Role of 12/15-lipoxygenase in the expression of MCP-1 in mouse macrophages

Yeshao Wen, Jiali Gu, George E. Vandenhoff, Xiaoping Liu, and Jerry L. Nadler

Diabetes and Hormone Center, University of Virginia, Charlottesville, Virginia

Submitted 2 March 2007; accepted in final form 29 January 2008

Wen Y, Gu J, Vandenhoff GE, Liu X, Nadler JL. Role of 12/15-lipoxygenase in the expression of MCP-1 in mouse macrophages. Am J Physiol Heart Circ Physiol 294: H1933–H1938, 2008. - Monocyte chemoattractant protein (MCP)-1 plays a key role in atherosclerosis and inflammation associated with visceral adiposity by inducing mononuclear cell migration. Evidence shows that mouse peritoneal macrophages (MPM) express a 12-lipoxygenase (12/15-LO) that has been clearly linked to accelerated atherosclerosis in mouse models and increased monocyte endothelial interactions in both rodent and human cells. However, the role of 12/15-LO products in regulating MCP-1 expression in macrophages has not been clarified. In this study, we tested the role of 12/15-LO products using MPM and the mouse macrophage cell line, J774A.1. We found that 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) increased MCP-1 mRNA and protein expression in J774A.1 cells and MPM. In contrast, 12(R)-HETE, a lipid not derived from 12/15-LO, did not affect MCP-1 expression. 15(S)-HETE also increased MCP-1 mRNA expression, but the effect was less compared with 12(S)-HETE. MCP-1 mRNA expression was upregulated in a macrophage cell line stably overexpressing 12/15-LO (Plox-86 cells) and in MPM isolated from a 12/15-LO transgenic mouse. In addition, the expression of MCP-1 was downregulated in MPM isolated from 12/15-LO knockout mice. 12(S)-HETE-induced MCP-1 mRNA expression was attenuated by specific inhibitors of protein kinase C (PKC) and p38 mitogen-activated protein kinase (p38). 12(S)-HETE also directly activated NADPH oxidase activity. Two NADPH oxidase inhibitors, apocynin and diphenyleneiodonium chloride, blocked 12(S)-HETE-induced MCP-1 mRNA. Apocynin attenuated 12(S)-HETE-induced MCP-1 protein secretion. These data show that 12(S)-HETE increases MCP-1 expression by inducing PKC and p38, and NADPH oxidase activity. These results suggest a potentially important mechanism linking 12/15-LO activation to MCP-1 expression that induces inflammatory cell infiltration.

12/15-lipoxygenase; monocyte chemoattractant protein-1; J774A.1 cells; mouse peritoneal macrophages

Therefore, MCP-1 directs the migration of circulating leukocytes to sites of inflammation or injury and induces the activation of inflammatory cells.

There is supporting evidence that mouse peritoneal macrophages (MPM) and murine macrophage cell lines contain a lipoxigenase (LO) that possesses both 12- and 15-LO activity (12/15-LO) (21, 25). 12/15-LO can hydrolyze arachidonic acid to form predominately 12(S)-hydroperoxy-5Z, -8Z, and -10E, 14Z-eicosatetraenoic acid [12(S)-HPETE], 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE], and smaller quantities of 15(S)-HPETE. 12(S)-HPETE and 12(S)-HETE have been shown to increase interleukin (IL)-6, tumor necrosis factor-α, IL-1β, and IL-12 mRNA and protein expression in macrophages (28). 12/15-LO can also catalyze stereoselective oxidation of linoleic acid at position 13 over position 9 to preferentially form 13(S)-hydroperoxyoctadecadienoic acid (13-HPODE), which is the predominant oxidized fatty acid in low-density lipoprotein (LDL). Evidence has shown that 13-HPODE upregulates the expression of MCP-1 in vascular smooth muscle cells (VSMC) (16, 3). There are several differences between 13-HPODE and 12(S)-HPETE. 13-HPODE is esterified to cholesterol as cholesteryl-HPODE in LDL particles, leading to the oxidative modification of LDL (32, 33, 1). In contrast, 12(S)-HETE is not typically taken up in LDL. Oxidation of LDL is believed to contribute to foam cell formation and lipid accumulation in lesions as well as to the formation of necrotic areas in the core of the plaque (31). 12(S)-HETE has been shown to increase monocyte binding to EC (18, 22) and VSMC (10). Because MCP-1 plays a role in the chemotaxis and binding of monocytes with EC or VSMC, it is possible that 12/15-LO products can regulate MCP-1 expression. However, it is unclear whether arachidonic acid metabolites of 12/15-LO participate in the regulation of MCP-1 expression in macrophages.

In this study, we tested the role of 12/15-LO products in the regulation of MCP-1 expression using MPM and the mouse macrophage cell line J774A.1. Our results demonstrate that treatment of MPM or J774A.1 cells with a low concentration of 12(S)-HETE increases MCP-1 mRNA and protein expression, whereas treatment of higher concentrations of 15(S)-HETE increases MCP-1 mRNA expression. We also observed increased MCP-1 expression in MPM from 12/15-LO transgenic (12/15-LO Tg) mice and in J774A.1 cells stably overexpressing 12/15-LO. In addition, reduced MCP-1 expression was observed in MPM from 12/15-LO null mice compared with that in MPM from control mice. These data suggest that the 12/15-LO pathway participates in the regulation of MCP-1 expression in mouse macrophages.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

J774A.1 cells were purchased from The American Type Culture Collection (ATCC; Manassas, VA). C57BL6 mice were from Jackson Laboratory (Bar Harbor, ME). 12/15-LO knockout (12/15-LO null) mice were originally a generous gift from Dr. Colin Funk and have been backcrossed to the C57BL6 background (22). 12/15-LO null and 12/15-LO Tg mice are maintained in the mouse core of the Cardiovascular Research Center in the University of Virginia. Thioglycollate, apocynin, diphenyleneiodonium chloride (DPI), cytochrome c, NADPH, and superoxide dismutase (SOD) were purchased from Sigma (St. Louis, MO). 12(S)-HETE and 15(S)-HETE (>98%) were purchased from Biomol (Plymouth Meeting, PA). The RNeasy Mini Kit was obtained from Qiagen (Valencia, CA). Mouse MCP-1 immunosassay kits were obtained from R & D System (Minneapolis, MN). Culture media and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from ATCC; RPMI-1640 and Hanks’ balanced salt solution (HBSS) were from Invitrogen (Carlsbad, CA). BSA (fraction V, fatty acid free) was obtained from ICN Biomedicals (Aurora, OH).

J774A.1 cell culture. J774A.1 cells were cultured following the instructions from ATCC. Plox-86 cells, which stably overexpress 12/15-LO on the J774A.1 cell background, and mock-transfected J774A.1 cells were established and kindly provided by Dr. Yoshimoto in Kanazawa (24). Before treatment with various agonists, cells were cultured in DMEM containing 0.5% FBS and 0.2% BSA (fraction V, fatty acid free) overnight. Cells were then cultured in the depleton medium, which was DMEM with 4 mM l-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose, and 0.2% BSA (free of fatty acid) without FBS for 2 h.

MPM isolation and culture. Eight- to ten-week-old male C57BL6 mice, 12/15-LO Tg mice, or 12/15-LO null mice (both on C57BL6 background) were injected intraperitoneally with 4% thioglycolate (free of fatty acid) without FBS for 2 h.

MCP-1 mRNA expression measurement. J774A.1 cells were cultured as described and treated with vehicle, ethanol, or 12(S)-HETE for different time periods, and then the J774A.1 cells were lysed in lysis buffer. Protein (100 µg) was distributed in 96-well flat-bottom culture plates (final volume 200 µl/well). Cytochrome c (500 µmol/l) and NADPH (100 µmol/l) were added in the presence or absence of SOD (200 U/ml) and incubated at room temperature for 30 min. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader.

RESULTS

Stimulatory effects of 12(S)-HETE on MCP-1 mRNA expression in macrophage cells. J774A.1 cells were cultured as described and treated with vehicle, ethanol, or 12(S)-HETE for 2 or 4 h. Figure 1A shows that 12(S)-HETE at 10 nM increased MCP-1 mRNA expression ~1.5-fold (P < 0.01) at 2 h with a further increase in MCP-1 expression to 3.0-fold (P < 0.01) at 4 h of treatment. 12(S)-HETE also increased MCP-1 mRNA expression in MPM isolated from wild-type C57BL6 mice (Fig. 1B). 12(S)-HETE (0.1 nM) increased MCP-1 mRNA expression 3.9-fold (P < 0.01) at 4 h. 12(S)-HETE (0.1 nM) seemed to be an optimal concentration since increases in MCP-1 mRNA expression were not as prominent when the concentration went up to 1 or 10 nM. In contrast, 12(R)-HETE, an inactive analog of 12(S)-HETE, did not have any effect on MCP-1 mRNA expression (Fig. 1C). 12(S)-HETE also increased MCP-1 protein secretion from J774A.1 cells 1.5 ± 0.2-fold (P < 0.05, n = 3) and ~2-fold in MPM (P < 0.01, n = 3). Another 12/15-LO product, 15(S)-HETE, also slightly increased MCP-1 mRNA expression in isolated mouse macrophages. Results showed that 1 nM 15(S)-HETE induced a 1.5 ± 0.10-fold increase in MCP-1 mRNA expression (n = 3, P < 0.03). The mRNA expression was not further increased (1.44 ± 0.34-fold over basal level, n = 3, P < 0.05) when the concentration increased to 10 nM, although the expression was similar to control levels when the 15(S)-HETE concentration declined to 0.1 nM (1.1 ± 0.02-fold over basal, n = 3).
Effects of overexpression or deficiency of 12/15-LO gene on MCP-1 mRNA expression in macrophages. We next evaluated the MCP-1 mRNA expression in Plox-86 cells, a J774A.1 cell line stably overexpressing porcine leukocyte-type 12/15-LO. Figure 2A shows that MCP-1 mRNA expression in Plox-86 cells was more than threefold ($P < 0.01$) higher than in mock-transfected cells. We then evaluated MCP-1 mRNA expression in macrophages isolated from 12/15-LO Tg mice (Fig. 2B). Macrophages isolated from 12/15-LO Tg mice expressed ~2.3-fold higher MCP-1 mRNA expression over that in MPM from wild-type C57BL6 mice. Interestingly, macrophages isolated from 12/15-LO null mice showed a significantly ($P < 0.01$) reduced MCP-1 mRNA expression (Fig. 2B).

Role of protein kinase C activity in 12(S)-HETE-induced MCP-1 mRNA expression. Our previous data have shown that 12(S)-HETE activates protein kinase C (PKC) activity in J774A.1 cells. To determine whether PKC activity is involved in 12(S)-induced MCP-1 expression, the relatively specific PKC inhibitors calphostin C (100 nM) and GF-109203X (100 nM) were used. Both calphostin C and GF-109203X at 100 nM concentration completely suppressed 12(S)-HETE-induced MCP-1 mRNA expression (Fig. 3).

Role of p38 mitogen-activated protein kinase in 12(S)-HETE-induced MCP-1 mRNA expression. Previous studies have shown that 12(S)-HETE activates p38 mitogen-activated protein kinase (MAPK) activity. To determine whether p38 MAPK activity is important for 12(S)-induced MCP-1 expression, the p38 MAPK inhibitor SB-202190 was used. The results (Fig. 3) show that 3 μM of SB-202190 completely suppressed 12(S)-HETE-induced MCP-1 mRNA expression.
The SB-202190 compound did not affect basal MCP-1 mRNA expression.

**NADPH oxidase activity is implicated in 12(S)-HETE-induced MCP-1 mRNA expression.** We tested whether 12(S)-HETE activates NADPH oxidase activity by measuring SOD-inhibitable cytochrome c reduction in macrophages. Figure 4A shows that 4 h treatment of 12(S)-HETE (1 nM) significantly increased NADPH oxidase activity ~24-fold over that in J774A.1 cells treated with vehicle alone. Figure 4 also shows that pretreatment with 1 mM apocynin completely suppressed 12-HETE-induced NADPH oxidase activity ($P < 0.01$). Although 1 mM apocynin alone increased basal superoxide release, 12(R)-HETE did not stimulate superoxide release. To determine whether NADPH oxidase-mediated oxidative stress plays a role in 12(S)-HETE-induced MCP-1 mRNA expression, two structurally distinct NADPH oxidase inhibitors, apocynin and DPI, were used. Figure 4B shows that 1 nM 12(S)-HETE increased MCP-1 mRNA expression in J774A.1 cells, and 0.1 mM apocynin significantly suppressed ~50% of 12(S)-HETE-induced effects ($P < 0.02$) without affecting basal MCP-1 expression. When the concentration of apocynin was increased to 1 mM, 12(S)-HETE-induced MCP-1 mRNA expression was fully suppressed, but this drug concentration reduced basal MCP-1 expression (Fig. 4B). We also tested another NADPH oxidase inhibitor (DPI). Figure 4C clearly indicates that 0.1 μM completely inhibited 12(S)-HETE-induced MCP-1 mRNA expression ($P = 0.004$). These data suggest for the first time that NADPH oxidase is implicated in 12(S)-HETE-induced MCP-1 mRNA expression. We next evaluated the effect of apocynin on 12(S)-HETE-induced MCP-1 protein secretion in MPM. Primary cultured MPM were treated with 12(S)-HETE (1 nM) in the absence or presence of apocynin for 24 h, and the supernatants were collected for analysis of MCP-1 protein. Figure 4D shows that 12(S)-HETE induced MCP-1 protein secretion twofold ($P < 0.001$) in MPM, and apocynin suppressed 12(S)-HETE-in-
duced MCP-1 protein secretion. Apocynin alone did not affect basal MCP-1 protein secretion.

DISCUSSION

In this study, we have demonstrated a link between the 12/15-LO pathway and the expression of a major proinflammatory chemokine in macrophages. The 12/15-LO product 12(S)-HETE has been recognized as an inflammatory compound (15, 29), and 12/15-LO in macrophages is clearly linked to atherosclerosis in the mouse (7). In this study, we first demonstrated that direct addition of 12(S)-HETE to either the J774A.1 macrophage cell line or primary cultured MPM induced a significant increase in MCP-1 mRNA expression. 12-HETE also increased MCP-1 protein secretion from both J774A.1 and cultured MPM. In contrast, 12(R)-HETE, an inactive analog of 12(S)-HETE, had no effect on MCP-1 mRNA expression, clearly suggesting a specific effect of 12/15-LO derived lipids. We then demonstrated that another 12(S)-LO product, 15(S)-HETE, also increases MCP-1 mRNA level 1.5-fold in mouse macrophages. Evidence has shown that MPM and murine macrophage cell lines contain a LO that possesses both 12- and 15-LO activity (12/15-LO) (21, 25, 28). The results suggest that both 12-LO and 15-LO activities contribute stimulatory effects on MCP-1 regulation, thus, the inflammatory effects on macrophages. To further demonstrate a stimulatory effect of the 12/15-LO pathway in the regulation of MCP-1 expression, we tested the effect of 12/15-LO overexpression on MCP-1 expression. We found that there was a significant increase of MCP-1 mRNA in Plox-86 cells, which are cultured J774A.1 cells stably overexpressing 12/15-LO. We also found that there was a significant increase of MCP-1 expression in primary cultured MPM isolated from 12/15-LO Tg mice compared with that in MPM isolated from control C57BL6 mice. In addition, MCP-1 mRNA expression was significantly reduced in MPM from 12/15-LO null mice compared with that in MPM from control C57BL6 mice.

These in vitro and in vivo data clearly demonstrate that the 12/15-LO pathway has direct regulatory effects on MCP-1 expression. To our knowledge, this is the first study demonstrating a link of the 12/15-LO pathway to MCP-1 expression and inflammatory macrophages. In a recent preliminary study, we found that macrophage infiltration in visceral fat is reduced in 12/15 null mice fed a high-fat diet (unpublished observation).

We next conducted studies on possible mechanisms of 12(S)-HETE-induced MCP-1 expression. Previous data have shown that 12(S)-HETE activates PKC (14) and p38 MAPK (30) in adrenals and cardiac fibroblasts stably overexpressing 12/15-LO. It has recently been shown that 12(S)-HETE activates these kinases in J774A.1 macrophage cells (28). To study whether PKC and p38 are important for 12(S)-HETE activation of MCP-1 mRNA expression, two structurally distinct PKC inhibitors, calphostin C (100 nM) and GF-109203X (100 nM), and the accepted p38 inhibitor SB-202190 (3 μM) were used. The data (Fig. 3) showed that the blockade of these two kinases inhibited 12(S)-HETE-induced MCP-1 mRNA expression without affecting basal MCP-1 expression. These results suggest that PKC and p38 are two key mediators of 12(S)-HETE-induced MCP-1 expression. Our results are consistent with previous studies showing a role of PKC and p38 MAPK in regulating MCP-1 expression in other cell types (5, 26). Because the 12/15-LO pathway is involved in IL-12 production in macrophages (28), it is possible that IL-12 participates in 12/15-LO action to increase MCP-1 expression since IL-12p40 can increase MCP-1 expression (29). However, additional studies will be needed to explore this hypothesis.

There is evidence demonstrating that the 12/15-LO pathway induces superoxide generation in the presence of NADPH and NADPH, indicating a direct link of 12/15-LO to superoxide generation (2). Evidence also indicates that levels of superoxide were reduced in 12/15-LO KO mouse mesangial cells compared with that in mesangial cells from wild-type mice (9). Therefore, we evaluated whether 12(S)-HETE activates NADPH oxidase activity. Our results demonstrated that 12(S)-HETE activated NADPH oxidase activity in J744A.1 cells; in contrast, 12-HETE did not stimulate NADPH oxidase activity. The 12(S)-HETE-induced increase in NADPH oxidase activity was almost suppressed by pretreatment of 1 mM apocynin, which is a well-characterized inhibitor of NADPH oxidase (19). These data are consistent with the results in mesangial cells from 12/15-LO null mice (9). Our data that pretreatment of J744A.1 cells with NADPH oxidase inhibitors apocynin and DPI suppressed 12(S)-HETE-induced MCP-1 mRNA expression in J744A.1 cells and apocynin suppressed 12(S)-HETE-induced MCP-1 mRNA and protein expression clearly suggest an involvement of NADPH oxidase in 12(S)-HETE regulation on MCP-1 expression.

We have found in this study that PKC, p38, and oxidative stress were involved in the regulation of 12(S)-HETE-induced MCP-1 expression. There are data showing either a linear signaling cascade or cross talk among some of these signaling cascades. However, we have not pursued the relationship among these signaling cascades in this study.

Because increased reactive oxygen species have been shown to be an important trigger for insulin resistance, type 2 diabetes, and atherosclerosis (13, 6), the results may suggest a possible mechanism of 12/15-LO products in the development of insulin resistance and type 2 diabetes. However, further detailed studies will be needed to test this hypothesis.

Summary

This is the first study that demonstrates that 12/15-LO products regulate for MCP-1 expression in macrophages. MCP-1 plays an important role not only in atherosclerosis but also in the inflammatory state seen in visceral obesity. The results suggest a potentially important mechanism linking 12/15-LO activation to MCP-1 expression and inflammatory cell infiltration.

ACKNOWLEDGMENTS

We thank Starr Palmore for secretarial help and James Fredrick for figure preparation.

GRANTS

This study was supported by National Institutes of Health Grants P01 HL-55798 and RO1 DK-55240.

REFERENCES