaptor protein Shc and its association with Grb2-Sos complex. Moreover, stimulation of RTE cells with AA resulted in significant phosphorylation of epidermal growth factor (EGF) receptor and its association with Shc. The effect of AA on EGF receptor phosphorylation, its association with Shc, and MAPK activation was similar to the effect of 1 ng/mL EGF. Tyrphostin AG1478, a specific inhibitor of EGF receptor tyrosine kinase activity, completely blocked the effects of AA and EGF but not phorbol ester on MAPK phosphorylation. These data suggest that in renal tubular epithelial cells, the mechanism of AA-induced MAPK activation involves tyrosine phosphorylation of EGF receptor and its association with Shc and Grb2-Sos complex. Given the critical role of AA in signaling linked to G protein–coupled receptors (GPCRs), these observations provide a mechanism for cross talk between GPCRs linked to phospholipases and the tyrosine kinase receptor signaling cascades. (Hypertension. 1998;32:1089-1093.)

Key Words: kinases ■ receptor, epidermal growth factor ■ Shc ■ phosphorylation ■ kidney

Arachidonic acid (AA) and its metabolites play a critical role in a variety of physiological and pathological processes within the kidney. It is released from phospholipids after activation of phospholipases in response to different extracellular signals linked to growth factors and G protein–coupled receptors. In renal proximal tubule epithelium, AA is an important second messenger in signaling linked to epidermal growth factor (EGF), angiotensin II (Ang II), bradykinin, and other hormones. Recently, AA and its lipoxygenase and cytochrome P450 derivatives have been implicated in mitogenesis as well as in activation of mitogen-activated protein kinase (MAPK) cascade, one of the most crucial pathways involved in induction of cell growth.

Activation of MAP kinase (extracellular signal regulated kinase [ERK]) requires its phosphorylation on tyrosine and threonine residues by MAP kinase by MEK, which in turn is phosphorylated by the serine/threonine kinase, Raf. This evolutionary conserved kinase cascade is a common pathway for both receptor tyrosine kinase– and G protein–mediated mitogenesis. Activation of Raf can be induced by different pathways involving protein kinase C (PKC)–dependent and –independent mechanisms. The latter involves tyrosine phosphorylation of adaptor protein Shc by receptor tyrosine kinases or intracellular tyrosine kinases, binding of Shc to another adaptor protein Grb2, which is in the complex with guanine nucleotide exchange factor Sos, recruitment of Sos to the membrane, and activation of small GTPase p21ras, which in turn activates Raf.

The purpose of this study was to examine the ability of AA to activate protein tyrosine phosphorylation in renal proximal tubule epithelial (RTE) cells and to identify the signaling molecules involved in this action of AA. We demonstrate that AA induces phosphorylation and activation of MAPK in RTE cells and provide the first evidence that phosphorylation of adaptor protein Shc and its association with Grb2 and Sos1 are linked to this process. Moreover, our data implicate the EGF receptor (EGFR) as a possible component of AA-induced MAPK signaling in RTE cells.

Methods

Materials
AA (20:4, N=6) was obtained from Biomol Research Laboratories; phorbol 12,13-didecanoate (PDD) from Research Biochemicals International; EGF and tyrphostin AG1478 from Calbiochem; and

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protease inhibitors from Boehringer Mannheim Biochemicals. Antibodies were from the following sources: phosphospecific anti-p42/p44 MAPK (Y204) from New England Biolabs; anti-Shc from Transduction Laboratories; anti-Grb2, anti-Sos1, and polyclonal anti-EGFR from Santa Cruz Biotechnology; and HRP-conjugated monoclonal anti-phosphotyrosine (αPY/HRP) from Calbiochem. Polyclonal antiphosphotyrosine antibodies (αPY) were kindly provided by Dr J. Schlessinger (New York, NY). Polyclonal antibodies against p42/p44 MAPK (αMAPK) were from Dr M. Dunn (Milwaukee, Wis).

Cell Culture
Rabbit RTE cells were isolated from male New Zealand White rabbits (Hazelton, Denver, Pa) as previously described. The standard growth medium for RTE cells was a 50:50 mixture of DMEM and Ham’s F12 media supplemented with 15 mmol/L HEPES (pH 7.4), 0.35 mg/mL L-glutamine, 0.6 mg/mL sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL bovine insulin, 5 µg/mL human transferrin, 0.5 µmol/L hydrocortisone, and 5% FBS. The first-passage RTE cells were serum-starved for 24 hours before the experiment.

Immunoprecipitation and Western Blot Analysis
After stimulation with desired agonists, the cells were washed twice with ice-cold PBS, lysed in a buffer containing 25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 1% Triton X-100, 1 mmol/L PMSF, 1 µmol/L leupeptin, 10 µg/mL pepstatin, 50 µg/mL bestatin, 200 µmol/L Na-orthovanadate, and 1 mmol/L NaF. The lysed cells were scraped and centrifuged at 14,000 rpm for 20 minutes in the microcentrifuge. The desired protein was immunoprecipitated by incubation of cleared cell lysates with specific antibodies at 4°C for 2 hours on rotator and then by incubation with protein A–conjugated Sepharose beads (Gibco BRL) for an additional 1 hour. The beads were then washed 3 times with the lysis buffer and boiled in Laemmli buffer. Western blotting with desired antibodies was performed after electrophoresis and transfer of proteins to Immobilon P membrane (Millipore) according to manufacturer’s protocol. MAPK activity was measured in MAPK immunoprecipitates with myelin basic protein (MBP) as a substrate, as described by Wang et al.

Results
Incubation of RTE cells with AA resulted in tyrosine phosphorylation of a number of proteins with estimated molecular weights of 42, 44, 52, 56, 85, and 170/180 kDa (Figure 1A). The phosphoproteins pp44 and pp42 were identified as 2 isoforms of MAPK (ERK1 and ERK2, respectively) by immunoblotting of cell lysates with phosphospecific anti-p42/p44 MAPK antibodies (Figure 1B). AA-induced phosphorylation of ERK was transient (Figure 1A), dose-dependent (Figure 1B), and accompanied by an increase in its activity similar to that induced by fetal calf serum (FCS) with MBP as substrate (Figure 1C).

The activation of MAPK induced by FCS is believed to be mediated primarily by growth factors and lysophosphatidic acids by the mechanism involving recruitment of adaptor proteins Shc and Grb2, guanine nucleotide exchange factor Sos, small GTPase p21ras, and activation of a kinase cascade (Raf, MEK) leading to phosphorylation of MAPK. In our experiments, AA and FCS stimulated tyrosine phosphorylation of proteins with the same molecular weight (Figure 1A), suggesting that in RTE cells AA and FCS might induce ERK activation by a similar mechanism. Therefore, we examined whether AA was able to stimulate tyrosine phosphorylation of Shc and its association with Grb2 and Sos1 by coinmunoprecipitation technique. The effect of AA was compared with that of phorbol ester (PDD), which activates the MAPK cascade directly through Raf-1, and EGF, which recruits adaptor proteins for activation of ERK.

As shown in Figure 2B, incubation of RTE cells with AA resulted in tyrosine phosphorylation of p52 and p46 isoforms of Shc as determined by immunoprecipitation of Shc from total cell lysates and then by immunoblotting with antiphosphotyrosine antibodies. Shc was confirmed to be equivalent in all lanes (Figure 2C). Phosphorylation of Shc induced by AA was accompanied by physical association of Shc with Grb2 (Figure 2D) and Sos1 (Figure 2E), as determined by coinmunoprecipitation of Shc with Grb2 and Sos1, respectively. As expected, the phorbol ester (PDD), which was as potent as AA in ERK phosphorylation (Figure 3F), failed to induce tyrosine phosphorylation of Shc and its association with Grb2 and Sos1 (Figure 2B through 2E).

During the conduct of these experiments, we consistently observed that AA induced tyrosine phosphorylation of proteins with high molecular weight (Figure 1A) close to that of growth factor receptors (170 to 180 kDa). Moreover, after addition of AA to RTE cells, a tyrosine phosphorylated protein with a molecular weight of 175 kDa coimmunoprecipitated with Shc (Figure 2A). The 175-kDa phosphoprotein also coimmunoprecipitated with Shc after stimulation of RTE cells with 100 ng/mL EGF (Figure 2A), suggesting that similar to EGFR signaling, AA-induced phosphorylation of Shc may be a consequence of tyrosine phosphorylation of EGFR and its subsequent association with Shc. To elucidate the possible role of EGFR in AA signaling, we investigated (1) whether AA was able to induce tyrosine phosphorylation...
Influence of tyrphostin AG1478 on AA- and EGF-induced tyrosine phosphorylation of EGFR, its association with Shc, and phosphorylation of Shc. Tyrosine phosphorylation of EGFR induced by AA represents the most important component of the present study. Previous studies have demonstrated EGF-independent phosphorylation of EGFR induced by G protein–coupled receptors. However, the mechanism and physiological significance of this phenomenon have not been determined. In our experiments, AA-stimulated tyrosine phosphorylation of EGFR (Figure 3B), which was accompanied by its association with and phosphorylation of adaptor protein Shc (Figures 2B and 3C). Moreover, tyrphostin AG1478, an inhibitor of EGFR tyrosine kinase activity, completely blocked AA-induced EGFR phosphorylation, its association with Shc, and activation of MAPK (Figure 3). This suggests an essential role of EGFR phosphorylation in the mechanism of AA-induced activation of MAPK. Thus, being a second messenger in the signaling of a variety of hormones, AA may provide a link between activation of G protein–coupled receptors (GPCRs) and phosphorylation of EGFR observed previously. Moreover, these data provide a mechanism for AA-induced growth regulation.

Discussion

The present study demonstrates for the first time that AA can induce MAPK activation by a mechanism common for receptor tyrosine kinase signaling, involving tyrosine phosphorylation of EGFR and its association with the adaptor protein Shc, and tyrosine phosphorylation of Shc, which recruits the Grb2/Sos1 complex. These data expand on previous observations from this laboratory, wherein AA was documented to promote activation of the small G protein p21ras in RTE cells.16

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Immunoprecipitation of EGFR from total lysates of RTE cells, followed by immunoblotting with antiphosphotyrosine antibodies (αPY), demonstrated a significant tyrosine phosphorylation of EGFR induced by AA, which was comparable to the effect of 1 ng/mL of EGF (Figure 3B). Moreover, AA-induced phosphorylation of EGFR resulted in association with Shc similar to that induced by 1 ng/mL of EGF, as determined by immunoblotting of EGFR immunoprecipitates with antiphosphotyrosine (αPY/HRP) antibodies (Figure 3C).

Although at higher concentrations, EGF stimulated more profound phosphorylation of EGFR (Figure 3B) and its association with Shc (Figure 2A and 3C), the effect of 1 ng/mL of EGF on ERK activation was maximal and was not increased at higher concentrations of EGF (Figure 3D). The equal protein loading in analyzed samples was confirmed by immunoblotting of EGFR immunoprecipitates with αEGFR antibodies (Figure 3A) and total cell lysates with αERK antibodies (Figure 3E).

Preincubation of RTE cells with tyrphostin AG1478, the specific inhibitor of EGFR tyrosine kinase activity, attenuated the effect of AA and EGF on phosphorylation of EGFR (Figure 3B), its association with Shc (Figure 3C), and activation of ERK (Figure 3D). Tyrphostin AG1478 had no significant effect on phorbol ester–induced phosphorylation of MAPK (data not shown).

Taken together, these data strongly suggest that tyrosine phosphorylation of EGFR mediates MAPK activation induced by AA in RTE cells.
Arachidonate-Induced EGF Receptor Phosphorylation

Previous studies have implicated PKC in the mechanism of AA-induced activation of MAPK in vascular smooth muscle cells' and liver epithelial cells. In our experiments, a nonselective PKC inhibitor, staurosporine, had no effect on AA-induced phosphorylation of ERK, whereas it completely inhibited phorbol ester–induced phosphorylation of ERK in RTE cells. Moreover, phorbol ester failed to induce tyrosine phosphorylation of Shc and its association with Grb2-Sos complex, whereas it was as potent as AA in activation of ERK (Figure 2). This strongly suggests that in RTE cells, the mechanism of AA-induced MAPK phosphorylation is PKC-independent.

Tyrosine phosphorylation of Shc is believed to be a point of convergence of receptor tyrosine kinase– and G protein–mediated signaling, Shc phosphorylation, as well as MAPK activation, has been shown to be dependent on phospholipase C (PLC) and calcium mobilization. However, in renal proximal tubule epithelium, the G protein–coupled Ang II receptors (AT2 subtype) are not linked to activation of PLC-2,21 but are very potent in stimulation of AA release22 and activation of p21ras13 and MAPK.18 This suggests an alternative PLC-independent mechanism of MAPK activation, wherein AA could mediate the effect of Ang II by stimulation of EGFR and Shc phosphorylation in RTE cells.

Of interest is the observation that the effect of AA on EGFR and Shc phosphorylation and EGFR-Shc association was similar to the effect of low (1 ng/mL) concentrations of EGF (Figure 3B and 3C) and was “minimal” but significant enough to induce the maximal activation of MAPK (Figure 3D). The increase in EGF concentration resulted in more profound phosphorylation of EGFR but did not elevate further phosphorylation of MAPK (Figures 2 and 3). This indicates that activation of only a small population of EGFR molecules is sufficient for maximal stimulation of ERK in RTE cells. The further activation of EGFR may be important for a longer duration of MAPK phosphorylation and/or for induction of other signaling pathways, which are mediated by distinct phosphotyrosine residues on EGFR.23–25 This raises an important question, whether AA- and/or GPCR-induced phosphorylation of EGFR mediates recruitment of other substrates of EGFR, such as PLCγ, GTase activating protein GAP, adaptor protein Nck, and phosphotyrosine phosphatase Syp.

Another important question concerns the mechanism of AA-induced EGFR phosphorylation. It is established that EGFR undergoes EGF-induced dimerization in the plane of plasma membrane, followed by induction of its intrinsic tyrosine kinase activity resulting in its autophosphorylation. However, using a sensitive method for assessment of EGFR dimerization, we were not able to detect the formation of EGFR dimers in response to AA (data not shown). This suggests that AA-induced EGFR phosphorylation may be mediated by other tyrosine kinases such as Src26 or Jak2.29 Finally, the role of AA metabolites in the mechanism of AA-induced phosphorylation of EGFR also needs to be investigated. In renal proximal tubule epithelium, cytochrome P450 products represent the major pathway of AA metabo-


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