Overall these results clearly demonstrate the ability of the exogenous addition of PAF to induce eosinophil recruitment *in vivo* in an eotaxin-dependent manner.

We have previously shown that eotaxin was released in the allergic pleurisy model and was greatly responsible for the eosinophil recruitment in response to antigen challenge (Klein *et al.*, 2001). Thus, it was of interest to examine whether endogenous production of PAF and action on PAF receptors could modulate the production of eotaxin and subsequent eosinophil recruitment in the allergic pleurisy in mice. Our results demonstrate that blockade of PAF receptors with UK-74,505 inhibited the recruitment of eosinophils in the allergic pleurisy model. Moreover, eosinophil recruitment in $PAFR^{-/-}$ animals was clearly lower than that observed in wild-type animals, albeit the inhibition observed was of lesser intensity than after UK-74,505 treatment. One distinct possibility that arises from these results is that chronic PAF receptor deficiency may trigger compensatory mechanisms not observed after acute treatment with the PAF receptor antagonist. These issues are being investigated in our laboratory at present.

Not only was eosinophil recruitment suppressed, but also the PAF receptor antagonist inhibited to a great extent the release of eotaxin after antigen challenge. Altogether, our results suggest that PAF is a major modulator of eotaxin production in the allergic pleurisy model and that blockade of eotaxin release may underlie the inhibitory effects of PAF receptor antagonists on the recruitment of eosinophils. Other studies have previously demonstrated the ability of PAF to modulate chemokine production by different cell types in response to a range of different stimuli (e.g. Maruoka *et al.*, 2000; Au *et al.*, 2001). However, this is, to the best of our knowledge, the first demonstration of the important role of PAF in modulating eotaxin release *in vivo*.

One final interesting observation was the synergistic effect of sub-threshold doses of PAF and eotaxin to induce the recruitment of eosinophils *in vivo*. These results are in agreement with our previous study demonstrating the ability of another lipid mediator, LTB_4, to cooperate with eotaxin to induce eosinophil recruitment *in vivo* (Klein *et al.*, 2001). In addition, one other study has also shown the synergistic effects of the administration of PAF and eotaxin on eosinophil recruitment (assessed as tissue content of eosinophil peroxidase) and airway hyperresponsiveness in the guinea-pig lung (Fukuyama *et al.*, 2000). One important suggestion that derives from these studies is that in an allergic reaction, smaller quantities of different mediators (e.g. PAF/LTB_4 and eotaxin) may be necessary and sufficient to mediate a full recruitment of inflammatory cells. Thus, although mediator redundancy does occur *in vivo*, a range of different mediators must cooperate to obtain a final adequate response, ie. eosinophil migration. The corollary of the latter affirmative is that blockade of one or other mediator may be sufficient to suppress the functional response observed. Thus and in addition to the coordinated (temporal) effects of mediator release (Lukacs *et al.*, 1999; Gonzalo *et al.*, 1998), mediator cooperation may explain the ability of distinct strategies to suppress completely eosinophil migration in several models of allergic inflammation.

In conclusion, the production of PAF in an allergic reaction could function in multiple ways to facilitate the recruitment and activation of eosinophils – by facilitating eotaxin release, by cooperating with eotaxin to induce greater recruitment of eosinophils (the present study), and by priming and activating the eosinophils which reached the tissues (van der bruggen *et al.*, 1994; Schweizer *et al.*, 1996; Liu *et al.*, 1998; Ishii & Shimizu, 2000). As eosinophils are thought to play a major role in allergic diseases and PAF appears to be a major regulator of eosinophil recruitment/function in experimental animals, it would be reasonable to suggest that PAF receptor antagonists would be an ideal therapeutic target for the treatment of these diseases. However, at least in the case of asthma, several clinical studies have failed to demonstrate a beneficial effect of PAF receptor antagonists (Kuitert *et al.*, 1995; Evans *et al.*, 1997; reviewed in Ishii & Shimizu, 2000). Having the latter trials in mind, it will be important to examine whether PAF receptor activation also plays a major role in the production of eotaxin (and other chemokines) following allergen challenge in other experimental models and in humans.

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Receptor-dependent Metabolism of Platelet-activating Factor in Murine Macrophages*

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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-acyl-phosphatidylcholine. In addition, release of PAF acetylhydrolase 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 acetylhydrolases (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 β_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 tool 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 [^{3}H]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,

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[1] The abbreviations used are: PAF, platelet-activating factor; PAF-AH, PAF acetylhydrolase; PAFR-KO, PAF receptor-deficient; BSA, bovine serum albumin; ConA, concanavalin A; GPCR, G-protein-coupled receptor; KO, knock-out; PAO, phenylarsine oxide; PC, phosphatidylcholine; PLA_2, phospholipase A_2; TBS-T, Tris-buffered saline (pH 8.0) containing 0.05% Tween 20; and WT, wild-type.

Japan). 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 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% thioglycollate. 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's/BSA) before assay. The numbers of cells recovered from PAFR-WT ($3.5 \pm 0.9 \times 10^7$ cells/mouse, mean ± S.D., $n = 4$) and -KO ($3.7 \pm 0.4 \times 10^7$ cells/mouse, $n = 4$) mice were not significantly different.

PAF Degradation Assay—Macrophages seeded onto 24-well plates were incubated with 2 nM [*acetyl*-^{3}H]PAF in Hepes/Tyrode's/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 [*alkyl*-^{3}H]PAF in Hepes/Tyrode's/BSA at 4 °C for 1 h. After washing three times with the same buffer at 4 °C, cells were lysed with 5% Triton X-100, and the radioactivity was counted. Nonspecific binding was determined in the presence of 1 μM PAF. Macrophages were also incubated with different concentrations of [^{3}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.

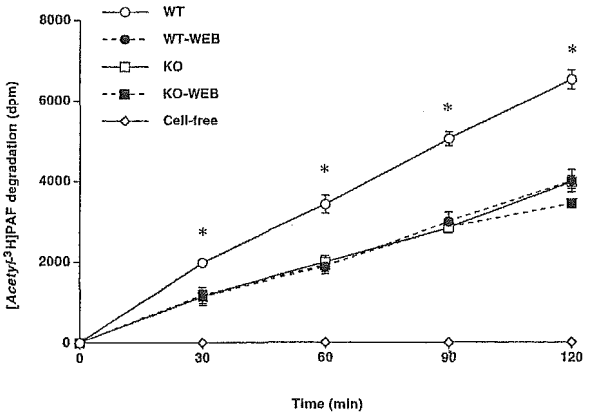

FIG. 1. **Time course of PAF degradation by macrophages from PAFR-WT and -KO mice.** Macrophages were incubated with 2 nM [*acetyl*-^{3}H]PAF. PAF degradation was measured by counting [^{3}H]acetic acid released into the medium. The concentration of WEB 2086 was 10 μM. In the absence of macrophages, PAF was not degraded. The results represent the means of triplicate samples. *Error bars* represent S.D. *, $p < 0.005$ *versus* other groups by unpaired *t* test. The data are the representative of four independent experiments that gave similar results.

Receptor-mediated PAF Internalization—Macrophages seeded onto 12-well plates were incubated with 2 nM [*alkyl*-^{3}H]PAF in Hepes/Tyrode's/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 were counted. Inhibitors for receptor internalization were added 20 min before incubation with [*alkyl*-^{3}H] 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 [*alkyl*-^{3}H]PAF.

Lipid Extraction and Thin Layer Chromatography—Macrophages seeded onto 12-well plates were incubated with 2 nM [*alkyl*-^{3}H]PAF in Hepes/Tyrode's/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 mM 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, lyso-PAF, and PC were used as authentic markers. The spots corresponding to PAF, lyso-PAF, and PC were scraped off. Each scraped silica gel was mixed with 10 ml of a liquid scintillation mixture, Atomlight, to determine the radioactivity.

Release of Metabolites—Macrophages seeded onto 12-well plates were incubated with 2 nM [*acetyl*-^{3}H]PAF or [*alkyl*-^{3}H]PAF in Hepes/Tyrode's/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 was 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 ml of 50 mM Tris-HCl (pH 7.4) containing 5 mM EDTA and 100 μM [*acetyl*-^{3}H]PAF. The reaction was stopped by adding 2.5 ml of chloroform/methanol (4:1, 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. After 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*-^{3}H]PAF (10 μM) and the incubation time (20 min).

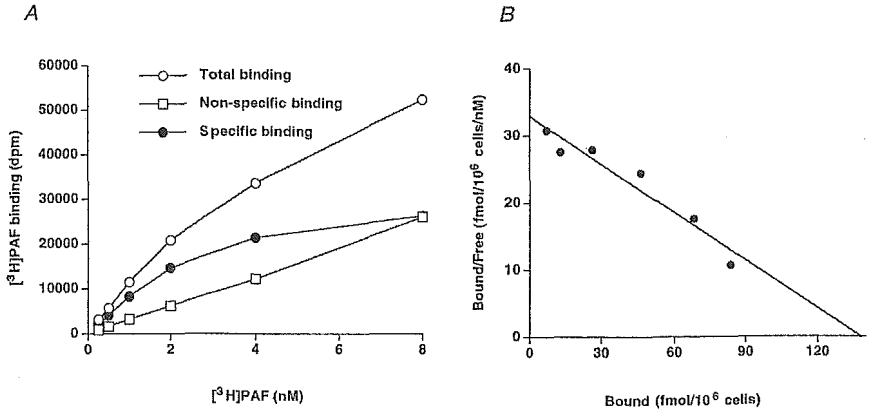

FIG. 2. **Ligand binding to PAF receptor.** *A*, macrophages were incubated for 1 h with [*alkyl*-^{3}H] PAF at 4 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled PAF. *B*, Scatchard analysis of specific [*alkyl*-^{3}H]PAF binding to macrophages. The K_d and B_{max} values are estimated as 4.2 nM and 8.3×10^4 sites/cell, respectively. The data are the means of duplicate samples.

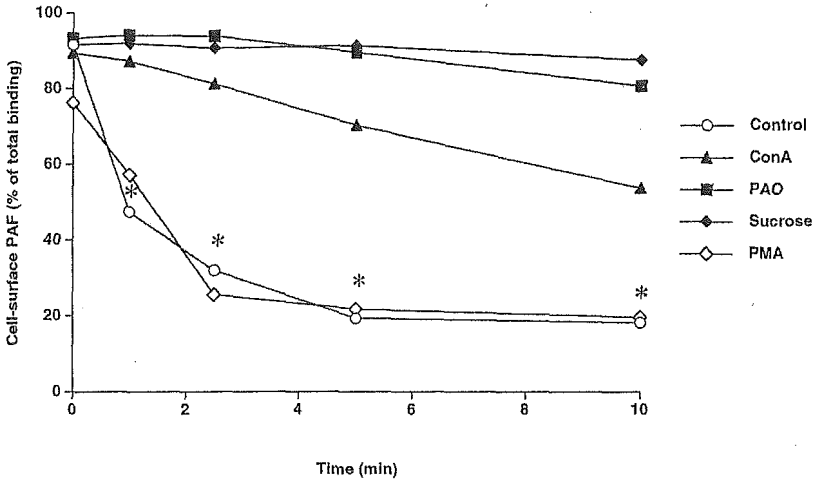

FIG. 3. **Internalization of PAF with PAF receptor.** Macrophages were incubated with 2 nM [*alkyl*-^{3}H]PAF at 4 °C for 1 h and washed with Hepes/Tyrode's/BSA. Upon incubation at 37 °C, PAF internalization was initiated. At each time, the PAF remaining bound to the cell surface receptors was recovered 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.* 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—Macrophages were homogenized in phosphate-buffered saline and applied to 12% SDS-polyacrylamide gels. The proteins were transferred to polyvinylidene 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 over night. After washing the membranes with TBS-T, a rabbit serum against guinea-pig plasma-type PAF-AH 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*-^{3}H]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 had no 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*-^{3}H]PAF for 1 h at 4 °C. The number of cell surface receptors (B_{max}) observed at 4 °C using [*alkyl*-^{3}H]PAF (Fig. 2) was nearly equal to that at room temperature using [^{3}H]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*-^{3}H]PAF were extracted and separated by thin layer chromatography, and the radioactivity was counted. Fig. 4*A* shows the time course of PAF degradation in macrophages. Once internalized, PAF was initially degraded to 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 about 20% of total [*alkyl*-^{3}H]PAF metabolites between 10 and 60 min. The PAF degradation in the cells was a slower reaction ($t_{1/2} \approx 20$ min) as compared with the PAF internalization ($t_{1/2} \approx 1$ min). To examine whether the receptor-mediated PAF internalization has a significant role on intracellular PAF metabolism, PAF degra-

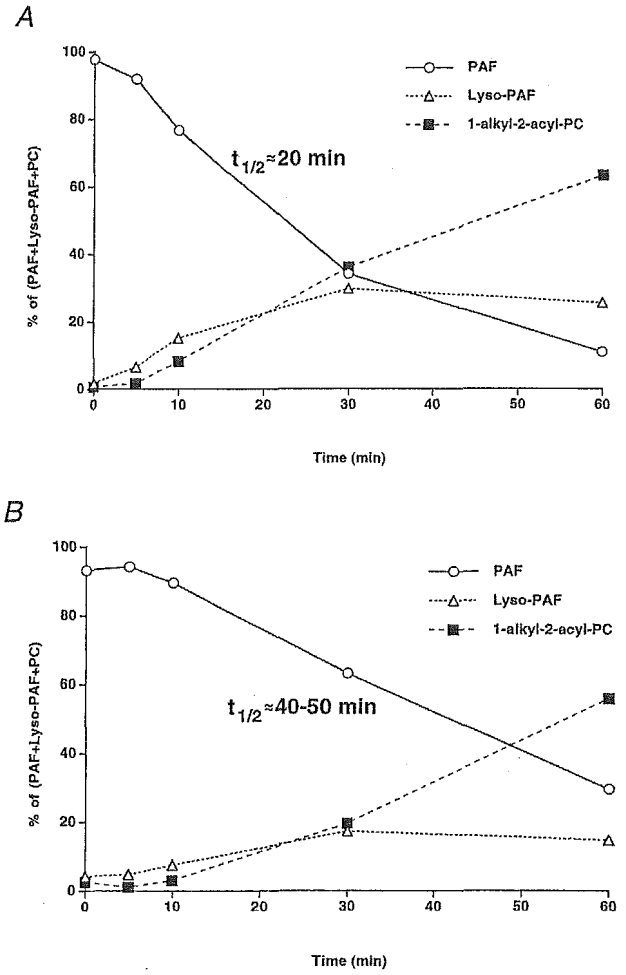

FIG. 4. **Intracellular metabolism of [*alkyl*-^{3}H]PAF.** Macrophages were incubated with 2 nM [*alkyl*-^{3}H]PAF at 4 °C for 1 h. After washing with Hepes/Tyrode's/BSA, cells were incubated at 37 °C. At each time, total lipids were extracted, and separated by thin layer chromatography. The relative radioactivity of PAF and the metabolites of PAF (lyso-PAF and PC) are expressed as the percent of the total. 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. *A* and *B*, time course of [*alkyl*-^{3}H]PAF metabolism in the absence (*A*) or presence (*B*) of ConA.

dation was measured using macrophages treated with ConA. The metabolic conversion of PAF to lyso-PAF and 1-alkyl-2-acyl PC was significantly decreased ($t_{1/2}$ ≈ 40–50 min) (Fig. 4*B*).

Release of the Acetyl Group from Macrophages—When macrophages were incubated with [*alkyl*-^{3}H]PAF, most of the internalized radioactivity was retained in the cells at least until 120 min (Fig. 5). On the other hand, about 90% of the internalized radioactivity of [*acetyl*-^{3}H]PAF was released into the medium at 120 min (Fig. 5). These observations imply that the acetyl group of PAF (either acetic acid or its derivatives), but not lyso-PAF or 1-alkyl-2-acyl PC, is released from macrophages.

PAF-induced Secretion of PAF-AH Activity—Macrophages are known to secrete plasma-type PAF-AH. Indeed, we observed the constitutive secretion of PAF-AH into the culture medium of WT macrophages, which was enhanced upon the stimulation with PAF (Fig. 6*A*). By contrast, PAF did not enhance the PAF-AH release for 30 min in PAFR-KO cells (Fig. 6*B*). The similar results were obtained when the medium was recovered after 60 min of stimulation (data not shown). The enhancement observed in WT cells was consistently blocked by the addition of WEB 2086 (data not shown). These results show that PAF-AH secretion is slightly but significantly increased

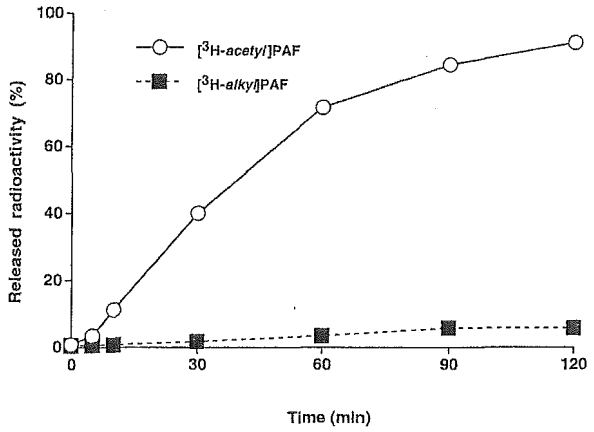

FIG. 5. **Release of metabolites of [^{3}H-*acetyl*]PAF.** Macrophages were incubated with 2 nM [*acetyl*-^{3}H]PAF or [*alkyl*-^{3}H]PAF at 4 °C for 1 h. After washing with Hepes/Tyrode's/BSA, the cells were incubated at 37 °C. At each time, the radioactivity in the medium and the cells were counted. The percentage of the radioactivity in the medium to the total radioactivity is shown as the quantity of PAF metabolites released. Data are the means of triplicate samples. Because S.D. values are within the symbol size, error bars are omitted.

from macrophages by the activation of PAF receptor.

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 ≈40 kDa was detected using an antiserum against plasma-type PAF-AH (Fig. 7*A*). 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. 7*B*). 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_2s 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 ($t_{1/2}$ ≈ 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 β_2-adrenergic receptor internalization, where the rates of internalization are different: T-lymphoma cell line ($t_{1/2}$ ≈ 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 ($t_{1/2}$ ≈ 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-

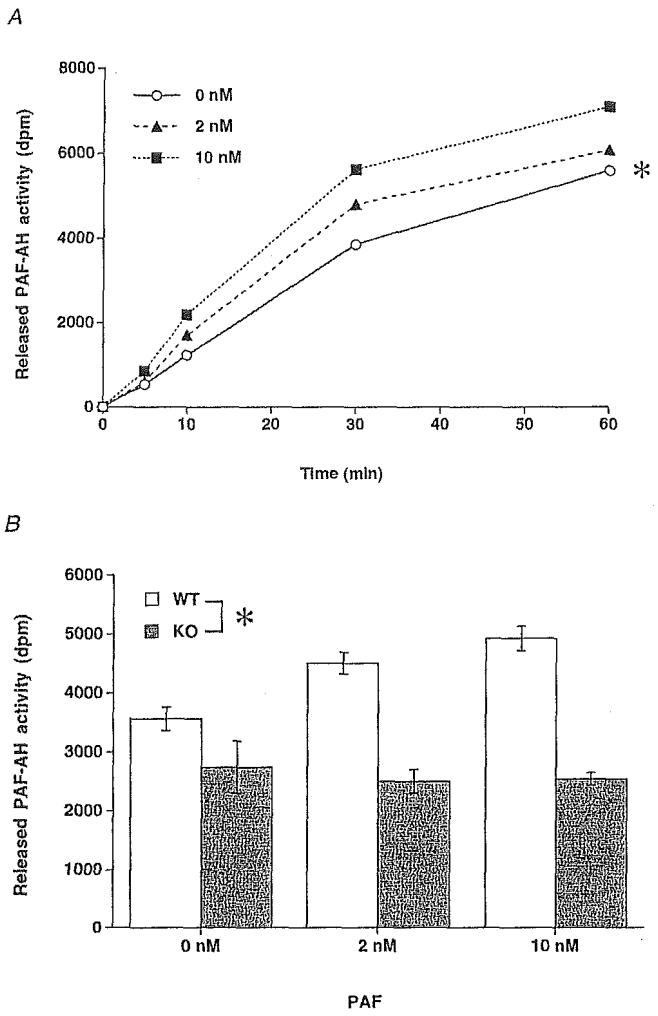

FIG. 6. **PAF-induced PAF-AH release via PAF receptor.** *A*, macrophages from PAFR-WT mice were stimulated with 2 or 10 nM PAF for 5, 10, 30, and 60 min. The PAF-AH activity in the medium was measured. Each value shows a mean of triplicate measurements. Error bars are omitted because S.D. values are within the symbol size. The statistical significance was evaluated by two-way factorial analysis of variance (*, $p < 0.0001$). *B*, macrophages from PAFR-WT and -KO mice were stimulated with 2 or 10 nM PAF for 30 min, and PAF-AH activity in the medium was measured. Each value shows a mean of triplicate samples. *Error bars* represent S.D. Data are representative of two independent experiments. The statistical significance of differences between WT and KO cells was evaluated by two-way factorial analysis of variance (*, $p = 0.0004$).

TABLE I
PAF acetylhydrolase activity in murine serum

Sera from PAFR-WT and -KO mice were incubated with 10 μM [^{3}H]PAF at 37 °C for 10 min. PAF-AH activity was determined by measuring [^{3}H]acetate liberated from PAF. Each experiment was done in triplicate. Values are the means ± S.D.

Genotype	PAF-AH activity	*n*
	pmol/μl serum/min	
PAFR-WT	415 ± 31.2	4
PAFR-KO	420 ± 42.2	4

nents of the endocytic machinery between macrophages and Chinese hamster ovary cells. Internalization of thromboxane A_2 receptor is accelerated when G protein-coupled receptor kinase 3 or arrestin 2 was co-transfected in HEK 293 cells (35). The internalization of β_2-adrenergic receptors is modified by Rab GTPases that involve in vesicular membrane transport (36).

The mechanism of PAF receptor internalization was studied

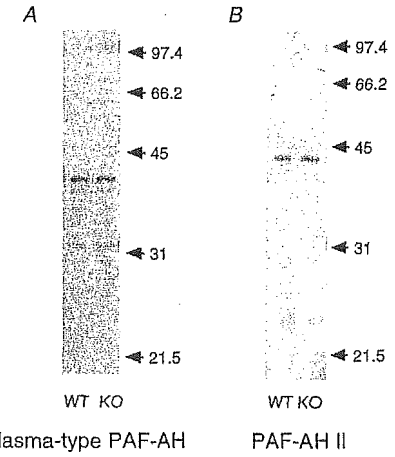

FIG. 7. **Detection of plasma-type PAF-AH and PAF-AH II in macrophages.** Lysates of macrophages from PAFR-WT and -KO mice (20 μg of protein) are subjected to immunoblot analysis using antisera against plasma-type PAF-AH (*A*) and anti-PAF-AH II monoclonal antibody (*B*).

using inhibitors against receptor internalization, PAO, ConA, hypertonic sucrose, and phorbol 12-myristate 13-acetate treatment (Fig. 3). PAO, a tri-arsenical compound, blocks endocytosis of macromolecules by cross-linking proteins with sulfur groups and inhibits internalization of various kinds of membrane receptors (34, 37, 38), including PAF receptor (20, 21). ConA or hypertonic sucrose also inhibits the internalization of many receptors including PAF receptor (20, 21). Hypertonic sucrose inhibits vesicle endocytosis by inhibiting the formation of clathrin lattice (39). Treatment of hypertonic sucrose here almost completely inhibited the PAF internalization (Fig. 2), whereas phorbol 12-myristate 13-acetate, an inhibitor of caveola-mediated receptor internalization, did not affect the PAF internalization (Fig. 3). The PAF binding decreased with ConA (8,767 dpm), PAO (7,495 dpm) or sucrose (4,090 dpm) compared with the control (18,800 dpm) (Fig. 3). The rates of PAF internalization, however, were similar at PAF concentrations of 0.5, 1.0, and 2.0 nM (data not shown), suggesting that the numbers of bound receptors do not affect the internalization rates. Thus, PAF upon binding to its receptor appears to internalize with clathrin-coated vesicles in macrophages. The rapid internalization of PAF receptor is likely to prevent excessive stimulation of macrophages by PAF. In fact, the PAF concentrations ($\approx 10^{-9}$ M) used here were sufficient for the PAF receptor activation and internalization in macrophages.

PAF degradation was decreased in macrophages from PAFR-KO mice (Fig. 1), suggesting the functional significance of the receptor-dependent degradation mechanism. A PAF antagonist, BN 52021, was previously reported to decrease PAF degradation activity of rabbit platelets (40, 41). By tracing [^{3}H]PAF, we showed that PAF was internalized very rapidly with PAF receptor (Fig. 3). It is likely that the receptor-bound PAF is degraded intracellularly; PAF internalization ($t_{1/2} \approx 1$ min) clearly preceded PAF degradation ($t_{1/2} \approx 20$ 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 ($t_{1/2} \approx 40$ min), whereas the latter was metabolized to 1-alkyl-2-acyl-PC intracellularly (Fig. 4*A*).

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 (Fig. 7). Thus, the expression levels of PAF-AHs are not major reasons for the difference of PAF degradation rates between 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 PLA_2s 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 known to be an autacoid that functions at restricted regions. In inflammatory sites, therefore, both receptor-dependent PAF internalization and PAF-AH release are probably important for the rapid removal of PAF from the extracellular space and the cell surface. The intracellular signals of PAF that lead to PAF internalization and PAF-AH exocytosis are yet to be determined.

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Platelet-Activating Factor Receptor

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Platelet-activating factor (PAF) is a pro-inflammatory lipid mediator possessing a unique 1-*O*-alkyl glycerophospholipid (GPC) backbone (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholin). Cloned PAF receptor, which belongs to the G protein–coupled receptor superfamily, transduces pleiotropic functions including cell motility, smooth muscle contraction, and synthesis and release of mediators and cytokines *via* multiple heterotrimeric G proteins. Pharmacological studies have suggested that PAF functions in a variety of settings including allergy, inflammation, neural functions, reproduction, and atherosclerosis. Establishment of PAFR$^{-/-}$ mice confirmed that the PAF receptor is responsible for pro-inflammatory responses, but that its roles in other settings remain to be clarified.

Key words: bronchial asthma, endotoxin shock, G protein–coupled receptors, oxidized phospholipids, platelet-activating factor.

Overview

Platelet-activating factor (PAF), a structurally unusual lipid autacoid possessing an intact 1-*O*-alkyl glycerophospholipid (GPC) backbone (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholin), was originally identified as a pro-inflammatory mediator in the late 1970s. Subsequent researches suggest that PAF, and structurally related GPC oxidatively fragmented at the *sn*-2 position, function as mediators in a variety of settings including atherosclelosis, neural functions and reproduction. Cloned PAF receptor (PAFR) possesses a typical structure of G protein–coupled receptors (GPCRs) with seven transmembrane helices, and it presumably signals through Gαq/11, Gαo, and Gαi, and also Gβγ. PAFR subtypes have not been identified. PAFR$^{-/-}$ mice apparently grow normally. Their phenotypes revealed that the cloned PAFR plays major roles in inflammatory responses including systemic anaphylaxis, but its roles in other biological functions should be clarified by further studies.

PAF, its synthesis, degradation, and cell-surface expression

Platelet-activating factor (PAF), initially recognized as platelet-stimulating activity from FεRI-engaged basophils (*1*), was structurally identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholin in the late 1970s (*2, 3*) (Fig. 1). In contrast to unsaturated fatty acid–derived major autacoid species [*e.g.*, prostanoids (PGs) and leukotriens (LTs)], PAF is unusual in its intact glycerophospholipid structure. The ether-bonded fatty alcohol with C16-18 chain length at the *sn*-1 position of the glycerol backbone, an acetyl residue at *sn*-2, and phosphocholine at *sn*-3 are all required for optimal PAF activity (reviewed in Ref. *4*).

Biological activity of PAF does not seem to be confined to pro-inflammatory functions. Recent works suggest its involvement in a variety of settings, including reproduction, central nervous system functions, and circulatory system disturbance such as atherosclerosis (reviewed in Refs. *5–7*).

The majority of PAF is synthesized from glycerophosphocholins (GPCs) with 1-*O*-alkyl moieties (Fig. 2). 1-*O*-Alkyl-GPCs are enriched with arachidonic acid at the *sn*-2 position (*4*). Upon cell activation, cytoplasmic phospholipase A_2 (cPLA$_2$) (*8*) simultaneously liberates arachidonic acid and Lyso-PAF, the direct precursor of PAF, providing the basis for interrelated synthesis of eicosanoids and PAF. PAF is finally synthesized by the action of acetyl CoA-lysoPAF acetyl transferase. This enzyme has not been purified, and its nature remains to be determined. The involvement of a cPLA$_2$-dependent "remodeling" pathway in bulk PAF synthesis in inflammatory cells was confirmed in cPLA$_2^{-/-}$ mice (*9, 10*). Another metabolic pathway dependent on phosphocholine transfer from CDP-choline to 1-*O*-alkyl-2-acetyl-glycerol was also reported ("*de novo*" pathway, reviewed in Ref. *4*), but its significance remains to be clarified.

PAF is hydrolyzed at the *sn*-2 position by PAF acetyl hydrolases (PAF-AH) to yield lyso-PAF. There exist at least three types of PAF-AH: two intracellular enzymes (tissue types I and II) and one secreted one (plasma type). Tissue type I is a heterotrimer containing the product of the LIS1 gene, which is genetically associated with a congenital brain agyria, Miller-Dieker lissencephaly (*11*). Tissue type II and plasma type PAF-AH are structurally related monomeric enzymes (*12*). Both possess activities hydrolyzing oxidized fatty acyl residues and acetyl residues from the *sn*-2 position of GPCs, and LCAT-like acetyl transferase activity (*13, 14*).

Besides PAF synthesized *via* the regulated pathway, oxidized 1-*O*-acyl GPCs, whose unsaturated fatty acyl resi-

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Abbreviations: PAF, platelet-activating factor; PAFR, PAF receptor; GPCR, G protein–coupled receptor; PG, prostaglandins; LT, leukotrienes; GPC, glycerophosphocholin; cPLA2, cytosolic phospholipase A2; PAF-AH, PAF acetyl hydrolase; TM, transmembranous region; Tg, transgenic.

dues at the *sn*-2 position are randomly fragmented by oxidization, also stimulate PAF receptor (PAFR) (*15, 16*). The oxidized GPCs possess hydroperoxy fatty acids of shorten chain length (C2–C4), resembling the short acetyl moiety at the *sn*-2 position of PAF (*17, 18*). The oxidized GPC species are implicated in atherogenesis: GPCs with short oxidized fatty acyl moieties are found in oxidized low-density lipoprotein (LDL) (*18, 19*), PAFR is expressed on atherosclerotic lesions in humans (*20*), and intervention of the PAF-like action with PAF-AH or with PAF antagonists successfully suppressed progression of atherosclerosis in model animals (*21, 22*).

Lipid autacoid release across the plasma membrane sometimes requires specific machinery as seen in LTC_4 transport *via* ATP-binding cassette transporter (*23*). In the case of PAF synthesized in vascular endothelial cells, its polar head translocates to the outer surface of the cell *via* undefined "flip-flop" mechanisms, with the saturated alkyl moiety being inserted into outer leaflet of plasma membrane. The cell-associated PAF functions as a juxtacrine liand stimulating adherent leukocytes (reviewed in Refs. *6* and *24*). These characteristics of PAF are reminiscent of fractalkine, a transmembranous chemokine expressed on the endothelial surface (*25*), which induces firm adhesion and trans-endothelial migration of leukocytes through "inside-out" integrin activation. Such short-range PAF signaling may represent mechanisms to avoid its accelerated conversion to inactive lyso-PAF by high activity of plasma-type PAF acetyl hydrolase (*26*).

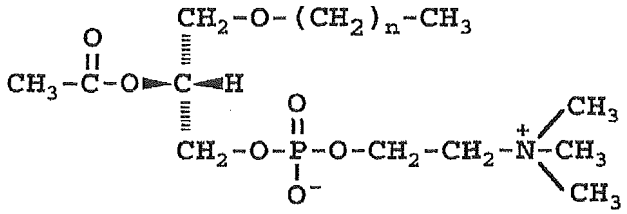

Fig. 1. **Structure of PAF.**

PAF receptor: structure–function analysis and regulated expression

As suggested by earlier findings that PAF specifically binds to and stimulates GTPase activity in polymorphonuclear leucocyte (PMN) membranes (*27*), cloned PAF receptors from various species possess a typical structure of G protein–coupled receptors (GPCRs) with seven transmembrane helices (TMs) (*28–30*) (Fig. 3). To date no other subtypes have been recognized. Specific binding of PAF or PAF antagonists has been detected in various cells including PMNs, platelets, macrophage-lineage cells (Mø, Kupffer cells and microglia), thoracheal epithelium, vascular endothelium, and myometrium (see references in Ref. *5*). PAFR expression in primary T and B lymphocytes is still controversial. PAFR mRNA is widely distributed in PMNs, spleen, kidney, liver, heart, skeletal muscle, and brain from various species. *In situ* hybridization detected PAFR mRNA in mesangial cells in rat kidney, blood vessels, smooth muscles, and alveolar wall in human lung, microglia and to a lesser extent in neurons in rat brain (*5*).

PAFR mutagenesis studies have provided several insights into G protein-coupling, ligand-binding, and activation states of the receptor (Fig. 3). Overexpression of PAFR 3rd intracellular loop, a putative Gq/11 coupling site in m3 muscarinic Ach receptor (*31, 32*), exerts dominant negative effects on PAFR functions (*33*). Mutagenesis of the amphipathic α helix at the 3rd loop [residues 210–220, IHTLLTRPVRQ (rat PAFR); see Fig. 3] disrupted the PAFR-phospholipase C cascade, thereby indicating that the 3rd loop is involved in G protein–coupling (*34*). In addition, A230E exchange at the C-terminal end of the 3rd loop interrupts

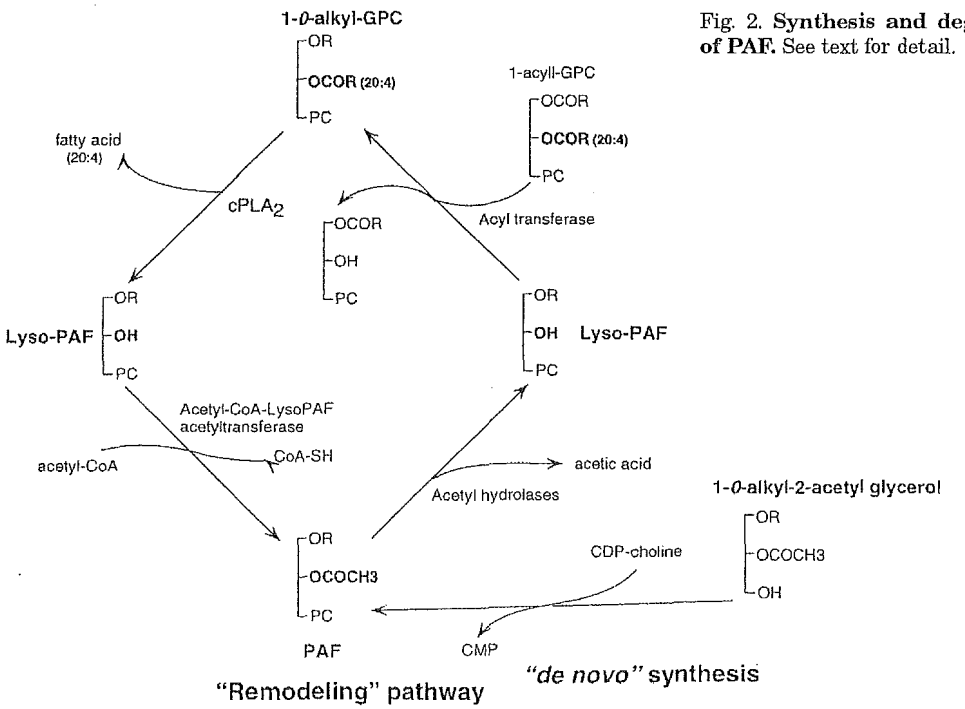

Fig. 2. **Synthesis and degradation of PAF.** See text for detail.

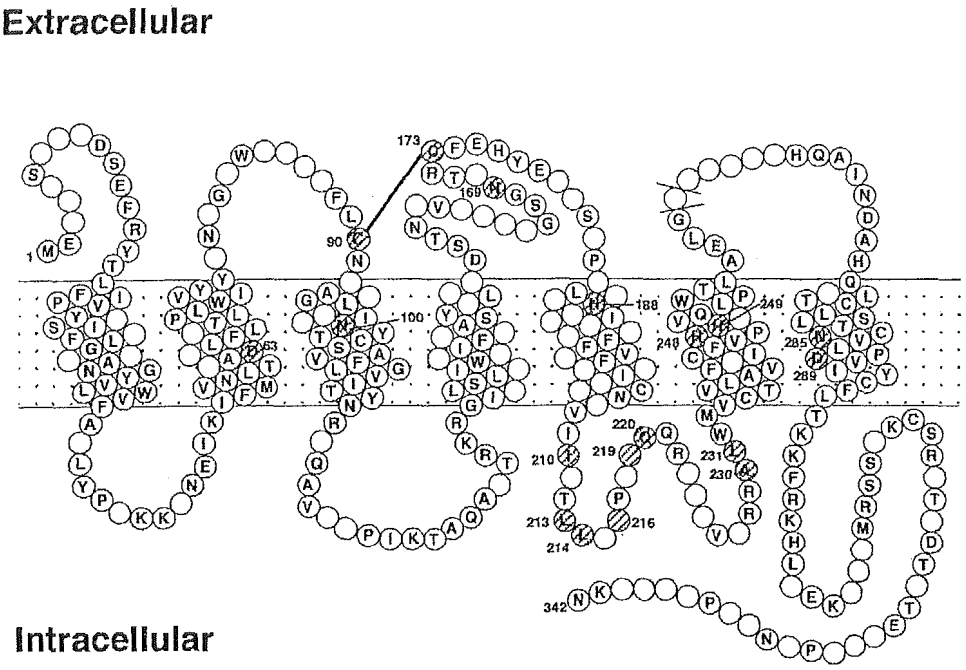

Fig. 3. **Transmembrane structure of PAF receptor.** Shaded amino acids indicate the sites of mutagenesis studies. See text for detail.

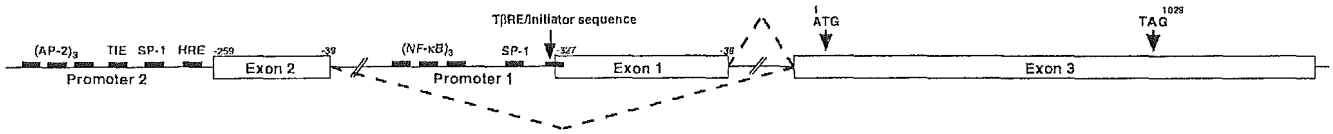

Fig. 4. **Genome structure of human PAF receptor.** Two 5′ non-coding exons (exons 2 and 1; the order is inverted for historical reasons) are spliced to exon 3, the entire coding sequence, yielding two PAFR transcripts. Their expression is regulated by two promoters (promoters 2 and 1). Promoter 2 contains AP-2, TIE (TGFβ-inhibitory element), SP-1 and HRE (hormone responsive element); and promoter 1, NF-κB SP-1, and TβRE (TGFβ responsive element).

PAFR-G protein–coupling (*35*). Interestingly, the adjacent L231R substitution created constitutively active PAFR with intact PAF responsiveness and higher affinity to PAF than wild-type PAFR (*35*). These data suggest that subtle structural changes at around the 3rd loop partially imitate an activation state of PAFR. N100A substitution in the 3rd TM was found to induce another constitutive PAFR activation with higher affinity to PAF (*36*). This seemingly remote effect suggests that G protein activation is defined in three dimensions as well.

Ishii et al. performed Ala-scanning mutagenesis of transmembranous polar amino acids (*36*). They showed that extinction of the polarities in the 2nd, 3rd, and 7th TMs induces higher PAF binding affinities than WT PAFR, whereas replacement of three His residues close to the outer surface in the 5th and 7th TMs critically decreased affinity to PAF (*36*). They proposed that the three His residues coordinately bind phosphate of PAF. These findings are consistent with the idea that ligand-binding pockets in GPCRs are composed in three dimensions of multiple TMs through polar and non-polar interactions. In the GPCR superfamily, D63 in the 2nd TM and N285 and D289 are well preserved (*37*) and hypothetically create a negatively charged binding pocket. This module was once presumed to create a choline-binding pocket (*38*). However, mutagenesis studies showed that these amino acids are not essential for PAF binding (*39*). The binding site for the choline residue of PAF is still undetermined.

PAFR is post-translationally modified by disulfide bonding at C90-C173 and by *N*-linked glycosylation at N169. These modifications are required for efficient cell surface expression of PAFR (*40*). The Ser and Thr cluster at the C-terminus is phoshorylated upon PAF binding, and this process, presumably catalyzed by G protein–coupled receptor kinase (GRK)–2, seems crucial for homologous desensitization and for facilitated internalization of PAFR (*41–44*). Common and downstream desensitization mechanisms are also noted in the PAFR system, including phospholipase Cβ3 (PLCβ3) phosphorylation by protein kinase C (PKC) (*45*) and Gq-mediated proteolysis of inositol 1,4,5-trisphosphate (IP3) receptor (*46*).

PAFR expression seems to be differentially regulated by two promoters (promoters 1 and 2) flanking two 5′-noncoding exons (exons 1 and 2) (*47*) (Fig. 4). These noncoding exons are spliced to an acceptor site on the exon 3 encoding entire PAFR open reading frame, yielding two PAFR transcripts (transcripts 1 and 2). PAFR transcript 1 is ubiquitously expressed and abundant in PMNs and monocytes. Transcript 2 is seen in organs including heart, lung, spleen, and kidney, but its expression is low in PMNs and monocytes (see references in Refs. *5* and *48*). Promoter 1 contains consensus sequences for NF-κB and Sp1 and a TGF-β responsive element, and PAFR expression is augmented in response to phorbol ester and TGF-β (*47, 49*). Promotor 2

contains a TGF-β inhibitory element and a hormone-responsive element, and transcript 2 levels are regulated negatively by TGF-β and positively by steroid hormones such as retinoic acid, triiodothyronine and estradiol (*50, 51*).

Signal transduction from PAF receptor

Selective PAFR coupling with heterotrimeric G proteins has been studied through various approaches. PAFR regulates initial GPCR 2nd messengers: it augments inositol 1,4,5-trisphosphate (IP_3) synthesis and calcium mobilization and suppresses forskolin-stimulated cAMP synthesis in CHO cells (*52*). The latter effect, a hallmark of Gαi species, is completely inhibited by pertussis toxin (PTX) (*52*). IP_3 synthesis is partially sensitive to PTX in CHO cells and in RBL mast cells (*42, 52*), and the PTX-insensitive portion is abolished when GDP-βS is incorporated into RBL cells, indicating that both PTX-insensitive and sensitive G proteins regulated this pathway. Recently PTX-insensitive Gαq was found to reconstitute the PAFR-IP_3 axis in COS cells (*53*), showing the roles of the Gαq/11 family.

Additional information was obtained from studies focusing on PAFR-induced Erk and p38 MAP kinase activation. PAFR-mediated Erk activation, and also Erk-dependent cytosolic phospholipase A2 activation, are largely sensitive to PTX in CHO cells (*52*). The Erk pathway is dependent on Gαo expression, and PAFR induces azido-GTP incorporation into Goα in CHO cells (*54*). Moreover, expression of a PTX-insensitive mutant of Gαo, but not of Gαi2 or 3, renders the pathway resistant to PTX (*54*). PAFR-induced p38 MAPK activation is insensitive to PTX in CHO cells and in PMNs (*54, 55*). This pathway is attenuated by RGS16 Gα GAP expression, and a QL mutant of Gα11 lacking GTPase activity overcomes the inhibitory effects in CHO cells (*54*). Therefore, it is conceivable that PAFR links to Gαq/11, Gαo, and Gαi G proteins. PLCβ activation is presumably transduced mainly by Gαq/11 and partly by Gαo, p38 by Gαq/11, and Erk by Gαq/11, Gαo, and also by Gβγ depending on cell types (see below).

Molecular mechanisms of the post-G protein signaling network that participate in cellular functions, *i.e.*, cell polarization, adhesion and motility, gene expression, and trophic effects, have been the focus of intensive research (reviewed in Ref. *56*) and are beyond the scope of this review. Noticeable characteristics of the network are that the post-G protein signaling is highly dependent on cell-context. For instance, the PAFR-Erk pathway, which probably regulates cell growth and gene expression including inflammatory cytokines, is Ras-independent and PKC-dependent in fibroblasts (*52, 54*), whereas PAFR activates the Ras-Erk pathway in PMNs, presumably through the Gq-Ras GRF pathway (*56*). In addition, PAFR utilizes transactivation of EGF receptor in Erk activation, which is theoretically transduced by Gβγ and forms part of the Ras pathway, in epidermal cells (*57*). The last example indicates PAFR-transactivation of receptor protein tyrosine kinases (PTKs) or non-receptor PTKs including Src family kinases (*58*), but the underlying mechanisms are still elusive. PAFR activates MEK1/2-Erk and MEK3 (and presumably MEK6)-p38 MAP kinases in various cells (*55, 59*), whereas c-Jun N-terminal kinase activation by PAF has been noted solely in primary hippocampal neurons (*60*). PAFR-mediated PIP3 synthesis, which presumably regulates cell polarization/motility and cell survival and growth, utilizes Gβγ-activatable PI3 kinase γ in a macrophage cell line (*61*), while PAFR signals *via* p85/p110 PI3Ks in an erythroleukemia cell line (*62*). PAFR is also reported to regulate other downstream signaling molecules, including PLD, PLCγ, and other small G proteins, Ral and Rap (*63, 64*).

Roles of PAF receptor in pathophysiological conditions: insights from PAF receptor-overexpressing, and PAF receptor$^{-/-}$ mice

Through a number of experiments in animal models, and in several cases in humans, PAF has been implicated in pathophysiological conditions including allergic asthma, endotoxin shock, acute pancreatitis and dermal inflammations such as psoriasis and pruritis (reviewed in Ref. *5*). Recent works suggests the roles of PAFR in atherogenesis (see above). These proposals are based on PAF-induced pathological responses, prevention of the pathological conditions by PAFR antagonists or by PAF acetylhydrolases, and measurement of PAF or PAF-related compounds in pathological regions. To date, however, PAF antagonists have not been applied clinically. Although PAF is conceivably involved in these conditions, it might play modifying roles in them.

Several reports suggest roles of PAF in implantation of embryos. Pre-implantation embryos synthesize PAF, and notably (*65*), pretreatment of embryos with PAF reportedly increases implantation rate in *in vitro* fertilization in humans (*65, 66*). PAF fulfils the requirements for retrograde messengers in neural synapses in that it is a small and diffusible molecule produced in CNS (*67*). Bazan and colleagues have proposed that hippocanpal LTP, and also memory function in animals, involves PAF-regulated events, based on the observations that a PAF antagonist inhibits LTP in the CA1 region and that *in vivo* infusion of an unhydrolizable PAF analog (methylcarbamoyl PAF) into dorsal hippocampus, amygdala, or entorhinal cortex improved memory functions in male Wistar rats (*68, 69*).

Creation of PAFR-transgenic (Tg) mice and PAFR$^{-/-}$ mice have provided insights into several, if not all, of the above-mentioned possibilities (*70, 71*). Since the PAFR-Tg construct used in the studies is driven by β-actin promoter, it should be kept in mind that PAFR transgene expression is different from that in intrinsic PAFR (*70*). PAFR-Tg spontaneously develops melanocyte tumors (*70*), suggesting an direct or indirect melanocyte proliferating potential of PAFR. PAFR$^{-/-}$ mice grow apparently normally. PAFR-Tg progeny are reproducibly smaller than the wild type when either male or female PAFR-Tg heterozygotes are mated with wild-type mice. However, PAFR$^{-/-}$ mice exhibited normal reproductive potential (*71*). Thus PAFR is not essential for reproduction, but an augmented (or ectopic) PAF signal both in embryos and in maternal systems appears to be disadvantageous for fertilization in mice (*70, 71*).

In PAFR$^{-/-}$ mice, intravenous PAF injection does not cause hypotension, and PAF challenge fails to induce calcium mobilization in PAFR$^{-/-}$ PMNs. Hence, these PAF functions are entirely ascribed to the cloned PAFR. PAFR-Tg and PAFR$^{-/-}$ mice display altered behaviors in response to immunological or inflammatory challenges. PAFR$^{-/-}$ mice are extremely resistant to antigen-induced systemic anaphylaxis, including bradycardia, circulatory shock, and lung edema (*71*). PAFR-Tg mice respond more severely to lipopolysaccharide (LPS)-induced endotoxin shock, while

$PAFR^{-/-}$ mice respond similarly to wild-type mice (*71*). These findings show that PAF plays major roles in type I (and/or III) allergic anaphylaxis and that it enhances the severity of endotoxin shock. PAFR-Tg mice show bronchial hyper-responsiveness to methacholine as well as PAF (*70*). PAFR-Tg mice are significantly sensitive to PAF injection in terms of bronchial constriction, and these effects seem to be indirectly mediated thromboxane A2 and leukotriene D4 (*70*). $PAFR^{-/-}$ mice are also more resistant to hydrochloric acid aspiration–induced lung edema (a model of aspiration pneumonia) than wild type mice (*72*).

Apparently contradictory to previous pharmacological studies (*69*), $PAFR^{-/-}$ mice exhibited normal LTP and showed no obvious abnormality in excitatory synaptic transmission in the hippocampal CA1 region (*73*). These discrepancies might suggest the existence of PAF receptors other than the cloned one, or that PAF antagonists and/or methylcarbamoyl PAF exert effects *via* a different pathway than PAFR, including PAF acetylhydrolase inhibition.

Conclusion

As the first lipid autacoid receptor to be cloned, the cloned PAFR has furnished information on the inflammatory and non-inflammatory actions of PAF and the signaling mechanisms of GPCRs. The accumulated information suggests that PAFR mediates fine modifications of a variety of biological functions in co-operation with other GPCRs such as chemokine and eicosanoid receptors.

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Host response of platelet-activating factor receptor-deficient mice during pulmonary tuberculosis

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SUMMARY

Platelet-activating factor (PAF) is a phospholipid with potent, diverse actions, which has been implicated as an important mediator in host defence against several intracellular pathogens. To determine the role of PAF in host defence in pulmonary tuberculosis, PAF receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice were infected intranasally with a virulent strain of *Mycobacterium tuberculosis*. Mycobacterial outgrowth in lungs and liver did not differ significantly between PAFR−/− and PAFR+/+ mice at 2 or 6 weeks postinfection. After 28 weeks, 86% of PAFR−/− mice and 79% of PAFR+/+ mice had died (non-significant). In addition, both mouse strains were indistinguishable with respect to histopathology, the recruitment and activation of lymphocytes, and cytokine concentrations in the lung. These data suggest that PAF is not involved in the protective immune response to tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a re-emerging disease, affecting patients in both developing and industrialized countries.[1] The increasing incidence of antibiotic resistance, together with synergism between human immunodeficiency virus (HIV) and TB, has increased our interest in this important infectious disease and in mechanisms contributing to antimicrobial host defence. Resistance to mycobacterial infections is mediated mainly by macrophages and T cells and requires the formation of granulomas, characterized by lymphocytes, macrophages and granulocytes.[2] Their interaction is dependent on the interplay of cytokines and chemokines produced by different inflammatory cells.[2]

Platelet-activating factor (PAF) is a potent phospholipid mediator that plays an important role in inflammatory and immune responses.[3] PAF is produced by a large number of cells, including platelets, endothelial cells, stromal cells, lymphoid tissue and neutrophils.[4] The biological activity of PAF is mediated through a specific G-protein-coupled receptor (PAFR) on the membrane of responsive cells, which has been identified on many haemopoietic cells, including neutrophils, dendritic cells, macrophages and monocytes.[4,5] Recent studies have suggested that endogenous PAF may play an important role in stimulating an adequate immune response to intracellular microorganisms, such as *Leishmania amazonensis and Trypanosoma cruzi*. Indeed, treatment with PAF antagonists was found to increase the outgrowth of microorganisms and mortality in murine models of these infections.[6,7] In accordance, PAF reduced the intracellular growth of *Leishmania* and *Trypanosoma* in macrophages.[6,7] Notably, *M. tuberculosis* is an intracellular microorganism that uses macrophages as its natural environment in the host, and many of the host defence mechanisms known to be important for the protection against *M. tuberculosis* are also involved in the protective immune response to other intracellular pathogens, including *Leishmania* and *Trypanosoma*.[2,8–10] These findings led us to hypothesize that PAF may also be important for host defence against *M. tuberculosis*. Therefore, in the present study we sought to determine the role of PAF in the immunopathology of TB.

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MATERIALS AND METHODS

Mice

PAFR gene-deficient (PAFR−/−) mice were generated as described previously.[11] For the experiments described here, female PAFR−/− mice, backcrossed seven times to a

C57BL/6 background, and female wild-type C57BL/6 (PAFR+/+) mice (Harlan Sprague Dawley Inc., Horst, the Netherlands), were used at 6–8 weeks of age. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands) approved all experiments.

Experimental infection

Pulmonary TB was induced exactly as described previously.[12–14] Briefly, a virulent laboratory strain of *M. tuberculosis* H37Rv was grown for 4 days in liquid Dubois medium containing 0·01% Tween-80. A replicate culture was incubated at 37°, harvested at mid-log phase, and stored in aliquots at −70°. For each experiment, a vial was thawed and washed twice with sterile 0·9% NaCl. Mice were anaesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and infected with 1×10^5 live bacilli in 50 μl of saline, as determined by viable counts on 7H11 Middlebrook agar plates. Bacterial administration was performed intranasally, as described previously.[12–14] Survival was monitored for 200 days in 14 PAFR−/− and 14 PAFR+/+ mice. In addition, groups of eight mice per time-point were killed 2 or 6 weeks postinfection, and the lungs and one lobus of the liver were removed aseptically. Organs were homogenized using a tissue homogeniser (Biospec Products, Bartlesville, OK), in 5 volumes of sterile 0·9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days of incubation at 37°. Numbers of colony-forming units (CFU) are provided as total in the lungs or as total/g of liver tissue. For cytokine measurements, lung homogenates were diluted 1 : 1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl.H_2O$, 1 mM $CaCl_2$, 1% Triton-X-100, 100 μg/ml pepstatin A, leupeptin, and aprotinin), and incubated on ice for 30 min. Supernatants were sterilized using a 0·22-μm filter (Corning, Corning, NY) and frozen at −20° until required.

Histological analysis

The right lungs of six PAFR−/− and six wild-type PAFR+/+ mice were removed 2 or 6 weeks after intranasal inoculation with *M. tuberculosis* and then fixed for 24 hr in 4% paraformaldehyde in phosphate-buffered saline (PBS). After embedding in paraffin wax, 4-μm-thick sections were stained with haematoxylin & eosin or the Ziehl–Neelsen (ZN) stain for acid-fast bacilli. All slides were coded and semiquantitatively scored for the total area of inflammation (percentage of surface of the slide) and granuloma format by a pathologist. In separate experiments, organs of six uninfected PAFR−/− and wild-type PAFR+/+ were harvested and examined as described above.

Fluorescence-activated cell sorter (FACS) analysis

For FACS analysis, pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and processed as described previously.[13] Cells from two mice per group ($n = 10$) were pooled for each time-point (yielding five samples per group for FACS analysis) and then adjusted to a concentration of 4×10^6 cells/ml of FACS buffer (PBS supplemented with 0·5% bovine serum albumin, 0·01% NaN_3, and 100 mM EDTA). Immunostaining for cell-surface molecules was performed for 30 min at 4° using antibodies (Abs) directly labelled against CD3 [anti-CD3 phycoerythrin (PE)], CD4 (anti-CD4 CyChrome), CD8 [anti-CD8 fluorescein isothiocyanate (FITC); anti-CD8 peridinin chlorophyll protein (PerCP)], CD25 (anti-CD25 FITC) and CD69 (anti-CD69 FITC). All Abs were used at concentrations recommended by the manufacturer (PharMingen, San Diego, CA). To correct for non-specific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. The number of positive cells was obtained by setting a quadrant marker for non-specific staining.

Cytokine measurements

Interferon-γ (IFN-γ) and interleukin (IL)-4 concentrations were measured using commercially available enzyme-linked immunosorbent assay (ELISA) reagents, according to the instructions of the manufacturer (R & D Systems, Abingdon, UK).

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Comparisons were performed using Mann–Whitney *U*-tests. For comparison of survival curves, Kaplan-Meier analysis with a log rank test was used. *P*-Values of $\leq 0{\cdot}05$ were considered statistically significant.

RESULTS

Survival

PAFR−/− and PAFR+/+ mice were inoculated intranasally with 10^5 live *M. tuberculosis* bacilli and their survival was monitored for a time-period of 200 days (Fig. 1). Although PAFR−/− mice tended to succumb to TB earlier than PAFR+/+ mice, the difference between the two strains was not significant. Overall, the survival rate was 14% for PAFR−/− mice and 21% for PAFR+/+ mice (not significant).

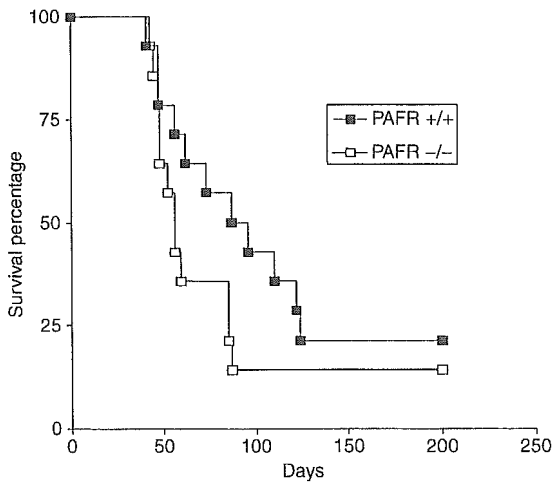

Figure 1. Platelet-activating factor (PAF) receptor deficiency does not influence survival during murine lung tuberculosis. Survival of PAF receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice infected intranasally with 10^5 *Mycobacterium tuberculosis* colony-forming units (CFU) ($n = 14$ per group). No significant difference was found in lethality between the two strains of mice.

Mycobacterial outgrowth

Next, the numbers of *M. tuberculosis* CFU were determined in lungs and livers of PAFR−/− and PAFR+/+ mice at 2 and 6 weeks after intranasal infection. Both organs contained a similar number of *M. tuberculosis* CFU in PAFR−/− and PAFR+/+ mice at each time-point (Fig. 2).

Cellular recruitment to lungs

The histology of parenchymatous organs of 8–10-week-old PAFR−/− and PAFR+/+ mice, without *M. tuberculosis* infection, was similar and displayed no signs of abnormalities (data not shown). Histopathological examination of lungs from PAFR−/− and PAFR+/+ mice at 2 and 6 weeks after intranasal infection with *M. tuberculosis* revealed no differences between the two mouse strains. Figure 3 shows representative slides of lungs from mice killed 6 weeks after intranasal infection. At this time-point, dense and diffuse infiltrates were found in the lungs of both mouse strains; the percentage of inflamed parenchyma was similar in both groups (data not shown). To obtain

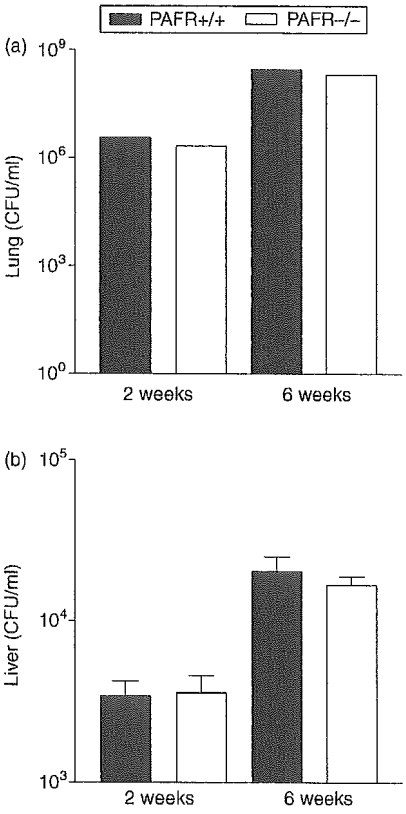

Figure 2. Platelet-activating factor (PAF) receptor deficiency does not influence mycobacterial outgrowth in lungs or liver during murine tuberculosis. Bacterial outgrowth is represented, in colony-forming units (CFU)/ml of organ, in PAF receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice in lungs (a) and livers (b) at 2 and 6 weeks after intranasal infection with 10^5 *Mycobacterium tuberculosis* CFU. Data represent mean values ± standard error (SE) (n = 8/group). SE values in Fig. 2(a) were too small to show on the figure. No significant differences were found in mycobacterial outgrowth.

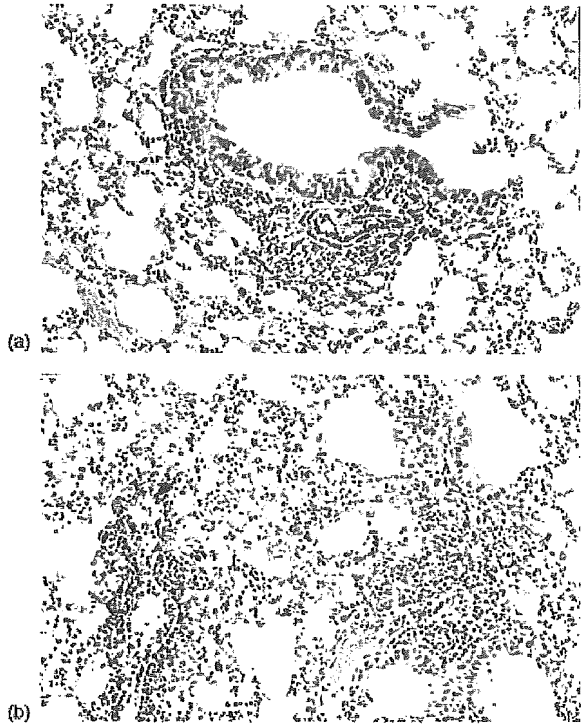

Figure 3. No differences in histopathology were observed between platelet-activating factor (PAF) receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice. (a) Representative slides of lung tissue of PAFR+/+ mice, 6 weeks after intranasal infection with 10^5 *Mycobacterium tuberculosis* colony-forming units (CFU), showed a diffuse inflammatory infiltrate which was almost confluent. Macrophages were the most predominant cell type observed, together with small number of lymphocytes (haematoxylin & eosin staining; original magnification × 25). A comparable picture was observed in PAFR−/− mice (b) 6 weeks postinfection (haematoxylin & eosin staining, original magnification × 25). Slides are representative for six mice per strain.

further insight into the cellular composition of the pulmonary infiltrates, we analysed whole lung cell suspension by FACS analysis. The percentages of $CD4^+$ and $CD8^+$ lymphocytes did not differ significantly between PAFR−/− and PAFR+/+ mice; furthermore, the surface expression of CD25 and CD69 on T cells was similar in both mouse strains (shown for the 6-week postinfection time-point in Table 1).

Lung IFN-γ and IL-4 concentrations

Cytokine concentrations in lung homogenates of 8–10-week-old PAFR−/− and PAFR+/+ mice, without *M. tuberculosis* infection, were either low or undetectable, with no differences between groups. IFN-γ and IL-4 concentrations in lung homogenates obtained at 2 and 6 weeks postinfection were similar in PAFR−/− and PAFR+/+ mice (shown for the 6-week postinfection time-point in Table 1).

DISCUSSION

PAF has been implicated as a protective mediator in the host response to several intracellular pathogens. The data presented

Table 1. Cellular composition and cytokine concentrations in lungs

Cells (10^4/ml)	PAFR+/+	PAFR−/−
Total cells	290·0 ± 31·1	278·0 ± 45
Cell subsets (percentage of total)		
$CD4^+$	67·2 ± 0·8	71·3 ± 1·2
$CD8^+$	27·1 ± 1·1	22·0 ± 0·9
$CD4^+/CD69^+$	9·9 ± 0·9	11·5 ± 2·4
$CD4^+/CD25^+$	8·6 ± 0·7	11·1 ± 1·6
$CD8^+/CD69^+$	16·7 ± 2·2	15·2 ± 0·6
$CD8^+/CD25^+$	1·7 ± 0·2	2·0 ± 0·4
Cytokines (ng/ml)		
IFN-γ	7·75 ± 0·64	7·68 ± 0·64
IL-4	5·96 ± 0·75	6·67 ± 0·64

Total cell counts and lymphocyte typing were performed on pulmonary cell suspensions 6 weeks postinfection, as described in the Materials and methods. Fluorescence-activated cell sorter (FACS) analysis was performed on pooled cells from two mice for each analysis from a total of 10 mice per group (i.e. yielding five samples per mouse strain). FACS results are expressed as the percentage of $CD4^+$, $CD8^+$, $CD25^+$ and $CD69^+$ within the $CD3^+$ population [i.e. for each of the five samples per mouse strain the percentage of positive cells relative to the total number of $CD3^+$ cells was determined, and from these data means ± standard error (SE) were calculated]. Cytokine data were obtained from eight mice per group and data are expressed as mean ± SE.

PAFR−/−, platelet-activating factor receptor gene-deficient mice; PAFR+/+, wild-type platelet-activating factor mice.

here argue against such a protective role of PAF in pulmonary TB. Indeed, intranasal infection with live *M. tuberculosis* was associated with similar mortality rates in PAFR−/− and PAFR+/+ mice, and the mycobacterial loads in lungs and liver, determined during the early phase of the infection when all animals were still alive, did not differ significantly between the two mouse strains.

Host defence against TB, at least in part, relies on $CD4^+$ and $CD8^+$ T cells.[2] We therefore determined the number of T cells in whole-lung cell suspensions and, in addition, obtained insight into their activation state by measuring the surface expression of CD25 and CD69. Theoretically, PAF can inhibit certain lymphocyte functions. Indeed, PAF has been found to reduce proliferation of $CD4^+$ T cells induced by phytohaemagglutinin, which is associated with a reduced expression of CD25.[15] PAF also suppresses the mitogen-stimulated production of IL-2 by human lymphocytes.[16] However, to our knowledge little, if anything, is known about the effects of PAF on lymphocyte activation *in vivo*. We here demonstrate that deficiency of the PAFR does not influence the recruitment or activation of $CD4^+$ and $CD8^+$ lymphocytes during pulmonary TB.

The clinical outcome of pulmonary TB is considered to be dependent on a type 1-mediated host response.[2] We therefore determined whether PAFR deficiency influences the type 1/type 2 balance by measuring the concentration of the type 1 cytokine IFN-γ and the type 2 cytokine, IL-4, in lung homogenates of infected PAFR−/− and PAFR+/+ mice. However, no differences in the pulmonary concentrations of these cytokines were found between these two strains.

Our assumption, that PAF could be involved in the protective immune response to TB, was primarily based on its reported protective role in experimental infections of mice with *L. amazonensis*, *T. cruzi* and *Candida albicans*.[6,7,17] From the present study it remains unclear why PAF does not contribute to protective immunity in TB. PAFR−/− mice are capable of producing PAF, yet PAF cannot exert any biological effect owing to the absence of its receptor.[4] Knowledge of the production of PAF in TB, either experimentally induced or in patients, is, to the best of our knowledge, not available. In this respect it is important to realize that PAF measurements do not necessarily provide insight into the production of this lipid mediator, as PAF that is synthesized remains predominantly in cell-associated form.[18] Clearly, further research is warranted to dissect the distinct molecular mechanisms that contribute to an adequate immune response to different intracellular pathogens.

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