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Inhibitory effect of arachidonic acid on platelet-activating factor production in rat neutrophils

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Abstract

Platelet-activating factor production in rat neutrophils in response to opsonized zymosan was suppressed dose dependently in the presence of 10–100 μM arachidonic acid. The amount of lyso-PAF was also reduced by these doses of arachidonic acid. Phospholipase A_2 activity was not changed by addition of arachidonic acid up to 100 μM, but acetyltransferase activity was slightly reduced at a 100 μM concentration of arachidonic acid. Pretreatment with indomethacin did not alter the inhibitory effect of arachidonic acid on PAF production, indicating no involvement of cyclooxgenase products. Triacsin C, an acyl-CoA synthetase inhibitor, reversed the arachidonic acid-induced suppression of lyso-PAF and PAF production, suggesting that arachidonic acid might exert its inhibition via the acylation pathway of lyso-PAF to reduce the availability of lyso-PAF.

Keywords: PAF (platelet-activating factor); Arachidonic acid; Acyltransferase; Triacsin C; Zymosan

1. Introduction

Platelet-activating factor (PAF) is a well-known potent mediator of inflammation involved in vascular permeability increases or leukocyte migration. In inflammatory cells, PAF is synthesized via the remodeling pathway, including activation of phospholipase A_2 and acetyl-CoA acetyltransferase (Hanahan, 1986; Snyder, 1987). We reported previously that one of the major factors regulating PAF biosynthesis is availability of lyso-PAF in rat neutrophils when stimulated with opsonized zymosan (Naraba et al., 1993). Furthermore, we found that opsonized zymosan caused increases in cytosolic phospholipase A_2 activity and free arachidonic acid in these cells, in addition to the activation of transacylation of lyso-PAF (Naraba et al., 1995). These enzymes, i.e., cytosolic phospholipase A_2 and acyltransferase, are known to have affinity for phospholipids containing arachidonic acid (Takayama et al., 1991; Sugiura et al., 1987).

There are several reports that arachidonic acid and its metabolites have some effects on PAF production. For example, Remy et al. (1989) reported that inhibition of PAF synthesis by arachidonic acid may be caused by inhibition of acetyltransferase in bovine neutrophils stimulated with ionophore A23187. Winkler et al. (1993) described that the addition of arachidonic acid altered phospholipase A_2 activity in human neutrophils. There is no general agreement in these reports. Therefore, in the present work we examined and sought to clarify the mechanism by which arachidonic acid inhibits PAF production by rat neutrophils stimulated with opsonized zymosan.

2. Materials and methods

2.1. Materials and methods

Platelet-activating factor (PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), lyso-platelet-activating factor (lyso-PAF, 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine), and arachidonic acid were purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Zymosan A, acetyl-coenzyme A (acetyl-CoA), indomethacin, prostaglandin E_2, and bovine serum albumin (essentially fatty acid-free) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Acetyl-CoA (1.56 Ci/mmol) was from New England Nuclear (Boston, MA, USA), and

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1-acyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphoethanolamine (55.1 mCi/mmol) was from Amersham International (Buckinghamshire, UK). 9-Anthryldiazomethane was a gift from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Triacsin C (1-hydroxy-3-(*E,E,E*,-2′,4′,7′-undecatrienylidine) triazene) was generously donated by Dr H. Tomoda and Dr S. Ōmura, Kitasato institute (Tokyo, Japan).

2.2. Preparation of rat neutrophils

Rat neutrophils were collected from male Sprague-Dawley rats (Japan SLC Co., Hamamatu, Japan) at 16 h after the intraperitoneal injection of 1% casein solution as described previously (Naraba et al., 1995). The cells were washed with Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution and then suspended in Hanks' balanced salt solution containing 0.1% essentially fatty acid-free bovine serum albumin. The cell suspension contained > 93% neutrophils as assessed by Giemsa staining, and their viability was > 99% as determined by trypan blue dye exclusion.

2.3. Stimulation of rat neutrophils

Rat neutrophils (10^7 cells/ml) were preincubated for 5 min at 37°C and then exogenous arachidonic acid (10–100 μM) and opsonized zymosan (500 μg/ml) were added. After incubation for the desired times, the reaction was terminated by the addition of methanol containing 2% acetic acid for the measurement of contents of PAF and lyso-PAF. For measurement of arachidonic acid metabolites and enzymatic activity, ice-cold buffer was used to terminate the reaction.

2.4. Measurement of PAF and lyso-PAF

PAF and lyso-PAF were extracted from the whole incubation mixture and separated by HPLC as previously reported (Hayashi et al., 1992). PAF content was measured in terms of its activity toward the aggregation of washed rabbit platelets (Hayashi et al., 1992). The lyso-PAF fraction from HPLC was chemically acetylated to PAF (Naraba et al., 1995), and the lyso-PAF content was then measured as PAF as above.

2.5. Measurement of arachidonic acid metabolites

The incubation mixture used for stimulation of the neutrophils was centrifuged at 1000 rpm for 5 min, and the supernatant was loaded on to a SEP-PAK C18 column (Waters Associates, Milford, MA, USA). The lipid fraction was eluted with methanol and derivatized with 9-anthryldiazomethane. After purification by passage through a SEP-PAK silica column (Waters Associates), the individual contents of ADAM derivatives of arachidonic acid metabolites were measured by reverse-phase HPLC as previously reported (Yamaki and Oh-ishi, 1992).

2.6. Measurement of enzyme activities

The cellular fraction separated from the reaction mixture was suspended in a sonication buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA) and sonicated three times with a Branson sonifer 250 (Danbury, CT) for 30 s each time. The activities of phospholipase A_2 and acetyltransferase in the cell lysates were measured. Phospholipase A_2 activity was assayed as the radioactivity of [^{14}C]arachidonic acid liberated from 2 μM 1-acyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphoethanolamine as described by Fujimori et al. (1993). Acetyltransferase activity was assayed as the radioactivity of [^{3}H]acetyl-CoA incorporated into PAF as described by Nakagawa et al. (1992).

2.7. Statistics

All data were expressed as means ± S.E.M., and individual groups were compared by means of Dunnett's *t*-test. A *P* value of less than 0.05 was considered to be significant.

3. Results

3.1. Effect of exogenous arachidonic acid on PAF and lyso-PAF production

As shown in Fig. 1, PAF and lyso-PAF produced in rat neutrophils in response to opsonized zymosan (500 μg/ml) was suppressed dose dependently by the addition of arachidonic acid at 10–100 μM to the incubation medium. Levels of lyso-PAF and PAF were assessed at their peak production time following stimulation, i.e., 5 min and 10

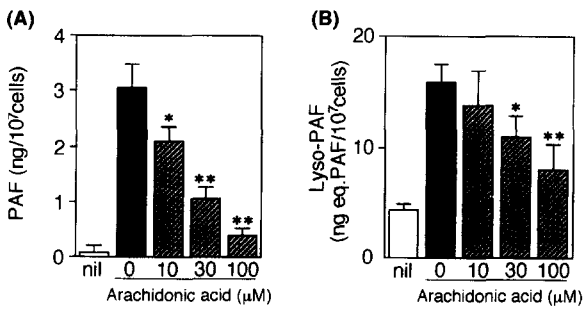

Fig. 1. Effect of exogenous arachidonic acid on PAF (A) and lyso-PAF (B) production in rat neutrophils stimulated with opsonized zymosan. Rat neutrophils (10^7 cells/ml) were stimulated with zymosan (500 μg/ml) in the absence (solid column) or presence (hatched column) of the indicated concentrations of exogenous arachidonic acid. In (A) PAF was extracted at the end of a 10-min incubation and was measured by the rabbit platelet aggregation assay referenced in Methods. In (B) lyso-PAF was extracted after a 5-min incubation, chemically converted to PAF, and measured by the same aggregation assay. Each value is the mean with S.E.M. of samples from 4–5 rats. *, ** Values that are significantly different at $P < 0.05$ and $P < 0.01$, respectively, from the value obtained in the absence of exogenous arachidonic acid.

Table 1
Effect of exogenous arachidonic acid on the activity of phospholipase A_2 and of acetyl-CoA acetyltransferase

Arachidonic acid (μM)	Enzyme activity (nmol/min/mg protein)	
	Phospholipase A_2	Acetyltransferase
No stimulation	0.126 ± 0.008	0.157 ± 0.018
0	0.453 ± 0.059	0.532 ± 0.088
10	0.449 ± 0.021	0.487 ± 0.093
30	0.441 ± 0.025	0.433 ± 0.101
100	0.446 ± 0.038	0.390 ± 0.075 *

Rat neutrophils were stimulated with opsonized zymosan (500 μg/ml) in the presence or absence of the indicated concentrations of arachidonic acid, and the cell lysates were obtained after sonication of the cells. The activities of phospholipase A_2 and acetyltransferase were assayed as described in Methods. Enzyme activities are expressed as the products formed/min/mg protein from their substrates, which were [^{14}C]arachidonoyl-glycero-3-phosphoethanolamine for phospholipase A_2 and [^{3}H]acetyl-CoA for acetyltransferase. * Significant difference at $P < 0.05$ from the value for 0 μM exogenous arachidonic acid. Each value is the mean ± S.E. from 4 rats.

min, respectively, according to previous data (Naraba et al., 1995). The addition of arachidonic acid (10–100 μM) by itself did not have any effect on the production of lyso-PAF or PAF production at the concentrations used in this study. Whereas 100 μM arachidonic acid caused a decrease in the viability of cells (62.8 ± 9.3%), there was no effect at the concentrations of 10 and 30 μM, at which the viabilities were 96.1 ± 3.6% and 92.1 ± 5.6%, respectively ($n = 4$).

3.2. Effect of exogenous arachidonic acid on the activities of phospholipase A_2 and acetyl-CoA acetyltransferase

To examine enzymatic activities, we prepared cell lysates from rat neutrophils stimulated with opsonized zymosan (500 μg/ml) in the presence of exogenous arachidonic acid (10–100 μM) and then measured the activities of phospholipase A_2 and acetyltransferase. Arachidonic acid did not affect phospholipase A_2 activity in the dose range used, as shown in Table 1. However, arachidonic acid slightly, and significantly, suppressed acetyltransferase activity at 100 μM.

3.3. Production of arachidonic acid metabolites from opsonized zymosan-stimulated rat neutrophils

Arachidonic acid metabolites in the supernatant were measured after rat neutrophils had been stimulated with opsonized zymosan (500 μg/ml) for 10 min in the presence or absence of exogenous arachidonic acid (30 μM). As shown in Table 2, the production of prostaglandin E_2, prostaglandin D_2, thromboxane B_2 and 5-hydroxyeicosatetraenoic acid was detected following stimulation with opsonized zymosan, and their levels were further increased in the presence of arachidonic acid. These increased levels of prostaglandin E_2, prostaglandin D_2 and thromboxane B_2 were almost completely suppressed by pretreatment with indomethacin (50 μM), whereas the amounts of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid were increased by treatment with this drug. This increased production of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid is in agreement with previous reports showing that cyclooxgenase inhibitors enhance the release of lipoxygenase products (Engineer et al., 1987).

3.4. Effect of arachidonic acid metabolites and indomethacin on PAF production from opsonized zymosan-stimulated rat neutrophils

To find if arachidonic acid metabolites had inhibited PAF production when the cells were stimulated with opsonized zymosan in the presence of arachidonic acid, we examined the effect of indomethacin on PAF production. Indomethacin (50 μM) did not affect either PAF production elicited by opsonized zymosan (500 μg/ml) alone, or that suppressed in the presence of arachidonic acid (30 μM) and opsonized zymosan, as shown in Fig. 2. This dose of indomethacin almost completely suppressed the products formed by cyclooxgenase activity, as shown in Table 2. However, addition of 300 pmol of prostaglandin

Table 2
Level of arachidonic acid metabolites produced by opsonized zymosan-stimulated rat neutrophils

	No stimulation [a]	Zymosan [b]	Zymosan + AA [c]	Zymosan + AA + indomethacin [d]
PGE_2	< 1.5	2.60 ± 0.59	17.17 ± 1.32	< 1.5
PGD_2	< 1.5	< 1.5	11.15 ± 0.43	< 1.5
TxB_2	< 1.5	1.68 ± 0.97	16.15 ± 1.94	< 1.5
LTB_4	< 1.5	1.81 ± 0.39	3.32 ± 0.10	6.35 ± 0.39
5-HETE	17.57 ± 5.53	50.07 ± 3.32	148.45 ± 17.34	176.05 ± 8.85

Rat neutrophils (10^7 cells/ml) were divided into 4 groups; [a] cells were incubated in a vehicle for 10 min, [b] cells were incubated with opsonized zymosan (500 μg/ml) for 10 min, [c] cells were incubated with opsonized zymosan in the presence of arachidonic acid (AA, 30 μM), and [d] cells were incubated with opsonized zymosan in the presence of arachidonic acid and indomethacin (50 μM). The supernatants of the incubation mixtures were processed and assayed for individual metabolites of arachidonic acid (PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; TxB_2, thromboxane B_2; LTB_4, leukotriene B_4; 5-HETE, 5-hydroxyeicosatetraenoic acid) by ADAM-HPLC as described in Methods. Values are expressed as pmol/10^7 cells of the means ± S.E. from 4–5 rats. Very low values below the detection limit are expressed as < 1.5.

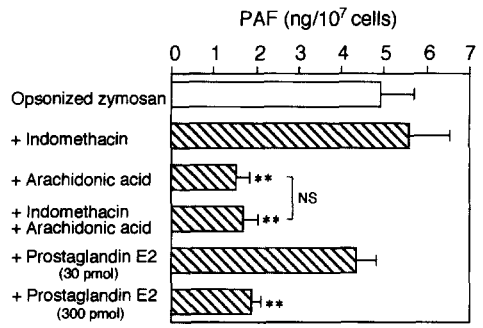

Fig. 2. Production of PAF in opsonized zymosan-stimulated neutrophils in the presence of various agents. The PAF level was assessed in rat neutrophils stimulated with opsonized zymosan for 10 min after preincubation in the presence of indomethacin (50 μM), arachidonic acid (30 μM) or PGE_2 (30 or 300 pmol) for 15 min. Details are in the Methods. Each value is the mean with S.E.M. of samples from 4 rats. ** Value significantly different at $P < 0.01$ from the value obtained with opsonized zymosan alone. NS: not significant.

E_2, but not of 30 pmol, inhibited significantly the PAF production from opsonized zymosan-stimulated cells, as also shown in Fig. 2. Other arachidonic acid metabolites, prostaglandin D_2, thromboxane A_2, leukotriene B_4, 5-hydroxyeicosatetraenoic acid, 12-hydroxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid (300 pmol) did not show any effect, as shown in Table 3.

3.5. Effect of triacsin C on the arachidonic acid-induced inhibition of PAF production in rat neutrophils

To determine if another mechanism was involved in the arachidonic acid-mediated inhibition of PAF production, we examined the effect of triacsin C, an acyl-CoA synthetase inhibitor, on the inhibitory effect of arachidonic acid on PAF production. Neutrophils were treated with triacsin C at the concentration of 500 nM for 60 min before the addition of opsonized zymosan, a condition previously described as sufficient to inhibit acyl-CoA synthetase (Hayashi et al., 1992). As shown in Fig. 3, triacsin C reversed the inhibition induced by arachidonic acid. In other words, in the presence of triacsin C, arachidonic acid did not suppress the increased level of PAF production by opsonized zymosan-stimulated cells.

Table 3
Effect of arachidonic acid metabolites on PAF production in opsonized zymosan-stimulated rat neutrophils

Arachidonic acid metabolites (300 pmol)	PAF (ng/10^7 cells)
Opsonized zymosan alone	3.54 ± 0.33
PGD2	4.09 ± 0.38
TXA2	3.39 ± 0.50
LTB4	3.55 ± 0.58
5-HETE	4.02 ± 0.66
12-HETE	3.67 ± 0.64
15-HETE	3.41 ± 0.29

The PAF level was assessed in rat neutrophils stimulated with opsonized zymosan for 10 min in the presence of prostaglandin D_2 (PGD_2), thromboxane A_2 (TxA_2), leukotriene B_4 (LTB_4), 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE and 15-HETE. Each value is the mean with S.E.M. of samples from 4 rats.

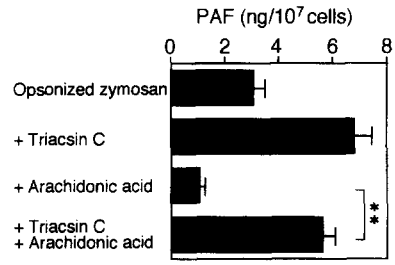

Fig. 3. Effect of triacsin C on the arachidonic acid-induced inhibition of PAF production in rat neutrophils. Rat neutrophils were incubated with or without triacsin C (500 nM) for 60 min and stimulated with opsonized zymosan (500 μg/ml) in the presence of 30 μM exogenous arachidonic acid. After incubation for 10 min, PAF was measured as in Fig. 1. Each value is the mean with S.E.M. of samples from 4 rats. ** Value significantly different at $P < 0.01$ from the value for opsonized zymosan alone.

4. Discussion

The addition of exogenous arachidonic acid at 30 μM significantly reduced the PAF and lyso-PAF production that had been induced in response to opsonized zymosan. At this concentration arachidonic acid did not suppress phospholipase A_2 and acetyltransferase activities, indicating that these two enzymes might not contribute to the reduction in PAF production. Although acetyltransferase was slightly inhibited at 100 μM arachidonic acid, the inhibition at 30 μM was not significant, and thus this enzyme may not be involved in the suppression of PAF. However, Remy et al. (1989) indicated that arachidonic acid inhibited PAF production in ionophore-stimulated bovine neutrophils through inhibition of acetyltransferase. Also, Winkler et al. (1993) found that arachidonic acid (10–30 μM) inhibited PAF production in ionophore-stimulated human neutrophils by altering phospholipase A_2 activity. This apparent discrepancy may exist because of differences in species or stimuli, but the exact reason is not known. The most plausible explanation is that the relative activity of each enzyme, such as phospholipase A_2, acetyltransferase or acyltransferase, could be differently regulated by each cell type in response to different stimuli. Regarding differences in experimental methods, Remy et al. (1989) examined acetyltransferase activity in cell lysates incubated with arachidonic acid, whereas we measured the activity in cell lysates prepared after the cells had been incubated with arachidonic acid and zymosan. Winkler et al. (1993) measured arachidonic acid release from the cells as the activity of phospholipase A_2. These differences in methods may also have caused the different result.

As for arachidonic acid metabolites, 300 pmol but not 30 pmol of exogenous prostaglandin E_2 inhibited PAF production, and the level of prostaglandin E_2 detected in the incubation mixture of neutrophils stimulated with opsonized zymosan in the presence of arachidonic acid was 17 pmol, that is, less than 30 pmol. Therefore, the concentration of prostaglandin E_2 in the incubation mixture incubated with arachidonic acid was too low to exert inhibition. Other cyclooxygenase metabolites examined, such as prostaglandin D_2 and thromboxane A_2, did not show inhibition of PAF production. Furthermore, indomethacin did not alter the inhibitory effect of arachidonic acid, even at the dose that almost completely suppressed the formation of cyclooxgenase-dependent products in the incubation mixture. Even though we did not try any lipoxygenase inhibitor, we could eliminate an effect of lipoxygenase products because addition of exogenous leukotriene B_4 and 5-, 12- and 15-hydroxyeicosatetraenoic acid did not show any effect on PAF production at a concentration of 300 pmol/ml. This concentration is higher than the actually measured levels of the products in the stimulated neutrophils as shown in Table 2. These results suggest that inhibition of PAF production may not be due to such arachidonic acid metabolites.

We previously reported that the acyl-CoA synthetase inhibitor, triacsin C, increased lyso-PAF and PAF levels in opsonized zymosan-stimulated rat neutrophils (Naraba et al., 1995). Treatment with triacsin C reversed the inhibitory effect of arachidonic acid, suggesting that the inhibition of PAF production by arachidonic acid may occur via acylation of lyso-PAF.

In conclusion, arachidonic acid inhibits PAF production in rat neutrophils in response to opsonized zymosan by increasing the transacylation of lyso-PAF, thereby decreasing the availability of PAF precursor.

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