Induction of Mitogen-activated Protein Kinase Phosphatase-1 by Arachidonic Acid in Vascular Smooth Muscle Cells*

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Arachidonic acid (AA) and its metabolites play important roles in a variety of biological processes, such as signal transduction, contraction, chemotaxis, and cell proliferation and differentiation. It was demonstrated recently that AA can activate mitogen-activated protein kinases (MAPKs), which are crucial for transducing signals initiating cell growth and apoptosis. Here we studied the effect of AA on the induction of MAPK phosphatase-1 (MKP-1) in vascular smooth muscle cells (VSMCs) and found that AA stimulated induction of MKP-1 mRNA and proteins in VSMCs in a time- and dose-dependent manner. Specific inhibitors of cyclooxygenase-, lipoxygenase-, and cytochrome P450-dependent metabolism did not affect AA-induced MKP-1 expression, indicating that eicosanoid biosynthesis was not involved in this process. The glutathione precursor N-acetylcysteine, an antioxidant, abolished AA-stimulated MKP-1 gene expression, whereas inhibition of protein kinase C by calphostin C had no influence on MKP-1 induction. VSMC pretreatment with genistein, a tyrosine kinase inhibitor, completely blocked AA-stimulated MKP-1 induction. MAPK kinase inhibitor PD 98059 did abolish AA-stimulated activation of extracellular signal-regulated kinases but not MKP-1 induction. Furthermore, agonists that increase AA release stimulated MKP-1 induction and activation of MAPKs, including extracellular signal-regulated kinases and c-Jun NH₂-terminal protein kinases or stress-activated protein kinases. Taken together, our findings demonstrate that AA induced MKP-1 expression in VSMCs via activation of tyrosine kinases involving AA-induced free radical generation, suggesting an important role for MKP-1 in the regulation of AA-initiated signal transduction in VSMCs.

Mitogen-activated protein kinases (MAPKs),¹ a ubiquitous group of serine/threonine kinases, are thought to play a crucial role in transmitting transmembrane signals required for cell growth, differentiation, and apoptosis (1–3). At least three distinct subfamilies of MAPK exist: extracellular signal-regulated kinases (ERKs), c-Jun NH₂-terminal protein kinases (JNKs) or stress-activated protein kinases (SAPKs), and p38 MAPKs (1–7). The ERKs, SAPKs, and p38 MAPKs are activated by the reversible dual threonine and tyrosine phosphorylation of a conserved TGY, TPY, or TGY motif, respectively (6–9). Although distinct and selective MAPK activators have been cloned and characterized (1–7), less is known about negative regulation of these kinases.

Several protein phosphatases with high specificity for MAPKs have been described, including the ubiquitously expressed rodent MAPK phosphatase-1 (MKP-1), its human homolog CL100, and the lymphocyte-specific PAC-1 protein (10–14). A number of studies have implicated MKP-1 in the regulation of mitogenesis (10, 14). MKP-1 has been shown to dephosphorylate phosphothreonine and phosphotyrosine residues of both ERKs and SAPKs, resulting in their inactivation, although it exhibits cell type specificity (10–14). Thus, the balance between MAPK activation and MKP-1 induction should be an important point in determining the fate of cells stimulated by environmental insults.

Arachidonic acid (AA) and its metabolites are critical to a variety of biological processes, such as chemotaxis, inflammation, and signal transduction (15, 16). In addition to these functions, several investigators in recent years have reported that some peptide growth factors such as epidermal growth factor and fibroblast growth factors stimulate AA production in a number of cell types (17–20). In fact, it was also found that induction of mitogenesis by these growth factors required AA metabolism (18–20). AA metabolites have been found to stimulate growth in many cell types, including vascular smooth muscle cells (VSMCs) when added exogenously (21–24). Recently, it was demonstrated that AA activates both ERKs and JNKs/SAPKs in VSMCs or epithelial cells (25–27), indicating an important role for AA and its metabolites in mitogenic signaling events. However, no data exist as to whether AA induces MKP-1 expression, which is important in regulating MAPK activity during cell proliferation. In the present study, we evaluated the potential effects of AA on MKP-1 induction in VSMCs using Western and Northern blot analyses and demonstrated that AA stimulation results in MKP-1 mRNA expression followed by increased protein induction. The mechanism appears to involve AA-stimulated superoxide generation, which activates tyrosine kinases and is independent of downstream eicosanoid biosynthesis and of protein kinase C (PKC) signal pathways.

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¹ The abbreviations used are: MAPKs, mitogen-activated protein kinase; AA, arachidonic acid; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; JNK, c-Jun NH₂-terminal protein kinase; MKP, mitogen-activated protein kinase phosphatase; MOPS, 4-morpholinepropanesulfonic acid; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SAPK, stress-activated protein kinase; VSMC, vascular smooth muscle cell.

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**EXPERIMENTAL PROCEDURES**

**Materials—**A rat MKP-1 cDNA was isolated from rat lung cDNA library by Liu et al. (14). Plasmids expressing MKP-1 were propagated in *Escherichia coli*, and cDNA was obtained by cutting the plasmids with EcoRI. Polyclonal antibodies against rat MKP-1, mammalian ERK2, and JNK1/SAPK were obtained from Santa Cruz Biochemicals (Santa Cruz, CA). The monoclonal antibody against phosphorylated ERK2 was obtained from New England Biolabs, Inc. (Beverly, MA). Arachidonic acid, linoleic acid, stearic acid (tissue culture grade), calf thymus DNA, bovine sericin, penicillin (100 units/ml) and streptomycin (100 µg/ml) were from Sigma (St. Louis, MO). Protein A-Sepharose 4B suspension (BA85; Schleicher and Schuell, Dassel, Germany). Indomethacin, angiotensin II, endothelin-1, herbimycin A, and inhibitors at 37 °C for the times indicated in figure legends. After two washes with cold (4 °C) phosphate-buffered saline, pH 7.4, cells were harvested on ice in buffer A containing 20 mM Hepes, pH 7.4, 2 mM EDTA, 1 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na3VO4, 1% Triton X-100, 10 mM sodium fluoride, 100 µM aprotinin, 400 µM phenylmethylsulfonyl fluoride. The suspension was incubated on ice for 20 min. Cells to AA resulted in a time-dependent induction of MKP-1 and was maximal at 20 µM (Fig. 1B). In addition, other fatty acids, including linoleic acid and stearic acid, did not significantly stimulate MKP-1 induction (data not shown).

**RESULTS**

**AA-stimulated MKP-1 Induction—** Growth-arrested VSMCs were exposed to 20 µM AA for varying times, and protein extracts were prepared. Equal amounts of proteins from control and experimental samples were used to test MKP-1 induction using Western blot analysis. As shown in Fig. 1A, exposure of cells to AA resulted in a time-dependent induction of MKP-1 which was evident at 20 min, peaked at 1 h, and declined thereafter. Fig. 1B summarizes MKP-1 induction as determined by quantification of optical densities from autoradiograms of three experiments. Exposure of cells to AA produced 10–20-fold changes in MKP-1 after 1 or 2 h in protein extracts of VSMCs. AA-stimulated MKP-1 induction was also dose-dependent in that an increment in the proteins was observed at 2 µM and was maximal at 20 µM (Fig. 1C). In addition, other fatty acids, including linoleic acid and stearic acid, did not significantly stimulate MKP-1 induction (data not shown).

MKP-1 mRNA levels in AA-treated VSMCs were analyzed by Northern blots. As shown in Fig. 2, AA treatment resulted in significantly increased MKP-1 mRNA in VSMCs. Kinetic analysis of the response revealed that AA-induced MKP-1 mRNA expression occurred as early as 20 min (Fig. 2A), with maximum levels (10-fold greater than untreated control) achieved between 20 min and 1 h after treatment and a return to basal levels thereafter. To establish further the relationship between AA treatment and MKP-1 expression, we performed a dose-response analysis of AA-induced MKP-1 mRNA accumulation. As shown in Fig. 2B, MKP-1 mRNA levels increased in a dose-dependent manner between 3 and 80 µM. The lower halves of Fig. 2 (A and B) show the amount of 28 S and 18 S RNA from the corresponding blot.

**MKP-1 Induction Is Independent of AA Metabolism—** In most mammalian cells, AA is oxidized through the cyclooxygenase and lipoxygenase pathways to yield eicosanoids that mediate biological effects. To evaluate the contributions of these metabolic pathways, VSMCs were treated with the cyclooxygenase inhibitor indomethacin, the lipoxygenase inhibitor cinnamyl-3,4-dihydroxy-a-cyanocinnamate, or the cytochrome P450 inhibitor ketoconazole for 30 min followed by stimulation with 20 µM AA. Indomethacin (20 µM) effectively inhibited 6-keto prostaglandin F1α production stimulated by exogenous AA in VSMCs (data not shown) and did not significa...
cantly stimulate MKP-1 induction (Fig. 3, upper panel). Two other inhibitors did not stimulate MKP-1 production at doses known to abrogate eicosanoid biosynthesis in mammalian cells (36–38) (Fig. 3). These inhibitors did not block the MKP-1 induction in AA-stimulated VSMCs but moderately enhanced this induction (Fig. 3). There is evidence that ketoconazole could effectively block ERK activation and calcium mobilization, but not JNK/SAPK activation, after AA stimulation (27, 39). Thus, our findings suggested that AA-stimulated MKP-1 induction in VSMCs did not require subsequent eicosanoid biosynthesis.

MKP-1 Induction Is PKC-independent—Early studies demonstrated that AA activates PKC (40, 41), and the PKC agonists PMA induces MKP-1 gene expression in several cell types. To determine whether PKC mediates AA-stimulated MKP-1 induction in VSMCs, PKC was either depleted by exposing VSMCs to 1 μM PMA for 24 h or inhibited by incubating the cells with calphostin C before AA stimulation. PMA pretreatment did not reduce MKP-1 or mRNA levels but enhanced the induction by AA (Fig. 4). Similar findings were obtained with the PKC specific inhibitor, calphostin C, i.e., no significant inhibition of AA-induced expression (Fig. 4). Together, these results indicate that the AA-stimulated MKP-1 induction is independent of PKC.

AA-induced MKP-1 via Free Radical Generation and Tyrosine Kinase Activation—Because it has been demonstrated that AA stimulation results in superoxide generation, independent of PKC, in phagocytes (42, 43) and VSMCs (44), we assessed whether AA-induced MKP-1 expression was mediated by released free radicals via AA-activated NADPH oxidase. The antioxidant N-acetylcysteine, shown to be a superoxide anion scavenger, blocked H$_2$O$_2$-induced ERK activation in HeLa cells (45). When N-acetylcysteine was added to the culture with adjusting pH of the medium, AA-stimulated MKP-1 production was inhibited in a dose-dependent manner (Fig. 5). More than 50% of AA-stimulated protein induction was inhibited at a concentration of 40 mM (Fig. 5, A and B), whereas 20 mM abolished the MKP-1 mRNA induction by AA (Fig. 5, C and D). Moreover, VSMCs treated with H$_2$O$_2$, resulted in MKP-1 gene expression and MKP-1 induction in a dose-dependent manner (Fig. 6), supporting the involvement of a redox mechanism of MKP-1 induction.

Tyrosine kinases have been shown to be involved in several signal transduction pathways such as growth factor-ras-
MAPKs and G-protein-dependent pathways (46, 47). Therefore, we pretreated VSMCs with genistein or herbimycin A to inhibit different types of tyrosine kinases. As shown in Fig. 7, genistein treatment completely blocked AA-stimulated MKP-1 induction of both proteins and mRNA in VSMCs, whereas herbimycin A had a moderate influence in this induction (Fig. 7). In addition, genistein alone stimulated a modicum of MKP-1 mRNA induction in VSMCs (Fig. 7D), although the mechanism of this induction remains unknown. Our data support the involvement of genistein-sensitive tyrosine kinases in AA-induced MKP-1 expression.

ERK-independent MKP-1 Induction—Both ERK and JNK/SAPK can be activated by AA (25–27), and MKP-1 induction was demonstrated to be dependent on JNK/SAPK and/or ERK cascades (48, 49). To investigate whether these kinases are involved in AA-induced MKP-1 expression, ERK activities and MKP-1 induction were determined simultaneously in VSMCs pretreated with PD 98059, a specific MAPK kinase (MEK1/2) inhibitor. A marked activation of ERK1/2 by AA was found (Fig. 8A), confirming previous observations (25–27). AA-activated ERK1/2 was inhibited by PD 98059 in a concentration-dependent manner (Fig. 8A, upper panel). ERK2 kinase activity was abolished completely by 50 μM PD 98059 (Fig. 8A, lower panel) but not JNK/SAPK activity (data not shown). MKP-1 induction was not affected by the inhibitor (Fig. 8B). These results support the notion that AA-induced MKP-1 production may be dependent on JNK/SAPK but not ERK activation.

Agnostists Inducing AA Release Stimulate MKP-1 Induction and MAPK Activation—It has been demonstrated that agonists binding to G protein-coupled receptors and PDGF receptors lead to release of AA, which serves as a second messenger during signaling in VSMCs and other cell types (50–53). We wanted to determine whether agonists inducing AA release stimulate ERK and JNK/SAPK activation as well as MKP-1 induction. When vasoactive agonists angiotensin II, endothelin-1, and bradykinin and tyrosine kinase receptor agonist PDGF-AB were added to quiescent VSMCs, marked activation of ERK and JNK/SAPK was observed (Fig. 9, A and B). The magnitude of ERK activation by agonists was similar to that of JNK/SAPK activation. Concomitantly, MKP-1 induction by the variety of agonists was observed 1 h after stimulation (Fig. 9C). These findings suggest the existence of a close relationship between MAPK activation and MKP-1 induction stimulated by agonists, in which released AA may play a central role in coordinating different signal pathways.

DISCUSSION

AA, an important lipid messenger, is generated in response to a variety of stimuli, including growth factors, vasoactive
agonists, inflammatory agents, and cytokines (15–20). AA modulates gene expression, protein synthesis, and cell growth and differentiation (17–20). In the present study we demonstrate that AA can also stimulate MKP-1 expression, which is crucial to regulation of MAPK activities in VSMCs. These results have several implications. First, understanding that AA induces MKP-1 may strengthen the notion that this fatty acid plays an important role in regulating VSMC growth. Whereas AA stimulates ERK and JNK/SAPK activation, which appears to be a component common to signaling pathways initiated by a wide range of growth-stimulating factors, including mitogens and hormones (Fig. 9; Refs. 54–56), MKP-1 serves as a negative regulator, controlling cell growth via inactivation of both MAPKs. Second, AA plays a pivotal role in the regulation of VSMC contraction (57, 58) induced by vasoactive agonists, which results in MKP-1 expression (10–14). In this process, MKP-1 might be involved in inactivation of other kinases that initiate cell contraction. Finally, AA-stimulated ERK activation is dependent on AA metabolites (25, 26), whereas MKP-1 induction by AA appears independent, suggesting a complicated network of AA-mediated signaling in VSMCs. Thus, our findings could significantly advance our understanding of the possible role of MKP-1 in physiologic conditions or pathologic changes, i.e. inhibition of VSMC growth or hypertrophy in response to AA and its metabolites.

Previous studies have established that AA stimulation results in induction of intracellular superoxide radical formation in several cell types (27, 46–48). Our data show that free radical scavenger N-acetylcysteine completely blocks MKP-1 mRNA induction and that exogenous H$_2$O$_2$ stimulates MKP-1 expression, supporting the notion that MKP-1 induction is largely dependent on AA-stimulated superoxide formation. Furthermore, differential activation of ERK and JNK/SAPK by H$_2$O$_2$ has been identified in VSMCs, HeLa cells, and astrocytes, respectively (Fig. 6; Refs. 49, 59, 60). These activated kinases may subsequently lead to the transcription factor activation responsible for MKP-1 gene expression. Our findings concomitant with other reports have demonstrated the role of H$_2$O$_2$ in stimulated MKP-1 expression in VSMCs (Fig. 6) and astrocytes (60).

Tyrosine kinases, such as Pyk2 and Src-related kinases, have been shown to be essential intermediates linking upstream kinases and MKP-1 gene expression. In our system, genistein blocked AA stimulation of MKP-1 (Fig. 7), although the identity of the tyrosine kinase(s) remains to be determined.

**Fig. 6.** H$_2$O$_2$ stimulates MKP-1 induction. Panel A, Western blot analysis for the induction of MKP-1 by H$_2$O$_2$. After treatment with H$_2$O$_2$ in the indicated concentrations for 1 h, VSMCs were harvested for protein extracts and Western blot analysis as described under “Experimental Procedures.” Panel B, Northern blot analysis for MKP-1 mRNA expression. Quiescent VSMCs were treated with H$_2$O$_2$ at 37 °C for 30 min and harvested for RNA isolation and Northern blot analysis as described in the legend to Fig. 2. The lower panel shows 28 S and 18 S RNA. AA, treated with arachidonic acid.

**Fig. 7.** Effects of genistein and herbimycin A on AA-stimulated MKP-1 induction. Quiescent VSMCs were pretreated for 1 h with 100 μM genistein or with 1 μM herbimycin A for 16 h. Cells were exposed to 20 μM AA for 1 h (panels A–C) or 30 min (panels D and E) and harvested for protein extracts or RNA isolation as described under “Experimental Procedures.” Panels A and C represent the results of Western blot analysis, panel B summarizes data from three independent experiments, and panels D and E show MKP-1 mRNA. * indicates a significant difference from other three groups, p < 0.001. Data in panels A, C, D, and E are an example from similar results of two or three independent experiments. S, fetal calf serum treatment as a positive control.
We have also observed that AA increases tyrosine phosphorylation of many as yet unidentified molecules (data not shown). Identifying the tyrosine kinases will greatly enhance our understanding of molecular events occurring in response to AA. One of the potential downstream effector molecules that can be activated by tyrosine kinases includes Shc, leading to recruitment and activation of the GRB2, mSOS, and Ras pathway (1–4). This pathway may be important in AA signaling because AA simultaneously activates both ERK and JNK/SAPK, which share a common point during activation via Ras (25–27). Interestingly, Bokemeyer et al. have recently demonstrated that JNK/SAPK activation is responsible for MKP-1 gene expression, at least in fibroblasts (48). Our data demonstrate that AA-induced MKP-1 production is correlated with JNK/SAPK but not ERK activation. Taken together, the results support the role of JNK/SAPK in AA-stimulated MKP-1 expression.

Growth and proliferation of VSMCs have been shown to be associated with a number of vascular disease states, including medial hypertrophy in hypertension, intimal thickening in atherosclerosis, and restenosis after angioplasty. Multiple factors, including growth factors, cytokines, mechanical stress, neurotransmitters, and hormones that activate MAPK signal pathways (61–63), are believed to contribute to the processes leading to VSMC growth (64–66) wherein AA may serve as a common second messenger orchestrating the negative and positive signal pathways. Thus, we postulate that the balance between MKP-1 and MAPK levels/activities stimulated by AA in VSMC is critical for maintaining homeostasis of the arterial wall. Further understanding of the mechanisms regulating MAPK and MAPK phosphatase activities by AA could lead to strategies for the prevention or treatment of vascular disorders.

**References**

1. Nishida, E., and Gotoh, Y. (1993) Trends Biochem. Sci. 18, 128–131
2. Ruderman, J. V. (1993) Curr. Opin. Cell Biol. 5, 207–213
3. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–745
4. Cano, E., and Mahadevan, L. C. (1995) Trends Biochem. Sci. 20, 117–122
5. Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1023–1037
6. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) Nature 369, 156–160
7. Davis, R. J. (1994) J. Biol. Chem. 269, 14553–14556
8. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
9. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zou, L. I. (1994) Nature 372, 794–798
10. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1995) Cell 75, 487–493
11. Charles, C. H., Sun, H., Lau, L. F., and Tonks, N. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 5292–5296
12. Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R. J., and Kelly, K. (1994) Nature 367, 651–654
13. Alessi, D. R., Smythe, C., and Keyse, S. M. (1993) Oncogene 8, 2015–2020
14. Liu, Y., Gunge, M., Yang, C., and Holbrook, N. J. (1993) J. Biol. Chem. 268, 8377–8380
15. Hanahan, D. J. (1986) Annu. Rev. Biochem. 55, 483–509
16. Axelrod, J., Burch, R. M., and Jelsma, C. L. (1988) Trends Neurosci. 11, 117–123
17. Peppelenbosch, M. P., Tertoolen, L. G., den Hertog, J., and deLaat, S. W. (1992) Cell 69, 295–305
18. Handler, J. A., Danilowicz, R. M., and Eling, T. E. (1990) J. Biol. Chem. 265, 3669–3673
19. Peppelenbosch, M. P., Tertoolen, L. G., den Hertog, J., and deLaat, S. W. (1992) Cell 69, 295–305
20. Selltzer, A., Udehthoven, W. M., Weber, P. C., and Bonventre, J. V. (1991) J. Biol. Chem. 266, 3809–3817
21. Faivre, F., Jiang, Z. P., and Bohlen, P. (1991) J. Cell. Physiol. 149, 277–283
22. Owen, N. E. (1986) Annu. Rev. Physiol. 58, 584–588
23. Palmberg, L., Chesson, H. E., and Thyberg, J. (1987) J. Cell Sci. 88, 151–159
24. Setty, R. N., Gruber, J. E., and Stuart, M. J. (1987) J. Biol. Chem. 262, 17613–17622
25. Harris, R. C., Homma, T., Jacobson, H. R., and Capdevila, J. (1990) J. Cell. Physiol. 144, 429–437
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