Mutations in the *SLC34A2* Gene Are Associated with Pulmonary Alveolar Microlithiasis

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Rationale: Pulmonary alveolar microlithiasis is an autosomal recessive disorder in which microliths are formed in the alveolar space.
Objectives: To identify the responsible gene that causes pulmonary alveolar microlithiasis.
Methods: By means of a genomewide single-nucleotide polymorphism analysis using DNA from three patients, we have narrowed the region in which the candidate gene is located. From this region, we have identified a gene that has mutations in all patients with pulmonary alveolar microlithiasis.
Measurements and Main Results: We identified a candidate gene, *SLC34A2*, that encodes a type IIb sodium phosphate cotransporter and that is mutated in six of six patients investigated. *SLC34A2* is specifically expressed in type II alveolar cells, and the mutations abolished the normal gene function.
Conclusion: Mutations in the *SLC34A2* gene that abolish normal gene function cause pulmonary alveolar microlithiasis.

Keywords: pulmonary alveolar microlithiasis; homozygosity mapping; GeneChip; single-nucleotide polymorphisms

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pulmonary alveolar microlithiasis is an autosomal recessive disorder in which microliths are formed in the alveolar space. However, the responsible gene has not been identified.

What This Study Adds to the Field

Mutation of the *SLC34A2* gene that encodes the sodium-dependent phosphate transporter causes pulmonary alveolar microlithiasis.

Pulmonary alveolar microlithiasis (PAM; OMIM [Online Mendelian Inheritance in Man] 265100) is a disease in which microliths are formed in the alveolar space (Figure 1A) (1–3). Ever since the first description by Puhr in 1933 (4), over 500 cases have been reported worldwide, including more than 100 cases in Japan (5). Patients remain symptom free until middle age when chronic respiratory failure and cardiopulmonary decompensation develop. In a chest X-ray image, diffuse fine nodular opacities formed by countless microliths are observed (Figure 1B). PAM has been considered to be an autosomal recessive disorder, because it transmits horizontally and inbreeding frequently coexists (3).

To identify the responsible gene for diseases with an autosomal recessive trait, the homozygosity mapping approach has been successfully applied (6). This method has identified the gene from fewer than 10 patients. Recent technological development has enabled high-density, genomewide single-nucleotide polymorphism (SNP) analysis. The numbers of SNPs genotyped are so great that fine mapping of the candidate region for the gene is anticipated. To efficiently use the SNP data, we have developed a novel algorithm based on the homozygosity mapping, and have used it to identify the gene responsible for PAM.

Preliminary (7–9) and final results of this study (10, 11) have been presented in the form of abstracts.

METHODS

Subjects and Ethical Considerations

The study was approved by the institutional review boards of the participating institutions. For all cases, written, informed consent was obtained from either the patient or from a family member.

Genotyping

We isolated genomic DNA either from blood samples or from paraffin-embedded tissues using standard protocols. For the whole-genome scan, we used the GeneChip Human Mapping 100k set (Affymetrix, Santa Clara, CA). The scan was performed at the Australian Genome Research Facility (Victoria, Australia) and AROS Applied Biotechnology (Aarhus Nord, Denmark). Computer analyses of the GeneChip results were performed using custom programs written in our laboratory (source codes available on request). The nucleotide sequencing of the

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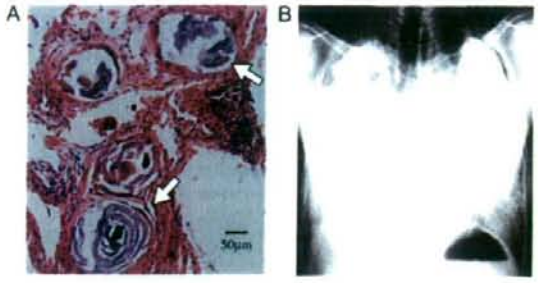

Figure 1. Microscopic and chest X-ray images of pulmonary alveolar microlithiasis (PAM). (*A*) A thin section of the decalcified lung tissue from patient 5 stained with hematoxylin and eosin. Concentric lamellar structures are decalcified remnants of microliths that occupied the entire alveolar space (*arrows*). Alveolar walls are thickened and many inflammatory cells have infiltrated into the parenchyma, indicating the presence of chronic inflammation. (*B*) A chest X-ray image of patient 2. Countless microliths form diffuse fine nodular opacities. The "snowstorm" appearance is very characteristic.

individual exons of *SLC34A2* was performed using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

Frequency of the Mutation of *SLC34A2* in the Japanese Population

Using a 5′ nuclease assay (for exon 7) or the PNA-LNA (peptide nucleic acid–locked nucleic acid) polymerase chain reaction clamp (for exon 8) (12), we investigated the presence of the mutations in genomic DNA from 188 normal volunteers. The amplification signals were detected using Smart Cycler II (Cepheid, Sunnyvale, CA).

Microinjection into *Xenopus* oocytes

We subcloned cDNA of the wild-type *SLC34A2* and its mutants seen in the patients into pcDNA3.1, and capped RNA was transcribed *in vitro* from the T7 promoter. Integrity of RNA was confirmed by gel electrophoresis. Expression of the proteins on the surface of *Xenopus* oocytes and functional study were performed as described (13). In short, after being microinjected with 46 nl of water containing 46 ng of RNA, oocytes were then incubated in modified Barth's solution at 18°C for 3 d. In the phosphate transport assay, oocytes, seven for each injection group, were washed in Na^+-free ND-100 solution containing 0.5 mM KH_2PO_4 (25 mCi/ml ^{32}P-orthophosphoric acid) for 60 min. After washing three times with cold Na^+-free ND-100 solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid [HEPES]), oocytes were lysed in 0.2 ml of 10% sodium dodecyl sulfate and the radioactivity was counted. In the electrophysiologic recording, whole-cell currents were measured using a conventional, 2-microelectrode-voltage clamp method with a holding potential of −50 mV. Under a continuous recording of current, oocytes were serially superfused with ND-100 solution (pH 6.6), ND-100 solution containing 1 mM NaH_2PO_4 (pH 6.6), and then ND-100 solution (pH 6.6).

In Situ Hybridization and Immunohistochemistry

Paraffin-embedded normal human lung tissue was serially thin sectioned, and we investigated the expression of *SLC34A2* RNA and surfactant protein A (SP-A). A part of the open reading frame of *SLC34A2* cDNA was amplified using primers tagged with the T7 promoter sequence. Fluorescent-labeled RNA probes were made using an RNA labeling kit (Hoffmann-La Roche, Basel, Switzerland). *In situ* hybridization was performed and the signal was detected by GenPoint fluorescein kit (Dako Cytomation, Glostrup, Denmark). SP-A protein was detected using a mouse anti-human SP-A monoclonal antibody (Dako Cytomation), together with a horseradish-labeled goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 3,3′-diaminobenzidine chromogen.

RESULTS

We identified 6 cases of PAM from the case reports in Japan. Three patients were alive. Three had died; however, their paraffin-embedded tissues were available (Table 1 and Figure E1 of the online supplement). We were able to obtain samples from six of these patients (three still alive and three dead). Of the three live patients, two (patients 1 and 2) were children of parents who were first cousins. The other (patient 3) was from a family in which three patients were clustered and thus inbreeding was strongly suspected, considering that the inbreeding rate has been historically high in Japan (14). These three patients were unrelated and from different parts of Japan. To identify the gene responsible for PAM (the PAM gene) from a small number of samples, genomic DNA isolated from the peripheral blood of three live patients was investigated using the GeneChip mapping set (Affymetrix), which has the ability to analyze 100,000 SNPs at one time. The results were analyzed according to the homozygosity mapping strategy (6), which anticipates the candidate gene will be located in the homozygosity segment in which both copies of homologous chromosomes are derived from a single ancestral chromosome. To apply the strategy to genomewide SNP genotyping data, we took the following approach. We considered that the homozygosity segment could be detected as a stretch of homozygous SNPs (SHS) in which every SNP type is homozygous. We also considered that if the significance of SHS is appropriately defined, the homozygosity segment will be clearly delineated, and this should allow us to narrow the candidate region for the PAM gene geometrically by serially obtaining intersections of SHS for each patient. We defined significant SHS as a run of homozygous SNPs where the product of the frequencies of homozygosity reported in the Asian population for the individual SNPs is less than 1 in 100,000 (*see* the online supplement). Significant SHS of patients 1 through 3 are shown in Figures 2B–2D. A mathematical calculation revealed that the probability that the PAM gene is contained in significant SHS is 0.999 for the child of the first-cousin marriage and 0.94 even for the child

TABLE 1. PATIENTS' CHARACTERISTICS

	Sex	Alive/Dead Status	Inbreeding	PAM in Other Family Members	Sample Used	Other Information
Patient 1	F	Alive	Yes	Yes	Peripheral blood	Older sister of patient 4
Patient 2	M	Alive	Yes	Yes	Peripheral blood	
Patient 3	F	Alive	Uncertain	Yes	Peripheral blood	
Patient 4	F	Dead	Yes	Yes	Paraffin-embeded tissue	Younger sister of patient 1
Patient 5	F	Dead	NA	NA	Paraffin-embeded tissue	
Patient 6	F	Dead	NA	Yes	Paraffin-embeded tissue	

Except for patients 1 and 4, all patients were from different families and unrelated. NA = information not available.

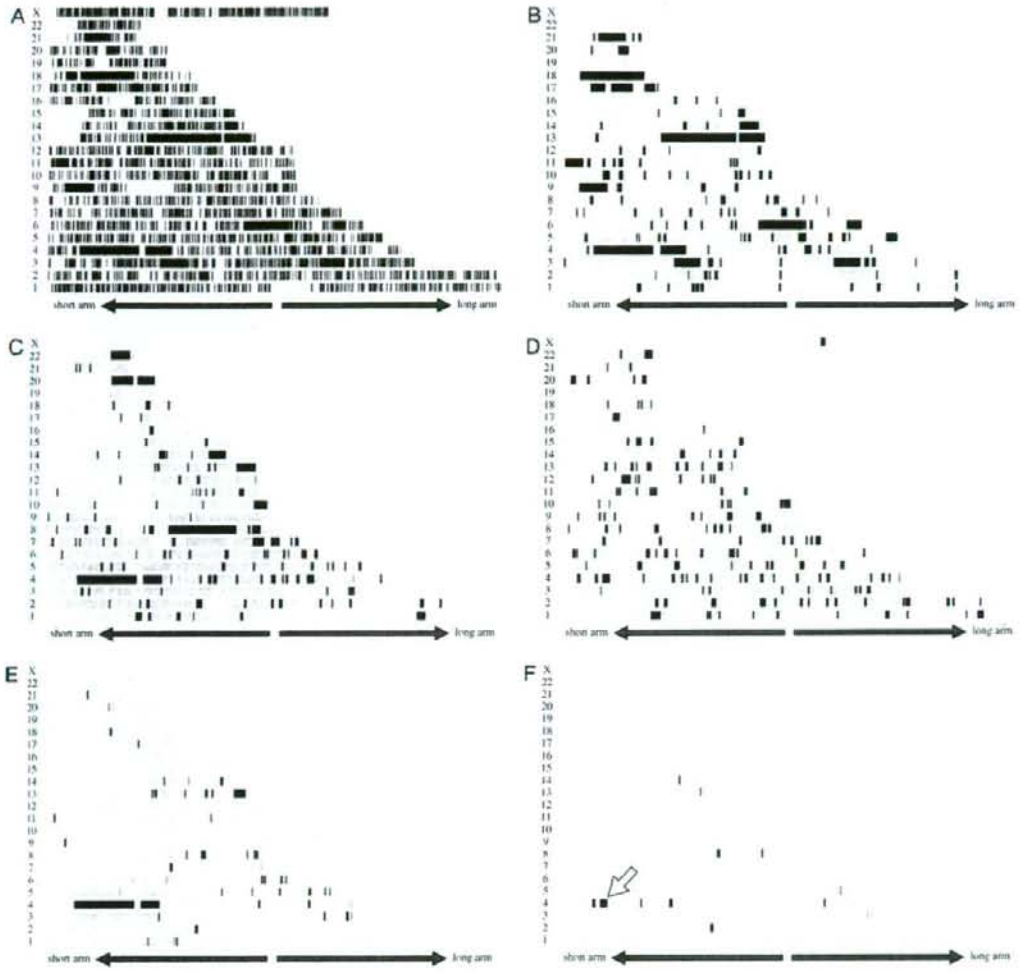

Figure 2. Narrowing the candidate chromosomal regions for the PAM gene using significant stretches of homozygous SNPs (SHS). (*A*) Result of SNP typing for patient 1. Each *vertical bar* indicates an SNP aligned according to its location on the chromosome. A homozygous SNP is shown in *black* and a heterozygous SNP is in *light gray*. *Gray arrows* under each panel indicate the direction of the short and the long arms of the chromosomes. (*B*) Significant SHS (shown in *black*) for patient 1 who is a child of a first-cousin marriage. (*C*) Significant SHS for patient 2. (*D*) Significant SHS for patient 3. (*E*) The overlap of significant SHS for patients 1 and 2. Data from only two patients considerably narrowed the candidate regions. (*F*) The overlap of significant SHS for patients 1, 2, and 3. An *arrow* indicates the significant SHS located on chromosome 4 that contained the PAM gene (*SLC34A2*).

of the 20th-cousin marriage (*see* Table E1). Consequently, the overlaps of significant SHS for patients 1 and 2 (Figure 2E) have a probability of 0.998 ($= 0.999^2$) of containing the PAM gene. We assumed the presence of the inbreeding in patient 3, and further narrowed the region, giving candidate regions with a total length of 12.4 Mb (*see* Table E2). The region contained 50 genes, of which 31 genes had known or suspected functions. *SLC34A2* (Unigene Hs. 479372: solute carrier family 34 [sodium phosphate], member 2) (15, 16), a transporter of sodium phosphate, was the only gene that was directly related to calcium or phosphate metabolism. We thus investigated the individual exons of *SLC34A2* and found that five of five patients had homozygous mutations, whereas 10 normal volunteers did not, indicating that a mutation in *SLC34A2* is significantly associated with PAM ($p < 2.5 \times 10^{-4}$ by the Fisher's exact test). To determine the frequency of the mutant genes in the Japanese population, we screened the genomic DNA from 188 volunteers (i.e., 376 control chromosomes) for the two mutations found, and all samples were negative. This indicates that the frequency of a chromosome with a mutant gene in the general population is less than 0.008 (a 95% confidence interval). In patients 3 and 5, an aberrant sequence replaced a part of exon 7 (Figure 3A), causing a frameshift that produces a truncated protein. In patients 1, 2, 4, and 6, a G nucleotide in the donor site of the splicing signal of exon 8 is

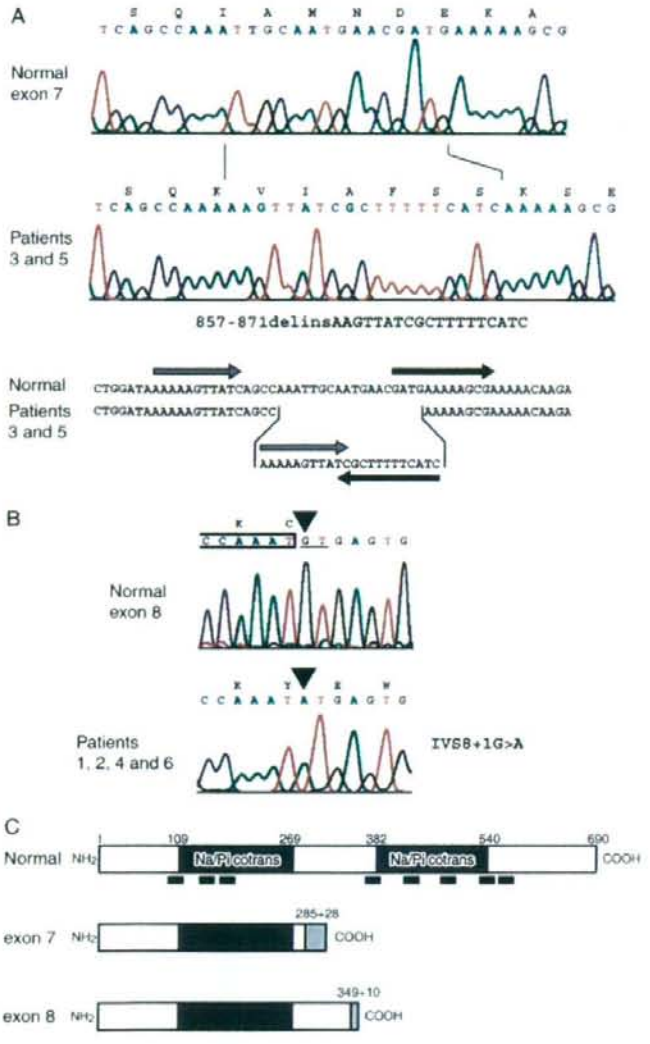

Figure 3. *SLC34A2* mutations in patients. (*A*) A deletion-insertion mutation in patients 3 and 5. Small duplications in the nucleotide sequence in the same (*gray arrows*) and in the opposite (*black arrows*) directions suggest a complicated mutation event. (*B*) Mutation observed in patients 1, 2, 4, and 6. The 3′ end of the exon 8 sequence is enclosed with a translated amino acid sequence shown *above*. A conserved splicing donor sequence (GT) is *underlined*. *Solid, inverted triangles* indicate the point of mutation resulting in a G to A transition. The nucleotide change abrogates normal splicing and the translation continues into intron 8 to add 10 aberrant amino acids before being terminated by an occasional stop codon. (*C*) Mutant proteins predicted from the mutant sequences. Structures of normal protein and protein produced by the mutation in exon 7 and in exon 8 are depicted. Na/Pi cotrans = a sodium phosphate cotransporter motif (pfam 02,690). *Black boxes* under normal protein are predicted transmembrane domains. Numbers shown on mutant proteins are (numbers of *SLC34A2*-derived amino acids in the mutant protein) plus (numbers of aberrant amino acids added by a frameshift resulting from a mutation). Sequences that exist only in the mutant proteins are indicated by *light-gray boxes*.

replaced with an A (Figure 3B), which causes splicing failure, leading to a premature termination of the protein. The truncated proteins are about half the size of the full-length protein (Figure 3C). Patients 1 and 2 shared a conserved haplotype in a 1.1-Mb–long region, whereas patients 3 and 5 shared a 150-kb–long region (Figure 4). This suggests that each mutation is derived from a single founder.

Because *SLC34A2* is a membrane protein with eight predicted transmembrane domains, proteins lacking five of these domains are likely to lose the normal function. To confirm this, we cloned the wild-type and the mutant *SLC34A2* cDNA individually into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Capped RNA was transcribed *in vitro* from the T7 promoter and microinjected into the oocytes of *Xenopus laevis*. The wild-type *SLC34A2* transported phosphate in the presence of sodium, whereas the mutants did not (Figure 5A). *SLC34A2* cotransports sodium and phosphate into the cells (15) with a stoichiometry of $3Na^+$:$1HPO_4^{2-}$, and thus produces inward current. The wild-type *SLC34A2* elicited inward current with the addition of NaH_2PO_4, whereas mutants did not (Figure 5B). The proteins with the mutations seen in the patients abolished the normal transporter function.

DISCUSSION

SLC34A2 is expressed mainly in lung and mammary gland and to a lesser extent in intestine, kidney, and prostate (15). *SLC34A2* is the only phosphate transporter that is highly expressed in the lung (15), where the expression is observed specifically in type II alveolar cells (Figure 6) (17). By immunohistochemistry, *SLC34A2* protein was observed in the apical pole of the cells (18). The type II cells produce pulmonary surfactant, of which phospholipids are essential constituents. Outdated surfactant is taken up by type II cells for recycling and degradation and by

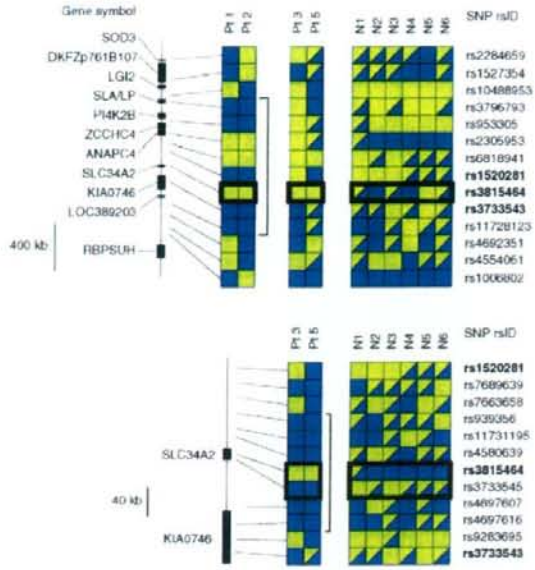

Figure 4. Haplotype analysis of the patients. Coarse scale (*upper panel*) and fine scale (*lower panel*) analyses are shown. We individually amplified each SNP site and genotyped by sequencing. Samples of patients 4 and 6 were excluded from the analysis: patient 4 is the younger sister of patient 1 and the same haplotype was expected. DNA of sufficient quality was not obtained from the paraffin-embedded tissue of patient 6 for completion of all SNP analyses. Regions conserved for patients 1 and 2 or for patients 3 and 5 are indicated by *brackets*. All SNPs have a minor allele frequency of more than 0.2. *Blue* and *yellow squares* show common alleles and rare alleles, respectively. Heterozygous sites are indicated by both colors. SNP sites included in the *SLC34A2* gene are *boxed*. SNP rsIDs used in both coarse and fine analyses are in *bold letters*. N = normal control; Pt = patient. The headings Gene symbol and SNP rsID indicate, respectively, the official symbol and an SNP ID used in the Genbank database (www.ncbi.nlm.nih.gov).

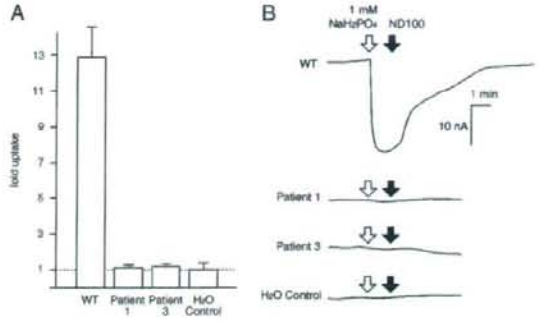

Figure 5. Functional analysis of *SLC34A2* mutants. (*A*) Phosphate transport assay. *Xenopus* oocytes microinjected with transcribed *in vitro* wild-type RNA, mutant RNA, or water were assayed for phosphate uptake. (*B*) Electrophysiologic measurement. Microinjected oocytes superfused in ND-100 solution (pH 6.6; 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid [HEPES]) were exposed to ND-100 solution containing 1 mM NaH_2PO_4 (pH 6.6) (*open arrows*) and then washed with ND-100 solution (pH 6.6) (*solid arrows*). Patient 1, Patient 3 = mutants observed in patients 1 and 3; WT = wild type.

alveolar macrophages for degradation (19). Degraded phospholipids release phosphate that should be cleared from the alveolar space. Dysfunction of *SLC34A2* may reduce the clearance of phosphate and may lead to the formation of microliths. In many cases of PAM, the lung is the only organ affected (1–3). Other organs may have phosphate transporters with redundant functions.

We have demonstrated that (*1*) homozygous inactivating mutations in *SLC34A2* are present in patients with PAM, (*2*) *SLC34A2* is highly expressed in alveolar type II cells, and (*3*) loss of the normal function of *SLC34A2* can explain the pathophysiology of PAM. On the basis of this evidence, we conclude that *SLC34A2* is the PAM gene.

Recently, Corut and colleagues (20) reported that *SLC34A2* is mutated in Turkish patients with PAM. The result is consistent with ours and confirms that *SLC34A2* is the causative gene for PAM.

No effective treatment for PAM currently exists, with the exception of lung transplantation. Disodium etidronate inhibits microcrystal growth of hydroxyapatite and thus inhibits ectopic calcification. This drug has been used to treat PAM, with little or no benefit (21–23). Our results suggest that remedies that

Figure 6. *In situ* hybridization. Cells expressing *SLC34A2* were investigated using serial sections of normal lung tissue. *Antisense probe*: *in situ* hybridization using *SLC34A2* antisense probe. *SP-A antibody*: immunohistochemical detection of SP-A (a marker of type II alveolar cells). *Arrows* indicate cells detected by both methods.

target phosphate metabolism rather than calcium metabolism may be beneficial for the treatment of PAM.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Theophylline Attenuates the Neutrophil-Dependent Augmentation of Eosinophil Trans-Basement Membrane Migration

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Key Words
Neutrophils · Eosinophils · Theophylline · Interleukin-8 · Cell migration

Abstract
Background: Recent evidence suggests that both neutrophilic and eosinophilic inflammation persist in the airways of patients with severe asthma. Neutrophils can secrete a variety of mediators which may augment the migration of eosinophils. We have reported that activated neutrophils augment the trans-basement membrane migration (TBM) of eosinophils in vitro. Theophylline has been shown to modulate some functions of both neutrophils and eosinophils. The objective of this study was to evaluate whether theophylline modulates the neutrophil-dependent augmentation of eosinophil TBM. ***Methods:*** Eosinophils and neutrophils were isolated from peripheral blood collected from healthy donors and were then preincubated with either 0.1 mM theophylline or the medium control. The TBM of eosinophils in response to IL-8 was evaluated in the presence or absence of neutrophils by using the chambers with a Matrigel®-coated Transwell® insert. The generation of O_2^- was evaluated by the cytochrome c reduction assay. ***Results:*** As previously reported, IL-8-stimulated neutrophils significantly augmented the TBM of eosinophils. Theophylline significantly attenuated the neutrophil-dependent augmentation of eosinophil TBM ($p < 0.001$) and did not directly modify the TBM of neutrophils in response to IL-8 or LTB4. Similarly, the LTB4-induced TBM of eosinophils was not modified by theophylline. Finally, theophylline attenuated the superoxide anion generation from IL-8-stimulated neutrophils on the Matrigel-coated plates. ***Conclusions:*** Our results show that theophylline can attenuate the neutrophil-dependent augmentation of eosinophil TBM. This effect is possibly attributable to the suppression of neutrophil activation provoked by the combination of basement membrane and IL-8.

Introduction

Traditionally, theophylline has been used as a bronchodilator in the treatment of asthma. However, this drug appears to reduce the tissue infiltration of eosinophils, predominant inflammatory cells in the airways of asthmatic patients [1–3]. For example, Sullivan et al. [1] reported that theophylline attenuates the airway accumulation of eosinophils in response to allergen inhalation in atopic asthma. More recently, Aizawa et al. [2] showed that theophylline eventually reduced the percentage of eosinophils in induced sputum from patients with

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mild-to-moderate asthma. Finally, Lim et al. [3] reported that theophylline reduced the number of eosinophils in sputa, biopsied specimens, and bronchoalveolar lavage fluids in mild asthmatic patients. Theophylline actually possesses a variety of in vitro effects on human eosinophils: induction of apoptosis [4, 5], inhibition of degranulation [5–7] and cell migration [8], and reduction of the surface expression of CD11b [6, 7, 9] or CD4 [8]. In this context, we have reported that theophylline attenuates eosinophil adhesion to endothelial cells in vitro at a clinically therapeutic concentration [9]. Furthermore, theophylline inhibits the expression of ICAM-1 and VCAM-1 on endothelial cells which had been stimulated with interleukin-4 (IL-4) plus tumor necrosis factor-α (TNF-α) [9, 10]. Thus, theophylline possibly exerts an inhibitory effect on both the adhesive property of eosinophils and the expression of adhesion molecules on endothelial cells [9, 10].

In a subgroup of patients with asthma, the accumulation of neutrophils is found in their airways even in the absence of infection. Asthma in such patients is often severe and chronic and is refractory to corticosteroid therapy [11–14]. Based upon a report from the European Network Study for Understanding Mechanisms of Severe Asthma (ENFUMOSA), patients with severe asthma have a higher percentage of neutrophils in sputum and present evidence of the continuing release of eosinophil-derived mediators in comparison to patients with mild-to-moderate asthma, suggesting that both neutrophilic and eosinophilic inflammation persist in the airways of patients with severe asthma [14]. In this context, we have reported a positive correlation between the concentrations of neutrophils and eosinophils in induced sputum from patients with severe persistent asthma who were treated with medicines including systemic corticosteroids [15]. Concentrations of IL-8 which acts as a chemoattractant for neutrophils have been shown to be correlated with the accumulation of neutrophils in the airways of patients with asthma [16], and this chemokine may therefore be an essential molecule responsible for the development of neutrophilic inflammation in asthma. We recently published that, when eosinophils were coincubated with neutrophils and stimulated with IL-8 in vitro, the trans-basement membrane migration (TBM) of eosinophils was significantly induced [17]. This augmented TBM of eosinophils by neutrophils was inhibited by a matrix metalloproteinase-9 inhibitor, a leukotriene B4 receptor antagonist, or platelet activating factor antagonists [17]. This provides a mechanism for the relationship between neutrophils and eosinophils in severe asthma and raises a possibility that therapies which suppress the interaction between neutrophils and eosinophils may be effective for severe asthma.

The objective of this study was to evaluate whether theophylline modulates the neutrophil-dependent augmentation of the eosinophil TBM.

Materials and Methods

Reagents

Percoll® was obtained from Pharmacia (Uppsala, Sweden). Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyl Biotec (Auburn, Calif., USA). RPMI 1640 medium, PBS, newborn calf serum (NCS) and fetal calf serum (FCS) were obtained from Life Technologies (Grand Island, N.Y., USA). Recombinant human (rh-) IL-8 was purchased from R&D Systems (Minneapolis, Minn., USA). Leukotriene B4 (LTB4) was purchased from Cayman Chemical (Ann Arbor, Mich., USA). O-Phenylenediamine (OPD) and BSA were obtained from Sigma (St. Louis, Mo., USA). The acetoxy methyl ester of 2′-7′-bis (2-carboxy-ethyl)-5(6)-carboxyfluorescein (BCECF-AM) was purchased from Dojin Laboratory (Kumamoto, Japan). Other reagents were purchased from Sigma (St. Louis, Mo., USA) unless otherwise specified.

Preparation of Neutrophils and Eosinophils

Neutrophils and eosinophils were isolated from peripheral blood collected from nonatopic healthy donors whose eosinophil content was less than 5% of their peripheral leukocytes [17]. Informed consent was obtained prior to the collection of each blood sample. Neutrophils and eosinophils were separated by the combination of Percoll density-gradient centrifugation and negative immunomagnetic bead selection as previously described [17]. Briefly, 40 ml of dextran were added to 160 ml of heparinized blood, and erythrocytes were removed as sediments. The remaining suspension of leukocytes was layered onto Percoll gradients of 1.080, 1.085, and 1.090 g/ml in density. After centrifugation at 700 *g* for 20 min, neutrophils (purity exceeded 95%) were collected from 1.085/1.090 g/ml interface and were then suspended in HBSS containing 0.2% BSA (HBSS/BSA buffer). Following the removal of Percoll, the red blood cells in the pellet were lysed by hypotonic shock and were then removed by washing with cold PBS. The remaining cells were washed with 4°C HBSS supplemented with 2% NCS (HBSS/NCS), incubated with anti-CD16 antibody-coated magnetic beads for 30 min at 4°C and were then filtered with a column containing steel wool placed in a magnetic field (Miltenyl Biotec). Eosinophils (> 98% purity and > 99% viability), which passed through the column, were collected and washed, and the number of cells was adjusted to 2.5×10^5 cells/ml by using the HBSS/BSA buffer.

Trans-Basement Membrane Migration

The TBM of neutrophils and eosinophils was examined using a modified Boyden's chamber method [17]. The study was conducted in duplicate. Briefly, neutrophils or eosinophils were precultured in the presence or absence of a clinically relevant concentration of 0.1 mM theophylline at 37°C for 20 min before the each study. Neutrophils were fluorescent-labeled with BCECF-AM. La-

beled neutrophils (0.5×10^5 cells), eosinophils (0.5×10^5 cells), or a combination thereof (0.5×10^5 cells plus 0.5×10^5 cells) in a 200-μl medium, was added to the upper compartment of a chamber with a Matrigel®-coated Transwell® insert (pore size 3 μm, Becton Dickinson Labware, N.Y., USA). Either the control medium (500 μl) or a medium which contained one of activators (IL-8 and LTB4) was added to the lower compartment of the chamber. After a 2-hour incubation in 5% CO_2 at 37°C, the medium in the upper compartment of the chamber and the inserts between the chambers were gently removed. The peroxidase activity of eosinophils in the medium in the lower compartment of the chamber was determined, and the number of migrated eosinophils was calculated from the activity of the standard media which contained known numbers (5×10^3, 1.5×10^4, 5×10^4, 1.5×10^5 and 5×10^5 cells) of eosinophils. To determine the peroxidase activity of eosinophils, the medium was incubated with a substrate (1 mM OPD, 1 mM H_2O_2, and 0.1 % Triton X-100 in Tris-HCl, pH 8.0) for 30 min at room temperature [17]. The reaction was stopped by adding 100 μl of 4 N H_2SO_4, and absorbance at 490 nm was determined. The numbers of migrated neutrophils were determined by the measurement of fluorescence in the medium using the Fluoromark® (Bio-Rad Laboratories, Calif., USA) microplate fluorometer [17]. The viability of both eosinophils and neutrophils after migration exceeded 98% by trypan blue exclusion.

Superoxide Anion (O_2^-) Generation

The neutrophil O_2^- generation was measured as the SOD-inhibitable reduction of ferricytochrome c, as described previously [18], in 96-well culture plates (Corning Inc., Corning, New York, N.Y., USA) coated with Matrigel. We initially added SOD (0.2 mg/ml in HBSS/gel; 20 μl) to SOD control wells and then HBSS/gel to all wells to obtain the final volume to 100 μl. Neutrophil density was adjusted to 1.25×10^6 cells/ml of HBSS/gel, and this suspension was mixed with cytochrome c at 4:1 (12 mg/ml of HBSS/gel). Neutrophils, which were preincubated with either 0.1 mM theophylline or the buffer alone (control) at 37°C for 20 min, were then added to all wells at a volume of 100 μl. To initiate the reaction, the cells were incubated with IL-8 (10 nM). Immediately after adding the activator, absorbance of the cell suspensions in the wells was measured at 550 nm in an IMMUNO-MINI (NJ-2300; Japan Intermed Co., Tokyo, Japan), followed by repeated readings over the next 240 min. Between readings, the plates were placed in a 5% CO_2 incubator at 37°C. Each reaction was performed in duplicate against an identical control reaction containing 20 μg/ml SOD. The results were adjusted to represent a 1-ml reaction volume, and the O_2^- generation was calculated at an extinction coefficient of 21.1×10^3 M/l^{-1} cm^{-1} as nanomoles of cytochrome c reduced per 1.0×10^6 cells per minute minus the SOD control. The maximum value during the incubation time was examined for the effects of drugs on eosinophil O_2^- generation. Cell viability as determined by trypan blue exclusion at the completion of each experiment remained at 95% after 240 min of incubation.

Statistical Analysis

Values are expressed as mean ± SEM. Repeated-measures ANOVA was conducted to compare variables. When the initial p value was below 0.05, Scheffé's posthoc test was conducted to determine the significant difference between groups. Student's t test was conducted to make paired comparisons. $p < 0.05$ was considered statistically significant.

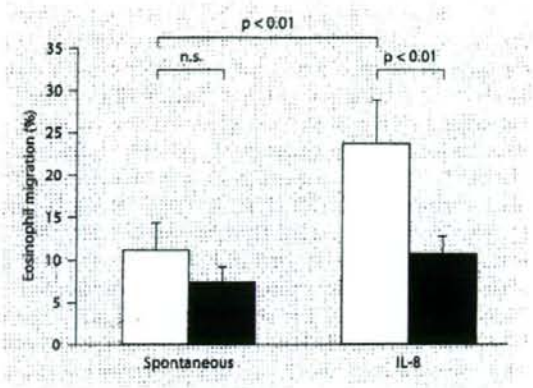

Fig. 1. Effects of 0.1 mM theophylline on the spontaneous (left panel) or 10 nM IL-8 (right panel)-induced trans-basement migration of eosinophils in the presence of neutrophils. Open bars represent the control (no drug), and closed bars represent cells incubated with theophylline. Values are expressed as mean ± SEM of 10 experiments.

Results

Effects of Theophylline on the TBM of Eosinophils Induced by a Combination of IL-8 and Neutrophils

As previously reported [17], IL-8 (10 nM) alone or the presence of neutrophils alone does not induce the TBM of eosinophils. However, a combination of IL-8 and neutrophils significantly induces the TBM of eosinophils (data not shown).

To evaluate whether theophylline modulates the neutrophil-dependent augmentation of the eosinophil TBM, neutrophils and eosinophils were precultured in the presence or absence of a clinically relevant concentration of 0.1 mM theophylline at 37°C for 20 min; the TBM assay was then performed. Consequently, the spontaneous TBM of eosinophils in the presence of neutrophils was not modified by 0.1 mM theophylline (fig. 1). However, the enhanced TBM of eosinophils induced by a combination of IL-8 and neutrophils was significantly reduced by 0.1 mM theophylline (fig. 1).

Theophylline Does Not Directly Modulate the TBM of Neutrophils or Eosinophils

To examine whether the inhibitory effect of theophylline on the neutrophil-dependent augmentation of the eosinophil TBM occurs through the attenuation of the TBM of either neutrophils or eosinophils, the effects of

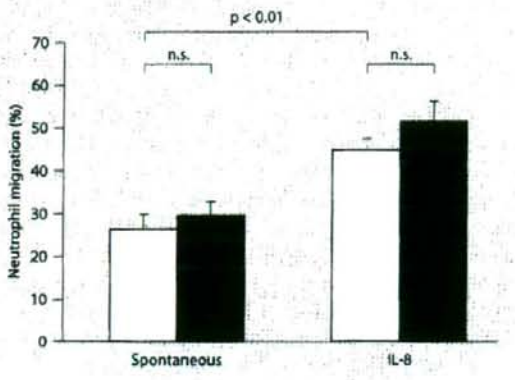

Fig. 2. Effects of 0.1 mM theophylline on the spontaneous (left panel) or 10 nM IL-8 (right panel)-induced trans-basement migration of neutrophils. Open bars represent the control, and closed bars represent cells incubated with 0.1 mM theophylline. Values are expressed as mean ± SEM of 6 experiments.

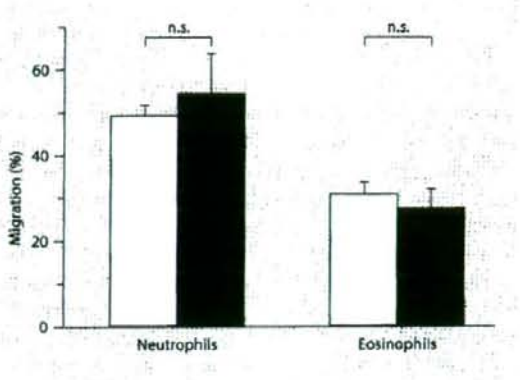

Fig. 3. Effects of 0.1 mM theophylline on the 30 nM LTB4-induced trans-basement migration of neutrophils (left panel) or eosinophils (right panel). Open bars represent the control, and closed bars represent cells incubated with 0.1 mM theophylline. Values are expressed as mean ± SEM of 6 experiments.

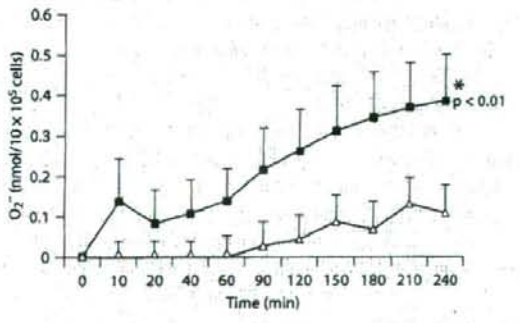

Fig. 4. Effects of theophylline on the superoxide anion generation from neutrophils incubated in 96-well culture plates coated with Matrigel and activated with 10 nM IL-8. Closed squares represent the control, and open triangles represent cells incubated with 0.1 mM theophylline. Values are expressed as mean ± SEM of 6 experiments.

theophylline on the TBM of each cell type were evaluated. Theophylline, at a concentration where it can attenuate the neutrophil-induced TBM of eosinophils, does not modify the spontaneous or IL-8-induced TBM of neutrophils (fig. 2). Similarly, theophylline does not modify the LTB4-induced TBM of neutrophils or eosinophils (fig. 3). Therefore, the inhibitory effect of theophylline on the TBM of eosinophils (fig. 1) is unlikely due to the direct effect on the migrative response of neutrophils or eosinophils.

Theophylline Inhibits the O_2^- Generation from Activated Neutrophils

Theophyline may reduce the TBM of eosinophils through the inhibition of activation and release of mediators from neutrophils. To test this hypothesis, neutrophils were preincubated in the presence or absence of 0.1 mM theophylline at 37°C for 20 min; subsequently, the O_2^- generation was evaluated in 96-well culture plates coated with Matrigel. In these experimental settings, neutrophils did not spontaneously generate O_2^-. However, neutrophils generated O_2^- in the presence of 10 nM IL-8, and the generation was significantly attenuated by theophylline (fig. 4).

Discussion

We found that a therapeutically relevant concentration of theophylline attenuated the TBM of eosinophils which is enhanced by neutrophils. The inhibitory effect of theophylline was not attributable to the direct effect on

the migratory response of neutrophils or eosinophils, since the drug modified the TBM of neither neutrophils induced by IL-8 or LTB4, nor eosinophils induced by LTB4. On the other hand, theophylline attenuated the IL-8-induced generation of O_2^- in the presence of a basement membrane component. Collectively, these results suggest that theophylline attenuates the neutrophil-dependent mechanism in the augmentation of the eosinophil TBM which may be important in the airways of patients with severe asthma [13–15].

Activated neutrophils can secrete a variety of mediators, e.g. matrix metalloproteinases, LTB4, platelet-activating factor, and TNF-α, which can induce the digestion of basement membrane or the migration or activation of eosinophils [19, 20], thus possibly attributing to the pathophysiology of asthma. Our observations indicated that a combination of IL-8 and a basement membrane component induced neutrophil respiratory burst which is inhibited by theophylline. The biological consequences of oxygen species released from inflammatory cells would modify either the morphological or functional status of the endothelium such as permeability, adhesion molecule expression, and adhesiveness to leukocytes [21–23]. More importantly, oxygen species alter the functional status of leukocytes. For example, we and others have reported that hydrogen peroxide promotes eosinophil and neutrophil adhesion via β_2 integrins [24, 25]. Therefore, the inhibitory effect of theophylline on the enhanced TBM of eosinophils seems, at least in part, attributable to the suppression of neutrophil activation and mediator release provoked by a combination of basement membrane and chemoattractants such as IL-8. Functions of neutrophils are not effectively suppressed by corticosteroids [26, 27], suggesting that neutrophils may play a role in the pathophysiology of asthma even under corticosteroid treatment. Classically, theophylline has been used as a bronchodilator to control the smooth muscle tone of the airways of asthmatics. More recently, however, theophylline has been shown to have a variety of inhibitory effects on inflammatory cells involved in asthmatic inflammation. In clinical settings, there is increasing evidence that the use of theophylline attenuates the eosinophil accumulation in the airways of asthmatic patients [1–3]. Our observations provide evidence of the novel modulatory effects of theophylline on the interactions between neutrophils and eosinophils as a mechanism for the attenuation of eosinophil accumulation in the airways of asthmatics, especially in patients with severe disease. Although the exact impact of this anti-inflammatory property of theophylline on its clinical effects remains to be elucidated, it is plausible that theophylline exerts inhibitory effects on both eosinophils directly and inflammatory networks such as the interaction between neutrophils and eosinophils. The combination of inhaled or oral corticosteroids and theophylline has been shown to provide additive clinical and physiological effects in moderate to severe asthma [28, 29], suggesting that these two classes of drugs act via differential mechanisms to modulate airway changes in asthma. Theophylline would alter both bronchial smooth muscle tone and the accumulation and/or functional state of eosinophils via a complex of mechanisms including intervention on the interaction between neutrophils and eosinophils, and may therefore provide clinical benefits additionally to corticosteroids in the treatment of asthma.

In conclusion, we found here that theophylline attenuates the neutrophil-dependent augmentation of the eosinophil TBM mainly via the suppression of neutrophil activation provoked by a combination of basement membrane and IL-8. This provides a novel mechanism of theophylline effects and raises a possibility that theophylline suppresses the interaction between neutrophils and eosinophils which may be important in the pathophysiology of severe asthma.

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A Single Enzyme Catalyzes Both Platelet-activating Factor Production and Membrane Biogenesis of Inflammatory Cells

CLONING AND CHARACTERIZATION OF ACETYL-CoA:LYSO-PAF ACETYLTRANSFERASE[*] [S]

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Platelet-activating factor (PAF) is a potent proinflammatory lipid mediator eliciting a variety of cellular functions. Lipid mediators, including PAF are produced from membrane phospholipids by enzymatic cascades. Although a G protein-coupled PAF receptor and degradation enzymes have been cloned and characterized, the PAF biosynthetic enzyme, acetyl-CoA:lyso-PAF acetyltransferase, has not been identified. Here, we cloned lyso-PAF acetyltransferase, which is critical in stimulus-dependent formation of PAF. The enzyme is a 60-kDa microsomal protein with three putative membrane-spanning domains. The enzyme was induced by bacterial endotoxin (lipopolysaccharide), which was suppressed by dexamethasone treatment. Surprisingly, the enzyme catalyzed not only biosynthesis of PAF from lyso-PAF but also incorporation of arachidonoyl-CoA to produce PAF precursor membrane glycerophospholipids (lysophosphatidylcholine acyltransferase activity). Under resting conditions, the enzyme prefers arachidonoyl-CoA and contributes to membrane biogenesis. Upon acute inflammatory stimulation with lipopolysaccharide, the activated enzyme utilizes acetyl-CoA more efficiently and produces PAF. Thus, our findings provide a novel concept that a single enzyme catalyzes membrane biogenesis of inflammatory cells while producing a prophlogistic mediator in response to external stimuli.

Platelet-activating factor (PAF[3]; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid mediator that activates a G protein-coupled receptor (1–3) and results in pleiotropic and potent biological effects, including platelet activation, airway constriction, and hypotension (1). PAF is synthesized in various cells and tissues via two distinct pathways, the *de novo* and remodeling pathways (2, 4, 5), and the latter is regulated by extracellular signals and plays a critical role in stimulus-coupled PAF biosynthesis (2, 4–6). PAF synthesis induced by extracellular signals has been reported in murine peritoneal cells stimulated by calcium ionophore (7) or by PAF (8), in human eosinophils stimulated by fMet-Leu-Phe (9), in human neutrophils stimulated by acid stress (10), and in murine peritoneal macrophages stimulated by lipopolysaccharide (LPS) (11). In the remodeling pathway, the precursor of PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF), is synthesized from 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (1-alkyl-phosphatidylcholine; PC) by the action of phospholipase A_2 (2, 4, 12, 13). Subsequently, lyso-PAF is converted to PAF by acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAF acetyltransferase) (EC 2.3.1.67) (14). PAF is then rapidly degraded to lyso-PAF by PAF acetylhydrolases (15). Alternatively, lyso-PAF is again transformed into PC by the action of lysophosphatidylcholine (LPC) acyltransferase (2.3.1.23) (16).

A G protein-coupled PAF receptor was cloned in our laboratory (17), and PAF acetylhydrolases have been cloned and characterized by others (18, 19). Lyso-PAF acetyltransferase was initially demonstrated and partially characterized by Wykle *et al.* (14) in 1980. Since this first report, the enzyme activity has been detected in microsomes of rat spleen and lung as well as porcine leukocytes (14, 20). Although several groups have attempted to identify and characterize the enzyme from various sources (2, 4, 14, 21, 22), it has not yet been purified nor cDNA-cloned.

PC is a major component of cellular membranes and also plays an important role as a PAF precursor. PC is formed from diacylglycerol by a *de novo* pathway, originally described by Kennedy (23) in 1961 but is also generated from LPC by a remodeling pathway. Rapid turnover of the *sn*-2 acyl moiety of glycerophospholipids was described by Lands (Lands' cycle) (24–26) and is attributed to activation of phospholipases A_2 and lysophospholipid acyltransferases. Recently, we (27) and Chen *et al.* (28) independently cloned one of LPC acyltrans-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB244716 (mouse) and AB244718 (human).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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[3] The abbreviations used are: PAF, platelet-activating factor; LPS, lipopolysaccharide; PC, phosphatidylcholine; lyso-PAF acetyltransferase, acetyl-CoA:lyso-PAF acetyltransferase; LPC, lysophosphatidylcholine; DEX, dexamethasone; ER, endoplasmic reticulum; PAFR, PAF receptor; mLysoPAFAT and hLysoPAFAT, mouse and human LysoPAFAT, respectively; siRNA, small interfering RNA; $DiOC_5(3)$, 3,3′-dihexyloxacarbocyanine iodide.

Supplemental Material can be found at: http://www.jbc.org/cgi/content/full/M609641200/DC1

ferases designated LPCAT1, which is highly expressed in alveolar type II cells. Since PC synthesis occurs in a variety of different tissues, additional LPC acyltransferases may be present for membrane biogenesis.

Using the previously reported LPCAT1 (27) and an extensive genomic data base search as well as 5′- and 3′-RACE, we have identified a lyso-PAF acetyltransferase gene. The enzyme is primarily expressed in inflammatory cells and is induced by LPS. Surprisingly, the enzyme also catalyzes incorporation of arachidonoyl-CoA to produce PAF precursor membrane glycerophospholipids (LPC acyltransferase activity). Thus, we designated this enzyme LysoPAFAT/LPCAT2. Although this enzyme possesses both acetyltransferase and acyltransferase activity, only the acetyltransferase activity was enhanced by acute inflammatory signals. To our knowledge, this is the first documentation of a cDNA for LysoPAFAT/LPCAT2, a critically important enzyme in the biogenesis of PAF and in membrane homeostasis of inflammatory cells.

EXPERIMENTAL PROCEDURES

Materials—Various lysophospholipids and acyl-CoAs were obtained from Avanti Polar Lipids (Alabaster, AL). LPS from *Salmonella minnesota*, 4-amidinophenylmethanesulfonyl fluoride, dexamethasone (DEX), estradiol-17β, and linoleoyl-CoA (C18:2) were purchased from Sigma. [^{3}H]Acetyl-CoA (185 GBq/mmol) and [^{3}H]lyso-PAF (6.25 TBq/mmol) were obtained from Amersham Biosciences (Buckinghamshire, UK). [1-^{14}C]Arachidonoyl-CoA (2.22 GBq/mmol) was purchased from Moravec Biochemicals (Mercury Lane, CA). ODN1826 and poly(I:C) were purchased from InvivoGen (San Diego, CA).

Cloning of LysoPAFAT/LPCAT2—The mLysoPAFAT/LPCAT2 gene was identified based upon sequence similarity to the LPCAT1 gene (27) and LPA acyltransferase ζ gene (29) through a comprehensive basic local alignment search tool (BLAST) search. A 1.6-kb cDNA clone encoding the full-length mLysoPAFAT/LPCAT2 (DDBJ accession number AB244716) was cloned by PCR amplification using the forward primer 5′-CTAGCTAGCCACCATGGATTACAAGGATGACGATGACAAGAACCGATGCGCCGAGGCGGCCGC-3′, the reverse primer 5′-CCGCTCGAGTCAGTCCACCTTTTTGTCTGAGGTGCCCTC-3′, and a mouse spleen cDNA library as a template. The FLAG epitope (DYKDDDDK) was attached to the N terminus of mLysoPAFAT/LPCAT2 by PCR using a forward primer. Amplified PCR products were cloned into the pCXN2.1 vector, a slightly modified version of pCXN2 (30) with multiple cloning sites, and sequenced. Similarly, hLysoPAFAT/LPCAT2 cDNA (DDBJ accession number AB244718) was amplified by PCR and inserted into the pCXN2.1 vector.

Quantitative Real Time RT-PCR—Mouse total RNAs were prepared using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene), and first strand cDNA was subsequently synthesized using Superscript II (Invitrogen). The PCRs were performed using FastStart DNA Master SYBR Green I (Roche Applied Science). The primers for mLysoPAFAT/LPCAT2 were designed to amplify a 167-bp fragment: forward primer, 5′-GTCCAGCAGACTACGATCAGTG-3′; reverse primer, 5′-CTTATTGGATGGGTCAGCTTTTC-3′. The primers for hLysoPAFAT/LPCAT2 were designed to amplify a 176-bp fragment: forward primer, 5′-TTGCTTCCAATTCGTGTCTTATT-3′; reverse primer, 5′-ATCCCATTGAAAAGAACATAGCA-3′.

Expression of FLAG-mLysoPAFAT/LPCAT2 in CHO-K1 Cells—After 48 h of transfection with FLAG-tagged enzyme using Lipofectamine 2000 (Invitrogen), cells in 10-cm dishes were scraped into 1 ml of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, and a proteinase inhibitor mixture, Complete (Roche Applied Science), and then sonicated three times on ice for 30 s. After centrifugation for 10 min at 800 × *g*, the supernatant was collected and centrifuged at 100,000 × *g* for 1 h. The resulting pellet was resuspended in buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM sucrose, and 1 mM EDTA. Protein concentration was measured by the method of Bradford (31), using a commercially prepared protein assay solution (Bio-Rad) and bovine serum albumin (fraction V, fatty acid-free; Sigma) as a standard.

Confocal Microscopy—CHO-K1 cells were seeded onto 6-cm dishes before transfection. Six μg each of pCXN2.1 vector or FLAG-mLysoPAFAT/LPCAT2 were transfected using Lipofectamine 2000. 48 h post-transfection, vector- or FLAG-tagged enzyme-transfected cells were incubated with 2.5 μg/ml 3,3′-dihexyloxacarbocyanine iodide (DiOC$_6$(3)) (Invitrogen) and 10 μg/ml M5 anti-FLAG mouse monoclonal antibody (Sigma) in ¼× permeabilization buffer (Beckman Coulter, Marseille, France). After washing, cells were incubated with 10 μg/ml Alexa Fluor 546 goat anti-mouse IgG (Eugene, OR) for 30 min. Confocal microscopy was performed with an LSM510 laser-scanning microscope (Carl Zeiss) equipped with a ×63 water immersion objective lens (numerical aperture = 1.2). FLAG-mLysoPAFAT/LPCAT2 was monitored by excitation at 543 nm with a helium/neon laser and by emission with a 585-nm long path filter. For the detection of DiOC$_6$(3), the excitation was at 488 nm with an argon laser, and emissions were taken with a 505–550-nm band pass filter.

Assay of Lyso-PAF Acetyltransferase—The lyso-PAF acetyltransferase activity was determined according to the method of Kume *et al.* (20), except for washing resin one time before and eight times after application of the reaction mixture. Briefly, 100 μM [^{3}H]acetyl-CoA (1.11 GBq/mmol) and protein were incubated at 37 °C for 10 min in the presence or absence of 20 μM lyso-PAF (Cayman). Subsequently, the product was bound to C8 resin (Millipore), washed, and eluted. The difference between the radioactivities obtained in the presence and absence of lyso-PAF corresponded to lyso-PAF acetyltransferase activity.

Assay of Lysophospholipid Acetyltransferase and Acyltransferase by TLC—The acyltransferase activity was measured in two ways: (i) conversion of [1-^{14}C]lyso-PAF (293 MBq/mmol) to PC in the presence of acetyl- and acyl-CoA and (ii) the transfer of [^{3}H]acetyl-CoA (1.11 GBq/mmol)- or [^{14}C]arachidonoyl-CoA (1.11–2.035 GBq/mmol) to lysophospholipids to form phospholipids. The reaction mixture contained 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$, 1 mg/ml PC, 5 mM 2-mercaptoethanol, 20 μM 4-amidinophenylmethanesulfonyl fluoride (Sigma), a proteinase inhibitor mixture, 10 or 100 μM acyl-CoA, 20 μM lysophospholipid, and enzyme in a total volume of 100

μl. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 0.3 ml of chloroform/methanol (1:2, v/v). Total lipids were extracted using the Bligh-Dyer method (32) and subsequently analyzed by TLC in chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v). Bands at positions corresponding to the expected product were visualized by I_2 vapor, cut from the plate, placed in Microscinti-O (Packard Bioscience), and analyzed in a liquid scintillation counter LS6500 (Beckman).

Radioligand Binding Assay—The method of PAF-PAF receptor (PAFR) binding assay was described previously (7, 33). Briefly, the membrane fraction containing 158 fmol of PAFR from hearts and skeletal muscles of PAFR transgenic mice (7, 34) were mixed with 25 nM [^{3}H]WEB 2086 and the lipid extract in a 96-well plate. After incubation at 25 °C for 90 min, receptor-bound [^{3}H]WEB 2086 was collected by filtration through a UniFilter-GF/C (PerkinElmer Life Sciences) using a MicroMate 196 simultaneous 96-well harvester (PerkinElmer Life Sciences), and the filter was washed and dried. Subsequently, the radioactivities were counted with a TopCount microplate scintillation counter (PerkinElmer Life Sciences).

Electrospray Ionization Mass Spectrometry Analysis of PAF—Extracted lipid from the acetyltransferase assay was identified by electrospray ionization mass spectrometry analysis. The analysis was performed using a 4000 Q-TRAP quadrupole-linear ion trap hybrid mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) with an Ultimate 3000 high pressure liquid chromatography system (DIONEX Co.) combined with an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The extracted lipids were subjected to electrospray ionization mass spectrometry analysis by flow injection without liquid chromatography separation. The solvent was acetonitrile, methanol, 50 mM ammonium formate, pH 7.4 (v/v/v, 45/50/5), and the flow rate was 10 μl/min. The scan range and speed were set at m/z 500–600 and 1000 Da/s, respectively. The trap fill time was set at 5 ms, and the ion spray voltage was set at −4500 V in the negative ion mode. Nitrogen was used as curtain and collision gas. The declustering potential was set at 20 V to minimize in-source fragmentation. Both Q1 and Q3 resolution were set to unit mass. The collision energy used was varied according to the desired experiment. The method to identify phosphatidylcholine species was described previously (35).

siRNA Transfection—hLysoPAFAT/LPCAT2 siRNAs (siRNA ID numbers 140446, 140447, and 140448; Ambion) and control siRNA (silencer negative control 1; Ambion) were transfected using siPORT amine transfection agent according to the manufacturer's protocol. The siRNA transfection was performed for 2 days in HEK293 cells.

Short Term LPS Stimulation—After transfection of RAW264.7 cells with LysoPAFAT/LPCAT2 using Lipofectamine 2000, cells were pretreated with or without 20 μM SB 203580 for 1 h and subsequently stimulated with 100 ng/ml LPS for 30 min. For preparation of cell extracts, the cells were scraped into 600 μl of an ice-cold buffer containing 20 mM Tris-HCl, (pH 7.4), 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol, 20 μM 4-amidinophenylmethanesulfonyl fluoride, and Complete, and the collected cells were sonicated twice on ice for 30 s. Intact cells, cellular debris, and mitochondria were removed by centrifugation at 9000 × g for 10 min at 4 °C. Enzyme activities were measured as described above.

Isolation and Stimulation of Mouse Peritoneal Cells—Mouse peritoneal macrophages induced by thioglycollate (Difco) was prepared as described in detail previously (11). The cells were treated with 100 ng/ml LPS, 0.8 μM ODN1826, or 1 μg/ml poly(I:C), in the presence or absence of 100 nM DEX or 100 nM estradiol-17β for 16 h. After treatment, the cells were washed with an ice-cold buffer containing 20 mM Tris-HCl (pH 7.4) and 300 mM sucrose. Cell extracts were prepared by the same method as described for experiments using RAW264.7 cells, and the enzyme activity was measured.

After treatment with microbial components for 16 h, total RNA was collected using the Absolutely RNA RT-PCR miniprep kit. Likewise, at 4 h after intraperitoneal injection with 2 ml of 2% casein, peritoneal exudate neutrophils were harvested from the peritoneal cavity, and their total RNA was prepared.

Statistics—Data are presented as mean ± S.E. or S.D. p values less than 0.05 were considered statistically significant. All statistical calculations were performed using Prism 4 (GraphPad Software) and StatView-J, version 5.0 (Abacus Concepts, Berkeley, CA).

Mice—C57BL/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan). Mice were maintained in a light-dark cycle with light from 8:00 to 20:00 at 21 °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.

RESULTS

Identification and Cloning of LysoPAFAT/LPCAT2—The mouse LysoPAFAT/LPCAT2 (mLysoPAFAT/LPCAT2) gene was identified based upon sequence homology with the previously reported LPCAT1 (27). The putative open reading frame of mLysoPAFAT/LPCAT2 encoded a 544-amino acid protein of 60.3 kDa, containing three putative transmembrane domains (36) and several conserved motifs found in members of the lysophospholipid acyltransferase family (27, 37). mLysoPAFAT/LPCAT2 contained putative EF-hand-like motifs (aa 374–494, E-value = $3e^{*}$-10) predicted by a conserved domain data base (available on the World Wide Web at www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (38) and showed 48.2% amino acid sequence homology to mouse LPCAT1. It exhibited 88.4% amino acid sequence homology to human LysoPAFAT/LPCAT2 (hLysoPAFAT/LPCAT2) (Fig. 1). The presence of the C-terminal sequence motif KK*XX* suggests that the protein is localized to the endoplasmic reticulum (ER) (39).

Tissue Distribution and Subcellular Localization of mLysoPAFAT/LPCAT2 mRNA—The tissue distribution of mLysoPAFAT/LPCAT2 was analyzed by quantitative PCR. We found the highest level of mLysoPAFAT/LPCAT2 expression in resident macrophages, casein-induced neutrophils, followed by skin, colon, spleen, and thioglycollate-induced macrophages (Fig. 2*A*). To facilitate immunocytochemical analysis of

FIGURE 1. **Amino acid sequence alignment of mouse and human LysoPAFAT/LPCAT2.** The predicted conserved lysophospholipid acyltransferase motifs (motifs 1–3) are *boxed*. The putative transmembrane domains are *underlined*. Amino acids conserved in both species are marked with *asterisks*. The ER localization sequence *KKXX* is present at the C terminus. The transmembrane motifs were determined using HMMTOP (available on the World Wide Web at www.enzim.hu/hmmtop/index.html).

mLysoPAFAT/LPCAT2, we constructed an expression vector encoding FLAG-tagged mLysoPAFAT/LPCAT2. It was transfected into CHO-K1, and the enzyme distribution was examined by confocal microscopy after 48 h. FLAG-mLysoPAFAT/LPCAT2 exhibited significant enzyme activities (Fig. 3, *A* and *B*). Cells were stained for ER-Golgi using $DiOC_6(3)$. The subcellular distribution pattern of FLAG-mLysoPAFAT/LPCAT2 was similar to that of $DiOC_6(3)$, suggesting that the enzyme is present mainly in the ER and Golgi (Fig. 2*B*).

Substrate Selectivity of mLysoPAFAT/LPCAT2—We next examined the acetyltransferase activity of mLysoPAFAT/LPCAT2 using a variety of 20 μM lysophospholipid acceptors and 100 μM [^{3}H]acetyl-CoA as a donor. mLysoPAFAT/LPCAT2 had detectable acetyltransferase activity toward LPC (Fig. 3*A*) and alkyl-LPC (Fig. 3*B*). The enzyme had a higher activity toward C16 lysophospholipids than C18 at the *sn*-1 moiety, which served as an acetyl acceptor (Fig. 3*B*). The acetyltransferase activity of mLysoPAFAT/LPCAT2 was linear for the first 40 min at 37 °C, and the enzyme exhibited calcium-dependent activity with a pH optimum around 7.4 (data not shown). To confirm whether the product of the acetyltransferase reaction is PAF, we first performed a competitive receptor binding assay using PAFR prepared from PAFR-transgenic mice (7, 34). Lipid extracts from the acetyltransferase reaction of mLysoPAFAT/LPCAT2 with lyso-PAF competed for binding of [^{3}H]WEB2086 to PAFR, indicating that the enzyme indeed produced PAF (supplemental Fig. 1). More definitively, the reaction product was identified by mass spectrometry (MS) using a 4000 Q-TRAP mass spectrometer (supplemental Fig. 2).

Human LysoPAFAT/LPCAT2 siRNA Decreases Lyso-PAF Acetyltransferase Activity—To investigate whether endogenous lyso-PAF acetyltransferase activity was decreased by transfection with an siRNA against hLysoPAFAT/LPCAT2, we transfected three hLysoPAFAT/LPCAT2 siRNAs (siRNA ID 140446, 140447, and 140448; Ambion) into HEK293 cells. We chose HEK293 cells, because the cells exhibit a high endogenous enzymic activity and high transfection efficiency (data not shown). All siRNAs decreased mRNA levels of hLysoPAFAT/LPCAT2 by 70–80% and lyso-PAF acetyltransferase activity by 50–60% (Fig. 3*C*). Control siRNA (silencer negative control 1; Ambion) had no apparent effect on either enzyme activity or mRNA expression. Thus, hLysoPAFAT/LPCAT2 appears to be the principal enzyme for PAF production in HEK293 cells.

LysoPAFAT/LPCAT2 Possesses LPC Acyltransferase Activity—Next, we examined the acyl-CoA selectivity of mLysoPAFAT/LPCAT2 using [^{3}H]lyso-PAF (C18) as an acceptor. At a high concentrations of acyl-CoAs (>20 μM), mLysoPAFAT/LPCAT2 showed both acetyltransferase and arachidonoyltransferase (acyltransferase) activities (Fig. 4). At a low concentration (<10 μM), arachidonoyl-CoA was a better substrate for LysoPAFAT/LPCAT2 than acetyl-CoA (Fig. 4). Medium chained fatty acyl-CoAs were poor substrates at both high and low concentrations of acyl-CoA (data not shown). These results suggest that mLysoPAFAT/LPCAT2 exhibits both lyso-PAF acetyltransferase and LPC acyltransferase activities. The apparent K_m values of the enzyme for acetyl-CoA and for arachidonoyl-CoA were 50.4 and 21.1 μM, respectively (Fig. 4).

Enzyme Activation by an Inflammatory Stimulus—Next, we investigated the activation of the enzyme by an inflammatory stimulus, such as LPS. To examine the response to LPS stimulation, mLysoPAFAT/LPCAT2 was transfected into the macrophage cell line, RAW264.7, which expresses the LPS receptor, Toll-like receptor 4 (TLR4), and cells were stimulated by LPS for 30 min in the presence or absence of the p38 mitogen-activated protein kinase inhibitor SB 203580 (Tocris Cookson). The acetyltransferase activity of mLysoPAFAT/LPCAT2 was

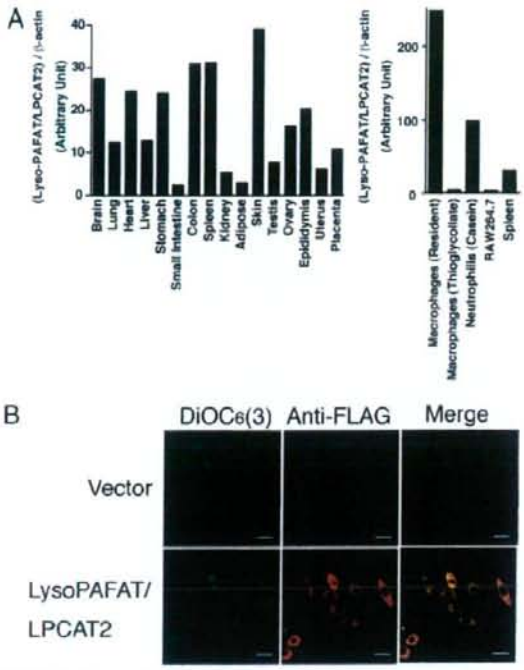

FIGURE 2. **Tissue distribution and subcellular localization of mLysoPAFAT/LPCAT2.** *A*, the expression levels of mLysoPAFAT/LPCAT2 and β-actin mRNA in 20 mouse tissues were analyzed by quantitative RT-PCR, and the levels of mLysoPAFAT/LPCAT2 mRNA were normalized to those of β-actin mRNA in each tissue. The highest level of mLysoPAFAT/LPCAT2 expression was observed in resident macrophages. *B*, CHO-K1 cells were transfected with FLAG-mLysoPAFAT/LPCAT2 and subjected to immunocytochemical analysis 48 h post-transfection. ER-Golgi and FLAG-mLysoPAFAT/LPCAT2 were visualized using $DiOC_6(3)$ (*green*) and the M5 anti-FLAG peptide antibody (*red*), respectively. The subcellular distribution pattern of FLAG-mLysoPAFAT/LPCAT2 was similar to that of $DiOC_6(3)$ (*Merge*), suggesting that the enzyme is present mainly in the ER and Golgi. $DiOC_6(3)$ is an ER-Golgi marker. The *scale bars* correspond to 20 μm. Results are representative of two independent experiments with similar results.

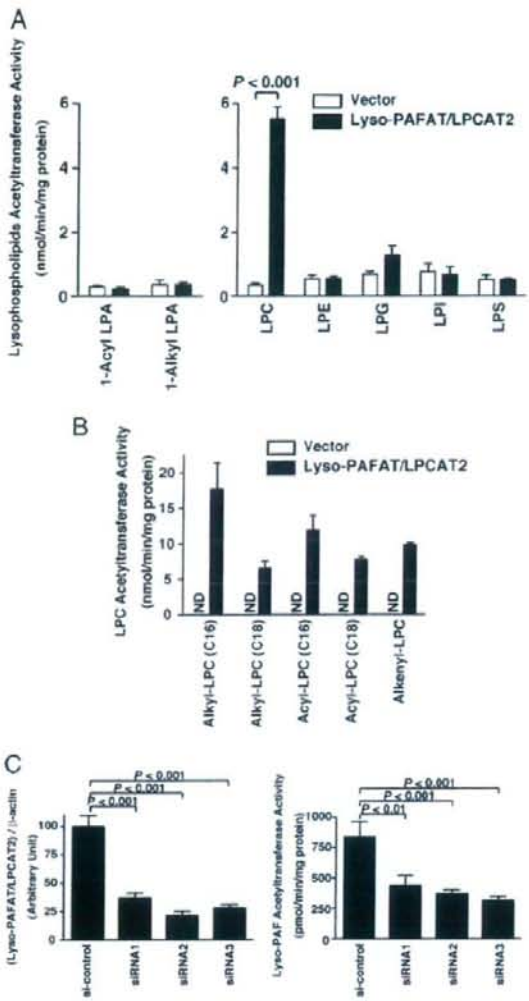

FIGURE 3. **Substrate selectivity of mLysoPAFAT/LPCAT2 and siRNA transfection with hLysoPAFAT/LPCAT2 siRNAs.** *A*, lysophospholipid acetyltransferase assays were performed by TLC with 20 μM lysophospholipid (1-acyl- and 1-alkyl-LPA, LPC, lysophosphatidylglycerol (*LPG*), lysophosphatidylethanolamine (*LPE*), lysophosphatidylinositol (*LPI*), or lysophosphatidylserine (*LPS*)) and 100 μM [^{3}H]acetyl-CoA in the presence of 1 μg of the microsomal fractions from vector-transfected (*open bars*) or mLysoPAFAT/LPCAT2-transfected (*closed bars*) cells. 1-*O*-Alkenyl-LPC (*heart*), LPI (*liver*), and LPS (*brain*) were from bovine tissues. Other lysophospholipids contained a palmitoyl group at the *sn*-1 position. *B*, several LPC (1-acyl-LPC C16, C18, 1-alkyl-LPC C16, C18, and 1-alkenyl-LPC) acetyltransferase activities were measured. mLysoPAFAT/LPCAT2 possessed lysophospholipid acetyltransferase activities toward 1-*O*-alkyl-LPC, 1-acyl-LPC, and 1-*O*-alkenyl-LPC. *ND*, not detectable. *C*, three hLysoPAFAT/LPCAT2 siRNAs and a control siRNA were transfected into HEK293 cells. After 48 h, mRNA levels (*left*) and lyso-PAF acetyltransferase activities (*right*) were measured, as described under "Experimental Procedures." Endogenous lyso-PAF acetyltransferase activity was reduced by each hLysoPAFAT/LPCAT2 siRNA. The data represent the mean ± S.D. of triplicate measurements. Statistical analyses were performed by analysis of variance and Tukey's multiple comparison test ($p < 0.01$). Two independent experiments were performed with similar results.

increased by LPS stimulation, but the effect was decreased in the presence of SB 203580; the acyltransferase activity of the enzyme was unchanged (Fig. 5). The endogenous lyso-PAF acyltransferase in RAW264.7 cells was activated by LPS, and this activation was blocked in the presence of SB 203580 (data not shown).

Induction of mLysoPAFAT/LPCAT2 mRNA—Next, we examined induction of mLysoPAFAT/LPCAT2 mRNA in response to long term treatment with Toll-like receptor agonists. Mouse thioglycollate-induced macrophages were treated with LPS (a TLR4 ligand), ODN1826 (a TLR9 ligand), or poly(I:C) (a TLR3 ligand) for 16 h in the presence or absence of DEX or estradiol-17β. As shown in Fig. 6*A*, the endogenous lyso-PAF acetyltransferase activity was enhanced 2.4- and 2.2-fold by LPS and ODN1826 treatment, respectively. Moreover, augmentation of the enzyme activity by LPS treatment was suppressed in the presence of DEX but not estradiol-17β. Similar results were obtained for the LPC acyltransferase activity (data not shown). The enzyme activation by ODN1826 also

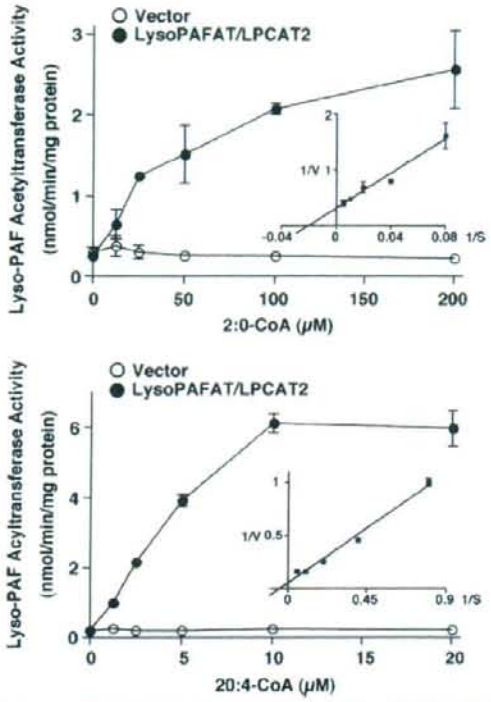

FIGURE 4. **Acetyltransferase and acyltransferase activities of mLysoPAFAT/LPCAT2.** Lyso-PAF acetyltransferase and acyltransferase assays were performed by TLC with the indicated concentrations of acetyl-CoA (2:0-CoA) and arachidonoyl-CoA (20:4-CoA) using 20 μM lyso-PAF. The *inset* shows a Lineweaver-Burk plot to calculate K_m values. The data represent the mean ± S.D. of triplicate measurements. The results are representative of two independent experiments with similar results.

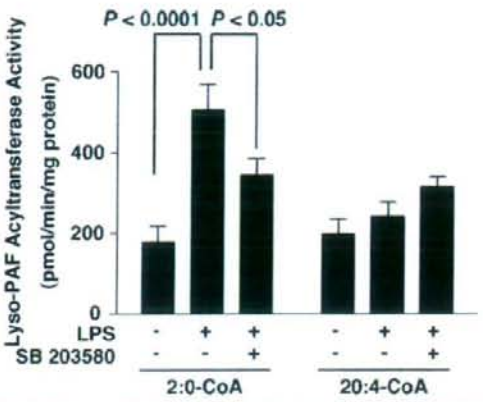

FIGURE 5. **Enzyme activation by an inflammatory stimulus.** RAW264.7 cells transfected with vector or mLysoPAFAT/LPCAT2 were stimulated with 100 ng/ml LPS in the presence or absence of SB 203580, and enzyme activity assays were performed subsequently. The difference between the activities obtained with vector and mLysoPAFAT/LPCAT2 corresponded to transfected enzyme activity. Endogenous acetyltransferase activities of nonstimulation, LPS stimulation, and LPS stimulation with SB203580 were 300.2, 469.0, and 259.4 pmol/min/mg protein, respectively. Endogenous acyltransferase activities were 604.0, 900.2, and 572.2 pmol/min/mg protein. Statistical analyses were performed by using analysis of variance with Fisher's projected least significant difference (PLSD) test ($p < 0.05$). The results are expressed as mean ± S.E. of three independent experiments, each performed in triplicate.

tended to be reduced by DEX. Poly(I:C) did not affect the lyso-PAF acetyltransferase activity in macrophages.

The mLysoPAFAT/LPCAT2 mRNA levels were increased 7.3- and 4.8-fold by LPS and ODN1826 treatment, respectively. Furthermore, induction of mLysoPAFAT/LPCAT2 by LPS was repressed by DEX treatment (Fig. 6*B*). ODN1826 also enhanced mLysoPAFAT/LPCAT2 levels, which tended to be reduced by DEX treatment. The expression level of mLysoPAFAT/LPCAT2 was not changed by poly(I:C) treatment, similar to the lack of effect on the enzyme activity (Fig. 6*A*). Induction of an IFNγ-inducible gene (IP-10) used as a positive control was observed by PCR after poly(I:C) stimulation under these conditions (data not shown).

DISCUSSION

This is the first report of isolation of a cDNA for LysoPAFAT/LPCAT2, a critically important enzyme in the biosynthesis of PAF. After long term treatments with a TLR4 or TLR9 agonist, the expression level of mLysoPAFAT/LPCAT2 mRNA was up-regulated, but not with a TLR3 agonist. Surprisingly, this enzyme catalyzed not only PAF biosynthesis (lyso-PAF acetyltransferase) but also generation of membrane glycerophospholipids (LPC acyltransferase), which are major membrane constituents and precursors of PAF. In the resting conditions, the enzyme prefers arachidonoyl-CoA to produce membrane lipids. However, under acute inflammatory stimulation by activating the TLR4, only the acetyltransferase activity of the enzyme was enhanced, and PAF production was augmented (Fig. 7).

Characterization of Lyso-PAF Acetyltransferase—mLysoPAFAT/LPCAT2 possessed lyso-PAF acetyltransferase activity but did not show 1-alkyl-LPA acetyltransferase activity, which catalyzes the first step of the *de novo* PAF biosynthesis pathway (Fig. 3, *A* and *B*) (2, 4). Using a heterologous overexpression system, we found that the enzyme was predominantly localized to the ER-Golgi complex (Fig. 2*B*). The exact localization of the endogenous protein in native cells remains to be determined. Additionally, LysoPAFAT/LPCAT2 possessed putative EF-hand-like motifs and showed calcium-dependent activity. These correlations remain to be further clarified. Using hLysoPAFAT/LPCAT2 siRNA transfection of HEK293 cells, both the mRNA and the enzyme activity of endogenous lyso-PAF acetyltransferase were significantly reduced (Fig. 3*C*), indicating that LysoPAFAT/LPCAT2 constitutes a major (50–80%) lyso-PAF acetyltransferase, in HEK293 cells. It is possible that other enzyme(s) are present also to catalyze PAF production in other tissues and cells.

LPC Acyltransferase Activity of LysoPAFAT/LPCAT2—mLysoPAFAT/LPCAT2 exhibited not only significant lyso-PAF acetyltransferase activity but also LPC acyltransferase activity (Fig. 4) *in vitro*, indicating that mLysoPAFAT/LPCAT2 can produce both PAF and PC. These data agree well with the previous studies showing that the lyso-PAF acetyltransferase activity in neutrophils was competed by long chain acyl-CoAs (40, 41). Recently, we and another group identified an LPC remod-

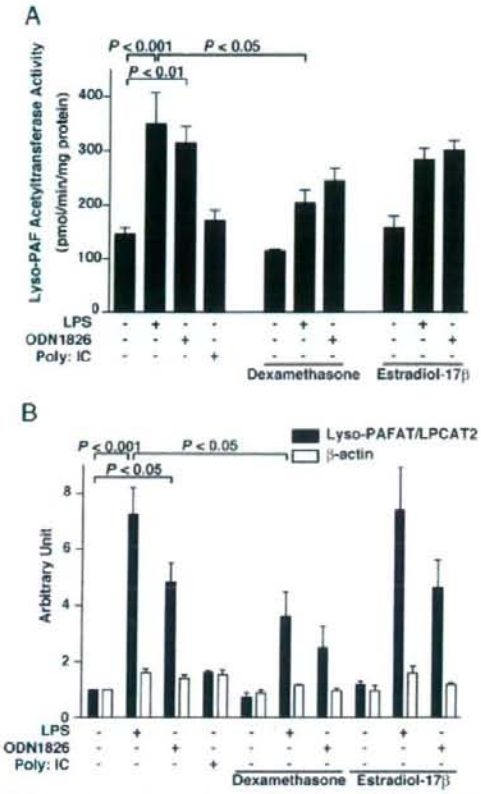

FIGURE 6. **Induction of LysoPAFAT/LPCAT2 by microbial components.** Thioglycollate-induced mouse macrophages were treated with 100 ng/ml LPS, 0.8 μM ODN1826, or 1 μg/ml poly(I:C) for 16 h in the presence or absence of 100 nM DEX or 100 nM estradiol-17β. Lyso-PAF acetyltransferase activity (*A*) and expression of mLysoPAFAT/LPCAT2 mRNA (*B*) were analyzed. The *open bars* indicate β-actin as a control. Statistical analyses were performed by analysis of variance and Tukey's multiple comparison test ($p < 0.05$). The results are expressed as mean ± S.E. of three independent experiments.

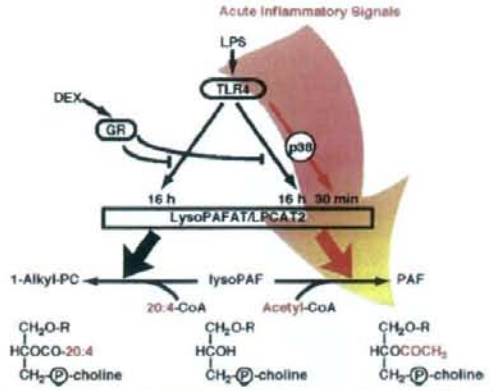

FIGURE 7. **Regulation of LysoPAFAT/LPCAT2 in mouse macrophages by inflammatory signals.** LysoPAFAT/LPCAT2 activity is increased by two distinct pathways in mouse macrophages. Although LysoPAFAT/LPCAT2 catalyzes both acetyltransferase and acyltransferase, only acetyltransferase activity was enhanced under acute inflammatory conditions. *GR*, glucocorticoid receptor; *p38*, p38 mitogen-activated protein kinase; *20:4-CoA*, arachidonoyl-CoA. See the first paragraph under "Discussion" for details.

eling enzyme, designated LPCAT1, which is highly expressed in lung (27, 28). In contrast, LysoPAFAT/LPCAT2 is predominantly expressed in inflammatory cells with modest expression in skin, brain, and colon. Because PC is biosynthesized in all cell types, a different class of LPCATs may exist in addition to LPCAT1 (27, 28) and LysoPAFAT/LPCAT2 (present study).

An Acute Inflammatory Response—Upon acute inflammatory stimulation by LPS, the acetyltransferase activity of mLysoPAFAT/LPCAT2 was enhanced (Fig. 5). Similar activation has been observed with endogenous lyso-PAF acetyltransferase in mouse peritoneal macrophages (11). Nixon *et al.* reported that lyso-PAF acetyltransferase in human neutrophils is directly activated by p38 mitogen-activated protein kinase (42). It is possible that LysoPAFAT/LPCAT2 is modified and activated by phosphorylation after LPS-stimulation. In contrast, the LPC acyltransferase activity of the enzyme was not changed by short term LPS stimulation (Fig. 5). The cellular signaling pathway mediating LysoPAFAT/LPCAT2 activation by LPS remains to be elucidated. In addition to putative conformational changes of the enzyme, both enzyme activities may be regulated by the ratio of two substrates (acetyl-CoA and arachidonoyl-CoA) in cells.

Induction of LysoPAFAT/LPCAT2 by Bacterial Components—Bacterial infections increase PAF production (1, 43, 44), and a TLR4 agonist (LPS) and a TLR9 agonist (ODN1826) induced the enzyme activity and mRNA levels of LysoPAFAT/LPCAT2. LPS activates both MyD88 (myeloid differentiation primary response gene 88) and TRIF (Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β) adaptor protein (45–47). These results suggest that LysoPAFAT/LPCAT2 expression is regulated in a MyD88-dependent manner in the innate immune system, because TLR9 and TLR3 mediate MyD88- and TRIF-dependent pathways, respectively (45, 48, 49). Induction of mLysoPAFAT/LPCAT2 was attenuated by DEX treatment (Fig. 6). Ogawa *et al.* (50) reported that glucocorticoid receptor signaling represses LPS-mediated up-regulation of a large set of related inflammatory response genes, such as cyclooxygenase-2 and interleukin-6. Furthermore, DEX inhibits LPS-induced plasma PAF release (51). It will be important to determine the transcriptional regulation and cis-elements of LysoPAFAT/LPCAT2 in future studies.

LPS-induced accumulation of LysoPAFAT/LPCAT2 increases acyltransferase activity in addition to the acetyltransferase activity. The biological significance of up-regulation of the acyltransferase activity may be related to the fact that under long term LPS stimulation, cytosolic and secretory phospholipase A_2 are activated, leading to increased release of free fatty acids and lysophospholipids from PC (52, 53). Lysophospholipids are toxic to cells because of their detergent effects. Alternatively, active membrane remodeling is required during inflammatory responses, such as phagocytosis or chemotaxis. LysoPAFAT/LPCAT2 may play an important role in the regulation of lysophospholipid and PAF levels and in the storage of PC as PAF precursor membrane glycerophospholipids.

Conclusion—We have isolated a new enzyme that catalyzes PAF production and membrane biogenesis (LysoPAFAT/LPCAT2). Further studies are needed to elucidate the roles of mLysoPAFAT/LPCAT2 *in vivo* and to determine its potential as a novel therapeutic target for various diseases involving PAF biosynthesis. It will be important to characterize both acetyltransferase and acyltransferase activities of the enzyme, including identification of binding sites for each substrate (acetyl-CoA and arachidonoyl-CoA) and differential regulation of individual enzyme activity. Molecular cloning and characterization of this first LysoPAFAT/LPCAT2 will enable us to better understand the biochemical mechanisms underlying PAF and phospholipid biosynthesis in inflammatory cells.

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