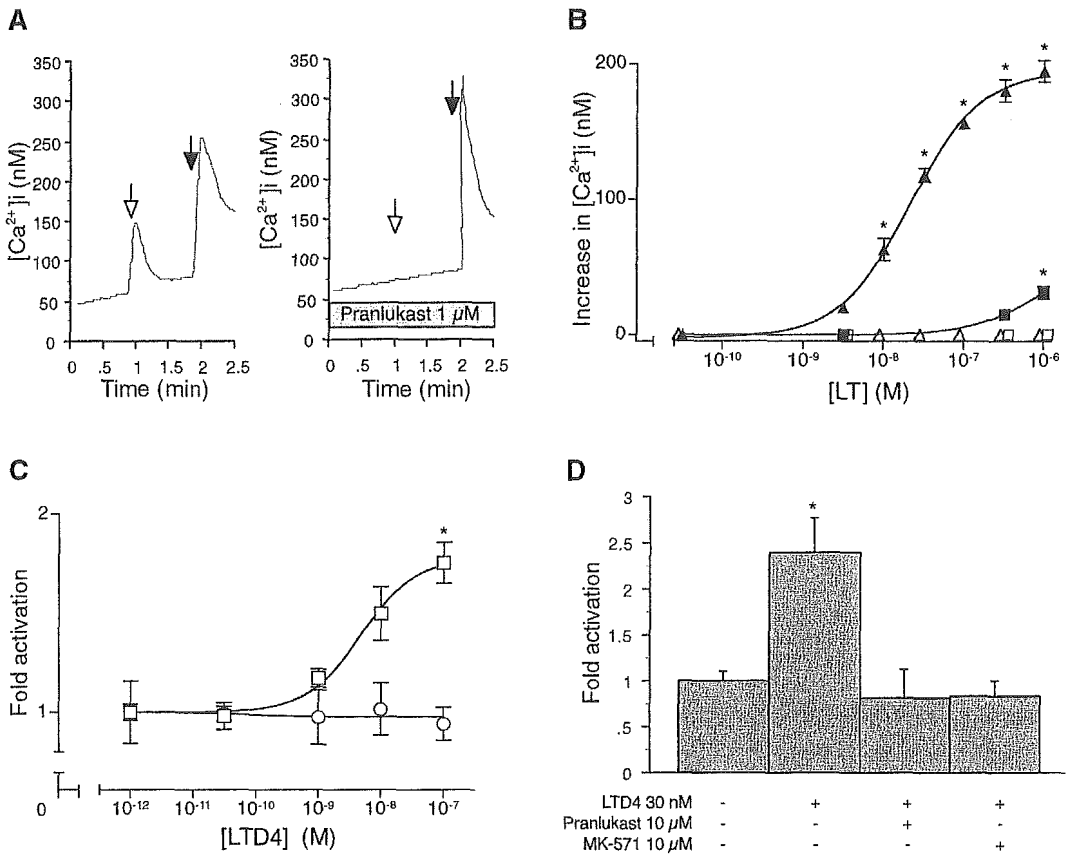


FIG. 2. **Ca^{2+} response and reporter gene expression using $mCysLT_1$.** *A* and *B*, Ca^{2+} mobilization in HEK-293 cells stably expressing $mCysLT_1$. *A*, the $mCysLT_1$-expressing cells (HEK 7-1) loaded with Fura2-AM were challenged with 100 nM LTD_4 (*open arrow*) or 10 μM ATP (*closed arrow*), and the change in $[Ca^{2+}]_i$ was measured. The *right panel* shows the response of the cells treated with 10 μM pranlukast 5 min before the challenge. The results are representatives of three independent experiments. *B*, HEK 7-1 cells were loaded with Fura2-AM and stimulated with various concentrations of LTC_4 (■) or LTD_4 (▲). Vector-transfected HEK-293 cells stimulated with LTC_4 (□) or LTD_4 (Δ) were used as negative controls. The differences of $[Ca^{2+}]_i$ before and after the challenges are shown ($n = 3$, means ± S.E.). Statistically significant differences between the vector control and HEK 7-1 are indicated. *, $p < 0.01$, unpaired *t* test. *C* and *D*, reporter gene assays of B103 cells transiently transfected with $mCysLT_1$ or vector alone. *C*, Cells transfected with $mCysLT_1$ (□) or vector alone (○) were stimulated with various concentrations of LTD_4. The data are expressed as fold activation over control (without LTD_4) and expressed as the means ± S.E. ($n = 3$). Statistically significant differences between the control and the LT-stimulated cells are indicated. *, $p < 0.05$, unpaired *t* test. *D*, the effects of two $CysLT_1$ antagonists are shown. The data are expressed as fold activation over the control (without LTD_4) and expressed as the means ± S.E. ($n = 4$). Statistically significant differences between the control and the drug-treated cells are indicated. *, $p < 0.05$, Bonferroni's multiple *t* test.

and *Renilla* luciferase activities were measured using PICAGENE Dual Seapansy and a Mini Lumat LB9506 luminometer (Berthold, Bad Wildbad, Germany). Firefly luciferase values were standardized to *Renilla* values.

Northern Blotting—Total RNA was extracted from 129+Ter/Sv Jcl (Clea Japan, Tokyo, Japan) and C57BL/6J Jcl (Clea Japan) mouse tissues including brain, heart, lung, liver, spleen, kidney, small intestine, skeletal muscle, and skin, using Isogen (Wako, Osaka, Japan). Poly(A)$^+$ RNA was isolated from 200 μg of the total RNA using a μMACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The RNA samples were denatured, electrophoresed on 0.7% formaldehyde-agarose gels, and transferred onto nylon membranes Hybond-N+ (Amersham Biosciences) as described (26). The membranes were hybridized with [α-^{32}P]dCTP-labeled ORF of $mCysLT_1$, $mCysLT_2$, or human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) at 65 °C for 2 h in a Rapid Hyb hybridization solution (Amersham Biosciences). The membranes were washed at 65 °C in 0.2× SSC, 0.1% SDS for 1 h and subjected to autoradiography for 5 days ($mCysLT_1$ and $mCysLT_2$) or overnight (G3PDH).

Quantitative Real Time Reverse Transcriptase-PCR—Total RNA was prepared as described above from 129 and C57BL/6 mouse adrenal gland, peritoneal macrophages, and spleen. For elicitation of peritoneal macrophages, the animals were injected with 2 ml of 4% thioglycollate broth 4 days prior to sacrifice and peritoneal lavage using ice-cold PBS with 2 mM EDTA. cDNA was synthesized from 1 μg of total RNA using Superscript II (Invitrogen) and 50 ng of random hexamers according to the manufacturer's protocol, and 2 μl of the cDNA was diluted in 38 μl of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) for PCR. PCR was carried out using a LightCycler System (Roche Molecular Biochemicals), and the products were detected by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. The PCR reactions were set up in microcapillary tubes in a volume of 20 μl. The reaction components were 1 μl of diluted cDNA, 1 × FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals), a final concentration of 3 mM $MgCl_2$, and 1 μM upstream and downstream primers. pc4HM-$mCysLT_1$, pc3.1-$mCysLT_2$, and an expressed sequence tag clone containing mG3PDH cDNA (GenBank™ accession number BF537941) purchased from IncyteGenomics (Palo Alto, CA) were used as standards. Primers were chosen so that they would yield PCR products identical in DNA sequence from 129 and C57BL/6 inbred strains. The following primers were used: mCys1-760+, 5′-CAACGAACTATCCACCTTCACC-3′; mCys1-923−, 5′-AGCCTTCTCCTAAAGTTTCCAC-3′; mCys2-662+, 5′-GTCCACGTGCTGCTCATAGG-3′; mCys2-843−, 5′-ATTGGCTGCAGCCATGGTC-3′; mG3PDH-879+, 5′-AGGTTGTCTCCTGCGACTTC-3′; and mG3PDH-1089−, 5′-CTTGCTCAGTGTCCTTGCTG-3′. These primer pairs result in PCR products of 164 ($mCysLT_1$), 182 ($mCysLT_2$), and 211 bp (G3PDH). The standards and the samples were simultaneously amplified using the same reaction master mixture. The reactions were incubated at 95 °C for 10 min to activate the polymerase, followed by 50 cycles of amplification. Each cycle of PCR included 3 s of denaturation at 95 °C, 3 s of primer annealing at 67 °C for G3PDH, 65 °C for $mCysLT_1$, and 68 °C for $mCysLT_2$, and 10 s of extension at 72 °C. The temperature ramp was 20 °C/s. At the end of the extension steps, the fluorescence of each sample was measured to allow quantification of the cDNAs. After cycling, melting curves of the PCR products were acquired by stepwise increase of the temperature from 5 °C above the annealing temperature to 95 °C. Using LightCycler analysis software, the SYBR Green I signal of each sample was plotted *versus* the

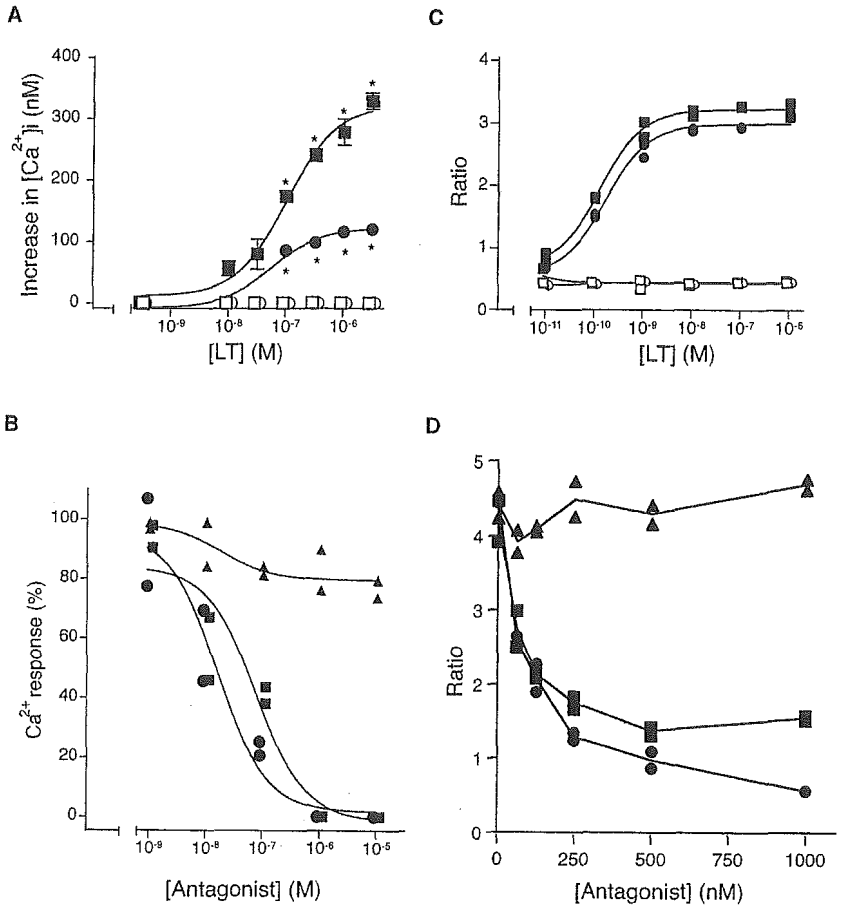


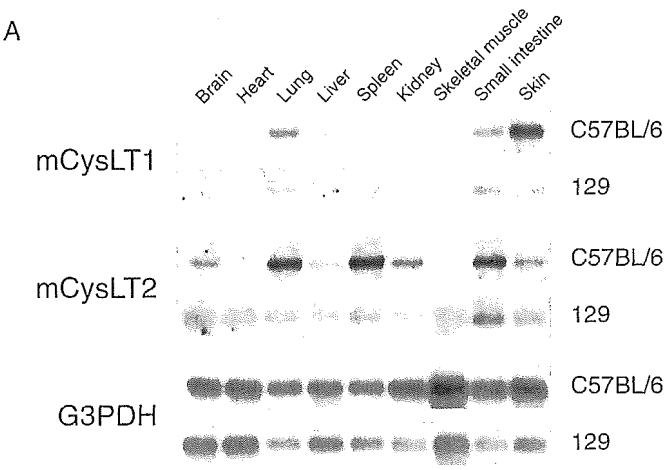
FIG. 3. **Ca^{2+} response and reporter gene expression using $mCysLT_2$.** *A* and *B*, Ca^{2+} mobilization in CHO cells stably expressing $mCysLT_2$. The cells were loaded with Fura2-AM and challenged with LTC_4 or LTD_4. The increase in $[Ca^{2+}]_i$ was calculated from the fluorescence ratio (340 nm/380 nm). *A*, $[Ca^{2+}]_i$ increases of $mCysLT_2$-expressing cells (CHO-7A1) challenged with LTC_4 (■) or LTD_4 (●) and vector control cells challenged with LTC_4 (□) or LTD_4 (○) are shown (n = 3, means ± S.E.). Statistically significant differences between the control and CHO-7A1 are indicated. *, $p < 0.01$, unpaired t test. *B*, effects of CysLT antagonists were examined. The increase in $[Ca^{2+}]_i$ after 100 nM LTC_4 stimulation is shown as a percentage to that of the cells without an antagonist (n = 2, each replicate shown). BAY u9773 (■) and pranlukast (●) inhibited the response to LTC_4, whereas MK-571 (▲) did not affect the response to the LTC_4 stimulation. Neither MK-571, pranlukast, nor BAY u9773 affected Ca^{2+} response to 10 μM ATP (n = 2, data not shown). *C* and *D*, reporter gene assay of PC12 cells transiently transfected with $mCysLT_2$ or vector alone. The ratios of firefly luciferase activity to *Renilla* luciferase activity are shown. *C*, the responses of $mCysLT_2$-transfected cells challenged with various concentrations of LTC_4 (■) or LTD_4 (●) and vector-transfected cells challenged with LTC_4 (□) or LTD_4 (○) are shown (n = 2, each replicate shown). The experiments using LTC_4 were performed in the presence of 5 mM serine and 10 mM borate. *D*, the responses to 10 nM LTD_4 in the presence of various concentrations of MK-571 (▲), pranlukast (●), or BAY u9773 (■) are shown (n = 2, each replicate shown).

number of cycles, and the crossing points were obtained. These crossing points correlate inversely with the log of the initial template concentration. The levels of mRNA were estimated by subtracting the initial levels of target DNA in PCR reactions without reverse transcription, which represents genomic contamination. Then the mRNA levels were normalized to the level of G3PDH mRNA.

In Situ Hybridization—Paraffin sections of the skin samples from 129 and C57BL/6 mice fixed in 10% formalin were investigated as described previously (27, 28) by using a slightly modified nonradioactive *in situ* hybridization technique with digoxigenin-labeled RNA probes. Briefly, paraffin-embedded tissues were cut to 4-μm-thin sections, mounted onto silane-coated slides, deparaffinized, and treated with proteinase K (5 μg/ml in PBS) for 10 min at 24 °C and glycine (2 mg/ml in PBS) for 15 min at 24 °C. Then the sections were acetylated with acetic anhydride (1 ml in 400 ml of 0.1 M triethanolamine, pH 8.0) for 15 min at 24 °C. After washing with PBS, the samples were soaked in 2× SSC with 50% formamide, subjected to hybridization. Fragments of cDNAs for mCysLTs ($mCysLT_1$ ORF at 687–887 and $mCysLT_2$ ORF at 18–222) were amplified by PCR using upstream primers with a recognition sequence for *Hin*dIII and downstream primers with a recognition sequence for *Eco*RI, and subcloned into pSPT18 by directional cloning. The plasmids were linearized using *Hin*dIII to prepare the antisense probes and *Eco*RI for the sense probes. The probes were labeled with digoxigenin-11-UTP using a DIG RNA labeling kit (Roche Molecular Biochemicals). The labeled RNA probes (1 μg/ml) in a mixture containing 50% formamide, 10% dextran sulfate, 2× SSC, 1 mg/ml tRNA, 1 mg/ml salmon sperm DNA, and 0.1% bovine serum albumin were placed on the slides and coverslipped. Hybridization was performed in a humidified chamber for 16 h at 42 °C for the $mCysLT_1$ probe and 50 °C for the $mCysLT_2$ probe. The slides were washed in 2× SSC with 50% formamide for 20 min three times at 42 °C. Nonhybridized probes were digested in 20 μg/ml RNase A, 500 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.0) for 30 min at 37 °C. They were then rinsed for 20 min in 0.1× SSC three times at 42 °C. The digoxigenin-labeled probes were visualized using a DIG nucleic acid detection kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. The slides were counterstained in methyl green for 10 min, washed in running tap water, and mounted.

RESULTS AND DISCUSSION

The Structure of $mCysLT_1$ and $mCysLT_2$—$mCysLT_1$ and $mCysLT_2$ were predicted to be polypeptides of 339 and 309 amino acid residues, respectively (Fig. 1*A*). The identities of the amino acid sequences between 129 mouse and human (16–18) CysLTs are shown in Fig. 1*B*. $mCysLT_1$ was longer than human $CysLT_1$ by two amino acid residues. $mCysLT_2$ was shorter than human $CysLT_2$ by 37 amino acid residues, being truncated at both the N and C termini. The sequence of $mCysLT_1$ was identical among 129, C57BL/6, and BALB/c mice, and there was a mismatch in $mCysLT_2$ sequences at the 213th amino acid residue between 129 (Val) and C57BL/6 (Ile). The preserved amino acids in the rhodopsin-like G protein-coupled



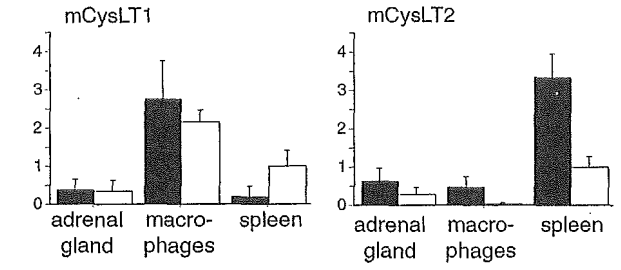


FIG. 4. **Expression of $mCysLT_1$ and $mCysLT_2$ mRNA in various tissues of C57BL/6 and 129 inbred strains.** *A*, Northern blot analysis. Poly(A)$^+$ RNAs (3 μg) were electrophoretically separated, transferred to nylon membranes, and hybridized with [α-^{32}P]dCTP-labeled ORFs of $mCysLT_1$, $mCysLT_2$, and human G3PDH. The shown data are representative of three experiments that gave similar results. *B*, quantitative real time reverse transcriptase-PCR. mRNA levels of C57BL/6 mice (*closed bar*) and 129 mice (*open bar*) were obtained as described under "Materials and Methods" and given as fold expression compared with the levels of 129 spleen (n = 3, means ± S.E.). The differences in expression levels between strains were seen in $CysLT_2$ of the spleen ($p < 0.05$).

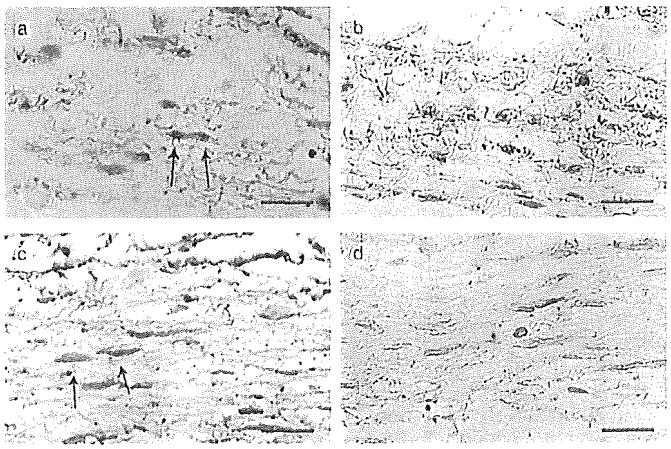


FIG. 5. ***In situ* hybridization of $CysLT_1$ and $CysLT_2$ mRNA in 129 mouse skin.** *a* and *b*, *in situ* hybridization of $CysLT_1$. *a*, antisense probe, showing expression in subcutaneous fibroblasts (*arrows*). *b*, sense control probe. *c* and *d*, *in situ* hybridization of $CysLT_2$. *c*, antisense probe, showing expression in subcutaneous fibroblasts (*arrows*). *d*, sense control probe. *Scale bars* are 50 μm.

receptor family, including two Cys residues in the first and second extracellular loops, Asp in transmembrane domain 2 (TM2), Trp in TM4, Tyr in TM5, and Pro in TM6, were all present in $mCysLT_1$ and $mCysLT_2$. $mCysLT_2$ had the Asn-Pro-Xaa_2-Tyr motif at the end of the TM7, whereas $mCysLT_1$ had an Asp residue instead of Asn in the motif. There was no Asp-Arg-Tyr motif at the TM3/intracellular loop 2 transition in $mCysLT_1$ nor $mCysLT_2$, although it is a highly conserved motif in the G protein-coupled receptor family. Both $mCysLT_1$ and $mCysLT_2$ had possible phosphorylation sites in intracellular loop 3 and the C terminus, and $CysLT_1$ had several possible *N*-glycosylation sites in the N terminus and extracellular loops.

Pharmacological Properties of $mCysLT_1$ and $mCysLT_2$—Mouse orthologues of $CysLT_1$ and $CysLT_2$ were identified as functional cysteinyl LT receptors by several methods. $CysLT_1$ and $CysLT_2$ are known to increase $[Ca^{2+}]_i$ (20, 29). LTD_4 evoked a dose-dependent increase in $[Ca^{2+}]_i$ in HEK-293 cell lines stably expressing $mCysLT_1$ (HEK 7-1 (Fig. 2, *A* and *B*) and HEK 7-3 (data not shown)). LTC_4 also evoked a slight increase in $[Ca^{2+}]_i$ (Fig. 2*B*), whereas LTB_4 or LTE_4 did not (data not shown). These cells pretreated with a $CysLT_1$ antagonist, pranlukast (Fig. 2*A*) or MK-571 (data not shown), did not respond to LTD_4, whereas they remained responsive to ATP. In a reporter gene assay, B103 cells transiently expressing $mCysLT_1$ increased luciferase activity in response to LTD_4 in a dose-dependent manner (Fig. 2*C*). The cells did not respond to either LTB_4 or LTE_4 at a concentration of 10 or 100 nM (data not shown). The LTD_4-induced response was inhibited by pranlukast or MK-571 (Fig. 2*D*).

In CHO cells stably expressing $mCysLT_2$ (CHO-7A1), LTC_4 and LTD_4 exhibited dose-dependent increases in $[Ca^{2+}]_i$ (Fig. 3*A*). The response was inhibited by BAY u9773, a nonselective antagonist of cysteinyl LT receptors (30), in a dose-dependent manner but was not inhibited by a $CysLT_1$-specific antagonist, MK-571 (Fig. 3*B*). The response of CHO-8B3 cells, another $mCysLT_2$-expressing clone, was similar to that of CHO-7A1 (n = 3; data not shown). ATP (10 μM) elicited the same level of increase in $[Ca^{2+}]_i$ in CHO-7A1, CHO-8B3, and the vector control (n = 3; data not shown). PC12 cells transiently expressing $mCysLT_2$ increased luciferase activities in response to LTC_4 and LTD_4 to the same extent in dose-dependent manners (Fig. 3*C*), and the responses were inhibited by BAY u9773 and not by MK-571 (Fig. 3*D*). Surprisingly, pranlukast, found to be a $CysLT_1$-specific antagonist from human studies (16, 17), inhibited the LTC_4-induced increase in $[Ca^{2+}]_i$ (Fig. 3*B*) and the LTD_4-induced luciferase activity (Fig. 3*D*) in the cells expressing $mCysLT_2$. Several reports showing that pranlukast does not antagonize human $CysLT_2$ (18, 20) imply a pharmacological difference of $CysLT_2$ between human and mouse likely because of significant difference in primary structure (Fig. 1*B*). BAY u9773 was partially agonistic on $mCysLT_2$ as is reported in human $CysLT_2$ (19) (data not shown).

Different Tissue Distribution of $CysLT_1$ and $CysLT_2$ mRNA in Two Mouse Inbred Strains—Hybridization of poly(A)$^+$ RNA from various mouse tissues detected transcripts of 3.0 and 5.5 kb for $CysLT_1$ and $CysLT_2$, respectively (Fig. 4*A*). As a whole, the expression levels of CysLTs were higher in C57BL/6 inbred strain than in 129 inbred strain, even though a slight difference in control hybridization (G3PDH) in Northern blotting was observed in some tissues. In C57BL/6 strain, the highest mRNA expression for $CysLT_1$ was observed in skin, lung, small intestine, and macrophages, and moderate expressions were found in other tissues; the expression of $CysLT_2$ was ubiquitous with higher expressions in spleen, lung, and small intestine (Fig. 4). Differential tissue expression between two strains suggests that regulatory polymorphism is present.

Given the importance of cysteinyl LTs in skin diseases including atopic dermatitis (31), we investigated the distribution of CysLTs in mouse skin by *in situ* hybridization. We chose 129 inbred strain because Goulet *et al.* (32) had reported the potent inflammatory response of the skin in 129 mice. No signals of

CysLTs were detected in epidermis (data not shown). In the subcutaneous connective tissues, however, high expressions of $CysLT_1$ (Fig. 5*a*) and $CysLT_2$ mRNA (Fig. 5*c*) were seen mostly in fibroblasts. No signal was obtained using the sense control (Fig. 5, *b* and *d*). It has been reported that cysteinyl LTs increase collagen synthesis in fibroblasts (33, 34), and our report is the first to demonstrate the expression of CysLTs in fibroblasts. Further study is needed to uncover yet unknown roles of cysteinyl LTs in wound healing and pathological collagen synthesis.

In conclusion, we have cloned $mCysLT_1$ and $mCysLT_2$ and found differences in the pharmacological characteristics between mouse and human $CysLT_2$. There are differences in mRNA expression of $CysLT_1$ and $CysLT_2$ between mouse strains, suggesting the importance of choosing a proper mouse strain for a disease model. We also discovered expression of both CysLTs in subcutaneous fibroblasts. These data are useful in interpreting and understanding the physiological and pathological roles of CysLTs in animal models of human diseases.

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REFERENCES

1. Murphy, R. C., Hammarstrom, S., and Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4275–4279
2. Shimizu, T., and Wolfe, L. S. (1990) *J. Neurochem.* **55,** 1–15
3. Samuelsson, B. (1983) *Science* **220,** 568–575
4. Samuelsson, B., Dahlen, S. E., Lindgren, J. A., Rouzer, C. A., and Serhan, C. N. (1987) *Science* **237,** 1171–1176
5. Welsch, D. J., Creely, D. P., Hauser, S. D., Mathis, K. J., Krivi, G. G., and Isakson, P. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9745–9749
6. Lam, B. K. (1997) *Front. Biosci.* **2,** 380–386
7. Penrose, J. F., and Austen, K. F. (1999) *Proc. Assoc. Am. Physicians* **111,** 537–546
8. Dahlen, S. E., Hedqvist, P., Hammarstrom, S., and Samuelsson, B. (1980) *Nature* **288,** 484–486
9. Piper, P. J. (1984) *Physiol. Rev.* **64,** 744–761
10. Lewis, R. A., Austen, K. F., and Soberman, R. J. (1990) *N. Engl. J. Med.* **323,** 645–655
11. Drazen, J. M. (1998) *Am. J. Respir. Crit. Care Med.* **158,** (suppl.) 193–200
12. Jones, T. R., Labelle, M., Belley, M., Champion, E., Charette, L., Evans, J., Ford-Hutchinson, A. W., Gauthier, J. Y., Lord, A., Masson, P., McAuliffe, M., McFarlane, C. S., Metters, K. M., Pickett, C., Piechuta, H., Rochette, C., Rodger, I. W., Sawyer, N., Young, R. N., Zamboni, R., and Abraham, W. M. (1995) *Can. J. Physiol. Pharmacol.* **73,** 191–201
13. Reiss, T. F., Chervinsky, P., Dockhorn, R. J., Shingo, S., Seidenberg, B., and Edwards, T. B. (1998) *Arch. Intern. Med.* **158,** 1213–1220
14. Suissa, S., Dennis, R., Ernst, P., Sheehy, O., and Wood-Dauphinee, S. (1997) *Ann. Intern. Med.* **126,** 177–183
15. Obata, T., Okada, Y., Motoishi, M., Nakagawa, N., Terawaki, T., and Aishita, H. (1992) *Jpn. J. Pharmacol.* **60,** 227–237
16. Lynch, K. R., O'Neill, G. P., Liu, Q., Im, D. S., Sawyer, N., Metters, K. M., Coulombe, N., Abramovitz, M., Figueroa, D. J., Zeng, Z., Connolly, B. M., Bai, C., Austin, C. P., Chateauneuf, A., Stocco, R., Greig, G. M., Kargman, S., Hooks, S. B., Hosfield, E., Williams, D. L., Jr., Ford-Hutchinson, A. W., Caskey, C. T., and Evans, J. F. (1999) *Nature* **399,** 789–793
17. Sarau, H. M., Ames, R. S., Chambers, J., Ellis, C., Elshourbagy, N., Foley, J. J., Schmidt, D. B., Muccitelli, R. M., Jenkins, O., Murdock, P. R., Herrity, N. C., Halsey, W., Sathe, G., Muir, A. I., Nuthulaganti, P., Dytko, G. M., Buckley, P. T., Wilson, S., Bergsma, D. J., and Hay, D. W. (1999) *Mol. Pharmacol.* **56,** 657–663
18. Heise, C. E., O'Dowd, B. F., Figueroa, D. J., Sawyer, N., Nguyen, T., Im, D. S., Stocco, R., Bellefeuille, J. N., Abramovitz, M., Cheng, R., Williams, D. L., Jr., Zeng, Z., Liu, Q., Ma, L., Clements, M. K., Coulombe, N., Liu, Y., Austin, C. P., George, S. R., O'Neill, G. P., Metters, K. M., Lynch, K. R., and Evans, J. F. (2000) *J. Biol. Chem.* **275,** 30531–30536
19. Nothacker, H. P., Wang, Z., Zhu, Y., Reinscheid, R. K., Lin, S. H., and Civelli, O. (2000) *Mol. Pharmacol.* **58,** 1601–1608
20. Takasaki, J., Kamohara, M., Matsumoto, M., Saito, T., Sugimoto, T., Ohishi, T., Ishii, H., Ota, T., Nishikawa, T., Kawai, Y., Masuho, Y., Isogai, T., Suzuki, Y., Sugano, S., and Furuichi, K. (2000) *Biochem. Biophys. Res. Commun.* **274,** 316–322
21. Maekawa, A., Kanaoka, Y., Lam, B. K., and Austen, K. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2256–2261
22. Henderson, W. R., Jr., Lewis, D. B., Albert, R. K., Zhang, Y., Lamm, W. J., Chiang, G. K., Jones, F., Eriksen, P., Tien, Y. T., Jonas, M., and Chi, E. Y. (1996) *J. Exp. Med.* **184,** 1483–1494
23. Jones, T. R., Zamboni, R., Belley, M., Champion, E., Charette, L., Ford-Hutchinson, A. W., Frenette, R., Gauthier, J. Y., Leger, S., Masson, P., McFarlane, C. S., Piechuta, H., Rokach, J., Williams, H., and Young, R. N. (1989) *Can. J. Physiol. Pharmacol.* **67,** 17–28
24. Yamazawa, T., Iino, M., and Endo, M. (1992) *FEBS Lett.* **301,** 181–184
25. Kawasawa, Y., Kume, K., Izumi, T., and Shimizu, T. (2000) *Biochem. Biophys. Res. Commun.* **276,** 957–964
26. Chomczynski, P. (1992) *Anal. Biochem.* **201,** 134–139
27. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Grotendorst, G. R., and Takehara, K. (1996) *J. Invest. Dermatol.* **106,** 729–733
28. Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Grotendorst, G. R., and Takehara, K. (1995) *J. Invest. Dermatol.* **105,** 280–284
29. Chan, C. C., Ecclestone, P., Nicholson, D. W., Metters, K. M., Pon, D. J., and Rodger, I. W. (1994) *J. Pharmacol. Exp. Ther.* **269,** 891–896
30. Coleman, R. A., Eglen, R. M., Jones, R. L., Narumiya, S., Shimizu, T., Smith, W. L., Dahlen, S. E., Drazen, J. M., Gardiner, P. J., Jackson, W. T., Jones, T. R., Krell, R. D., and Nicosia, S. (1995) *Adv. Prostaglandin. Thromboxane. Leukot. Res.* **23,** 283–285
31. Hishinuma, T., Suzuki, N., Aiba, S., Tagami, H., and Mizugaki, M. (2001) *Br. J. Dermatol.* **144,** 19–23
32. Goulet, J. L., Byrum, R. S., Key, M. L., Nguyen, M., Wagoner, V. A., and Koller, B. H. (2000) *J. Immunol.* **164,** 4899–4907
33. Phan, S. H., McGarry, B. M., Loeffler, K. M., and Kunkel, S. L. (1988) *Biochemistry* **27,** 2846–2853
34. Abe, M., Kurosawa, M., Ishikawa, O., and Miyachi, Y. (2000) *J. Allergy Clin. Immunol.* **106,** (suppl.) 78–84

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Platelet-activating factor drives eotaxin production in an allergic pleurisy in mice

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1 The activation of eosinophils *via* G-protein-coupled seven transmembran receptors play a necessary role in the recruitment of these cells into tissue. The present study investigates a role for PAF in driving eotaxin production and eosinophil recruitment in an allergic pleurisy model in mice.

2 The intrapleural injection of increasing doses of PAF (10^{-11} to 10^{-9} moles per cavity) induced a dose- and PAF receptor-dependent recruitment of eosinophils 48 h after stimulation.

3 Intrapleural injection of PAF induced the rapid (within 1 h) release of eotaxin into the pleural cavity of mice and an anti-eotaxin antibody effectively inhibited PAF-induced recruitment of eosinophils.

4 Eosinophil recruitment in the allergic pleurisy was markedly inhibited by the PAF receptor antagonist UK-74,505 (modipafant, 1 mg kg^{-1}). Moreover, recruitment of eosinophils in sensitized and challenged PAF receptor-deficient animals was lower than that observed in wild-type animals.

5 Blockade of PAF receptors with UK-74,505 suppressed by 85% the release of eotaxin in the allergic pleurisy.

6 Finally, the injection of a sub-threshold dose of PAF and eotaxin cooperated to induce eosinophil recruitment *in vivo*.

7 In conclusion, the production of PAF in an allergic reaction could function in multiple ways to facilitate the recruitment of eosinophils – by facilitating eotaxin release and by cooperating with eotaxin to induce greater recruitment of eosinophils.

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Abbreviations: LTB_4, Leukotriene B_4; OVA, ovalbumin; PAF, platelet-activating factor; $PAFR^{-/-}$, PAF receptor-deficient; PBS, phosphate-buffered saline; SCF, stem cell factor

Introduction

Eosinophils are typically tissue-dwelling cells and appear to play an important role in the pathogenesis of allergic diseases, such as asthma and atopic dermatitis (Cara *et al.*, 1999). There has been much interest in the understanding of the mechanisms underlying eosinophil recruitment *in vivo* as this knowledge may aid in the development of novel strategies for the treatment of allergic disorders (Teixeira *et al.*, 1995; Giembycz & Lindsay, 1999). The activation of eosinophils *via* G-protein-coupled seven transmembrane receptors play a necessary role in the recruitment of these cells into tissue and may, thus, be good targets for drug development (Teixeira *et al.*, 1995; 1997a,b).

We have recently shown that the cytokine stem cell factor (SCF) played an important role for the migration of eosinophils in an allergic pleurisy model in mice (Klein *et al.*, 2000). Of interest, SCF appeared to drive the local production of LTB_4 which cooperated with eotaxin to induce eosinophil recruitment (Klein *et al.*, 2001). Whereas SCF induced significant eotaxin production, the blockade of SCF did not inhibit the release of eotaxin following allergen challenge of sensitized animals (Klein *et al.*, 2001). Thus, it was not clear from our studies the mediator(s) underlying local eotaxin production and release.

Several studies have demonstrated the ability of the lipid mediator platelet-activating factor (PAF) to induce eosinophil recruitment and activation *in vivo* and *in vitro* (e.g. Silva *et al.*, 1991; Teixeira *et al.*, 1994; 1997a; Alves *et al.*, 1996). Indeed, PAF induces chemoattraction, activation of several other functions and priming of eosinophils and other cell types (e.g. van der bruggen *et al.*, 1994; Schweizer *et al.*, 1996; Ishii & Shimizu, 2000). Moreover, endogenous production of PAF may be involved in the effector function of eosinophils (e.g.: Tool *et al.*, 1992; Bartemes *et al.*, 1999) and in the modulation of the production of chemokines (e.g.: Maruoka *et al.*, 2000; Au *et al.*, 2001) in response to several stimuli. Here, using a PAF receptor antagonist (UK-74,505) and PAF receptor-deficient animals ($PAFR^{-/-}$), we investigated whether PAF participated in the cascade of events leading to mediator release and eosinophil recruitment in our allergic pleurisy model.

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Methods

Animals

Male BALB/C mice (18–22 g) were used throughout these experiments. Animals were housed in a temperature-controlled room with free access to water and food. All experimental procedures have been subjected to evaluation and were approved by the local Animal Ethics Committee. PAF receptor deficient animals were generated as previously described (Ishii *et al.*, 1998) and intercrossed for at least seven generations to establish the BALB/c strain.

Drugs and reagents

Recombinant murine eotaxin was purchased from Peprotech (London, U.K.). Eotaxin was dissolved in water, diluted further in phosphate buffered saline (PBS, pH 7.4) containing 0.01% bovine serum albumin and stored at −20°C until use. Bovine serum albumin, ovalbumin (OVA) and control rabbit serum were purchased from Sigma. PAF was purchased from Calbiochem. The specific and long-acting PAF receptor antagonist UK-74,505 (Modipafant, 4-(chlorophenyl)-1,4-dihydro-3-ethoxycarbonyl- 6-methyl-2- [4-(2-methylimidazol[4, 5-c]phenyl-5-[N-(2-pyridyl)carbomoyl]pyridine) (Alabaster *et al.*, 1991; Jezequel *et al.*, 1996) was a gift from Pfizer Global Research and Development, Kent, U.K. UK-74,505 was dissolved in HCl 0.01 N and further diluted in PBS. Control animals received drug vehicle.

Sensitization

Animals were immunized with OVA adsorbed to aluminium hydroxide gel as previously described (Das *et al.*, 1997). Briefly, mice were injected s.c. on days 1 and 8 with 0.2 ml of a solution containing 100 μg of OVA and 70 μg of aluminium hydroxide (Reheiss, Dublin, Ireland).

Leukocyte migration into the pleural cavity induced by PAF or antigen

PAF (10^{-11} to 10^{-9} mol per cavity) was injected intrapleurally (i.pl.) in naïve mice, and animals killed at 48 h after the i.pl. injection. In some experiments, low doses of eotaxin and PAF were mixed prior to their i.pl. injection. Sensitized mice were challenged with antigen (OVA) or PBS. The cells present in the pleural cavity were harvested by injecting 2 ml of PBS and total cell counts performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytospin preparations (Shandon III) stained with May-Grumwald-Giemsa using standard morphologic criteria to identify cell types. The results are presented as the number of cells per cavity.

PAF receptor antagonist or anti-eotaxin pretreatment

In order to investigate the role of endogenous PAF on the eosinophil recruitment induced by PAF or ovalbumin in immunized animals, the PAF receptor antagonist UK-74,505 (0.1 to 1.0 mg kg^{-1}) was administered i.p. 60 min prior to the stimulus, and the number of infiltrating eosinophils was assessed 48 hours after. This dose range has been shown to be effective at blocking PAF receptors in rodents (Miotla *et al.*, 1998; Borges *et al.*, 2000). A rabbit polyclonal anti-eotaxin antibody was prepared and purified over protein A column as previously described (Ruth *et al.*, 1998). This antibody is specific for eotaxin and not shown to cross-react to other known murine chemokines (Ruth *et al.*, 1998). Anti-eotaxin was administered i.p. at the dose of 100 μg per mouse 60 min prior to the i.pl. administration of PAF. In some experiments, the eosinophil recruitment 48 h after antigen challenge in $PAFR^{-/-}$ mice was compared to that of age- and sex-matched wild-type animals.

Measurement of eotaxin

Frozen supernatants obtained from pleural cavity washes at different times (1–24 h) after challenge with PAF (10^{-9} moles per cavity) were used for eotaxin detection. The effects of the PAF receptor antagonist was assessed by injecting UK-74,505 (1 mg kg^{-1}, i.p.) 60 min prior to antigen challenge. Six hours later, the pleural cavity was washed and the levels of eotaxin assessed on the supernatant.

The concentration of eotaxin protein in pleural effluents was measured by specific ELISA using commercially available antibody pairs and as specified by the supplier (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All results are presented as the mean ± s.e.mean. Normalized data were analysed by one-way ANOVA, and differences between groups were assessed using the Student-Newman-Keuls post-test. A P value <0.05 was considered significant.

Results

PAF induces eosinophil recruitment and eotaxin production in the pleural cavity of mice

The intrapleural injection of increasing doses of PAF (10^{-11} to 10^{-9} moles per cavity) induced a dose-dependent recruitment of eosinophils 48 h after stimulation (Figure 1). At this time point, a significant recruitment of mononuclear cells, but not neutrophils, was also observed (data not shown). These effects of PAF were PAF receptor-dependent as demonstrated by the ability of the PAF receptor antagonist UK-74,505 to abrogate PAF-induced eosinophil recruitment (PBS, 0.2 ± 0.1 eosinophils $\times 10^5$ per cavity; PAF 10^{-9} moles, 1.4 ± 0.3; PAF + UK-74,505 0.1 mg kg^{-1}; 0.4 ± 0.1; PAF + UK-74,505 1.0 mg kg^{-1}, 0.2 ± 0.1; $n=5$ in each group, $P<0.01$).

Next, we examined whether PAF could induce the release of eotaxin into the pleural cavity of mice. As seen in Figure 2A, significant levels of eotaxin were detected as early as 1 h after the injection of PAF in the pleural cavity of naïve mice. Elevated concentrations of eotaxin were also detected at 3 h and levels had dropped to background 6 h after PAF injection (Figure 2A). Not only was eotaxin induced after injection of PAF, but, more importantly, an anti-eotaxin antibody effectively inhibited the recruitment of eosinophils observed after injection of PAF (Figure 2B).

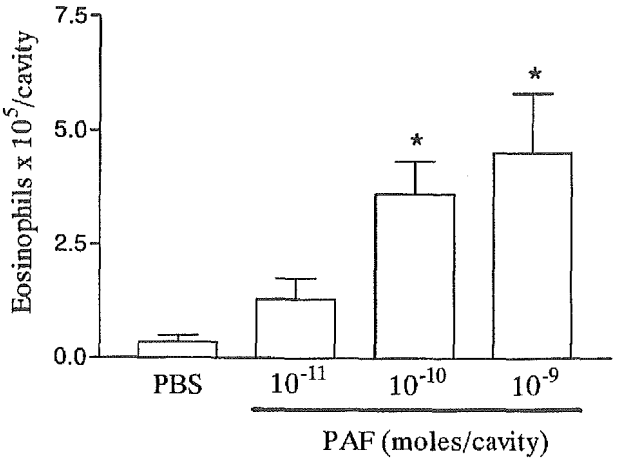

Figure 1 Dose-dependent effects of PAF on the recruitment of eosinophils to the pleural cavity of mice. In dose-response experiments, phosphate-buffered saline (PBS, 100 μl) or PAF (10^{-11} to 10^{-9} moles per cavity) were administered into the pleural cavity of naïve mice and the number of infiltrating eosinophils assessed 48 h after injection. The results are expressed as mean±s.e.mean of six mice in each group. *$P<0.01$ when compared to PBS-injected animals.

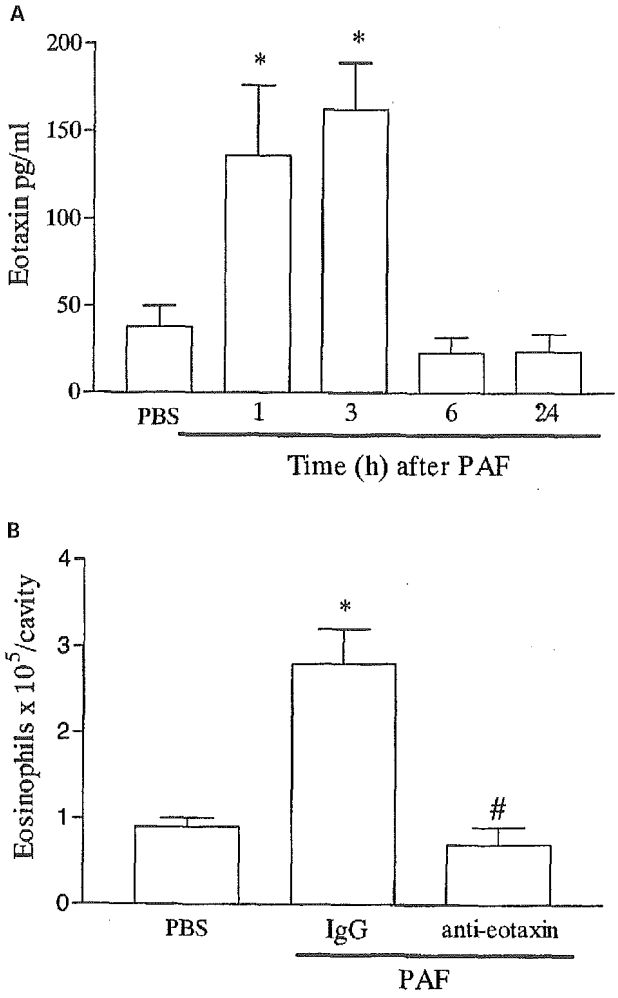

Figure 2 Time-course of the release of eotaxin into the pleural cavity after administration of PAF and effects of an anti-eotaxin polyclonal antibody on PAF-induced eosinophil recruitment. (A) Naïve mice were given an i.pl. injection of PAF (10^{-9} moles per cavity) and at different times after challenge (1–24 h), the pleural cavity of animals were washed, the cells centrifuged, and the supernatants used for the determination of eotaxin using a specific ELISA. (B) Naïve mice were pretreated with non-immune IgG (100 μg, i.p.) or purified anti-eotaxin polyclonal antibody (100 μg, i.p.) 30 min before the i.pl. injection of PAF (10^{-9} moles per cavity) and the number of infiltrating eosinophils assessed after 48 h. Results are expressed as mean±s.e.mean of six mice in each group. *$P<0.01$ when compared to PBS-injected mice and #$P<0.01$ when compared to animals treated with non-immune IgG.

Blockade of PAF receptors regulate the release of eotaxin and eosinophil recruitment in the allergic pleurisy

The possibility that endogenous production of PAF modulated eosinophil recruitment in the allergic pleurisy was investigated by using the PAF receptor antagonist UK-74,505. Pretreatment with UK-74,505 (1 mg kg^{-1}) significantly suppressed by 87% the recruitment of eosinophils observed 48 h after administration of antigen to sensitized mice (Figure 3A). As PAF induced significant local release of eotaxin, we examined whether blockade of PAF receptors would modulate eotaxin production in the allergic pleurisy. Our previous studies have demonstrated that eotaxin release peaks after 6 h in sensitized animals challenged with antigen (Klein *et al.*, 2001). Here, we demonstrate that pretreatment with UK-74,505 inhibited antigen-induced eotaxin production by 85% (Figure 4).

To confirm an important role of PAF receptors for the migration of eosinophils *in vivo*, PAFR$^{-/-}$ and wild-type mice were sensitized and challenged with antigen. As demonstrated in Figure 3B, eosinophil recruitment in PAFR$^{-/-}$ mice was inhibited by 50% as compared to wild-type mice. In PAFR$^{-/-}$ mice, the injection of PAF failed to induce a significant recruitment of eosinophils as compared to PBS-injected animals (wild-type: PBS, $0.2\pm0.1\times10^5$ eosinophils per cavity; PAF 10^{-9} moles per cavity, 2.7 ± 0.8; PAFR$^{-/-}$, PAF 10^{-9} moles per cavity, 0.4 ± 0.1, $P<0.05$, $n=4$).

Cooperation between PAF and eotaxin

Next, we investigated whether sub-threshold doses of PAF and eotaxin could cooperate to induce eosinophil recruitment *in vivo*. As seen in Figure 5, a sub-threshold dose of eotaxin (10 ng per cavity) and PAF (10^{-11} moles per cavity) failed to induce eosinophil recruitment in the pleural cavity of naïve mice when injected alone. Nevertheless, concomitant injection of PAF and eotaxin induced significant eosinophil recruitment that was greater than the somation of either mediator when injected alone (Figure 5). The injection of the mediators alone or in combination failed to affect significantly the levels of circulating eosinophils at 1, 4, 24 and 48 h after challenge, as compared to PBS-injected mice (data not shown).

Discussion

There are many experimental and clinical studies supporting a role for eosinophil recruitment and function in the pathophysiology of allergic diseases (Cara *et al.*, 1999). Thus, strategies which limit eosinophil migration and/or function *in vivo* may be relevant as novel therapy for the treatment of

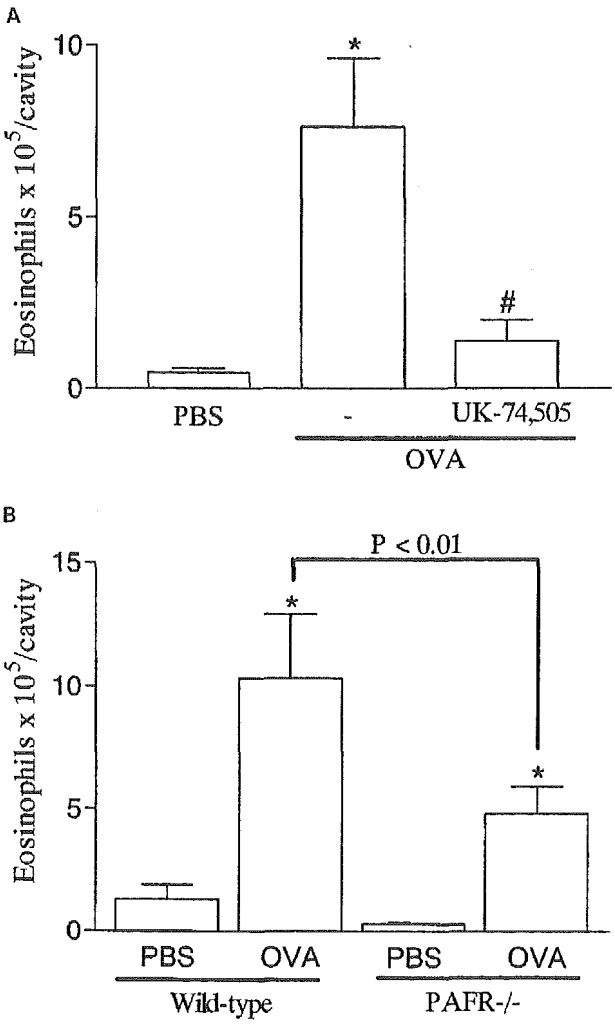

Figure 3 Effects of the PAF receptor antagonist, UK-74,505, and of PAF receptor deficiency on the eosinophil recruitment induced by allergen challenge in sensitized mice. (A) Mice were pretreated with UK-74,505 (1 mg kg^{-1}, i.p.) 60 min before the i.pl. injection of antigen (OVA, 1 μg per cavity) in sensitized mice and the number of infiltrating eosinophils assessed after 48 h. (B) Immunized wild-type or $PAFR^{-/-}$ received an i.pl. injection of antigen (OVA, 1 μg per cavity) and the number of infiltrating eosinophils assessed after 48 h. Results are expressed as means$\pm$s.e.mean of six mice in each group. *$P<0.01$ when compared to PBS-injected mice and #$P<0.01$ when compared to animals treated with drug vehicle.

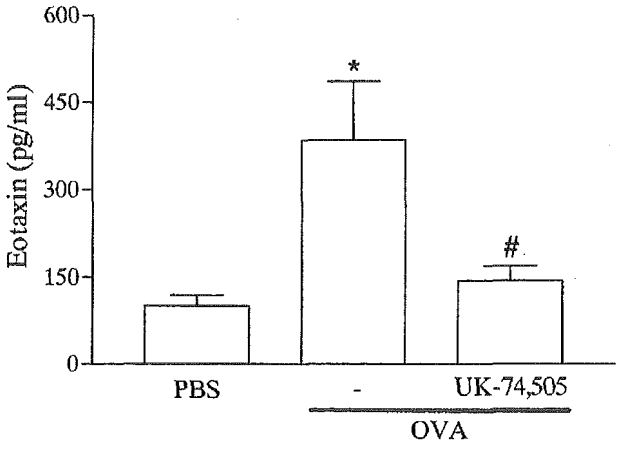

Figure 4 Effects of the PAF receptor antagonist, UK-74,505, on the release of eotaxin induced by allergen challenge in sensitized mice. Mice were pretreated with UK-74,505 (1 mg kg^{-1}, i.p.) 60 min before the i.pl. injection of antigen (OVA, 1 μg per cavity) in sensitized mice and the levels of eotaxin on the pleural wash assessed after 6 h. Results are expressed as means$\pm$s.e.mean of six mice in each group. *$P<0.01$ when compared to PBS-injected mice and #$P<0.01$ when compared to animals treated with drug vehicle.

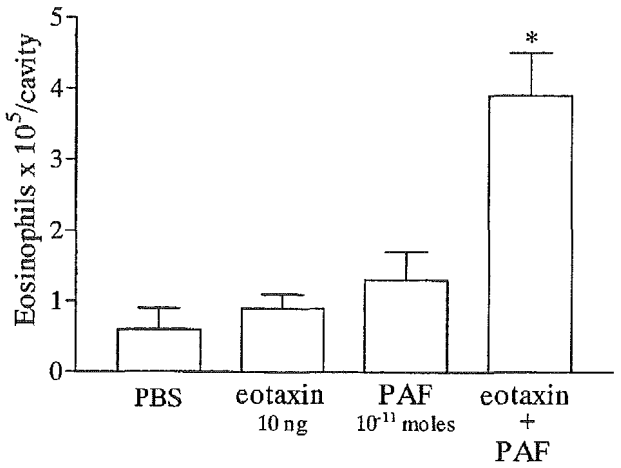

Figure 5 Cooperative effects of the injection of sub-threshold doses of eotaxin and PAF on eosinophil recruitment into the pleural cavity of mice. Mice were injected with phosphate-buffered saline (PBS, 100 μl), eotaxin (10 ng), PAF (10^{-11} moles) or with PAF and eotaxin and the number of infiltrating eosinophils assessed after 48 h. The results are expressed as means$\pm$s.e.mean of six mice in each group. *$P<0.01$ when compared to mice injected with PBS or the mediators alone.

allergic diseases (Teixeira *et al.*, 1995; Giembycz & Lindsay, 1999). Chemokines are among the mediators which are thought to play an important role for the migration of eosinophils (and other leukocytes) *in vivo* (Murphy *et al.*, 2000). We have been particularly interested in understanding the mediators of the inflammatory process which regulate chemokine production and how chemokines interact with other mediators to induce the recruitment of eosinophils *in vivo*. We have previously demonstrated a role for the cooperation between SCF-driven LTB_4 release and eotaxin in mediating eosinophil recruitment *in vivo* (Klein *et al.*, 2000; 2001). Here, we investigated a role for PAF in driving eotaxin production and eosinophil recruitment in the allergic pleurisy model.

In agreement with *in vivo* studies in experimental animals and in humans (Henocq & Vargaftig, 1986; Silva *et al.*, 1991; Teixeira *et al.*, 1994; 1997a), injection of PAF induced a significant recruitment of eosinophils into the pleural cavity of mice. Interestingly, PAF-induced eosinophil recruitment was preceded by a rapid increase in the release of eotaxin into the pleural cavity. The kinetics of eotaxin release following PAF is slightly faster than that observed after antigen challenge in the allergic pleurisy and other models (Gonzalo *et al.*, 1996; Humbles *et al.*, 1997; Klein *et al.*, 2001). Moreover, PAF-induced eosinophil recruitment was suppressed by pretreatment with an anti-eotaxin antibody.

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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References

ALABASTER, V.A., KEIR, R.F., PARRY, M.J. & DE SOUZA, R.N. (1991). UK-74505, a novel and selective PAF antagonist, exhibits potent and long lasting activity *in vivo*. *Agents Actions Suppl.*, **34**, 221–227.

ALVES, A.C., PIRES, A.L., CRUZ, H.N., SERRA, M.F., DIAZ, B.L., CORDEIRO, R.S., LAGENTE, V. & MARTINS, M.A. (1996). Selective inhibition of phosphodiesterase type IV suppresses the chemotactic responsiveness of rat eosinophils in vitro. *Eur. J. Pharmacol.*, **312**, 89–96.

AU, B.T., TEIXEIRA, M.M., COLLINS, P.D. & WILLIAMS, T.J. (2001). Blockade of PAF receptors controls IL-8 production by regulating the activation of neutrophil CD11/CD18. *Eur. J. Pharmacol.*, **425**, 65–71.

BARTEMES, K.R., MCKINNEY, S., GLEICH, G.J. & KITA, H. (1999). Endogenous platelet-activating factor is critically involved in effector functions of eosinophils stimulated with IL-5 or IgG. *J. Immunol.*, **162**, 2982–2989.

BORGES, C.M., SILVEIRA, M.R., BEKER, M.A., FREIRE-MAIA, L. & TEIXEIRA, M.M. (2000). Scorpion venom-induced neutrophilia is inhibited by a PAF receptor antagonist in the rat. *J. Leukoc. Biol.*, **67**, 515–519.

CARA, D.C., NEGRAO-CORREA, D. & TEIXEIRA, M.M. (1999). Mechanisms underlying eosinophil trafficking and their relevance *in vivo*. *Histol. Histopathol.*, **15**, 899–920.

DAS, A.M., FLOWER, R.J., HELLEWELL, P.G., TEIXERA, M.M. & PERRETTI, M. (1997). A novel murine model of allergic inflammation to study the effect of dexamethasone on eosinophil recruitment. *Br. J. Pharmacol.*, **121**, 97–104.

EVANS, D.J., BARNES, P.J., CLUZEL, M. & O'CONNOR, B.J. (1997). Effects of a potent platelet-activating factor antagonist, SR27417A, on allergen-induced asthmatic responses. *Am. J. Respir. Crit. Care Med.*, **156**, 11–16.

FUKUYAMA, S., INOUE, H., AIZAWA, H., OIKE, M., KITAURA, M., YOSHIE, O. & HARA, N. (2000). Effect of eotaxin and platelet-activating factor on airway inflammation and hyperresponsiveness in guinea pigs in vivo. *Am. J. Respir. Crit. Care Med.*, **161**, 1844–1849.

GIEMBYCZ, M.A. & LINDSAY, M.A. (1999). Pharmacology of the eosinophil. *Pharmacol. Rev.*, **51**, 213–340.

GONZALO, J.A., LLOYD, C.M., KREMER, L., FINGER, E., MARTINEZ, C., SIEGELMAN, M.H., CYBULSKY, M. & GUTIERREZ-RAMOS, J.C. (1996). Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J. Clin. Invest.*, **98**, 2332–2345.

GONZALO, J.A., LLOYD, C.M., WEN, D., ALBAR, J.P., WELLS, T.N.C., PROUDFOOT, A.E., MARTINEZ-A., C., DORF, M., BJERKE, T., COYLE, A.J. & GUTIERREZ-RAMOS, J.C. (1998). The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med.*, **188**, 157–167.

HENOCQ, E. & VARGAFTIG, B.B. (1986). Accumulation of eosinophils in response to intracutaneous PAF-acether and allergens in man. *Lancet*, **14**, 1378–1379.

HUMBLES, A.A., CONROY, D.M., MARLEAU, S., RANKIN, S.M., PALFRAMAN, R.T., PROUDFOOT, A.E., WELLS, T.N., LI, D., JEFFERY, P.K., GRIFFITHS-JOHNSON, D.A., WILLIAMS, T.J. & JOSE, P.J. (1997). Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *J. Exp. Med.*, **186**, 601–612.

ISHII, S., KUWAKI, T., NAGASE, T., MAKI, K., TASHIRO, F., SUNAGA, S., CAO, W.H., KUME, K., FUKUCHI, Y., IKUTA, K., MIYAZAKI, J., KUMADA, M. & SHIMIZU, T. (1998). Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating receptor. *J. Exp. Med.*, **187**, 1779–1788.

ISHII, S. & SHIMIZU, T. (2000). Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. *Prog. Lipid Res.*, **39**, 41–82.

JEZEQUEL, S.G., UDEN, S. & WASTALL, P. (1996). Modipant, a new PAF antagonist: pharmacokinetics and disposition in rat, dog and man. *Xenobiotica*, **26**, 963–975.

KLEIN, A., TALVANI, A., CARA, D.C., GOMES, K.L., LUKACS, N.W. & TEIXEIRA, M.M. (2000). SCF plays a major role in the recruitment of eosinophils in allergic pleurisy in mice via the production of LTB_4. *J. Immunol.*, **164**, 4271–4276.

KLEIN, A., TALVANI, A., SILVA, P.M., MARTINS, M.A., WELLS, T.N., PROUDFOOT, A., LUCKACS, N.W. & TEIXEIRA, M.M. (2001). Stem cell factor-induced leukotriene B(4) production cooperates with eotaxin to mediate the recruitment of eosinophils during allergic pleurisy in mice. *J. Immunol.*, **167**, 524–531.

KUITERT, L.M., ANGUS, R.M., BARNES, N.C., BARNES, P.J., BONE, M.F., CHUNG, K.F., FAIRFAX, A.J., HIGENBOTHAM, T.W., O'CONNOR, B.J. & PIOTROWSKA, B. (1995). Effect of a novel potent platelet-activating factor antagonist, modipafant, in clinical asthma. *Am. J. Respir. Crit. Care Med.*, **151**, 1331–1335.

LIU, L., ZUURBIER, A.E., MUL, F.P., VERHOEVEN, A.J., LUTTER, R., KNOL, E.F. & ROOS, D. (1998). Triple role of platelet-activating factor in eosinophil migration across monolayers of lung epithelial cells: eosinophil chemoattractant and priming agent and epithelial cell activator. *J. Immunol.*, **161**, 3064–3070.

LUKACS, N.W., OLIVEIRA, S.H. & HOGABOAM, C.M. (1999). Chemokines and asthma: redundancy of function or a coordinated effort? *J. Clin. Invest.*, **104**, 995–999.

MARUOKA, S., HASHIMOTO, S., GON, Y., TAKESHITA, I. & HORIE, T. (2000). PAF-induced RANTES production by human airway smooth muscle cells requires both p38 MAP kinase and Erk. *Am. J. Respir. Crit. Care Med.*, **161**, 922–929.

MIOTLA, J.M., JEFFERY, P.K. & HELLEWELL, P.G. (1998). Platelet-activating factor plays a pivotal role in the induction of experimental lung injury. *Am. J. Respir. Cell. Mol. Biol.*, **18**, 197–204.

MURPHY, P.M., BAGGIOLINI, M., CHARA, I.F., HERBERT, C.A., HORUK, R., MATSUSHIMA, K., MILLER, L.M., OPPENHEIM, J.J. & POWER, C.A. (2000). International union of Pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.*, **52**, 145–176.

RUTH, J.H., LUKACS, N.W., WARMINGTON, K.S., POLAK, T.J., BURDICK, M., KUNKEL, S.L., STREITER, R.M. & CHENSUE, S.W. (1998). Expression and participation of eotaxin during mycobacterial (type 1) and schistosomal (type 2) antigen-elicited granuloma formation. *J. Immunol.*, **161**, 4276–4282.

SCHWEIZER, R.C., VAN KESSEL-WELMERS, B.A., WARRINGA, R.A., MAIKOE, T., RAAIJMAKERS, J.A., LAMMERS, J.W. & KOENDERMAN, L. (1996). Mechanisms involved in eosinophil migration. Platelet-activating factor-induced chemotaxis and interleukin-5-induced chemokines are mediated by different signals. *J. Leukoc. Biol.*, **59**, 347–356.

SILVA, P.M., MARTINS, M.A., FARIA NETO, H.C., CORDEIRO, R.S. & VARGAFTIG, B.B. (1991). Generation of an eosinophilotactic activity in the pleural cavity of platelet-activating factor-injected rats. *J. Pharmacol. Exp. Ther.*, **257**, 1039–1044.

TEIXEIRA,, M.M., GIEMBYCZ, M.A., LINDSAY, M.A. & HELLEWELL, P.G. (1997a). Pertussis toxin reveals distinct early signalling events in platelet-activating factor, leukotriene B_4- and C5a-induced eosinophil homotypic aggregation *in vitro* and recruitment in vivo. *Blood*, **89**, 4566–4573.

TEIXEIRA, M.M., REYNIA, S., ROBINSON, M., SHOCK, A., WILLIAMS, T.J., WILLIAMS, F.M., ROSSI, A.G. & HELLEWELL, P.G. (1994). Role of CD11/CD18 in mediating cutaneous inflammation in the guinea pig. *Br. J. Pharmacol.*, **111**, 811–818.

TEIXEIRA, M.M., WELLS, T.N.C., LUKACS, N.W., PROUDFOOT, A.E.E., KUNKEL, S.L., WILLIAMS, T.J. & HELLEWELL, P.G. (1997b). Chemokine-induced eosinophil recruitment: Evidence of a role for endogenous eotaxin in an *in vivo* allergy model in mouse skin. *J. Clin. Invest.*, **100**, 1656–1666.

TEIXEIRA, M.M., WILLIAMS, T.J. & HELLEWELL, P.G. (1995). Mechanisms and pharmacological manipulation of eosinophil accumulation in vivo. *Trends Pharmacol. Sci.*, **16**, 418–423.

TOOL, A.T., KOENDERMAN, L., KOK, P.T., BLOM, M., ROOS, D. & VERHOEVEN, A.J. (1992). Release of platelet-activating factor is important for the respiratory burst induced in human eosinophils by opsonized particles. *Blood*, **79**, 2729–2732.

VAN DER BRUGGEN, T., KOK, P.T., RAAIJMAKERS, J.A., LAMMERS, J.W. & KOENDERMAN, L. (1994). Cooperation between Fc gamma receptor II and complement receptor type 3 during activation of platelet-activating factor release by cytokine-primed human eosinophils. *J. Immunol.*, **153**, 2729–2735.

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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.

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).

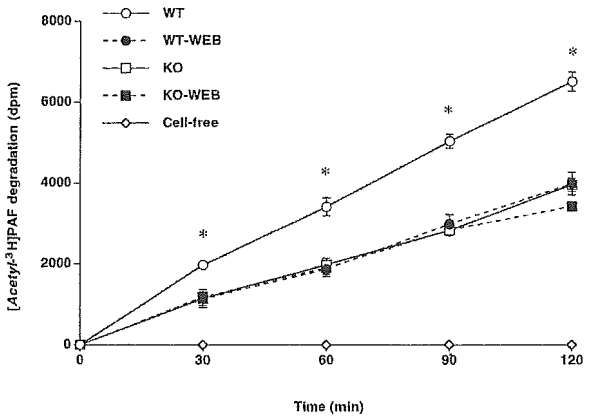

FIG. 1. **Time course of PAF degradation by macrophages from PAFR-WT and -KO mice.** Macrophages were incubated with 2 nM [*acetyl*-^{3}H]PAF. PAF degradation was measured by counting [^{3}H]acetic acid released into the medium. The concentration of WEB 2086 was 10 μM. In the absence of macrophages, 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.

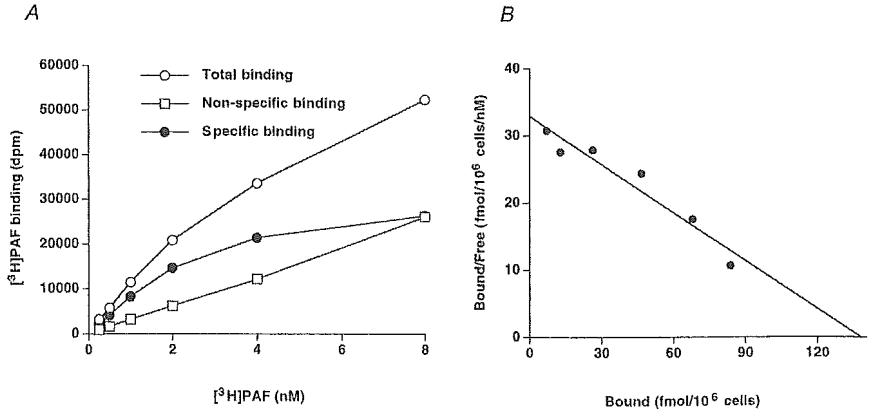

FIG. 2. **Ligand binding to PAF receptor.** *A*, macrophages were incubated for 1 h with [*alkyl*-^{3}H] PAF at 4 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled PAF. *B*, Scatchard analysis of specific [*alkyl*-^{3}H]PAF binding to macrophages. The K_d and B_{max} values are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

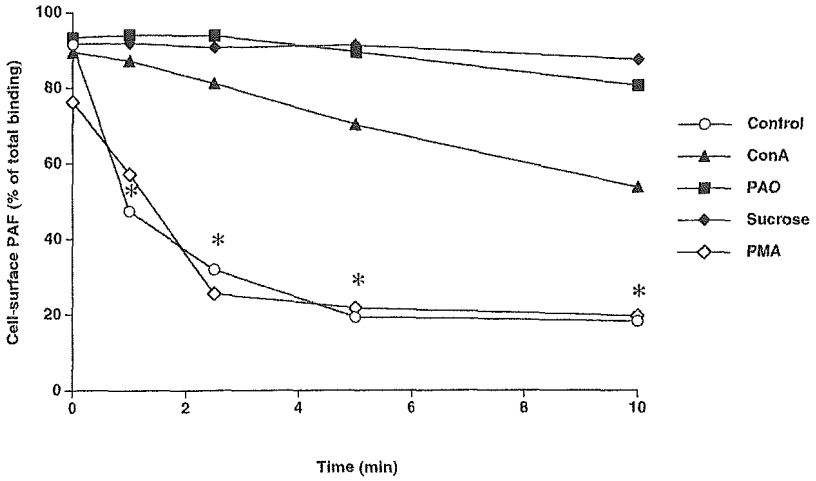

FIG. 3. **Internalization of PAF with PAF receptor.** Macrophages were incubated with 2 nM [*alkyl*-^{3}H]PAF at 4 °C for 1 h and washed with Hepes/Tyrode's/BSA. Upon incubation at 37 °C, PAF internalization was initiated. At each time, the PAF remaining bound to the cell surface receptors was recovered 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-

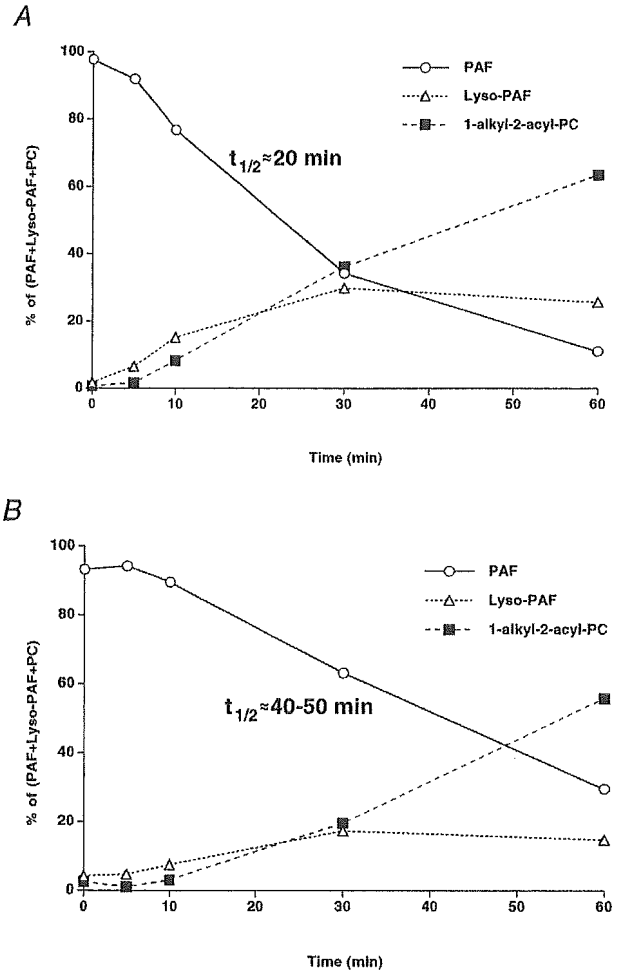

FIG. 4. **Intracellular metabolism of [*alkyl*-^{3}H]PAF.** Macrophages were incubated with 2 nM [*alkyl*-^{3}H]PAF at 4 °C for 1 h. After washing with Hepes/Tyrode's/BSA, cells were incubated at 37 °C. At each time, total lipids were extracted, and separated by thin layer chromatography. The relative 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

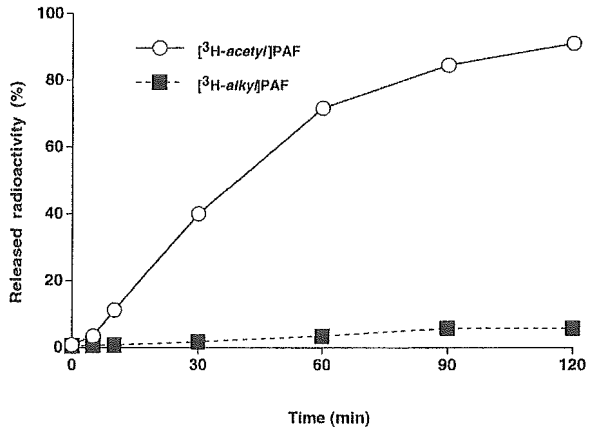

FIG. 5. **Release of metabolites of [^{3}H-*acetyl*]PAF.** Macrophages were incubated with 2 nM [*acetyl*-^{3}H]PAF or [*alkyl*-^{3}H]PAF at 4 °C for 1 h. After washing with Hepes/Tyrode's/BSA, the cells were incubated at 37 °C. At each time, the radioactivity in the medium and the cells were counted. 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-

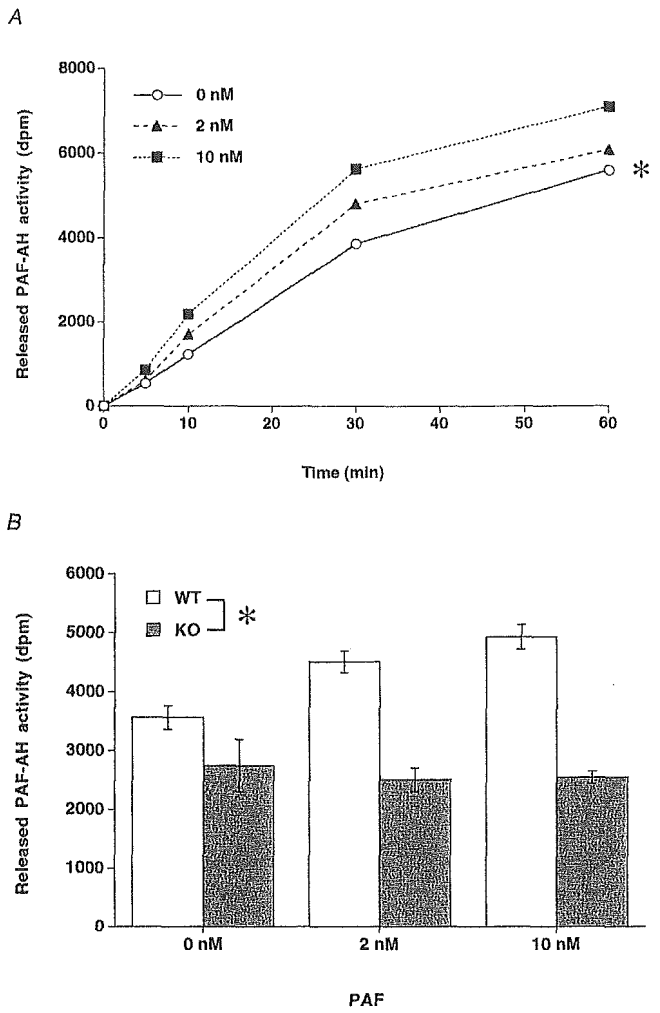

FIG. 6. **PAF-induced PAF-AH release via PAF receptor.** *A*, macrophages from PAFR-WT mice were stimulated with 2 or 10 nM PAF for 5, 10, 30, and 60 min. The PAF-AH activity in the medium was measured. Each value shows a mean of triplicate measurements. Error bars are omitted because S.D. values are 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

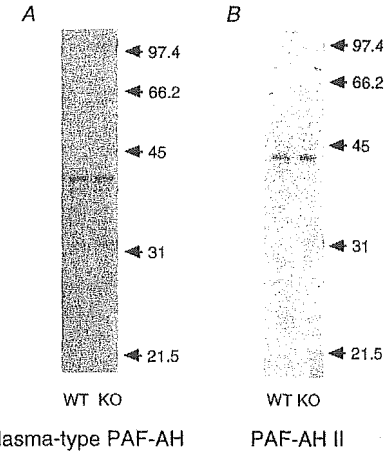

FIG. 7. **Detection of plasma-type PAF-AH and PAF-AH II in macrophages.** Lysates of macrophages from PAFR-WT and -KO mice (20 μg of protein) are subjected to immunoblot analysis using antisera against plasma-type PAF-AH (*A*) and anti-PAF-AH II monoclonal antibody (*B*).

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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REFERENCES

1. Izumi, T., and Shimizu, T. (1995) *Biochim. Biophys. Acta* **1259,** 317–333
2. Ishii, S., and Shimizu, T. (2000) *Prog. Lipid Res.* **39,** 41–82
3. Prescott, S. M., Zimmerman, G. A., Stafforini, D. M., and McIntyre, T. M. (2000) *Annu. Rev. Biochem.* **69,** 419–445
4. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., and Shimizu, T. (1991) *Nature* **349,** 342–346
5. Nakamura, M., Honda, Z., Izumi, T., Sakanaka, C., Mutoh, H., Minami, M., Bito, H., Seyama, Y., Matsumoto, T., Noma, M., and Shimizu, T. (1991) *J. Biol. Chem.* **266,** 20400–20405
6. Ye, R. D., Prossnitz, E. R., Zou, A. H., and Cochrane, C. G. (1991) *Biochem. Biophys. Res. Commun.* **180,** 105–111
7. Kunz, D., Gerard, N. P., and Gerard, C. (1992) *J. Biol. Chem.* **267,** 9101–9106
8. Ishii, S., Matsuda, Y., Nakamura, M., Waga, I., Kume, K., Izumi, T., and Shimizu, T. (1996) *Biochem. J.* **314,** 671–678
9. Ishii, S., Kuwaki, T., Nagase, T., Maki, K., Tashiro, F., Sunaga, S., Cao, W. H., Kume, K., Fukuchi, Y., Ikuta, K., Miyazaki, J., Kumada, M., and Shimizu, T. (1998) *J. Exp. Med.* **187,** 1779–1788
10. Nagase, T., Ishii, S., Kume, K., Uozumi, N., Izumi, T., Ouchi, Y., and Shimizu, T. (1999) *J. Clin. Invest.* **104,** 1071–1076
11. Hattori, M., Arai, H., and Inoue, K. (1993) *J. Biol. Chem.* **268,** 18748–18753
12. Hattori, K., Hattori, M., Adachi, H., Tsujimoto, M., Arai, H., and Inoue, K. (1995) *J. Biol. Chem.* **270,** 22308–22313
13. Tjoelker, L. W., and Stafforini, D. M. (2000) *Biochim. Biophys. Acta* **1488,** 102–123
14. Miwa, M., Miyake, T., Yamanaka, T., Sugatani, J., Suzuki, Y., Sakata, S., Araki, Y., and Matsumoto, M. (1988) *J. Clin. Invest.* **82,** 1983–1991
15. Hiramoto, M., Yoshida, H., Imaizumi, T., Yoshimizu, N., and Satoh, K. (1997) *Stroke* **28,** 2417–2420
16. Lefkowitz, R. J. (1998) *J. Biol. Chem.* **273,** 18677–18680
17. Tolbert, L. M., and Lameh, J. (1996) *J. Biol. Chem.* **271,** 17335–17342
18. Conchon, S., Monnot, C., Teutsch, B., Corvol, P., and Clauser, E. (1994) *FEBS Lett.* **349,** 365–370
19. Garland, A. M., Grady, E. F., Payan, D. G., Vigna, S. R., and Bunnett, N. W. (1994) *Biochem. J.* **303,** 177–186
20. Ishii, I., Saito, E., Izumi, T., Ui, M., and Shimizu, T. (1998) *J. Biol. Chem.* **273,** 9878–9885
21. Le Gouill, C., Parent, J. L., Rola-Pleszczynski, M., and Stankova, J. (1997) *J. Biol. Chem.* **272,** 21289–21295
22. Brown, M. S., and Goldstein, J. L. (1986) *Science* **232,** 34–47
23. Koenig, J. A., Kaur, R., Dodgeon, I., Edwardson, J. M., and Humphrey, P. P. (1998) *Biochem. J.* **336,** 291–298
24. Prpic, V., Uhing, R. J., Weiel, J. E., Jakoi, L., Gawdi, G., Herman, B., and Adams, D. O. (1988) *J. Cell Biol.* **107,** 363–372
25. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) *Nature* **390,** 618–622
26. Bligh, E. C., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
27. McIntyre, T. M., Zimmerman, G. A., Satoh, K., and Prescott, S. M. (1985) *J. Clin. Invest.* **76,** 271–280
28. Stafforini, D. M., McIntyre, T. M., and Prescott, S. M. (1990) *Methods Enzymol.* **187,** 344–357
29. Karasawa, K., Kuge, O., Kawasaki, K., Nishijima, M., Nakano, Y., Tomita, M., Yokoyama, K., Setaka, M., and Nojima, S. (1996) *J. Biochem. (Tokyo)* **120,** 838–844
30. Mahan, L. C., Koachman, A. M., and Insel, P. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 129–133
31. Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) *J. Biol. Chem.* **268,** 337–341
32. Kallal, L., Gagnon, A. W., Penn, R. B., and Benovic, J. L. (1998) *J. Biol. Chem.* **273,** 322–328
33. Pippig, S., Andexinger, S., and Lohse, M. J. (1995) *Mol. Pharmacol.* **47,** 666–676
34. Jimenez, E., Caro, M. C., Marsigliante, S., and Montiel, M. (1999) *Biochem. Pharmacol.* **57,** 1125–1131
35. Parent, J. L., Labrecque, P., Orsini, M. J., and Benovic, J. L. (1999) *J. Biol. Chem.* **274,** 8941–8948
36. Seachrist, J. L., Anborgh, P. H., and Ferguson, S. S. (2000) *J. Biol. Chem.* **275,** 27221–27228
37. Beaumont, V., Hepworth, M. B., Luty, J. S., Kelly, E., and Henderson, G. (1998) *J. Biol. Chem.* **273,** 33174–33183
38. Hertel, C., Coulter, S. J., and Perkins, J. P. (1985) *J. Biol. Chem.* **260,** 12547–12553
39. Hansen, S. H., Sandvig, K., and van Deurs, B. (1993) *J. Cell Biol.* **121,** 61–72
40. Lamant, V., Mauco, G., Braquet, P., Chap, H., and Douste-Blazy, L. (1987) *Biochem. Pharmacol.* **36,** 2749–2752
41. Lachachi, H., Plantavid, M., Simon, M. F., Chap, H., Braquet, P., and Douste-Blazy, L. (1985) *Biochem. Biophys. Res. Commun.* **132,** 460–466
42. Elstad, M. R., Stafforini, D. M., McIntyre, T. M., Prescott, S. M., and Zimmerman, G. A. (1989) *J. Biol. Chem.* **264,** 8467–8470
43. Satoh, K., Imaizumi, T., Kawamura, Y., Yoshida, H., Hiramoto, M., Takamatsu, S., and Takamatsu, M. (1991) *J. Clin. Invest.* **87,** 476–481
44. Svetlov, S. I., Howard, K. M., Miwa, M., Flickinger, B. D., and Olson, M. S. (1996) *Arch. Biochem. Biophys.* **327,** 113–122
45. Chang, S. W., Feddersen, C. O., Henson, P. M., and Voelkel, N. F. (1987) *J. Clin. Invest.* **79,** 1498–1509

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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*).

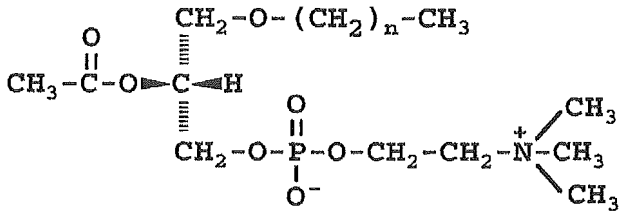

Fig. 1. **Structure of PAF.**

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

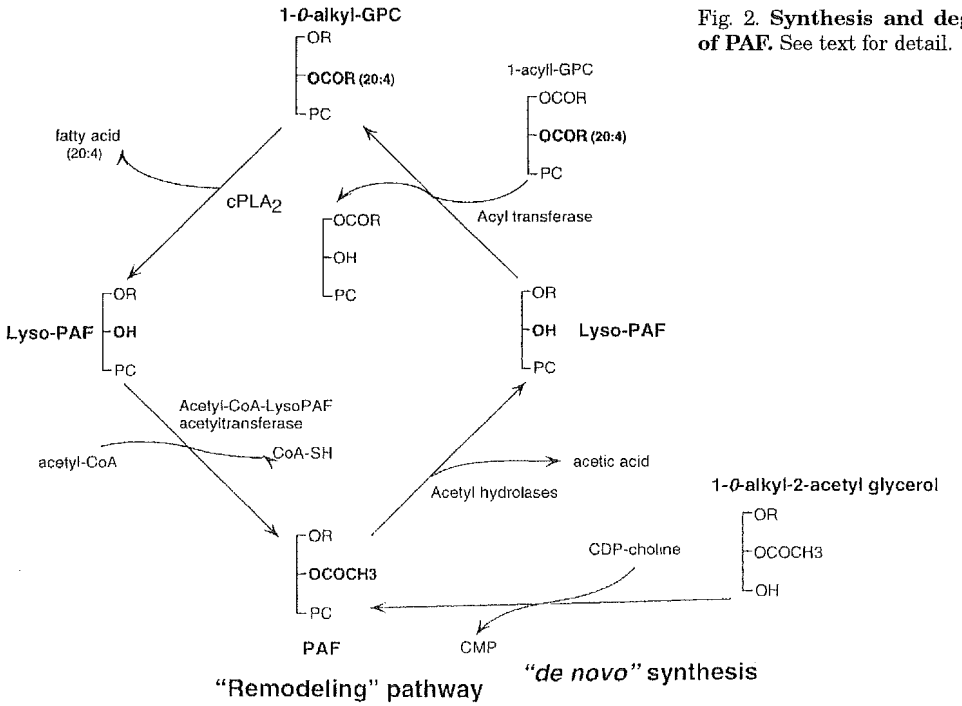

Fig. 2. **Synthesis and degradation of PAF.** See text for detail.

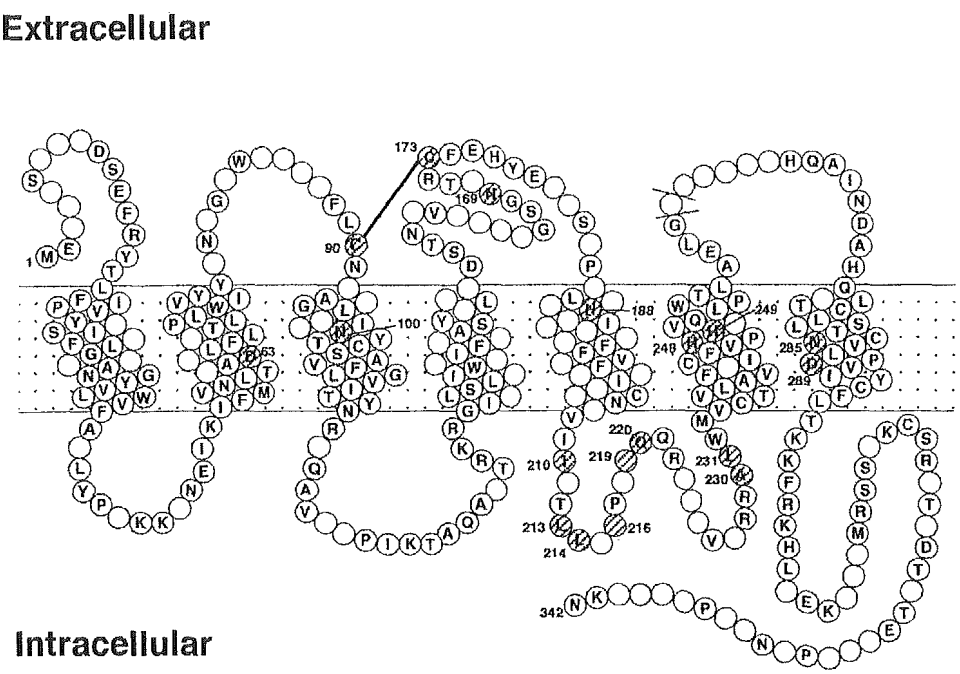

Fig. 3. **Transmembrane structure of PAF receptor.** Shaded amino acids indicate the sites of mutagenesis studies. See text for detail.

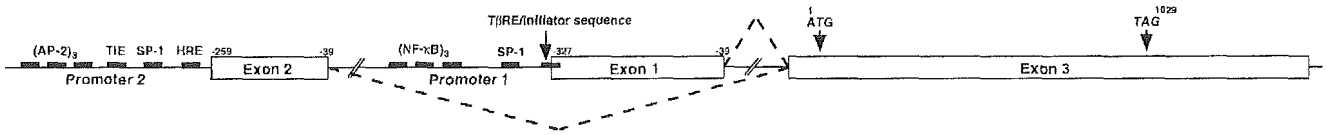

Fig. 4. **Genome structure of human PAF receptor.** Two 5′ non-coding exons (exons 2 and 1; the order is inverted for historical reasons) are spliced to exon 3, the entire coding sequence, yielding two PAFR transcripts. Their expression is regulated by two promoters (promoters 2 and 1). Promoter 2 contains 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