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新規生体膜生合成酵素と生理活性脂質（PAF）生合成酵素の機能解析

（Ｈ２０−免疫−若手−０２８）

平成２２年度　総括研究報告書

研究代表者　進藤　英雄

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厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
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新規生体膜生合成酵素と生理活性脂質（PAF）生合成酵素の機能解析

研究代表者　進藤　英雄　　東京大学大学院医学系研究科　助教

細胞は生体膜に覆われており、その生体膜は形態や機能に影響を与えている。生体膜の主成分はリン脂質であり、組織によって多種多様であり、約1000種類あると考えられている。この多様なリン脂質はリゾリン脂質アシル転移酵素によって形成されることが1950年代に報告され、その生合成経路はランズ回路と呼ばれた。それから約50年間その遺伝子群は不明であったが、近年私達はリゾリン脂質アシル転移酵素ファミリーを発見した。また、強力な免疫アレルギーのメディエーターである血小板活性化因子(PAF)もリン脂質であり、その生合成酵素もリゾリン脂質アシル転移酵素であり、LPCAT1とLPCAT2と名付けた。これら新規に同定されたリン脂質生合成酵素を解析し、マクロファージなどの炎症細胞における生体膜生合成やPAF生合成メカニズムの解明を進める。

A．研究目的

生体膜の主成分であるリン脂質は組織や細胞によって多種多様であり、血小板活性化因子(PAF)のような炎症性物質も含まれる。PAFは炎症や免疫を惹起する強力なメディエーターである。この生体膜の多様性は、脂肪酸を再結合させるリゾリン脂質アシル転移酵素によるリン脂質の代謝回転によって作られ、50年前にランズ回路と名付けられている。ランズ回路発見以来進展が難しかった分野であるが、近年リゾリン脂質アシル転移酵素分子が次々と同定され、急展開をみせている。私達はそのうち6種類の同定に成功した。中には血小板活性化因子（PAF）生合成酵素を含んだ。さらに、このうち3種類は新たなアシル転移酵素遺伝子ファミリーを形成していた。PAF生合成酵素は2種類存在し、リゾホスファチジルコリンアシル転移酵素（LPCAT）1とLPCAT2である。LPCAT2はマクロファージにおいてリポポリサッカライド（LPS）刺激で活性化や遺伝子誘導されることもわかった。これらの酵素群の機能解析を行うことによって、マクロファージなどの炎症細胞での生体膜形成メカニズムやPAF産生メカニズムを解明する。そして、免疫、アレルギーに強い細胞環境（膜）の解明やPAF関連疾患の治療を目指す。本研究年度では、LPCAT2のリン酸化を解析することにより、アシル転移酵素の理解を深めることを目的とした。

B．研究方法

リゾリン脂質アシル転移酵素はリゾリン脂質にアシルCoAから脂肪酸を転移する活性を持つ。例えばリゾホスファチジルコリンアシル転移酵素（LPCAT）はリゾホスファチジルコリン（LPC）からホスファチジルコリン（PC）を生合成する酵素である。これ

まで、我々が同定したリゾリン脂質アシル転移酵素は、（i）肺に多くPAFと呼吸に必須な肺サーファクタント脂質を合成するLPCAT1と（ii）マクロファージに多いPAF生合成酵素であるLPCAT2、（iii）精巣に多くアラキドン酸を貯蔵するリゾホスファチジン酸アシル転移酵素3（LPAAT3）、さらに、新規に発見した遺伝子ファミリーからは恒常的に生体膜を合成すると考えられる酵素（iv）LPCAT3、(v) LPCAT4、(vi)リゾホスファチジルエタノールアミンアシル転移酵素1 (LPEAT1)である。これら6分子を中心に、全てのリゾリン脂質アシル転移酵素をターゲットとして研究を進めている。

3年目として以下の実験を行った

LPCAT2のリン酸化の解析

RAW264.7細胞にLPCAT1とLPCAT2を過剰発現させてリポポリサッカライド（LPS）で30分間刺激した。これらの試料をphos-tag SDS-PAGEで解析した。phos-tagはリン酸化タンパク質と結合しSDS-PAGE上バンドをシフトさせるものである。

シフトしたバンドを切り出し回収し、質量分析計で解析し、リン酸化部位を34番目のSerと特定した。34番目のSer近辺の配列はほ乳動物に非常に良く保存されていた。S34A変異体とS34D変異体を作製しリン酸化と酵素活性を調べた。酵素の発現量に差は無かった。

LPCAT2をリン酸化する分子を調べるためLPSシグナル下流分子であるTAK1、p38 MAPK、MK2の阻害剤の効果を調べた。MK2 siRNAも用いて同様に調べた。

酵素活性測定は放射ラベルされた基質を用いて反応し、Bligh-Dyer法で反応産物であるリン脂質を抽出し、薄層クロマトグラフィー（TLC）で分離後、放射活性をカウントした。遺伝子変動はtotal RNAを抽出し定量的PCRを行い解析した。

C．研究結果

LPCAT2のリン酸化の解析

LPCAT1はLPSによって活性化されなかったがLPCAT2は活性が上昇した。phos-tag SDS-PAGE上でもLPCAT2は刺激依存的にバンドシフトを示した。S34A変異体は活性が低下し、LPSによる活性化もされなかった。S34D変異体は無刺激の活性が上昇し、活性化されなかった。PAF生合成活性（リゾPAFアセチル転移活性）とPC生合成活性（リゾPAFアシル転移活性）とで同様の結果が得られた。

TAK1の阻害剤でp38 MAPK、MK2、LPCAT2のリン酸化が阻害された。p38 MAPKの阻害剤SB203580でも、MK2、LPCAT2のリン酸化が抑制された。MK2の阻害剤でもLPCAT2リン酸化が阻害された。MK2 siRNAも同様の効果を示し、LPCAT2リン酸化が抑制された。PAF生合成活性（リゾPAFアセチル転移活性）はコントロールでは活性化されたが、MK2 siRNAでは変動しなかった。PAF生合成活性（リゾPAFアセチル転移活性）とPC生合成活性（リゾPAFアシル転移活性）とで同様の結果が得られた。

D．考察

LPCAT2のリン酸化の解析

LPCAT2の活性化はリン酸化によるものであり、その部位がSer34だとわかった。また、LPS刺激によるリン酸化はMK2依存的な経路で起こることもわ

かった。保存性の高いコンセンサス配列からMK2によって直接リン酸化されている可能性が高い。LPCAT2の結晶構造の解明が待たれる。
活性化型LPCAT2がわかり、創薬のターゲットになるであろう。活性化型LPCAT2のみを認識する阻害剤等が対象となる。また、もう一つのPAF生合成酵素であるLPCAT1は活性化も誘導もされない。LPCAT1とLPCAT2の比較も興味深い。本研究でPAF生合成メカニズムの一端が解明された。

E．結論

本研究では生体膜生合成酵素とそれに類似したPAF生合成酵素の機能解析から、炎症性細胞等の感染時における生体膜変化の解明を目的としている。一年目は、恒常的に働く新規PAF生合成酵素の発見や、活性中心の同定等に成功した。また、PAF生合成酵素の自然免疫応答による誘導の解明も進んだ。二年目にはヒトLPCAT1の解析から肺と同じような進化を遂げていることがわかり、PAF生合成能との関係が興味深いことがわかった。今後、2種類のPAF生合成酵素（恒常型LPCAT1と誘導型LPCAT2）のそれぞれに特異的な阻害剤などの開発が望まれる。PAFの生合成メカニズムの解明は炎症やアレルギーの新しい治療方法の開発につなげられるであろう。

また、今回新たにLPCAT2のリン酸化部位が明らかになった。活性化型LPCAT2の解明によりLPCAT2活性化メカニズムが解析された。

F．健康危険情報

なし

G．研究発表

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(2) Morimoto R, **Shindou H (corresponding author)**, Oda Y, Shimizu T. (2010) Phosphorylation of lysophosphatidylcholine acyltransferase 2 at Ser34 enhances platelet-activating factor production in endotoxin-stimulated macrophages J Biol Chem. 285, 29857-29862

2.　学会発表

（発表誌名巻号・頁・発行年等も記入）

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H．知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表
雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Morimoto R, **Shindou H (corresponding author)**, Oda Y, Shimizu T.	Phosphorylation of lysophosphatidylcholine acyltransferase 2 at Ser34 enhances platelet-activating factor production in endotoxin-stimulated macrophages	***J Biol Chem***	285	29857-29862	2010
Koeberle A., **Shindou H. (corresponding author)**, Harayama T., and Shimizu T.	Role of lysophosphatidic acid acyltransferase 3 for the supply of highly polyunsaturated fatty acids in TM4 Sertoli cells	***FASEB J***	24	4929-4938	2010

Phosphorylation of lysophosphatidylcholine acyltransferase 2 at Ser34 enhances platelet-activating factor production in endotoxin-stimulated macrophages*

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Running title: Phosphorylation of LPCAT2 for PAF biosynthesis

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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 mitogen-activated protein kinase (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) is a potent proinflammatory lipid mediator that triggers various cellular functions through its G protein-coupled receptor (PAF[5] receptor; PAFR) (1,2). It is proposed that PAF is synthesized in various cells and tissues via two distinct pathways, the *de novo* and remodeling pathways. Through the remodeling pathway, PAF is rapidly synthesized in response to extracellular stimuli. Under such conditions, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF), the precursor of PAF, is

synthesized from 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phos phocholine (1-alkyl phosphatidylcholine, PC) by the action of phospholipase A_2 (PLA_2). 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, lysophosphatidylcholine acyltransferase (LPCAT) 1 (8), and an inducible lyso-PAFAT, LPCAT2 (9). In these previous reports, endogenous lyso-PAFAT in inflammatory cells was activated by prophlogistic 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 Toll-like receptor (TLR) 4, which plays a central role in the activation of the innate immune system. Through its association with different combinations of four adaptors, the TLR4 signaling pathway leads to the phosphorylation of MAPKs; p38, ERK, and JNK. Subsequently, activated p38 phosphorylates MAPK-activated protein kinase 2 (MK2) (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 (St. Louis, MO). Lyso-PAF was from Cayman Chemical Co. (Ann Arbor, MI). Arachidonoyl-CoA was from Avanti Polar Lipids (Alabaster, AL). [^{3}H] Acetyl-CoA (129.5 GBq/mmol), horseradish peroxidase-linked anti-rabbit IgG, and horseradish peroxidase-linked anti-mouse IgG were from GE Healthcare (Buckinghamshire, UK). [1-^{14}C] Arachidonoyl-CoA (2.22 GBq/mmol) was from Moravec Biochemicals (Brea, CA). Thin layer chromatography (TLC) silica gel plates (type 5721) were from Merck (Darmstadt, Germany). 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). (5*Z*)-7-Oxozeanol was from TOCRIS Bioscience (Ellisville, MO). SB202474, SB203580, and MK2 inhibitor III were from Calbiochem (San Diego, CA). 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). The proteinase inhibitor mixture, EDTA-free Complete, was from Roche (Mannheim, Germany).

Mice

C57BL/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan). Mice were maintained in a light-dark cycle with lights on from 0800-2000 h at 22°C. Mice were fed with a standard laboratory diet and water ad libitum. All animal studies were

conducted in accordance with the guidelines for Animal Research at The University of Tokyo and were approved by The University of Tokyo Ethics Committee for Animal Experiments.

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 (5*Z*)-7-oxozeaenol (TAK1 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 re-suspended with ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol, 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 are described were S34A (Forward: CGC CAG GCG GCC TTC TTC CCG CCG C, Reverse: GCG GCG GGA AGA AGG

CCG CCT GGC G); and S34D (Forward: CGC CAG GCG GAC TTC TTC CCG CCG C, Reverse: GCG GCG GGA AGA AGT CCG CCT GGC G).

Transfection into RAW264.7 cells

RAW264.7 cells (5×10^6 cells), 100 μl of Nucleofector solution V, and 5 μg of each DNA of vector, FLAG-mLPCAT2, S34A, or S34D were mixed. The mixture in the cuvette was set onto the amaxa Nucleofector, and electroporated with program D-032. Then, cells were seeded onto 6 cm dishes. Twenty-four hours after transfection, cells were stimulated with 100 ng/ml LPS for 30 min. The siRNA transfection was performed similarly. The mixture in the cuvette contained 120 pmol siRNA.

Production of anti-LPCAT2 and anti-phospho-LPCAT2 antibodies

Anti-LPCAT2 antiserum was generated at Immuno-Biological Laboratories (Gunma, Japan). The C-terminal peptide, SNKVSPESQEEGTSDKKVD was used to immunize rabbits. Anti-LPCAT2 antibody was purified from the anti-LPCAT2 antiserum using activated thiol Sepharose 4B binding to the LPCAT2 epitope. Anti-phospho-LPCAT2 antibody was generated by SCRUM (Tokyo, Japan) using a phospho-peptide, RQA(pS)FFPPP, at the N-terminus of LPCAT2.

Western blot analysis

Western blot analyses were performed as previously described (18). To detect the band shift, which represents phosphorylated protein, an SDS-PAGE gel containing 50 μM Phos-tag acrylamide with 100 μM Mn^{2+} was used.

Assay of lyso-PAF acetyltransferase and LPCAT

Lyso-PAF acetyltransferase and LPCAT assays were performed as previously described (8,9).

Quantitative real-time PCR

Total RNAs were prepared using the RNeasy Mini Kit (QIAGEN), and first strand cDNA was subsequently synthesized using Superscript III (Invitrogen). The PCRs

were performed using Fast Start DNA Master SYBR Green I (Roche Applied Science). The primers for MK2 designed to amplify a 185-bp fragment were (Forward: GGA TCT TCG ACA AGA GAA CCC AG. Reverse: GAG ACA CTC CAT GAC AAT CAG CA).

Software

All statistical calculations were performed using Prism 4 (GraphPad Software). Alignment of mammal LPCAT2 was performed using GENETYX-MAC Version 13.0.6 (GENETYX Corporation). Sequences of mouse (BAF47695), human (BAF47696), bovine (XP_592529), dog (XP_854080), and rat (XP_001064713) LPCAT2 are available in the DDBJ/EMBL/GenBank databases.

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 amaxa Nucleofector transfection kit V. Since 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. While the LPCAT1 activity was unchanged after LPS-stimulation, the LPCAT2 activity was enhanced 4-fold compared to 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 Mn^{2+} 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 overexpressesing 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 (IMAC) enrichment of phosphopeptides (21), only one phospho-LPCAT2 peptide candidate (^{32}QApSFFPPPVPNPFVQQTTISASR^{54}) 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 IMAC 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 human, 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. 2B 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.

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 (Hyd-X-R-X-X-S) of MK2 substrates (22) is conserved around Ser34 (VPRQAS) in LPCAT2 (Fig. 1C), where Hyd represents a hydrophobic residue and X represents any amino acid. 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: tumor growth factor-β activated kinase-1 (TAK1), 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 (5*Z*)-7-oxozeaenol (a TAK1 inhibitor) abolished the phosphorylation of p38 MAPK, MK2, and

LPCAT2 (Fig. 4A). SB203580 (a p38 MAPK inhibitor) also inhibited the phosphorylation of MK2 and LPCAT2, while the inactive analogue, SB202474, did not affect their phosphorylation (Fig. 4B). Pyrrolopiridine (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).

Suppression of LPCAT2 phosphorylation by MK2-siRNA

The involvement of MK2 in LPCAT2 phosphorylation was further examined using the MK2 knockdown (KD) of RAW264.7 cells stably expressing LPCAT2. MK2-siRNA was transiently transfected into RAW264.7 cells by amaxa Nucleofector transfection kit V. After 48 h, the cells were treated with LPS for 30 min. The level of MK2-mRNA expression was decreased by 70-80% in MK2-siRNA-transfected cells (MK2-KD) compared to 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. 5C 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 that is an inducible lyso-PAFAT (9), and LPCAT1 that has constitutive lyso-PAFAT activity (8). LPCAT2 mRNA in macrophages is also upregulated 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-stimulation (Fig.3), consistent with the characteristics of endogenous lyso-PAFAT (6,9).

In this study, phosphorylated LPCAT2 was detected with the Phos-tag Western blot, by mobility shift (Fig. 1B and 2A). Through mass spectrometric analysis of the phosphorylated enzyme, Ser34 was identified as a phosphorylation site. Both the band shift and the activation were observed in WT LPCAT2, while the S34A mutant displayed neither characteristic (Fig. 2). Since mutagenesis at Ser34 did not abolish the basal activities, it is proposed that Ser34 is located in a regulatory region of LPCAT2. Moreover, the mutagenesis study indicated that Ser34 was the only target of the phosphorylation that led to the enzymatic activation of LPCAT2. Furthermore, Ser34 phosphorylation enhanced both the

lyso-PAFAT and LPCAT activities of LPCAT2 (Fig. 2).

The activation of LPCAT2 in LPS-stimulated RAW264.7 cells was dependent on MK2 located in the downstream of p38 MAPK. Both p38α and p38δ are mainly expressed in macrophages (27), and p38α and p38β signals are inhibited by SB203580. Thus, Fig. 3 and 4 suggest an LPCAT2 phosphorylation mediated by p38α–MK2 axis. MK2 induces the phosphorylation of its substrates with the consensus sequence (Hyd-X-R-X-X-S) (12,22). Near the N-terminus of LPCAT2, ^{29}VPRQAS^{34} was detected as corresponding to the consensus sequence. Thus, MK2 may directly phosphorylate LPCAT2, although it is possible that other kinases are present to link the two proteins. Future determination of the three dimensional structure of LPCAT2 should definitively clarify this activation mechanism.

LPCAT2 has lyso-PAFAT and LPCAT activities, both of which are enhanced by LPS-stimulation. The endogenous LPCAT activity in RAW264.7 cells was much higher than its lyso-PAFAT activity (Fig. 2B and C). Thus, activated LPCAT2 may function as a lyso-PAFAT to produce PAF. It is also possible that the LPCAT activity of LPCAT2 plays an important role in the storage of phospholipid precursors of PAF and eicosanoids (1). LPCAT2 catalyzes the membrane biogenesis (LPCAT activity) of inflammatory cells, while producing PAF (lyso-PAFAT activity) in response to external stimuli. Further studies are needed to elucidate the physiological and pathological importance of these dual activities.

This is the first report on the posttranslational modification of lysophospholipid acyltransferases (LPLATs) functioning in Lands' cycle. Our results showed that LPCAT2, a member of LPLATs, can produce lipid mediator and may contribute to membrane dynamics in response to extracellular stimuli.

This study will aid in the development of new anti-inflammatory drugs that inhibit PAF production by exogenous insults while maintaining the constitutive levels of the mediator. Because of the physiologically important roles of PAF,

PAFR antagonists have encountered several adverse effects during drug development. Inhibition of inducible PAF production by phospho-LPCAT2, but not unphospho-LPCAT2 or LPCAT1 (constitutive lyso-PAFAT) could serve as a potential target of medical interventions. These findings improve our understanding of both inflammatory responses and membrane biogenesis.

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