Potential Role for Mitogen-Activated Protein Kinase Phosphatase-1 in the Development of Atherosclerotic Lesions in Mouse Models

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Objective—Mitogen-activated protein kinase phosphatase-1 (MKP-1) is one of several oxidized-l-α-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC)-induced genes identified in human aortic endothelial cells (HAEC). We previously reported that MKP-1 activity is required for Ox-PAPC–mediated endothelial/monocyte interactions; however, an in vivo role of MKP-1 in atherogenesis has not been investigated.

Methods and Results—We now report that MKP-1 protein is expressed in the atherosclerotic lesions of mice. MKP-1 mRNA expression is highly induced in C57BL6/J mice on an atherogenic diet, low-density lipoprotein receptor (LDLR) (−/−) mice on a Western diet, and 10-week or older APOE (−/−) mice on a chow diet. In APOE (−/−) mice treated with 1 mg/mL of sodium orthovanadate (NaOV), a specific inhibitor of tyrosine phosphatases including MKP-1, total phosphatase activity and MKP-1 protein were decreased in both the aortic lesions and liver lysates. In 3 animal models of atherosclerosis [C57BL6/J mice on an atherogenic diet for 15 weeks, LDLR (−/−) mice on a Western diet for 10 weeks, and APOE (−/−) mice on a chow diet for 8 weeks], mice treated with NaOV had significantly smaller atherosclerotic lesions when compared with the control group.

Conclusion—MKP-1 expression is associated with hypercholesterolemia and atherosclerosis, and inhibition of MKP-1 activity may prevent atherosclerotic lesion development in mice. (Arterioscler Thromb Vasc Biol. 2004;24:1676-1681.)

Key Words: lipoprotein oxidation ■ MKP-1 ■ atherosclerosis ■ phosphatase inhibitor

Atherosclerosis is a chronic inflammatory condition in which the migration of circulating monocytes into the vessel wall is an important step.1 There is considerable evidence that oxidized phospholipids present in low-density lipoprotein (LDL) play an important role in the recruitment of monocytes and are one of the important mediators of inflammatory pathways leading to the development of atherosclerosis.1,2 Oxidized-l-α-1-palmitoyl-2-arachidonoyl-sn-glycero-phosphorylcholine (Ox-PAPC), a mixture of phospholipid oxidation products, contributes major bioactive components to minimally modified LDL (MM-LDL) present in human atherosclerotic lesions and several other inflammatory sites.3,4

Using suppressive subtractive hybridization, we previously identified a number of genes induced in human aortic endothelial cells (HAEC) in response to Ox-PAPC.5 One of the Ox-PAPC–inducible genes, mitogen-activated protein kinase phosphatase-1 (MKP-1) belongs to a family of nuclear dual-specificity phosphatases that dephosphorylate both threonine-serine and tyrosine residues on target proteins.6 To date, 11 dual-specificity phosphatases have been identified, and MKP-1 was the first identified mammalian dual-specificity phosphatase initially cloned and characterized as an oxidative stress and heat-shock–inducible gene.7 Because a number of kinases known to be involved in the mitogen-activated protein kinase (MAPK) and stress-activated protein kinase pathways are regulated by phosphorylation of serine/threonine and tyrosine residues, MKP-1 and its family members are considered to play a regulatory role in the MAPK and stress-activated protein kinase signaling pathways.

We have previously reported that MKP-1 plays an important role in endothelial/monocyte interactions and is necessary for Ox-PAPC–mediated induction of monocyte chemotactic activity by HAEC.8 The majority of the monocyte chemotactic activity in Ox-PAPC–treated HAEC is mediated by monocyte chemoattractant protein–1 (MCP-1), and MKP-1 is required for MCP-1 protein synthesis.8 Based on our previous results, we hypothesized that MKP-1 plays a key role in atherosclerosis and other chronic inflammatory conditions and may be a potential therapeutic target. In this article, we show that: (1) MKP-1 protein is expressed in mouse atherosclerotic lesions; (2) MKP-1 expression corre-

Received June 9, 2004; revision accepted June 29, 2004.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000138342.94314.64

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lates with hypercholesterolemia; and (3) atherosclerotic lesion development in vivo, and inhibition of tyrosine phosphatase activity and MKP-1 protein reduces the development of atherosclerotic lesions in mouse models of atherosclerosis.

Materials and Methods

Animal Studies and Experimental Treatments

C57BL6/J, ApoE (−/−), and LDL receptor (LDLR) (−/−) mice were obtained from Jackson Animal Laboratory (Bar Harbor, ME). For atherosclerotic lesion studies, each group of mice were placed on the following diet for an indicated period of time: C57BL6/J mice were placed on atherogenic diet for 15 weeks; LDLR (−/−) mice were placed on Western diet for 12 weeks; and ApoE (−/−) mice were placed on chow diet for 8 weeks (Harlan Teklad). In phosphatase inhibitor studies, along with the diet, sodium orthovanadate (NaOV) (Sigma) was either included or excluded in the drinking water at a concentration of 1 mg/mL. For dimyristoyl phosphatidyl choline (DMPC) studies, ApoE (−/−) mice (n=8) were treated with or without DMPC at 1 mg/mL (Sigma, MO) for 1 week. At the end of the study, mice were euthanized and analyzed for aortic lesions, serum lipid profiles, hepatic MKP-1 expression, and phosphatase activity.

Immunohistochemistry for MKP-1, Phospho-JNK, Phospho-p38, and CD-68

MKP-1 expression in atherosclerotic lesions was determined by immunohistochemical analysis. Frozen sections of mouse atherosclerotic plaque were air-dried and fixed in acetone for 10 minutes at room temperature. For staining, DAKO EnVision+ System (Carpinetra) was used following the manufacturer’s protocol. Briefly, sections were blocked for 20 to 30 minutes in 0.25% casein (DAKO) before primary antibody application. MKP-1 polyclonal antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, Calif) and used at a 1:200 dilution in antibody diluent (DAKO). Phospho-JNK and phospho-p38 polyclonal antibodies were obtained from Cell Signaling (Massachusetts) and used at a 1:200 dilution. For controls, sections were incubated with rabbit preimmune serum containing protein concentrations that matched the primary antibody. In CD68 staining for identification of macrophage infiltration, sections were analyzed for CD68 using the avidin-biotin-peroxidase technique (VECTASTAIN ABC-AP Kit; Vector Laboratories). Frozen sections were fixed in acetone for 10 minutes, blocked in 5% normal goat serum in phosphate-buffered saline with 3% bovine serum albumin, and incubated with biotinylated rat anti-mouse CD68 primary antibody (Serotec Inc, Raleigh, NC) at a 1:100 dilution for 1 hour. The avidin-biotin-peroxidase technique (Vectastain Elite ABC Kit, Vector Laboratories) was used following the manufacturer’s instructions. Sections were incubated with biotinylated goat anti-rabbit secondary antibody (Alexa Fluor 594; Invitrogen, Carlsbad, CA) at a 1:5000 dilution. Antibodies against phospho-JNK, JNK38, p38, and phospho-p38 were all obtained from Cell Signaling (Beverly, Mass).

Other Methods

Lipoprotein analyses were performed at the Lipid Core laboratory in the Atherosclerosis Research Unit at University of California at Los Angeles. Northern blot preparation and quantification of MKP-1 in the liver tissues were performed as previously described.10

Statistical Analysis

Data are expressed as means (±SEM). Differences between experimental groups were evaluated for statistical significance using Student’s t test. P<0.05 was considered to be statistical significance.

Results

MKP-1 Protein Is Expressed in Atherosclerotic Lesions of ApoE (−/−) Mice

MKP-1 is an inducible immediate early gene, which is either absent or expressed at very low levels in most tissues under physiological conditions. MKP-1 message and protein are not detected in any other areas of the aorta. Moreover, MKP-1 protein expression is localized only to the atherosclerotic lesion areas in ApoE (−/−) mice as determined by Oil-Red-O staining and immunohistochemistry using CD-68 antibody on serial sections (Figure 1). MKP-1 protein expression is not detected in any other areas of the aorta.
MKP-1 mRNA Expression Increases in C57BL6/J Mice Fed an Atherogenic Diet

C57BL6/J mice on an atherogenic diet for >10 weeks become hypercholesterolemic and develop atherosclerotic lesions. Because MKP-1 expression is localized to the atherosclerotic lesions in ApoE (−/−) mice, we examined the effect of an atherogenic diet on the expression of MKP-1 gene in wild-type C57BL6/J mice fed an atherogenic diet. Hepatic MKP-1 mRNA expression is very low in C57BL6/J mice on a chow diet, whereas hepatic MKP-1 mRNA is strongly induced in C57BL6/J mice on the atherogenic diet (Figure 2).

MKP-1 protein is also expressed in the atherosclerotic lesions of C57BL6/J mice fed an atherogenic diet for 15 weeks (data not shown). These data suggest that MKP-1 message and protein are elevated in response to atherogenic diet and hypercholesterolemia.

MKP-1 Expression Increases With Age and Gender in ApoE (−/−) Mice

To determine whether MKP-1 expression increased with increase in hypercholesterolemia, we examined MKP-1 mRNA expression in livers of 4-, 10-, and 25-week-old female ApoE (−/−) mice. Hepatic MKP-1 mRNA expression is elevated in female ApoE (−/−) mice as they get older (Figure 3). We next examined whether MKP-1 expression correlates with increased atherosclerotic lesions found in ApoE (−/−) female mice relative to ApoE (−/−) male mice. Fourteen-week-old ApoE (−/−) mice were euthanized and analyzed for aortic lesion sizes and hepatic MKP-1 mRNA expression in the liver. MKP-1 mRNA levels were significantly higher in the female mice compared with the male mice (Figure 4A). The MKP-1 levels correlate with the extent of hypercholesterolemia as indicated by lipoprotein measurement (not shown) and lesion size in female mice compared with male mice (Figure 4B).

MKP-1 Expression Decreases in ApoE (−/−) Mice Treated With Dimyristoyl Phosphatidyl Choline

We have recently reported that the oral synthetic phospholipid, DMPC raises high-density lipoprotein cholesterol levels, improves high-density lipoprotein function, and markedly reduces atherosclerosis in ApoE (−/−) mice.11 To determine the effect of DMPC on MKP-1 expression, ApoE (−/−) mice were treated with DMPC (1 mg/mL, in drinking water) for 1...
week and their livers were analyzed for MKP-1 expression and total phosphatase activity. ApoE (−/−) mice treated with DMPC have significantly lower levels of MKP-1 mRNA (Figure 1A, available online at http://atvb.ahajournals.org), protein (not shown), and phosphatase activity (Figure 1B) when compared with control mice.

**Total Phosphatase Activity and MKP-1 Protein Are Decreased While Phospho-JNK and Phospho-p38 Phosphorylation/Activation Are Elevated in ApoE (−/−) Mice Treated With NaOV**

In cell culture studies, NaOV and antisense oligonucleotide directed against MKP-1 are very similar in their ability to inhibit oxidized phospholipid-mediated endothelial/monocyte interactions. We examined whether NaOV prevented MKP-1 protein expression and phosphatase activity, in vivo. Total phosphatase activity (Figure 5A) and MKP-1 protein expression (Figure 5B) were inhibited in ApoE (−/−) mice treated with NaOV (1 mg/mL in drinking water). Because MAP kinases JNK and p38 are substrates for MKP-1, we next examined by immunohistochemistry (in aortic lesions) and Western blotting (in liver lysates) whether phosphorylation/activation of JNK and p38 are affected in ApoE (−/−) mice treated with NaOV. Phosphorylation of JNK and p38 were significantly elevated in both the aortic lesions (Figure II, available online at http://atvb.ahajournals.org) and liver lysates (Figure III, available online at http://atvb.ahajournals.org) of ApoE (−/−) mice treated with NaOV, compared with the control group.

**Inhibition of Tyrosine Phosphatase Activity by NaOV Prevents the Development of Atherosclerotic Lesions in 3 Mouse Models of Atherosclerosis**

To further examine whether inhibition of MKP-1 has any role in the development of atherosclerotic lesions, C57BL6/J mice on an atherogenic diet, LDLR (−/−) mice on a Western diet, and ApoE (−/−) mice on a chow diet were given NaOV (1 mg/mL) in drinking water for 15, 10, and 8 weeks, respectively. Administration of NaOV (1 mg/mL) did not significantly affect the lipoprotein profiles in any of the 3 animal models tested (data not shown). However, in all 3 models, atherosclerotic lesions are significantly reduced (Figure 6), suggesting that inhibition of tyrosine phosphatase activity including MKP-1 prevented the development of atherosclerotic lesions in these animal models.

**Discussion**

The migration of circulating monocytes into the vessel wall is an important step in the pathology of atherosclerosis. Oxidized phospholipids present in MM-LDL play an important role in the recruitment of monocytes. Ox-PAPC is the active component of MM-LDL–mediated activation of endothelial cells as well as MM-LDL–mediated promotion of endothelial...
MKP-1 expression was detected only in the atherosclerotic lesion areas (Figure 1), suggesting that MKP-1 protein is transiently induced. MAPKs are activated through the reversible phosphorylation of both tyrosine and threonine residues of the TXY motif located in the catalytic domain. The MAPK phosphatases are a family of dual-specificity phosphatases that can dephosphorylate both phosphorylase and phosphotheonine residues. A major function assigned to the MKP family of proteins is the inactivation of activated MAPKs. MKP-1 is the archetypal member of this family of proteins. MKP-1 is an immediate early gene induced by a variety of agents including oxidized phospholipids and growth factors. MKP-1 has been implicated in a number of physiological processes such as apoptosis and cell proliferation, and pathological processes such as inflammation and cancer. To date, a specific physiological role for MKP-1 has not been defined.

MKP-1 is a primary response gene and is not expressed unless induced by appropriate stimuli. In the mouse aorta, MKP-1 expression was detected only in the atherosclerotic lesion areas (Figure 1), suggesting that MKP-1 protein is induced by factors in the atherosclerotic milieu. A number of ligands including growth factors, oxidized lipids, and cytokines are associated with the atherosclerotic lesions, and MKP-1 induction has been reported for all these factors in cell culture studies. Moreover, it is interesting to note that in all of the animal models we tested, MKP-1 expression correlates with atherosclerotic lesion development and hypercholesterolemia (Figures 2 through 4 and Figure I). It is unclear at this time whether MKP-1 protein expression in the atherosclerotic lesions is pro- or anti-atherogenic. Dorfman et al. previously reported that MKP-1 knockout mice developed normally, were fertile, and cells cultured from MKP-1 knockout mice were not impaired in either MAPK activation or inactivation. Consequently, it was concluded that MKP-2 must be able to substitute for MKP-1. However, in the studies by Dorfman et al., the MKP-1 knockout mice were not subjected to oxidative stress and there was no determination of their susceptibility to atherosclerosis. It is possible that in the basal state MKP-2 can substitute for MKP-1. We are currently developing transgenic and knockout models to determine the role of MKP-1 in animal models of atherosclerosis.

We have previously shown that in artery wall cells MKP-1 is a critical component of Ox-PAPC-mediated endothelial/monocyte interactions and MCP-1 secretion. However, very little is known about the in vivo role of MKP-1 in relation to endothelial/monocyte interactions and atherosclerosis. Unfortunately, animal models are not readily available to study the role of MKP-1 in atherosclerosis. We have previously shown that in artery wall cells, inhibition of MKP-1 using NaOV (a tyrosine phosphatase inhibitor that is known to inhibit MKP-1 activity) or selective antisense oligonucleotides directed against MKP-1 mRNA were similar in inhibiting Ox-PAPC-induced endothelial/monocyte interactions and MCP-1 protein secretion. We therefore decided to test NaOV in our animal studies. We used NaOV concentrations that were previously used in mice, which did not result in toxicity. In preliminary studies, we tested concentrations ranging from 1 to 10 mg/mL and did not find a significant effect on the lipid and lipoprotein levels (triglycerides, total cholesterol, high-density lipoprotein cholesterol, and free fatty acids) in NaOV-treated mice compared with control mice receiving water alone. The livers were normal and no adverse changes in behavior, eating, or drinking patterns were noticed.

One of the surprising findings from our studies is the effect of NaOV on MKP-1 protein expression (Figure 5). Although NaOV has been shown to inhibit MKP-1 activity, it has never been shown to prevent MKP-1 protein expression. Our results, for the first time to our knowledge, suggest that NaOV can not only inhibit the phosphatase activity associated with MKP-1 but can also prevent the accumulation of MKP-1 protein. We have initiated studies to determine whether NaOV can inhibit MKP-1 protein accumulation in artery wall cell cultures, whether NaOV inhibits the accumulation of other phosphatases, and to determine the mechanism(s) behind the inhibition of MKP-1 protein accumulation by NaOV. However, it must be pointed out that the use of NaOV has its own limitations. First of all, in vivo NaOV can have nonspecific inhibitory activity on phosphatases other than MKP-1. Secondly, NaOV can affect the phosphorylation/activation of several tyrosine kinase receptors, as implicated in studies by Hadari et al. that could have influenced the lesion data in the animal models we tested. Finally, without corroborating data from MKP-1-selective inhibitors...
and/or MKP-1–specific animal models, it would be very difficult to implicate MKP-1 alone as the candidate phosphatase responsible for the decrease in lesions in our animal studies. We are, therefore, developing transgenic and genetically targeted mice to further determine the role of MKP-1 in atherosclerosis. We are also exploring ways to identify novel and selective inhibitors of MKP-1 to further investigate the validity of MKP-1 inhibition as a therapeutic target for the prevention of atherosclerosis.

In summary, our data suggest that MKP-1 expression correlates with hypercholesterolemia and the development of atherosclerosis in mouse models of atherosclerosis. Inhibition of tyrosine phosphatase activity dramatically reduces the development of atherosclerotic lesions in all 3 animal models tested. Development of MKP-1–specific animal models and identification of selective MKP-1 inhibitors will determine whether inhibition of MKP-1 is sufficient to prevent the development of atherosclerosis.

Acknowledgments
We thank the technical assistance provided by David Wadleigh. This work was supported in part by USPHS grants HL 30568, the Laubisch, Castera, and M.K. Gray Funds at University of California Los Angeles.

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Arterioscler Thromb Vasc Biol. 2004;24:1676-1681; originally published online July 8, 2004; doi: 10.1161/01.ATV.0000138342.94314.64
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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http://atvb.ahajournals.org/content/24/9/1676

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Figure I. MKP-1 expression is decreased in ApoE(-/-) mice treated with dimyristoyl phosphotidylcholine (DMPC). ApoE (-/-) mice (n=8 per group) were treated with DMPC (1 mg/ml, in drinking water) for 1 week. The mice were sacrificed and their livers were analyzed for A) MKP-1 mRNA expression and B) total phosphatase activity as described under Materials and Methods.

Figure I

A. B.
Figure II. Phospho JNK and phospho p38 phosphorylation/activation are elevated compared to the control group in the aortic lesions of ApoE (-/-) mice treated with NaOV. ApoE (-/-) mice on a chow diet (8 weeks) were given either drinking water alone or drinking water containing 1mg/ml of NaOV. At the end of the study, mice were sacrificed and aortic lesions were analyzed by immunohistochemistry for MKP-1, phospho JNK and phospho p38.
Figure III. Phospho JNK and phospho p38 phosphorylation/activation are elevated compared to the control group in the liver lysates of ApoE (-/-) mice treated with NaOV. ApoE (-/-) mice on a chow diet (8 weeks) were given either drinking water alone or drinking water containing 1mg/ml of NaOV. At the end of the study, mice were sacrificed and total lysates from livers were analyzed for phospho JNK, JNK, phospho p38 by and p38 western analysis (A). Protein bands on the western blots were quantified by densitometry and represented as phosphorylated to unphosphorylated proteins for each of the MAP kinases from control and NaOV treated groups (B).