Platelet-activating factor (PAF) is a potent proinflammatory phospholipid mediator that elicits various cellular functions under physiological and pathological conditions. We have recently identified two enzymes involved in PAF production: lysophosphatidylcholine acyltransferase-1 (LPCAT1) and LPCAT2. We found that LPCAT2 is highly expressed in inflammatory cells and is activated by lipopolysaccharide (LPS) treatment through Toll-like receptor 4. However, the molecular mechanism for the activation remains elusive. In this study, Phos-tag SDS-PAGE revealed the LPS-induced phosphorylation of LPCAT2. Furthermore, mass spectrometry and mutagenesis analyses identified Ser34 of LPCAT2 as the phosphorylation site to enhance the catalytic activities. The experiments using inhibitors and siRNA against MAPK cascades demonstrated that LPCAT2 phosphorylation through LPS-TLR4 signaling may directly depend on MAPK-activated protein kinase 2 (MAPKAP kinase 2 or MK2). These findings develop a further understanding of both PAF production and phospholipid remodeling triggered by inflammatory stimuli. Specific inhibition of the PAF biosynthetic activity by phosphorylated LPCAT2 will provide a novel target for the regulation of inflammatory disorders.

Platelet-activating factor (PAF); 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (lyso-PAF), the precursor of PAF, is synthesized from 1-O-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine (1-alkyl phosphatidylcholine; PC) by the action of phospholipase A2. Lyso-PAF is subsequently converted to PAF by acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAFAT).

Endogenous lyso-PAFAT activity was initially demonstrated in 1980 (3) and partially characterized (4–7). Recently, we identified two molecular entities of lyso-PAFATs: a constitutively expressed lyso-PAFAT, LPCAT1 (lysophosphatidylcholine acyltransferase 1) (8), and an inducible lyso-PAFAT, LPCAT2 (9). In these previous reports, endogenous lyso-PAFAT in inflammatory cells was activated by proinflammatory stimuli (6) and LPCAT2 in mouse peritoneal macrophages was indeed activated by lipopolysaccharide (LPS) stimulation (9). However, the exact mechanisms for LPCAT2 activation remain unknown.

LPS activates TLR4 (Toll-like receptor 4), which plays a central role in the activation of the innate immune system. Through its association with different combinations of four adapters, the TLR4 signaling pathway leads to the phosphorylation of MAPKs: p38, ERK, and JNK. Subsequently, activated p38 phosphorylates MK2 (MAPK-activated protein kinase 2) (10, 11), which can induce inflammatory cytokines (12, 13) and lipid mediators (14, 15).

LPCAT2 also possesses LPCAT activity to produce the major membrane phospholipid, PC, which mainly contains polyunsaturated fatty acids (PUFAs) at the sn-2 position. This biosynthetic pathway of phospholipids, known as Lands’ cycle or remodeling pathway, is responsible for generating the membrane diversity (16). PUFAs in phospholipids may affect membrane curvature and fluidity and store lipid mediator precursors that are converted to eicosanoids, such as prostaglandins, leukotrienes, and lipoxins (1). PC plays an important role as a precursor of both eicosanoids and PAF.

By mass spectrometry and mutagenesis studies, we demonstrated that LPCAT2 is activated by Ser34 phosphorylation in mouse peritoneal macrophages and RAW264.7 cells with LPS treatment. Consensus sequence and experiments with an MK2 inhibitor and siRNA suggested that MK2 might directly phosphorylate and activate LPCAT2. These findings contribute to a better understanding of the regulatory mechanisms of PAF biosynthesis in inflammatory cells.

EXPERIMENTAL PROCEDURES

Materials—PC from frozen egg yolk, LPS from Salmonella minnesota, and anti-FLAG M2 antibody were from Sigma.
Lyso-PAF was from Cayman Chemical Co. (Ann Arbor, MI). Arachidonoyl-CoA was from Avanti Polar Lipids (Alabaster, AL). [3H]Acetyl-CoA (129.5 GBq/mmol), horseradish peroxidase-linked anti-rabbit IgG, and horseradish peroxidase-linked anti-mouse IgG were from GE Healthcare. [1-14C]Arachidonyl-CoA (2.22 GBq/mmol) was from Moravec Biochemicals (Brea, CA). Thin layer chromatography (TLC) silica gel plates (type 5721) were from Merck. Cell line Nucleofector kit V was from LONZA (Basel, Switzerland). Acetyl-CoA, DMSO, and acrylamide/bis (29:1) were from WAKO (Osaka, Japan). Phos-tag acrylamide was from NARD Institute, Ltd. (Hyogo, Japan). (5Z)-7-Oxozeanol was from TOCRIS Bioscience (Ellisville, MO). SB20474, SB203580, and MK2 inhibitor III were from Calbiochem. The siRNAs (ON-TARGETplus Non-targeting Pool D-001810-10-20 and ON-TARGETplus SMARTpool L-040135-00-0005) were from Thermo Scientific (Dharmacon) (Waltham, MA). Anti-MK2, anti-phospho-MK2, anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies were from Cell Signaling Technology (Beverly, MA). Anti-MK2, anti-phospho-MK2, anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies were from Cell Signaling Technology (Beverly, MA). The proteinase inhibitor mixture, EDTA-free Complete, was from Roche Applied Science.

**Isolation of Mouse Peritoneal Macrophages**—Mouse peritoneal macrophages were isolated as previously described (6). Cells were cultured for 16 h before stimulation.

**Preparation of Cell Lysates**—Cells were pretreated with or without 20 μM MK2 inhibitor III, 20 μM SB203580 (p38 MAPK inhibitor), or 1 μM (5Z)-7-oxozeanol (TAK1 (tumor growth factor-β-activated protein kinase 1) inhibitor) for 1 h and then stimulated with 100 ng/ml LPS for 30 min. After stimulation, cells (peritoneal macrophages or RAW264.7 cells) were washed with ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 0.3 M sucrose, and 1 mM sodium orthovanadate and collected in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol, and 1× EDTA-free Complete. Subsequently, cells were sonicated twice on ice for 30 s each time and centrifuged at 9,000 × g for 10 min at 4 °C to remove cellular debris, intact cells, and mitochondria. For primary cultured mouse peritoneal macrophages, the resultant supernatant at 9,000 × g was centrifuged at 100,000 × g for 1 h at 4 °C. The resultant pellet was resuspended with ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol, and 1× EDTA-free Complete. The concentration of each protein was measured by the Bradford method (17), using protein assay solution (Bio-Rad). Bovine serum albumin (fraction V, fatty acid-free; Sigma) served as a standard.

**Site-directed Mutagenesis of LPCAT2**—Mouse LPCAT2 mutants (S34A and S34D) were constructed by overlap extension PCR. The amplified PCR products were cloned into the pCXN2.1 vector, and the sequence was confirmed. The primer sets utilized were S34A (forward, GCAGCAGCGGCGTATTCCGCGGCGC; reverse, GCGCGGAGAGAAGGCGCTGACC; and S34D (forward, CCGCAGGCGGACGACGTCCTCCCGCGC; reverse, GCGCGGAGAGAAGGCGCTGACC). The amplification PCR products were cloned into the pCXN2.1 vector, and the sequence was confirmed. The primer

**RESULTS**

**Phosphorylation of LPCAT2 by LPS Stimulation**—To examine the different characteristics of the two lyso-PAFATs (LPCAT1 and LPCAT2), FLAG-tagged LPCAT1 and LPCAT2 were transiently transfected into the mouse macrophage cell line RAW264.7 using the Amaza Nucleofector transfection kit V. Because RAW264.7 cells express TLR4 signaling molecules, cells were stimulated with LPS for 30 min, and the lyso-PAFAT activity was examined using the supernatant at 9,000 × g for 10 min. The lyso-PAFAT activities of LPCAT1 and LPCAT2 were
measured by radioisotope assays. Although the LPCAT1 activity was unchanged after LPS stimulation, the LPCAT2 activity was enhanced 4-fold compared with non-stimulated LPCAT2 (Fig. 1A). Lyso-PAFAT activity in the vector-transfected cells was slightly increased by LPS stimulation, possibly due to the presence of endogenously expressed LPCAT2 in RAW264.7 cells.

The mechanism of LPCAT2 activation was investigated using Phos-tag acrylamide gel electrophoresis. Phos-tag makes a complex with two Mn²⁺ ions and acts as a phosphate-binding molecule (19). The complex is used for phosphate affinity SDS-PAGE, which results in the mobility shift of the phosphorylated proteins. A shifted band of FLAG-LPCAT2, but not FLAG-LPCAT1, was observed after LPS stimulation (Fig. 1B). The upper band may represent the phosphorylated form of LPCAT2. This result suggests that LPCAT2 is phosphorylated and activated by extracellular stimuli.

To identify the phosphorylated amino acid residue(s) of LPCAT2, RAW264.7 cells stably overexpressing FLAG-LPCAT2 were established using Fugene HD in the presence of Geneticin. The cells were stimulated with LPS for 30 min, and the pellet at 100,000 × g for 1 h was analyzed by Phos-tag SDS-PAGE. The position corresponding to the shifted band in the Phos-tag Western blot was cut and subjected to in-gel trypsin digestion (20). After immobilized metal affinity chromatography enrichment of phosphopeptides (21), only one phospho-LPCAT2 peptide candidate (32QApSFFPPPVPNFPFVQQTTISASR54) was detected by liquid chromatography-mass spectrometry (LTQ, Thermo Electron, San Jose, CA) (data not shown). Peptides containing unphosphorylated Ser³⁴ were not detected in the phosphopeptide-enriched fraction. The flow-through fraction of immobilized metal affinity chromatography contained several other unphosphorylated peptides derived from LPCAT2. Although the Mascot score was 38, which is not significant, these results suggest that Ser³⁴ of LPCAT2 is a candidate residue of the phosphorylation induced by LPS stimulation. Ser³⁴ of mouse LPCAT2 is well conserved among mammals, such as bovine, dog, and rat (Fig. 1C).

Site-directed Mutagenesis of LPCAT2—To confirm Ser³⁴ as the target of phosphorylation, site-directed mutagenesis of LPCAT2 was performed. Ser³⁴ was substituted for alanine (S34A) and aspartate (S34D). These constructs were transiently transfected into RAW264.7 cells using Amaxa, and the cells were stimulated with LPS for 30 min. In the Phos-tag Western blot analysis using the M2 anti-FLAG antibody, a mobility shift was detected in wild-type (WT) LPCAT2 but not in the S34A or S34D mutant (Fig. 2A).

Next, we examined the effect of phosphorylation on the dual activities of LPCAT2 (lyso-PAFAT and LPCAT). Both activities of mutants were measured by radioisotope assays. Lyso-PAFAT and LPCAT activities were enhanced in WT LPCAT2 with LPS stimulation (Fig. 2, B and C). The enzyme activity of S34A was similar to WT but was not increased by LPS stimulation. In contrast, S34D exhibited a higher enzyme activity than WT, but no further stimulation was observed (Fig. 2B). The expression level of each mutant was similar to that of WT (Fig. 2A). These results indicate that both lyso-PAFAT and LPCAT activities were enhanced by the Ser³⁴ phosphorylation of LPCAT2.

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**Signaling Pathway for LPCAT2 Phosphorylation**—To investigate the time course of LPCAT2 phosphorylation, thioglycolate-induced murine peritoneal macrophages were stimulated with LPS for varying times (0–120 min). Each microsomal protein (pellet at 100,000 × g for 1 h) was analyzed by Western blot using anti-LPCAT2 and anti-Phospho-LPCAT2 antibodies. The amount of total LPCAT2 was nearly equal among the samples. The most intense phospho-LPCAT2 signal was detected at 15–30 min and decreased as the incubation continued until 120 min (Fig. 3). This is consistent with lyso-PAFAT activation in our previous study (6). Similarly, MK2 phosphorylation reached a peak at 15–30 min. The consensus phosphorylation sequence (HydXRXXS; where Hyd represents a hydrophobic residue) of MK2 substrates (22) is conserved around Ser³⁴ (VPRQAS) in LPCAT2 (Fig. 1C). These results suggest that LPCAT2 is one of the protein substrates of MK2. Murine MK2 has two splice variant proteins (23), and thus MK2 appeared at the positions of 45 and 55 kDa by the Western blot.

The signal transduction pathway for LPCAT2 phosphorylation was studied using several inhibitors of TLR4 signaling molecules: TAK1 (tumor growth factor-β-activated kinase-1), p38 MAPK, and MK2 (see Fig. 6). RAW264.7 cells
overexpressing FLAG-LPCAT2 were pretreated with each inhibitor for 1 h and stimulated with LPS for 30 min. Treatment with (5Z)-7-oxozeaenol (a TAK1 inhibitor) abolished the phosphorylation of p38 MAPK, MK2, and LPCAT2 (Fig. 3A). SB203580 (a p38 MAPK inhibitor) also inhibited the phosphorylation of MK2 and LPCAT2, whereas the inactive analogue, SB202474, did not affect their phosphorylation (Fig. 3B). Pyrrolopiridine (MK2 inhibitor III) (24) treatment diminished the phosphorylation of LPCAT2 (Fig. 3C). Combined with the consensus sequence of the MK2 substrates, these data strongly suggested that LPS-induced phosphorylation of LPCAT2 is dependent on MK2, a downstream kinase of TAK1 and p38 (see Fig. 6).

**Suppression of LPCAT2 Phosphorylation by MK2 siRNA—** The involvement of MK2 in LPCAT2 phosphorylation was further examined using the MK2 knockdown (MK2-KD) of RAW264.7 cells stably expressing LPCAT2. MK2 siRNA was transiently transfected into RAW264.7 cells by the Amaxa Nucleofector transfection kit V. After 48 h, the cells were treated with LPS for 30 min. The level of MK2 mRNA

**FIGURE 2. Site-directed mutagenesis of LPCAT2.** RAW264.7 cells transfected with vector, wild-type (WT), S34A, or S34D were stimulated with 100 ng/ml LPS for 30 min. A, in Phos-tag Western blot analysis, only WT with LPS stimulation showed the shifted band. The two mutants showed no shift. Lyso-PAFAT (B) and LPCAT (C) activities were measured. S34A and S34D did not show the activation, and the activity of S34D was already high without LPS. Open bars and closed bars indicate vehicle or LPS stimulation, respectively. Results are expressed as the mean ± S.D. (error bars) of an experiment performed in triplicate. Four independent experiments were performed with similar results. Statistical analyses were performed by analysis of variance and Tukey’s multiple comparison test.

**FIGURE 3. Time course for LPCAT2 phosphorylation.** Mouse thioglycollate-induced peritoneal macrophages were stimulated with 100 ng/ml LPS for the indicated periods. Microsomal proteins (100,000 × g pellets for 60 min) were analyzed by Western blot. Phosphorylated LPCAT2 and MK2 appeared within 15–30 min. The arrowheads indicate MK2 splice variants. Three independent experiments were performed with similar results.

**FIGURE 4. Signaling pathway for LPCAT2 phosphorylation.** RAW264.7 cells transfected with LPCAT2 were preincubated with or without each inhibitor for 1 h and subsequently stimulated with 100 ng/ml LPS for 30 min. See also Fig. 5. Supernatants (9,000 × g for 10 min) were subjected to Western blot analysis. 1 μM (5Z)-7-oxozeaenol (A), 20 μM SB203580 (B), and MK2 inhibitor III (C) abolished LPCAT2 phosphorylation. SB202474 is an inactive analogue of SB203580. The arrowheads indicate MK2 splice variants. Three independent experiments were performed with similar results.

4A). SB203580 (a p38 MAPK inhibitor) also inhibited the phosphorylation of MK2 and LPCAT2, whereas the inactive analogue, SB202474, did not affect their phosphorylation (Fig. 4B). Pyrrolpapiridine (MK2 inhibitor III) (24) treatment diminished the phosphorylation of LPCAT2 (Fig. 4C). Combined with the consensus sequence of the MK2 substrates, these data strongly suggested that LPS-induced phosphorylation of LPCAT2 is dependent on MK2, a downstream kinase of TAK1 and p38 (see Fig. 6).
expression was decreased by 70–80% in MK2 siRNA-transfected cells (MK2-KD) compared with cells transfected with negative control (NC) siRNA (Fig. 5A). The supernatant at 9,000 × g for 10 min was analyzed by Western blot using anti-MK2, anti-phospho-MK2, anti-LPCAT2, and anti-phospho-LPCAT2 antibodies. The amounts of total MK2 and phosphorylated MK2 were decreased in MK2-KD cells (Fig. 5B), consistent with their MK2 mRNA levels. Although the amount of total LPCAT2 was nearly equal in both MK2-KD and NC cells, that of phospho-LPCAT2 was significantly diminished in MK2-KD cells.

We also performed enzymatic assays and examined the effect of MK2 siRNA on LPCAT2 activation. Both lyso-PAFAT and LPCAT activities were enhanced by LPS stimulation in the NC cells; however, both activations were abolished in MK2-KD cells (Fig. 5, C and D). These results are consistent with the effect of the MK2 inhibitor on LPCAT2 phosphorylation (Fig. 4C) and thus indicate the MK2-dependent phosphorylation of LPCAT2.

**DISCUSSION**

Here, we present the activation mechanism of PAF biosynthetic enzyme by endotoxin stimulation. In response to inflammatory stimuli, LPCAT2 was phosphorylated and activated in mouse peritoneal macrophages and RAW264.7 cells. Mass spectrometry and mutagenesis analyses identified Ser34 of LPCAT2 as the phosphorylation site to enhance the enzymatic activities. MK2 inhibitor and siRNA suppressed LPCAT2 phosphorylation, suggesting that LPCAT2 might be directly phosphorylated by MK2 to promote PAF and PC biosynthesis (Fig. 6).

In 1980, the lyso-PAFAT activity as the PAF biosynthetic enzyme was reported (3). Since then, several groups have attempted to characterize the enzyme. Lyso-PAFAT is rapidly activated in response to extracellular stimuli, such as calcium ionophore (4), acid stress (7), and LPS (16). However, neither the lyso-PAFAT cDNA sequence nor the mechanism of lyso-PAFAT activation had been elucidated. Recently, we identified two types of lyso-PAFATs: LPCAT2, which is an inducible lyso-PAFAT (9), and LPCAT1, which has constitutive lyso-PAFAT activity (8). LPCAT2 mRNA in macrophages is also up-regulated by LPS treatment for 16 h (9). The difference between LPCAT1 and LPCAT2 resembles that of cyclooxygenase-1 and -2 to produce prostaglandins (25, 26). In mouse peritoneal macrophages, LPCAT2 is activated within 30 min by LPS stim-
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Our findings suggest that LPCAT2 is a potential target of MAPK activation in RAW264.7 cells. The phosphorylation of LPCAT2 by MAPK leads to an increase in both lyso-PAFAT and LPCAT activities, which is consistent with its role in PAF biosynthesis.

LPCAT2 has been shown to be involved in PAF biosynthesis in macrophages. Our study further established a role for LPCAT2 in the regulation of PAF production by RAW264.7 cells. The phosphorylation of LPCAT2 by MAPK enhances both the lyso-PAFAT and LPCAT activities of RAW264.7 cells, which is consistent with previous studies showing that LPCAT2 plays a crucial role in PAF biosynthesis.

In conclusion, our findings provide new insights into the role of LPCAT2 in PAF biosynthesis and the regulation of PAF production by RAW264.7 cells. These results suggest that targeting LPCAT2 might be a promising strategy for the development of new anti-inflammatory drugs.

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