Enhanced Production of Platelet-Activating Factor in Stimulated Rat Leukocytes Caused by the Blockade of Lysophospholipid Acylation

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ABSTRACT—We have previously reported that triacsin C, an acyl-CoA synthetase inhibitor, enhanced the production of platelet-activating factor (PAF) in calcium ionophore-activated rat polymorphonuclear leukocytes (PMNs). In this report, we further demonstrated that the production of PAF by PMNs in response to opsonized zymosan was significantly enhanced by pretreatment with triacsin C and also by the pretreatment with merthiolate, which was reported to be an inhibitor of acyl-CoA/lysolecithin acyltransferase. Pretreatment with triacsin C or merthiolate also enhanced the lyso-PAF content in the stimulated PMNs. Addition of lyso-PAF in the incubation mixture of PMNs in the presence of opsonized zymosan augmented the production of PAF. The enhancement of PAF production by lyso-PAF has been reported by several authors, and the importance of lyso-PAF in the remodeling pathway of PAF synthesis has been generally recognized. Therefore, from the above findings, it is assumed that blockades of the reacylation of lyso-phospholipids, by inhibitors such as triacsin C and merthiolate, might lead to accumulation of lyso-PAF and might result in the enhancement of PAF production when the remodeling pathway is active.

Keywords: Platelet-activating factor, Lyso-platelet-activating factor, Zymosan (opsonized), Merthiolate, Triacsin C

Platelet-activating factor (PAF) is one of the potent mediators of inflammation and allergy (1, 2). It has been reported that the production of PAF in various cells could be stimulated by various modulators, such as opsonized zymosan (OPZ) (3, 4), phorbol myristate acetate (5), Ca-ionophore A23187 (6, 7) and formyl-methionyl-leucyl-phenylalanine (8). PAF production in inflammatory cells occurs mainly by the action of the remodeling pathway in which phospholipase A₂ and acetyltransferase are key enzymes to form PAF from its precursor 1-alkyl-2-acyl-glycerophosphocholine (1-alkyl-2-acyl-GPC) (2).

We have proposed that reacylation of lyso-PAF may also be an important regulating pathway for PAF biosynthesis, since enhancement of arachidonic acid release (9) and PAF production (10) in A23187-stimulated rat PMNs were found after treatment with triacsin C, a novel inhibitor of acyl-CoA synthetase (11). Therefore, to prove the above hypothesis in this paper, we further determined if PAF production in response to zymosan in rat leukocytes is enhanced by pretreatment with another reacylation blocking agent, merthiolate, which was reported to be inhibitor of acyl-CoA/lysolecithin acyltransferase in human platelets (12), rat macrophages (12) and human neutrophils (13).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu) and Japanese white rabbits (Doken, Ibaraki) were purchased. The leukocytes were collected from peritoneal washings of rats 16 hr after the intraperitoneal injection of 30 ml of 1% casein solution, as previously reported (14). The leukocytes thus collected were mostly polymorphonuclear leukocytes (PMNs) (85–88%).

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Agents
C16-Platelet-activating factor (C16-PAF, 1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphocholine, 1-O-hexadecyl-2-O-acetyl-GPC, Bachem Fine Chemicals, Switzerland) and C16-lyso-PAF (1-O-hexadecyl-GPC, Bachem Fine Chemicals, Switzerland) were purchased from Funakoshi Pharm. Co. 1-O-[3H]Alkyl-(mixture of octadecyl- and hexadecyl-)-lyso-PAF (1665 GBq/mmol, NEN), zymosan (Sigma), and merthiolate (ethylmercurisalicylate, Sigma) were purchased. Triacsin C (1-hydroxy-3-(E,E,E,2',4',7'-undeca-trienylidine) triazene) was a gift from Drs. Tomoda and Omura (11).

Stimulation of PMNs with OPZ
OPZ was prepared as follows: zymosan was washed with physiological saline and autoclaved at 121°C for 15 min in a suspension of physiological saline, and then it was incubated in rat serum (10 mg/ml) for 30 min at 37°C and washed with saline solution twice.

PMNs (10⁷ cells in 0.9 ml), suspended in Hanks' balanced salt solution (HBSS), were preincubated with or without merthiolate for 10 min, because the effect of merthiolate reached a plateau by 10 min. Pretreatment with or without triacsin C was performed for 60 min, because its inhibitory effect on acyl-CoA synthetase reached a plateau by 60-min incubation as previously reported (10). Then the indicated amount of OPZ was added into the incubation mixture, which brought the total volume of the mixture to 1 ml, and further incubated for 5 or 10 min. To terminate the mixture to the reaction, 2.5 ml ice-cold methanol containing 2% acetic acid was added.

Measurement of PAF and lyso-PAF
PAF in the above incubation mixture of PMNs was extracted, separated by HPLC and assayed by rabbit platelet aggregation as reported previously (8). The produced lyso-PAF was also separated by HPLC (8), acetylated to PAF by acetic anhydride and pyridine (approximately 70–80% yield), and assayed as PAF as previously reported (10).

Measurement of reacylation of lyso-PAF
According to the method of Sugiura et al. (15), the microsomal fraction of rat PMNs (50 μg protein), which was obtained as described previously (5), was incubated with 1-O-[3H]Alkyl-lyso-PAF (ca. 200,000 dpm in 5 μM cold lyso-PAF) in the presence of merthiolate (0 to 10 μM) or triacsin C (0 to 500 nM) for 60 min at 37°C. Fractions of 1-alkyl-2-acyl-GPC and lyso-PAF in the incubation mixture were separated by thin layer chromatography (15), and the radioactivity in each fraction was counted.

Statistics
Statistical evaluation was performed by Student's t-test. Differences with P<0.05 and P<0.01 were considered significant.

RESULTS

Dose-response of zymosan in the PAF production of rat PMNs
We examined the PAF production of rat PMNs in response to various concentrations of OPZ as shown in Fig. 1. PAF production by PMNs was increased dose-dependently with OPZ in the concentration range of 0–2000 μg/ml. Incubation of PMNs with OPZ in this experiment was performed for 10 min, since we observed that PAF production by rat PMNs in response to OPZ peaked around 10–20 min and decreased slowly after 30 min (data not shown).

Effect of merthiolate and triacsin C on PAF production of OPZ stimulated PMNs
To examine the enhancing effect of merthiolate on PAF production in the following experiments, we chose an OPZ concentration of 500 μg/ml, which was a submaximal dose as shown in Fig. 1. As shown in Fig. 2, preincubation with merthiolate dose-dependently increased the PAF production, in the concentration range of 1–10 μM, by PMNs in response to OPZ (500 μg/ml). However, merthiolate alone did not cause PAF production. Pretreatment with triacsin C (0.05–5 μM) also significantly enhanced the PAF production by stimulation with OPZ. Again, triacsin C alone did not cause significant PAF production (Fig. 2).
Fig. 2. Production of PAF in rat PMNs in response to OPZ after preincubation of merthiolate or triacsin C. Rat PMNs, suspended in HBSS (10^7 cells/ml), were preincubated with merthiolate (0, 1, 3 and 10 μM) for 10 min or triacsin C (0, 50 and 500 nM, and 5 μM) for 60 min, and then they were stimulated with (•) or without (□) OPZ (500 pg/ml) for 10 min. Data are the means from 3–4 experiments with standard deviation. ** indicates significant difference at P<0.01.

Fig. 3. Production of lyso-PAF in rat PMNs in response to OPZ after preincubation with merthiolate or triacsin C. Rat PMNs, suspended in HBSS (10^7 cells/ml), were preincubated with merthiolate (10 μM) for 10 min or triacsin C (500 nM) for 60 min, and then they were stimulated with (•) or without (□) OPZ (500 pg/ml) for 5 min. Samples of vehicle (control) or OPZ alone were preincubated for 60 min. Data are the means from 3–4 experiments with standard deviations. ** indicates significant difference at P<0.01.

Effect of merthiolate and triacsin C on lyso-PAF production of zymosan stimulated PMNs

Lyso-PAF production of rat PMNs was also enhanced by pretreatment with merthiolate at 10 μM or triacsin C at 500 nM, as shown in Fig. 3. However, merthiolate alone or triacsin alone did not cause significant lyso-PAF production.

Effect of exogenous lyso-PAF on PAF production by PMNs

The effect of exogenous lyso-PAF on PAF production of PMNs was examined as shown in Fig. 4. Addition of lyso-PAF at 10 μM alone or OPZ at 500 μg/ml alone induced PAF production about 2–3 ng/10^7 cells. Synergistic enhancement in PAF production by PMNs was observed with the concomitant addition of lyso-PAF and OPZ in the incubation mixture as shown in Fig. 4.

Effect of merthiolate and triacsin C on reacylation of lyso-PAF

The microsomal fraction of rat PMNs could convert 1-
O-[3H]alkyl-lyso-PAF to its 2-acylated product without addition of CoA or acyl-CoA, and this activity was significantly suppressed in the presence of 10 pM merthiolate, as shown in Fig. 5. However, triacsin C did not show any inhibition of this transacylation.

**DISCUSSION**

Pretreatment with merthiolate enhanced PAF production by rat PMNs in response to OPZ, while merthiolate alone did not cause PAF production as shown in Fig. 2. Furthermore, by stimulation with OPZ, PMNs pretreated with merthiolate produced an increased amount of lyso-PAF (Fig. 3), which is a precursor of PAF in the remodeling pathway of PAF-biosynthesis (1, 2).

It has been commonly accepted that in remodeling pathway of PAF synthesis, production of lyso-PAF may be increased by enhancement of phospholipase A2 activity (1, 2). Merthiolate has been reported to be an inhibitor of acyl-CoA/lysolecithin acyltransferase (16) and also reported to suppress the uptake of arachidonic acid into various cells including human neutrophils (13). Furthermore, it was observed that merthiolate did not modulate phospholipase A2 activity at the dose that could suppress arachidonic acid uptake in rat macrophages (12). Therefore, our finding of lyso-PAF accumulation by pretreatment with merthiolate in the OPZ-stimulated PMNs may possibly be caused by the inhibition of lyso-PAF reacylation, as shown in Fig. 5.

Addition of lyso-PAF into the incubation mixture of PMNs with OPZ significantly increased PAF production. This result is consistent with several reports (17–20); for instance, the PAF production by human neutrophils was enhanced when they were stimulated with A23187 in the presence of lyso-PAF (17). Therefore, a cause of the enhanced production of PAF by merthiolate could be an increase in lyso-PAF, a precursor of PAF, whose accumulation resulted from the interference of reacylation. The mechanism of the enhanced production of PAF by lyso-PAF; i.e., whether lyso-PAF can be incorporated into cells or whether lyso-PAF activates acetyltransferase, is not known, and its elucidation remains for future studies.

Triacsin C did not suppress reacylation of lyso-PAF by the microsomal fraction of PMNs without addition of CoA in the incubation mixture (Fig. 5), indicating that triacsin C did not inhibit acyltransferase activity directly. However, triacsin C also augmented PAF production and lyso-PAF production in OPZ-stimulated rat PMNs, in a similar manner to merthiolate. Triacsin C has been reported to be an inhibitor of long chain fatty acid/acyl-CoA synthetase (11), and it was also reported that it inhibited 14C-arachidonic acid incorporation into rat PMNs (9). Therefore, triacsin C could suppress the acyl-CoA dependent reacylation by inhibiting acyl-CoA synthesis, and this could result in the enhanced production of lyso-PAF and PAF (10).

Taken these findings together, it can be assumed that the reacylation pathway is an important regulating pathway for PAF biosynthesis by converting lyso-PAF to 1-alkyl-2-acyl-GPC into membrane lipids (15). Also this pathway could regulate the free fatty acid pool, including arachidonic acid, in addition to the regulation by phospholipase A2 (20). The above conclusion is in line with the previous report (9, 10), in which we proposed that acylation of lyso-PAF may control PAF biosynthesis by using triacsin C.

Further precise examinations on the effects of merthiolate and triacsin C on all enzymes involved in the pathway would be necessary to prove the role of acylation in PAF synthesis and to elucidate the site of action of merthiolate in the reacylation pathway. Nevertheless, merthiolate and triacsin C could be useful modifiers for elucidation of the importance of the transacylation mechanism in the remodeling pathway of the inflammatory cells.

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REFERENCES

1. Hanahan, D.: Platelet activating factor: A biologically active phosphoglyceride. Annu. Rev. Biochem. 55, 483–509 (1986)
2. Snyder, F.: Enzymatic pathways for platelet-activating factor, related alkyl glycerolipids, and their precursors. In Platelet-Activating Factor and Related Lipid Mediators, Edited by Snyder, F., pp. 89–113, Plenum Publishing Co., New York (1987)
3. Damas, J.: Involvement of platelet-activating factor in the hypotensive response to zymosan in rats. J. Lipid Med. 3, 333–344 (1991)
4. Imai, Y., Hayashi, M. and Oh-ishi, S.: Key role of complement activation and platelet-activating factor in exudate formation in zymosan-induced rat pleurisy. Japan. J. Pharmacol. 57, 225–232 (1991)
5. Hayashi, M., Imai, Y. and Oh-ishi, S.: Phorbol ester stimulates PAF synthesis via the activation of protein kinase C in rat leukocytes. Lipids 26, 1054–1059 (1991)
6. Camussi, G., Aglietta, M., Malavas, F., Tetta, C., Piacibello, W., Sanavio, F. and Bussolino, F.: The release of platelet-activating factor from human endothelial cells in culture. J. Immunol. 131, 2397–2403 (1983)
7. Lee, T.-C., Lenihan, D.J., Malone, B., Roddy, L.L. and Wasserman, S.I.: Increased biosynthesis of platelet-activating factor in activated human eosinophils. J. Biol. Chem. 259, 5526–5530 (1984)
8. Hayashi, M., Kimura, J., Yamaki, K., Suwabe, Y., Dozen, M., Imai, Y. and Oh-ishi, S.: Detection of platelet-activating factor in exudates of rats with phorbol myristate acetate-induced pleurisy. Thromb. Res. 48, 299–310 (1987)
9. Igarashi, K., Abe, M., and Tomoda, H.: The acyl-CoA synthetase inhibitor triacsin C enhanced eicosanoid release in leukocytes. Japan. J. Pharmacol. 59, 417–418 (1992)
10. Hayashi, M., Imai, Y., Narabe, H., Tomoda, H., Omura, S. and Oh-ishi, S.: Enhanced production of platelet-activating factor in stimulated rat leukocytes pretreated with triacsin C, a novel acyl-CoA synthetase inhibitor. Biochem. Biophys. Res. Commun. 188, 1280–1285 (1992)
11. Tomoda, H., Igarashi, K., Cyong, J.C. and Omura, S.: Evidence for an essential role of long chain acyl-CoA synthetase in animal cell proliferation. Inhibition of long chain acyl-CoA synthetase by triacsin C caused inhibition of RAJI cell proliferation. J. Biol. Chem. 266, 4214–4219 (1991)
12. Goppelt-Strube, M., Koerner, C.-F., Hausmann, G., Gemsa, D. and Resch, K.: Control of prostanoid synthesis: Role of reincorporation of released precursor fatty acids. Prostaglandins 32, 373–385 (1986)
13. Hatzelmann, A., Haurand, M. and Ulrich, V.: Involvement of calcium in the thimerosal-stimulated formation of leukotriene B4 in human polymorphonuclear leukocytes. Biochem. Pharmacol. 39, 559–567 (1990)
14. Yamaki, K. and Oh-ishi, S.: Release of leukotriene B4 and 6-keto-prostaglandin F1α from rat leukocytes in response to platelet-activating factor or Ca-ionophore A23187. J. Lipid Med. 2, 317–327 (1990)
15. Sugiuara, T., Masuzawa, Y., Nakagawa, Y. and Waku, K.: Transacylation of lyso platelet-activating factor and other lysophospholipids by macrophage microsomes. Distinct donor and acceptor selectivities. J. Biol. Chem. 262, 1199–1205 (1987)
16. Hansch, G.M., Gemsa, D. and Resch, K.: Induction of prostanoid synthesis in human platelets by the late complement components C5b-9 and channel forming antibiotic nystatin: inhibition of the reacylation of liberated arachidonic acid. J. Immunol. 135, 1320–1324 (1985)
17. Chilton, F.H., Ellis, J.M., Olson, S.C. and Wykle, R.L.: 1-O-Alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine. A common course of platelet-activating factor and arachidonate in human polymorphonuclear leukocytes. J. Biol. Chem. 259, 12014–12019 (1984)
18. Kawasaki, T. and Snyder, F.: Synthesis of a novel acetylated neutral lipid related to platelet-activating factor by acyl-CoA:1-o-alkyl-2-acetyl-sn-glycerol acyltransferase in HL-60 cells. J. Biol. Chem. 263, 2593–2596 (1988)
19. Sugiuara, T., Fukuda, T., Masuzawa, Y. and Waku, K.: Ether-lipid-induced production of platelet-activating factor in human polymorphonuclear leukocytes. Biochim. Biophys. Acta 1047, 223–232 (1990)
20. Snyder, F., Lee, T.-C. and Blank, M.L.: The role of transacylases in the metabolism of arachidonate and platelet activating factor. Prog. Lipid Res. 31, 65–86 (1992)