Lysophosphatidylcholine Activates p38 and p42/44 Mitogen-Activated Protein Kinases in Monocytic THP-1 Cells, but Only p38 Activation Is Involved in Its Stimulated Chemotaxis

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Abstract—Oxidized LDLs (OxLDLs) have been shown to be involved in recruitment of blood monocytes into the arterial subendothelial space, which is the earliest step in atherogenesis, but the underlying molecular mechanisms are poorly understood. The present study demonstrated that lysophosphatidylcholine (LPC), a major phospholipid component of OxLDL, strongly evoked phosphorylation and activation of p38 and p42/44 mitogen-activated protein kinases in monocytic cells. The stimulation of p38 and p42/44 occurred in a dose- and time-dependent manner, reaching the maximal activation at 25 μg/mL LPC within 5 minutes. Interestingly, inhibition of p38 activation by OxLDL or LPC, using its selective inhibitors (SB203580 and SKF86002), completely blocked OxLDL- or LPC-stimulated chemotaxis of THP-1 cells, which was measured in a transwell chemotaxis assay. In contrast, inhibition of p42/44 activation by its potent inhibitor (PD98059) did not block OxLDL- or LPC-stimulated chemotaxis. Moreover, expression of a p38 dominant-negative mutant (p38AF) reduced cell chemotaxis significantly. In addition, activation of p38 by LPC was apparently mediated neither by scavenger receptors nor by tyrosine kinase receptors. It was, however, effectively blocked by pertussis toxin and substantially reduced by phospholipase C inhibitor (U73122) and phosphatidylinositol 3-kinase inhibitors (wortmannin and LY294002). LPC also inhibited forskolin-stimulated cAMP accumulation in a pertussis toxin–sensitive manner, indicating that Gi/Go proteins likely mediated the effects of LPC. Our results suggested that OxLDL/LPC efficiently activated both p38 and p42/44, but only the activation of p38 was functionally associated with OxLDL-/LPC-induced chemotaxis in THP-1 cells. (Circ Res. 2000;87:52-59.)

Key Words: atherosclerosis ■ lysophosphatidylcholine ■ mitogen-activated protein kinase ■ monocytes ■ chemotaxis

One of the earliest events in atherosclerotic lesion formation has been recognized as the attraction of blood monocytes to the endothelium followed by accumulation of blood monocytes in the subendothelium, where the monocyte-derived macrophages subsequently develop into lipid-laden foam cells. Oxidized LDLs (OxLDLs), produced by oxidative modification of LDLs in the subendothelium, have been demonstrated to be critically involved in atherogenesis.1–3 During the early stage of atherosclerosis, OxLDL is shown to promote expression of adhesion molecules and chemokines, suppress production of NO and prostacyclin, and induce various proinflammatory cytokines in most vascular cells. OxLDL is a potent chemoattractant for monocytes4 and T lymphocytes,5 which may contribute to the initiation of atherosclerosis. Recently, several lines of evidence have indicated that lysophosphatidylcholine (LPC), as a prominent phospholipid component of OxLDL, may be responsible for various biological activities of OxLDL, such as regulation of adhesion molecules,6 NO,7 cytokines, and growth factors.8,9 LPC itself is also a potent chemoattractant factor for monocytes,10 T lymphocytes,7 and vascular smooth muscle cells.11 However, molecular mechanisms underlying the chemotactic activity of either OxLDL or LPC are still under investigation.

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that perform important functions as mediators of cellular responses to a variety of extracellular stimuli. The following 4 major subfamilies of structurally related MAPKs have been identified in mammalian cells12–14: the extracellular signal–regulated kinases (ERK1/ERK2, also termed p42/44 MAPK), the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), big MAPK1 (BMK1), and p38 MAPK (p38), a more recently described member of the family. The subfamily of p42/44 is characteristically activated by various growth factors and associated with cell proliferation and hypertrophy. The subfamily of p38, containing at least 4 members (α, β, γ, and δ),15–19 is strongly activated in response to stress stimuli and proinflam-
matory cytokines. Some evidence suggests that activation of p38 triggers several growth factor–directed endothelial cell migrations. Recent reports have shown that OxLDL and LPC are able to activate p42/44 in several cultured cell lines, and our previous study demonstrated that OxLDL strongly activated p38 in vascular smooth muscle cells, but it is unclear yet whether LPC can stimulate both kinds of MAPKs in monocytes. The current study, therefore, was undertaken to investigate the possible MAPK activation by OxLDL/LPC and the potential involvement of MAPK activation in OxLDL/LPC-stimulated chemotaxis of monocytic THP-1 cells.

Materials and Methods

Materials

The sources of most of the reagents used for the present study have been previously described.26

Cell Culture

Monocytic THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mMol/L glutamine. Human monocytes were freshly isolated from healthy donor blood by Ficoll-Hypaque centrifugation and by an adherence test described previously.26 The obtained monocytes were treated with different agents for 5 minutes at 37°C in serum-free medium and then were lysed for Western blot analysis.

Lipoprotein Isolation and Oxidation

LDLs (density 1.019 to 1.063 g/mL) were separated from freshly drawn normal human plasma by sequential ultracentrifugation as described previously.26 The LDLs obtained were subjected to oxidative modification by Cu²⁺ incubation (5 μM/L CuSO₄, 20 hours at 37°C).

Western Blot Analysis

THP-1 cells were cultured at a density of 10⁶/mL. The growth medium was removed and replaced with medium containing 0.1% serum for 24 hours. After being treated with different agents at 37°C in serum-free medium, the cells were lysed and then analyzed by SDS-PAGE, essentially as described previously.26

Immunoprecipitation and p38 and p42/44 Kinase Assay

After being stimulated with OxLDL or LPC in serum-free medium, the cells were lysed with lysis buffer. For p38 activity assay, cell lysates were subjected to immunoprecipitation to detect p38 kinase activity using the p38 kinase assay kit according to the manufacturer’s instructions.26 This protocol measures p38-induced phosphorylation of recombinant ATF-2 fusion protein, as assessed by Western blotting using phospho-ATF-2 antibody. p42/44 kinase activity was measured using myelin basic protein as a substrate, as described previously.26

Chemotaxis Assay

Chemotaxis was assessed using a transwell cell chemotactic assay as described previously.26 During treatments and chemotaxis assay, no significant cellular toxicity using various agents, including SB203580, SKF86002, and PD98059 was observed, as assessed by trypan blue exclusion and lactate dehydrogenase leakage measurement.

Plasmids and Transient Transfection

Flag-tagged wild-type p38 and its dominant-negative mutant, p38AF (TGY→AGF), cDNAs were in the pcDNA3 vector (Invitrogen) as described and were purified with an EndoFree Plasmid Maxi kit (Qiagen). THP-1 cells were transfected with p38 constructs by electroporation using the Gene Pulser II electroporation system with radio frequency module (Bio-Rad) according to the manufacturer’s instructions. Two days after transfection, cells were subjected to chemotaxis assay, and cellular extracts were obtained for Western blot analysis. The similar transfection efficiency (~50%) was verified with fluorescent microscope and flow cytometry by cotransfection with control green fluorescent protein (GFP) cDNA.

cAMP Assay

Cells were challenged with OxLDL or LPC in the presence of 10 μmol/L forskolin and 500 μmol/L 3-isobutyl-1-methylxanthine at 37°C for 10 minutes. The cAMP level of each sample was determined using radioimmunooassay as described previously.26

Statistical Analysis

Results of the experiments are expressed as mean±SD. The Student t test was used for statistical analysis of the results. Values of P<0.05 were considered to be significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

The Phospholipid Component LPC OxLDL Strongly Stimulated Phosphorylation and Activation of p38 and p42/44 in Monocytic Cells

To investigate whether in monocytic cells OxLDL can effectively induce MAPK phosphorylation and activation, we first examined the phosphorylation of p38 and p42/44 in THP-1 cells using anti–dual-phosphorylated MAPK antibodies. As shown in Figure 1A, exposure of THP-1 to OxLDL (50 μg/mL) for 5 minutes strongly stimulated phosphorylation of both p38 and p42/44. In contrast, LDL (50 μg/mL), the precursor of OxLDL, displayed a weaker ability to stimulate phosphorylation of p38 and p42/44. We simultaneously tested which component of OxLDL was responsible for the stimulation of p38 and p42/44 phosphorylation. The results (Figure 1A) revealed that among these OxLDL components, only LPC could effectively stimulate phosphorylation of p38 and p42/44, whereas the others (PC, cholesterol, 25-hydroxycholesterol, or oxidized albumin) failed to do so. The results from the same samples probed with total p38 or total p42/44 antibody showed that the acute treatment of THP-1 cells with these agents did not change the total amount of p38 or p42/44 (Figure 1A).

It has been established that p38 is activated by dual phosphorylation of Thr180 and Tyr182 residues and that p42/44 is activated by phosphorylation of Thr202 and Tyr204 residues. Therefore, the phosphorylation of MAPK has been widely used to represent its activation. MAPK activity after OxLDL or LPC stimulation was further measured in THP-1 cells. The results showed that OxLDL and LPC significantly stimulated both p38 and p42/44 activity (Figure 1B) using an in vitro kinase assay, which was consistent with the results from the MAPK phosphorylation assay. The activity of p38 was effectively blocked by pretreatment of the cells with SB203580, a specific inhibitor of p38 (Figure 1B, top), whereas the p42/44 phosphorylation and activation were inhibited by PD98059, a selective inhibitor of MAPK/ERK kinase 1 (MEK1), which was the upstream activator of p42/44 (data not shown), as shown previously.26
In addition, in freshly isolated human peripheral blood monocytes, OxLDL/LPC also efficiently induced phosphorylation of p38 and p42/44 (Figure 1C).

**LPC Dose and Time Dependently Stimulated Phosphorylation of p38 and p42/44**

Phosphorylation of both p38 and p42/44 was stimulated by LPC in a dose-dependent manner, with the maximal response (6.9 ± 0.7- and 7.3 ± 0.8-fold versus control) at 25 μg/mL and the half-maximal response at ~2.5 μg/mL (Figure 2A). The time course of the LPC effects showed that a significant stimulation of p38 and p42/44 phosphorylation occurred rapidly, within 1 minute of exposure to LPC, and that the maximal stimulation of MAPK was achieved at 5 minutes (Figure 2B). Then, phosphorylation of both kinds of MAPK declined but remained at a detectable level by 60 minutes. Additionally, there appeared to be some difference in dose curve and time course between phosphorylation of p38, p42, and p44 as stimulated by LPC.

**Inhibition of p38 but Not p42/44 Reduced OxLDL-/LPC-Induced Chemotaxis**

It has been reported that both OxLDL and LPC can induce chemotaxis of monocytes and T lymphocytes. The current study demonstrated that treatment of THP-1 cells with OxLDL (0 to 50 μg/mL) and LPC (0 to 25 μg/mL) also produced a significant dose-dependent chemotaxis, whereas LDL or phosphatidylcholine (PC) exerted no such effects (Figures 3A and 3B). The potential correlation between MAPK activation and chemotaxis stimulated by OxLDL/LPC was further tested. Pretreatment of THP-1 cells with the p38 inhibitors (SB203580 and SKF86002) greatly inhibited OxLDL-/LPC-stimulated chemotaxis (Figure 3C). In contrast, pretreatment with PD98059 did not affect the OxLDL-/LPC-induced chemotactic effects (Figure 3C). Furthermore, SB203580 dose dependently both inhibited OxLDL-/LPC-stimulated dual phosphorylation of p38 (data not shown) and blocked p38 activity using an in vitro p38 kinase assay kit (Figure 4A) with reported potency (IC50) of 0.6 μmol/L. SB203580 also dose dependently inhibited OxLDL-/LPC-
induced chemotaxis (Figure 4B) at similar potency (IC_{50} ≈ 1 μmol/L).

Expression of Dominant-Negative p38 (p38AF) Significantly Attenuated OxLDL-/LPC-Stimulated Chemotaxis

To further confirm the effects of p38 in the chemotaxis, we transfected wild-type p38 or its dominant-negative mutant p38AF construct in THP-1 cells with electroporation. Transfection of both p38 constructs was verified by monitoring of cotransfected GFP (data not shown) or by Western blot analysis using anti-flag monoclonal antibody (Figure 5A, left). The transfected wild-type p38 could be activated by LPC stimulation as detected by anti-phospho-p38 antibody, whereas the p38AF failed to be activated by LPC (Figure 5A, right). Consequently, expression of wild-type p38 significantly increased OxLDL-/LPC-stimulated chemotaxis of THP-1 cells, whereas expression of p38AF markedly reduced directed chemotaxis of these cells (Figure 5B). The fact that chemotaxis was not completely blocked by p38AF might be due to the limitation of transfection efficiency.

p38 Activation by LPC Was Mediated by Pertussis Toxin (PTX)-Sensitive G Proteins

The possible signal transduction pathway mediating LPC activation of p38 was further investigated. Pretreatment of THP-1 cells with polyinosinic acid (poly I) or dextran sulfate (50 μg/mL, data not shown), each of which is an LDL receptor/scavenger receptor inhibitor, displayed no effect on the stimulation of p38 phosphorylation by LPC (Figure 6A). In addition, with preincubation with tyrphostin 51 or...
genistein (25 μmol/L, data not shown), the tyrosine kinase inhibitors did not affect the LPC stimulation of p38 phosphorylation (Figure 6A). However, p38 phosphorylation induced by LPC was strongly inhibited when cells were preincubated with PTX (Figure 6A), an effective blocker of Gi/Go proteins. As control, pretreatment of cells with cholera toxin (CTX) hardly affected LPC-induced phosphorylation of p38 (Figure 6A). OxLDL-induced phosphorylation of p38 was also subjected to similar regulation (Figure 6B). Moreover, LPC as well as OxLDL could significantly inhibit forskolin-stimulated accumulation of cAMP, which was also PTX sensitive (Figure 6C). Taken together, these data indicate that the effects of OxLDL/LPC were most likely mediated by PTX-sensitive Gi/Go proteins but not through the LDL receptors, scavenger receptors, or tyrosine kinase receptors.

Phospholipase C (PLC) and Phosphatidylinositol 3-Kinase (PI3K) Were Involved in the LPC-Induced Effects

PLC has been shown to be an important component of the signaling pathways in activation of p38.27 As presented in Figure 7A, U73122, a PLC inhibitor that has been shown to specifically inhibit phosphoinositide hydrolysis, considerably blocked p38 phosphorylation induced by LPC. As control, U73343, the inactive structural analogue of U73122, failed to block the LPC-induced p38 activation. Neither compound displayed any significant effect on basal p38 phosphorylation. The data suggested that PLC might be a signaling molecule involved in the LPC-induced p38 activation. In addition, as presented in Figure 7B, treatment of cells with selective PI3K inhibitors (wortmannin and LY294002) significantly reduced LPC-stimulated p38 phosphorylation, suggesting that PI3K might participate in the p38 activation by LPC.

PTX-Sensitive G Proteins, PLC, and PI3K Were Also Included in the OxLDL-/LPC-Induced Chemotaxis

Further experiments were performed to test whether these agents, which were involved in OxLDL-/LPC-stimulated p38 activation, also exert effects on OxLDL-/LPC-induced che-
motaxis. The data showed that PTX, U73122, and wortmannin effectively reduced OxLDL-/LPC-stimulated chemotaxis of THP-1 cells (Figure 8A), indicating that the events of p38 activation and chemotaxis are parallel with a causal correlation. Additionally, U73122, but not wortmannin, exhibited a likely synergistic effect with reduced concentration (1 μmol/L) of SB203580, suggesting that the PLC pathway is in a parallel relationship, and the PI3K pathway is in a sequential relationship, with p38 signaling.

Discussion

It has been shown that when LDL is oxidized to OxLDL, its phospholipid moiety PC is converted into LPC, which is present in OxLDL.22,24 It has been speculated that the oxidation of LDL into OxLDL in the subendothelium is involved in the initiation of atherosclerosis because of its recruitment of monocytes and T lymphocytes into subendothelial space. The present study revealed that OxLDL and LPC, but not their precursors LDL and PC, could effectively induce the chemotactic migration of THP-1 cells, thus providing molecular evidence for the functional role of LDL oxidation in the pathology of atherogenesis.

Scavenger receptors, a family of trimeric membrane glycoproteins at the surface of monocytes, can specifically mediate the uptake of OxLDL as well as LPC to exert their biological activities. So far, several different scavenger receptors with a single membrane-spanning domain for OxLDL uptake have been identified,23,28 but none of these receptors is reported to couple to any kind of heterotrimeric G proteins. Recently, OxLDL was reported to induce a rapid and transient rise in [Ca\(^{2+}\)], and suppress activation of nuclear factor κB in macrophages,29 which are both sensitive to PTX. LPC has also been shown to stimulate PLC via PTX-sensitive G proteins in HL-60 cells.30 Our data more clearly demonstrated that acute activation of p38 by OxLDL/LPC was not mediated by these scavenger receptors in THP-1 cells. Instead, OxLDL/LPC could effectively stimulate PTX-sensitive G proteins, which in turn mediated inhibition of cAMP accumulation. The finding that both p38 activation and chemotaxis induced by OxLDL/LPC were blocked by PTX pretreatment of cells further supports the mediation of the observed effects by PTX-sensitive G proteins. It is not known yet whether OxLDL/LPC directly activate G proteins via interaction with cell membranes or whether they activate G proteins via the unknown G protein–coupled receptors. Furthermore, whether p38 can be activated directly or indirectly via uptake of OxLDL/LPC into the cells through the scavenger receptors warrants further investigation. Recent reports revealed that PI3K participates in activation of p38,27 and PI3K is required for VSMC migration.31 The present data further indicated that PI3K and p38 were involved in chemotaxis. Additional intensive investigation needs to be done to elucidate the relationship among PI3K, PLC, and p38 in signaling, and their definite effects in cellular chemotaxis or migration.

It has been established that p38 or p42/44 activation induces different transcription factors to exert its distinctive biological functions. p38 can be activated by several kinds of growth factors and cytokines, which subsequently induces actin reorganization and endothelial cell migration,20,21,32 as well as neutrophil chemotaxis.33–35 p38 is also critical for endothelial expression of monocyte chemotactant protein-1,36 which is a potent chemokine for monocytes. In the present study, p38 activation was shown to functionally associate with and be critically involved in monocyteic THP-1 cell chemotaxis induced by OxLDL/LPC. In addition, because of the p38 isoform–inhibitory selectivity of SB203580 (inhibiting p38\(^{a}\) and \(-\beta\), but not \(-\gamma\) or \(-\delta\)),37 the effects of LPC may act via activation of the first 2 isoforms. In the present study, LPC activation was shown to specifically associate with and be critically involved in monocytic THP-1 cell chemotaxis induced by OxLDL/LPC. In addition, because of the p38 isoform–inhibitory selectivity of SB203580 (inhibiting p38\(^{a}\)) and \(-\beta\), but not \(-\gamma\) or \(-\delta\)),37 the effects of LPC may act via activation of the first 2 isoforms. In the present study, LPC was able to activate transfected exogenous p38 (p38\(^{a}\)). A recent report indicates that p38\(^{a}\) cannot be detected in monocytes and macrophages.38 Our preliminary data also demonstrated that LPC failed to activate transfected exogenous p38\(^{a}\) (data not shown). All of these findings indicated that only p38\(^{a}\) might be responsible for the LPC-induced monocyteic chemotaxis. Also, it is not clear whether JNK/SAPK, which can be activated by LPC,25,39 is involved in the LPC-directed chemotaxis, and this warrants further investigation.

In the current study, our data showed for the first time that in monocytic cells, OxLDL, which has been demonstrated to be critically involved in atherogenesis, could...
strongly stimulate phosphorylation and activation of both p38 and p42/44. Our results also demonstrated that the phospholipid LPC, among several moieties of OxLDL, was the major active component responsible for these activities of OxLDL. More interestingly, although OxLDL and LPC were able to efficiently activate both kinds of MAPK, only inhibition of p38, not of p42/44, markedly reduced OxLDL-/LPC-induced chemotaxis in THP-1 cells. Our results also revealed that the effects of OxLDL/LPC were apparently not mediated by the classical scavenger receptors for OxLDL or by the general tyrosine kinase receptors, but were likely mediated by PTX-sensitive G proteins and somehow involved PLC and PI3K. Thus, this study not only demonstrated activation of p38 and p42/44 by OxLDL/LPC but also established the functional importance of OxLDL/LPC activation of p38 in the OxLDL-/LPC-induced chemotaxis in monocyctic THP-1 cells. All of the evidence, taken together, implies that p38 may play an active role in atherogenesis and that selective inhibition of p38 in monocytes may help to prevent the initiation and progression of atherosclerosis.

Acknowledgments

This work was supported by grants from the National Basic Research Program of China (G1999053907 and G2000056905), the National Natural Science Foundation of China (39600063, 39625015, and 39630130), the Chinese Academy of Sciences (KY951-A1-301 and KJ951-B1-608), and the German Max-Planck Society. We thank Professor Lan Ma for her kind direction and Jian Zhao, Xu-Min Zhang, and Pei-Hua Wu for their kind assistance.

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