Degradation of platelet-activating factor (PAF) was examined by incubating PAF with macrophages from PAF receptor-deficient mice. The degradation rate was halved as compared with wild-type mice. The reduction of the rate was comparable with the presence of a PAF antagonist WEB 2086 in wild-type cells. PAF was internalized rapidly ($t_{1/2} \approx 1$ min) into wild-type macrophages. The PAF internalization was inhibited by the treatment of 0.45 M sucrose but was not affected by phorbol 12-myristate 13-acetate, suggesting that PAF internalizes into macrophages with its receptor in a clathrin-dependent manner. Internalized PAF was degraded into lyso-PAF with a half-life of 20 min. Treatment of concanavalin A inhibited the conversion of PAF into lyso-PAF, suggesting that uptake of PAF enhances PAF degradation. Lyso-PAF was subsequently metabolized into 1-alkyl-2-acetyl-phosphatidylcholine. In addition, release of PAF acylethanolamide from macrophages was enhanced when wild-type macrophages were stimulated with PAF but not from macrophages of PAF receptor-deficient mice. Thus, the PAF stimulation of macrophages leads to its degradation through both intracellular and extracellular mechanisms.

Platelet-activating factor (PAF),$^1$ 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a lipid mediator with versatile biological activities including platelet activation, leukocyte activation, airway constriction, and vascular hyperpermeability (see reviews in Refs. 1–3). PAF is thought to play an important role in pathological processes, such as inflammation and allergic disorders, through its G-protein-coupled receptor (GPCR) (4–8). Recent studies of PAF receptor-deficient (PAFR-KO) mice have revealed that PAF is involved in anaphylactic shock (9) and acute lung injury (10).

Degradation of PAF has been extensively studied in various cell types and tissues. PAF is degraded by PAF acylethanolamidases (PAF-AHs), isozymes with a Ca$^{2+}$-independent phospholipase $A_2$ ($PLA_2$) activity, which remove the acetyl moiety at the sn-2 position of PAF (11–13). The PAF inactivation is an important process because pathological conditions may deteriorate with an excess amount of PAF. In fact, plasma-type PAF-AH deficiency worsens respiratory symptoms in asthmatic children (14) and stroke (15).

In response to ligand stimulation, many GPCRs such as $\beta_2$-adrenergic receptor, muscarinic receptor, angiotensin II receptor, and substance P receptor (16–19) internalize into cells with their ligands. Upon stimulation, PAF receptor also internalizes with clathrin-coated vesicles (20, 21). Internalized PAF receptor is thought to move into early endosomes and to be recycled to cell surface membrane (20). PAF receptor internalization and the subsequent processes are felicitous to desensitization and resensitization of the receptor (20). The intracellular movement of the ligand-receptor complex is thought to have an important role in ligand degradation as reported previously for low density lipoprotein (22) or peptide ligands (19, 23). However, no evidence has yet been provided for various lipid mediators including PAF.

The aim of present study is to reveal the role of the PAF receptor in PAF degradation and subsequent metabolic pathways. Murine peritoneal macrophages rich in both PAF receptor and PAF production were used (9, 24, 25). We employed a method to quantify the internalization of a lipid ligand with its receptor by washing cells with an acidic buffer containing 1% bovine serum albumin (BSA). PAFR-KO mice provide us a useful method for examining the precise role of PAF receptor in PAF metabolism. We demonstrate here that PAF receptor internalization and receptor-dependent exocytosis of PAF-AH are important for the PAF degradation in macrophages.

EXPERIMENTAL PROCEDURES

Materials—PAF C-16 (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) and lyso-PAF C-16 (1-O-hexadecyl-sn-glycero-3-phosphocholine) were obtained from Cayman (Ann Arbor, MI). PAF antagonist WEB 2086 was a generous gift from Boehringer Ingelheim (Ingelheim, Germany). [Acetyl-$^3$H]PAF C-16 (370 GBq/mmol), [alkyl-$^3$H]PAF C-16 (2157 GBq/mmol), and [H]$^3$WEB 2086 (703 GBq/mmol) were purchased from PerkinElmer Life Sciences. Phosphatidylcholine (PC) from egg yolk, phenylarsine oxide (PAO), and concanavalin A (ConA) were purchased from Sigma. BSA (very low endotoxin, fatty acid-free) and thioglycollate were from Serological Proteins (Kankakee, IL) and Difco, respectively. A rabbit serum against guinea pig plasma-type PAF-AH was a kind gift from Dr. K. Karasawa (Teikyo University, Kanagawa, Japan). Monoclonal anti-PAF-AH I and anti-PAF-AH II antibodies were kind gifts from Drs. H. Arai and J. Aoki (University of Tokyo, Japan). The ECL Western blotting analysis system was from Amersham Biosciences, Inc. All other reagents, unless otherwise stated, were of analytical grade and were from Wako (Osaka, Japan) or Sigma.

Cell Preparation and Culture—Specific pathogen-free C57BL/6 mice (8–12 weeks old) were purchased from Charles River Japan (Tokyo, Japan) and bred in our institution. The mice were housed under barrier conditions with free access to food and water. The experimental procedures involving animal use were approved by the Tokyo Metropolitan Institute of Medical Sciences, Inc. and conformed to the Guide for the Care and Use of Laboratory Animals (Japan).
Receptor-dependent Metabolism of PAF in Murine Macrophages

In the experiments with PAFR-KO mice (8–16 weeks old), sex- and age-matched wild-type (WT) mice were used as controls. Mice back-crossed seven times with each with C57BL/6 were used for experiments. Peritoneal exudate macrophages were obtained by washing the peritoneal cavity three times each with 2 ml of ice-cold phosphate-buffered saline 3 days after intraperitoneal injection of 2 ml of sterile 4% thiglycollate. After centrifugation at 250 × g, the cells were suspended in RPMI 1640 (Sigma) supplemented with 100 international units/ml penicillin, 100 μg/ml streptomycin (Roche Molecular Biochemicals), and 10% heat-inactivated fetal bovine serum (Sigma). They were cultured in 12-well (1 × 10^6 cells/well) or 24-well plates (0.5 × 10^6 cells/well) in 5% CO_2 at 37°C. After overnight incubation, non-adherent cells were removed by washing three times with phosphate-buffered saline. Cells were incubated for 2 h at 37°C with Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3, 5.6 mM d-glucose, 0.49 mM MgCl_2, and 0.37 mM NaH_2PO_4) containing 10 mM Hepes-NaOH (pH 7.4) and 0.1% of BSA (Hepes/Tyrode/BSA) before assay. The numbers of cells recovered from PAFR-WT (3.7 ± 0.9 × 10^6 cells/mouse, mean ± S.D., n = 4) and -KO (3.7 ± 0.4 × 10^6 cells/mouse, n = 4) mice were not significantly different.

**PAF Degradation Assay**—Macrophages seeded onto 24-well plates were incubated with 2 nM [acetyl-^3H]PAF in Hepes/Tyrode/BSA for 30–120 min at 37°C. The medium was recovered, and the lipids were extracted by the Bligh and Dyer method (26). The radioactivity in the aqueous phase was regarded as PAF degradation products. The radioactivity was counted with an LS 6500 scintillation system (Beckman Instruments) and a liquid scintillation mixture (Atomlight; Packard, Meriden, CT).

**Ligand Binding to PAF Receptor**—Macrophages seeded onto 12-well plates were incubated with different concentrations of [acetyl-^3H]PAF in Hepes/Tyrode/BSA at 4°C for 1 h. After washing three times with the same buffer at 4°C, the cells were lysed with 5% Triton X-100, and the radioactivity was counted. Nonspecific binding was determined in the presence of 1 μM unlabeled PAF. Macrophages were also incubated with different concentrations of [H]WEB 2086 at 25°C for 1 h to determine WEB 2086 binding to macrophages in the presence or absence of 10 μM WEB 2086.

**Receptor-mediated PAF Internalization**—Macrophages seeded onto 12-well plates were incubated with 2 nM [acetyl-^3H]PAF in Hepes/Tyrode/BSA at 4°C for 1 h. After washing three times with the same buffer at 4°C, the cells were incubated at 37°C in Tyrode's buffer containing 10 mM Hepes-NaOH (pH 7.4) (Hepes/Tyrode's) for 1, 2.5, 5, and 10 min and then washed twice for 15 min with 200 mM sodium acetate buffer (pH 4.5) containing 25 mM NaCl and 1% BSA at 4°C to remove PAF bound to the cell surface receptors. This acid wash procedure was used to separate cell-surface (acid-sensitive) PAF from internalized (acid-resistant) PAF. After the cells were washed, they were lysed with 5% Triton X-100 containing 50 mM NaOH to measure the internalized PAF. At each time, the radioactivity of both the cell surface PAF and the internalized PAF was counted. Inhibitors for receptor internalization were added 20 min before incubation with [acetyl-^3H]PAF. Although ConA (250 μg/ml) or sucrose (0.45 M) were present in the medium throughout the assays, PAO (80 μM) was removed before the incubation with [acetyl-^3H]PAF.

**Lipid Extraction and Thin Layer Chromatography**—Macrophages seeded onto 12-well plates were incubated with 2 nM [acetyl-^3H]PAF in Hepes/Tyrode/BSA at 4°C for 1 h. After washing three times with the same buffer at 4°C, the cells were incubated at 37°C with Hepes/Tyrode's for 10, 20, 30, and 60 min. At each time, cells were scraped off from the plates by a cell lifter (Corning, Corning, NY) in 50 μM acetic acid in methanol/water (1:2, v/v) (27). Total lipids were obtained from the cells by the Bligh and Dyer method (26) and developed on thin layer chromatography plates (20 × 20 cm Silica gel 60; Merck) in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, v/v) (28). PAF, lypo-PAF, and PC were used as authentic markers. The spots corresponding to PAF, lypo-PAF, and PC were scraped off. Each scraped silica gel was mixed with 30 ml of a liquid scintillation mixture, Atomlight, to determine the amount of acetyl moieties liberated from PAF.

**Release of Metabolites**—Macrophages seeded onto 12-well plates were incubated with 2 nM [acetyl-^3H]PAF or [acetyl-^3H]PAF in Hepes/Tyrode/BSA at 4°C for 1 h. After washing three times with the same buffer at 4°C, the cells were incubated at 37°C with Hepes/Tyrode's for 5, 10, 30, 60, 90, and 120 min. At each time the medium was recovered, and its radioactivity was counted.

**PAF-AH Assay**—Mouse blood samples were obtained from the femoral artery and vein and were placed at room temperature to form clots for 1 h. Sera were prepared by centrifugation at 2,500 × g for 10 min at room temperature. PAF-AH assays were performed under the same conditions as reported previously with minor modifications (11). Briefly, the serum was diluted to 50 μl and incubated at 37°C for 30 min with 0.20 or 0.50 mM Tris- HCl (pH 7.4) containing 5 mM EDTA and 100 μM [acetyl-^3H]PAF. The reaction was stopped by adding 5.5 ml of chloroform/methanol/acetic acid/water (50:25:8:4, v/v) and 0.25 ml of water. The radioactivity of an aliquot (0.6 ml) of each aqueous phase was measured with a liquid scintillation mixture, Atomlight, to determine the amount of acetyl moiety liberated from PAF.

For the detection of PAF-AH activity released from macrophages, macrophages seeded onto 6-well plates were stimulated with 2 or 10 nM PAF for 60 min, the medium was collected, and its PAF-AH activity was measured. All assay conditions were the same as those of the serum PAF-AH assay, except for the concentration of [acetyl-^3H]PAF (10 μM) and the incubation time (20 min).
Macrophages were incubated with 2 nM \([alkyl-^3H]\) PAF at 4 °C for 1 h and washed with Heps/Tyrode's/BSA. Upon incubation at 37 °C, PAF internalization was initiated. At each time, the PAF remaining bound to the cell surface receptors was recovered with an acidic buffer containing 1% BSA, and cells were lysed with 5% Triton X-100 to quantify PAF internalization. Cell-surface PAF is expressed as a percentage of the total PAF, i.e. the sum of radioactivity of the acid-washable (cell surface) PAF and the acid-resistant (internalized) PAF. The PAF binding varied with each treatment: control (18,800 dpm), ConA (8,767 dpm), PAO (7,495 dpm), sucrose (4,090 dpm), and phorbol 12-myristate 13-acetate (PMA) (10,311 dpm). Data are the means of triplicate samples and are representative of two independent experiments that gave similar results. Because S.D. values are within the symbol size, error bars are omitted. The values from the treatment of ConA, PAO, or sucrose are significantly different at 1, 2.5, 5, and 10 min as compared with the control (*, \(p < 0.0001\) versus each group by analysis of variance with Fisher’s test).

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—**
Macroglides were homogenized in phosphate-buffered saline and applied to 12% SDS-polyacrylamide gels. The proteins were transferred to polyvinylidine difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were then washed once with Tris-buffered saline (pH 8.0) containing 0.05% Tween 20 (TBS-T) and then blocked with nonfat milk (BlockAce; Dainippon Medical, Osaka, Japan) at 4 °C overnight. After washing the membranes with TBS-T, a rabbit serum against guinea-pig plasma-type PAF-TH was added at a dilution of 1:1,000 in TBS-T and incubated for 1 h at room temperature. Then, the membranes were washed three times with TBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences, Inc.) (1:5,000 dilution) in TBS-T for 1 h at room temperature. After three washes, the protein bands were visualized using an ECL Western blot analysis system. For the detection of PAF-AH II, anti-PAF-AH II monoclonal antibody and horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Inc.) were used.

**Statistics—**Statistical analysis was performed using StatView (version 4.0) software (SAS Institute, Cary, NC). A p value less than 0.05 was considered to be statistically significant.

**RESULTS**

**PAF Receptor-dependent PAF Degradation by Peritoneal Macrophages—**We analyzed PAF degradation activity of peritoneal macrophages obtained from PAFR-WT and -KO mice. When cells were incubated with 2 nM \([acetyl-^3H]\) PAF, the aqueous degradation products of PAF, i.e. acetate or its derivatives, gradually accumulated in the culture medium in a time-dependent manner (Fig. 1). After a 120-min incubation, about 50% of the total PAF added had been degraded by WT cells. In PAFR-KO macrophages, PAF degradation was significantly slower as compared with WT cells. At 60 min, PAF degradation by KO cells was almost half that by WT cells. Under cell-free conditions, PAF was not degraded for at least 120 min.

To confirm whether the reduced PAF inactivation in KO cells was specific, we examined the effects of a PAF antagonist WEB 2086. In the presence of 10 μM WEB 2086, PAF degradation activity in WT cells was decreased to the same level as observed in KO cells (Fig. 1). In KO cells, WEB 2086 was without further effects on PAF degradation. These results suggest that PAF degradation by murine macrophages is accelerated by PAF receptor.

**Rapid Internalization of PAF—GPCRs are known to internalize by ligand stimulation.** Thus, we examined the internalization of PAF and PAF receptor in macrophages at 37 °C after treating with \([alkyl-^3H]\) PAF for 1 h at 4 °C. The number of cell surface receptors (\(B_{max}\)) observed at 4 °C using \([alkyl-^3H]\) PAF (Fig. 2) was nearly equal to that at room temperature using \([^3H]\) WEB 2086 (data not shown), showing that PAF binding to PAF receptor was not blocked at 4 °C. As previously reported for some GPCRs, ligands that bind to the cell-surface receptors are released by acidic pH treatment (19). Once the ligands internalize with receptors, they become tolerant to the acid-washing procedures. Therefore, PAF-PAF receptor complexes remaining on the cell surface are separated effectively by washing with sodium acetate buffer (pH 4.5) containing 1% BSA. Most of the cell-associated PAF (91 ± 0.04%, \(n = 3\)) was recovered by acid treatment at 0 min (Fig. 3). However, within 1 min after the temperature-shift to 37 °C, about half of PAF transferred to acid-resistant fraction (Fig. 3). This demonstrates that PAF together with PAF receptor internalized very rapidly into cells. When cells were pretreated either with ConA, PAO, or hypertonic shock with 0.45 M sucrose to block the receptor internalization, the rates of PAF internalization were significantly decreased (Fig. 3). Phorbol 12-myristate 13-acetate, known to inhibit caveola-mediated processes, had no effect on the rates of internalization (Fig. 3). These results suggest that PAF internalize with PAF receptor in a clathrin-dependent pathway.

**Metabolic Fate of PAF after Binding to PAF Receptor—**To examine the destiny of internalized PAF, we analyzed the metabolic fate of PAF after binding to its specific receptor. Degraded products of \([alkyl-^3H]\) PAF were extracted and separated by thin layer chromatography, and the radioactivity was counted. Fig. 4A shows the time course of PAF degradation in macrophages. Once internalized, PAF was initially degraded into lyso-PAF, which was subsequently converted into 1-alkyl-2-acyl PC by acylation at the sn-2 position. The percentage of lyso-PAF was maintained in equilibrium at around 20% of total \([alkyl-^3H]\) PAF metabolites between 10 and 60 min. The PAF degradation in the cells was a slower reaction (\(t_{1/2} \sim 20\) min as compared with the PAF internalization (\(t_{1/2} \sim 1\) min). To examine whether the receptor-mediated PAF internalization has a significant role on intracellular PAF metabolism, PAF degra-
Receptor-dependent Metabolism of PAF in Murine Macrophages

SERUM PAF-AH ACTIVITY FROM WT AND KO MICE

Serum PAF-AH activities from WT and KO mice were measured (Table I). PAF-AH activity in the serum from WT mice was almost the same as that from KO mice. Thus, PAF receptor does not appear to regulate the PAF-AH activity in blood at least under physiological conditions.

CHARACTERIZATION OF PAF-AHS IN MACROPHAGES

The total cell lysates from PAFR-WT and -KO macrophages were subjected to SDS-polyacrylamide gel electrophoresis. A major protein band with a molecular mass of \( \approx 40 \) kDa was detected using an antiserum against plasma-type PAF-AH (Fig. 7A). Similar expression levels of plasma-type PAF-AH were observed in WT and KO macrophages. The observed molecular size appears to correspond to the size of non-glycosylated plasma-type PAF-AH (29). A protein band with a molecular mass of 42 kDa was detected using a mouse monoclonal anti-PAF-AH II antibody (Fig. 7B). The expression levels of PAF-AH II were similar in WT and KO macrophages. Thus, the intracellular and extracellular degradation of PAF in macrophages might be due to the PAF-AH II and the plasma-type PAF-AH, respectively.

Type I PAF-AH was not detected in murine macrophages by Western blot analysis (data not shown). It is, however, possible that other PLA\(_2\)s are also involved in PAF degradation.

DISCUSSION

As a result of agonist binding, many GPCRs undergo rapid internalization with their agonists. We have previously reported that PAF receptor internalization in Chinese hamster ovary cells overexpressing PAF receptors occurred with a \( t_{1/2} \) of about 30–40 min after 100 nM PAF stimulation (20). On macrophages, however, the internalization was much faster (\( t_{1/2} \approx 1 \) min), even with low concentrations of PAF (0.5–2.0 nM). These results suggest that the rates of receptor internalization vary with the cell types. This is consistent with studies on the agonist-induced \( \beta_2 \)-adrenergic receptor internalization, where the rates of internalization are different: T-lymphoma cell line (\( t_{1/2} \approx 1 \) min) (30), Chinese hamster ovary cells \( (t_{1/2} < 10 \) min) (31), HeLa cells \( (t_{1/2} > 30 \) min) (32), and human A431 cells \( (t_{1/2} > 30 \) min) (33). In the case of substance P receptor expressed in rat epithelial cells (19) and angiotensin II receptor in rat hepatocytes (34), the rates of receptor internalization (\( t_{1/2} \approx 2–3 \) min) are comparable with that of the PAF receptor internalization in macrophages shown in this study. Quantitative and qualitative differences may be present in the expression of some compo-
were stimulated with 2 or 10 nM PAF for 30 min, and PAF-AH activity measuring [3H]acetate liberated from PAF. Each experiment was done
macrophages from PAFR-WT mice were stimulated with 2 or 10 n M
kinase 3 or arrestin 2 was co-transfected in HEK 293 cells (35).
A2 receptor is accelerated when G protein-coupled receptor
components of the endocytic machinery between macrophages and
Chinese hamster ovary cells. Internalization of thromboxane
receptor (Fig. 3). It is likely that the receptor-bound
PAF is degraded intracellularly; PAF internalization (t½ ≈ 1
min) clearly preceded PAF degradation (t½ ≈ 20 min) (Figs. 3
and 4). Furthermore, when internalization was blocked by
ConA, PAF degradation was delayed (Fig. 4). Internalized PAF
was converted to acetic acid and lyso-PAF. The former was
liberated extracellularly (t½ ≈ 40 min), whereas the latter was
metabolized to 1-alkyl-2-acyl-PC intracellularly (Fig. 4A).
Finally, we found that PAF-AH release from macrophages
was enhanced by PAF stimulation (Fig. 6) and contributed to
receptor-dependent degradation of PAF (Fig. 1). Plasma-type
PAF-AH is expressed in macrophages (42). In HepG2 cells (43)
and rat hepatocytes (44), PAF has been shown to stimulate the
release of the plasma-type PAF-AH. On the other hand,
PAF-AH activity in serum was not significantly different between PAFR-WT and -KO mice under physiological conditions (Table I). Thus, although the base-line release of PAF-AH appears to be unaffected by PAF receptor, a difference in serum PAF-AH activity between PAFR-WT and -KO mice may be seen under pathological conditions such as endotoxemia that increases blood PAF levels (45). Western blotting showed that the expression levels of plasma-type PAF-AH and PAF-AH II were almost the same in PAFR-WT and -KO macrophages. Because we need a number of mice and cells, we were unable to identify the isoforms of PAF-AH in the medium by Western blot analysis. Roles of other PLA2s on the receptor-dependent degradation of PAF also remain to be clarified.

In conclusion, PAF receptor in macrophages significantly enhances PAF degradation by enhancing internalization of receptor-bound PAF and the release of PAF-AH. PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is released from inflammatory cells, including neutrophils, eosinophils, and monocytes, and is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1). PAF is synthesized at inflammatory sites by macrophages, neutrophils, and eosinophils, all of which also express PAF receptor (1).