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Enhancement by cyclo-oxygenase inhibitors of platelet-activating factor production in thapsigargin-stimulated macrophages

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1 Thapsigargin stimulated the accumulation of cell-associated platelet-activating factor (PAF) and extracellular prostaglandin E_2 (PGE_2) in rat peritoneal macrophages. PAF in the conditioned medium was less than the detectable amount. To obtain further insight into the mechanism of PAF accumulation, the role of PGE_2 in PAF accumulation was investigated.

2 When macrophages were incubated in medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and cyclo-oxygenase inhibitors such as indomethacin, naproxen or ibuprofen, the PAF content of the cells at 10 min was increased in a concentration-dependent manner in accordance with inhibition of PGE_2 production. The stimulation of PAF accumulation by cyclo-oxygenase inhibitors was significant at 10 min. Without stimulation by thapsigargin, cyclo-oxygenase inhibitors did not increase PAF accumulation.

3 In thapsigargin-stimulated macrophages, when PGE_2 (10^{-7} M) was added to the medium, the cyclo-oxygenase inhibitor-induced stimulation of PAF accumulation at 10 min was markedly inhibited.

4 The accumulation of PAF induced by thapsigargin alone at 10 min was also inhibited by exogenous PGE_2 (10^{-8} and 10^{-7} M), or arachidonic acid (10^{-6} and 10^{-5} M) in accordance with the increase in PGE_2 production.

5 The accumulation of PAF induced by thapsigargin alone or by thapsigargin and indomethacin (10^{-6} M) was inhibited by dibutyryl cyclic AMP.

6 These results indicate that the concurrently produced PGE_2 in thapsigargin-stimulated macrophages down-regulates PAF accumulation by increasing intracellular cyclic AMP levels, and that cyclo-oxygenase inhibitors increase PAF accumulation by inhibiting PGE_2 production.

Keywords: Platelet-activating factor; prostaglandin E_2; thapsigargin; indomethacin; naproxen; ibuprofen; arachidonic acid; cyclic AMP; macrophage

Introduction

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Demopoulos *et al.*, 1979; Prescott *et al.*, 1990) has several pro-inflammatory activities that include an increase in vascular permeability (Stimler *et al.*, 1981; Humphrey *et al.*, 1982b; 1984; Morley *et al.*, 1983), local infiltration of leucocytes (Humphrey *et al.*, 1982a), as well as the activation of neutrophils (O'Flaherty *et al.*, 1981; Shaw *et al.*, 1981), eosinophils (Kimani *et al.*, 1988; Kroegel *et al.*, 1989) and platelets (Henson & Oades, 1976; Henson, 1977; McManus *et al.*, 1981). Furthermore, various inflammatory cells such as neutrophils (Lynch *et al.*, 1979), macrophages (Mencia-Huerta & Benveniste, 1979; Drapier *et al.*, 1983), lymphocytes (Malavasi *et al.*, 1986), basophils (Benveniste, 1974; Hanahan *et al.*, 1980), and vascular endothelial cells (Camussi *et al.*, 1983; Prescott *et al.*, 1984) produce PAF. Therefore, PAF has been implicated as a potent chemical mediator of inflammation and allergy. We reported (Watanabe *et al.*, 1990; 1994) that several PAF antagonists inhibit neutrophil infiltration in the allergic inflammation model in rats and suggested that PAF plays a significant role in neutrophil infiltration.

PAF production by inflammatory cells in response to various stimuli is thought to proceed mainly via the remodelling pathway (Albert & Snyder, 1983), in which the membrane phospholipid 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine is hydrolyzed by phospholipase A_2 releasing acyl fatty acids, predominantly arachidonic acid, and the resultant lyso-PAF is acetylated to become PAF by acetyltransferase (Wykle *et al.*, 1980). The released arachidonic acid is then metabolized to prostanoids by cyclo-oxygenase and lipoxygenase. Of interest is the fact that two different lipid mediators, namely PAF and prostanoids are produced from a common precursor in membrane phospholipids when PAF is produced via the remodelling pathway.

Thapsigargin, a hexa-oxygenated and tetra-acylated sesquiterpene lactone isolated as a potent skin-irritating constituent from the root of the umbelliferous plant, *Thapsia garganica* L. (Apiaceae) (Christensen *et al.*, 1982; Christensen & Norup, 1985), releases histamine from mast cells (Rasmussen *et al.*, 1978; Ali *et al.*, 1985; Ohuchi *et al.*, 1986), promotes carcinogenesis in mouse skin (Hakii *et al.*, 1986), and inhibits endomembrane Ca^{2+}-ATPase (Thastrup *et al.*, 1987). We showed (Ohuchi *et al.*, 1987; 1988) that thapsigargin stimulates the release of arachidonic acid and the production of PGE_2 at concentrations of 10 to 100 ng ml^{-1} (15.4 to 154 nM) in rat peritoneal macrophages. Furthermore, we found that intracellular accumulation of PAF is also enhanced by 10 ng ml^{-1} (15.4 nM) thapsigargin in rat peritoneal macrophages (Watanabe *et al.*, 1992). In the present investigation, the stimulant effects of thapsigargin on the production of PAF and prostaglandin E_2 (PGE_2) were further examined and we found that PAF production is transient, whereas that of PGE_2 is sustained by thapsigargin. We speculated that simultaneously produced PGE_2 down-regulates PAF production. Therefore, we carried out further studies to confirm this notion, using cyclo-oxygenase inhibitors.

We found that, in thapsigargin-stimulated macrophages, the inhibition of PGE_2 production by cyclo-oxygenase inhibitors such as indomethacin, naproxen, and ibuprofen, further increases the PAF accumulation. In addition, the cyclo-oxygenase inhibitor-induced increase in PAF accumulation

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was inhibited by exogenous PGE_2. PAF accumulation induced by thapsigargin alone was also inhibited by exogenous PGE_2. Furthermore, arachidonic acid also inhibited thapsigargin-induced PAF accumulation in accordance with the increase in PGE_2 production. These results indicated that PGE_2 produced by thapsigargin stimulation down-regulates PAF accumulation, and that cyclo-oxygenase inhibitors increase PAF accumulation by inhibiting PGE_2 production.

Methods

Preparation of rat peritoneal macrophages

A solution of soluble starch (Wako Pure Chemical Ind., Osaka, Japan) and bacto peptone (Difco Laboratories, Detroit, MI, U.S.A.), 5% (w/v) each, was injected intraperitoneally into male Sprague-Dawley rats (300–350 g, specific pathogen-free, Charles River Japan Inc., Kanagawa, Japan) at a dose of 5 ml per 100 g body weight. Four days later, the rats were killed by cutting the carotid artery under diethylether anaesthesia and peritoneal cells were harvested (Ohuchi *et al.*, 1985).

Macrophage culture

The peritoneal cells were suspended in Eagle's minimal essential medium (Nissui Inc., Tokyo, Japan) containing 10% (v/v) calf bovine serum (Flow Laboratories, North Rydge, N.S.W., Australia), penicillin G potassium (18 $\mu g\ ml^{-1}$) and streptomycin sulphate (50 $\mu g\ ml^{-1}$) (Meiji Seika Co., Tokyo, Japan). The macrophages were seeded at a density of 6×10^6 cells per 60 mm plastic tissue culture dish in 4 ml of the medium and incubated for 2 h at 37°C. The dishes were then washed three times with the medium to remove non-adherent cells (Ohuchi *et al.*, 1986). The adherent cells were further incubated for 20 h at 37°C in 4 ml of the medium and used for the following experiments.

Incubation of macrophages with drugs

After washing three times with the medium containing no calf serum, the cells were incubated at 37°C for the indicated periods in 4 ml of the medium containing thapsigargin (LC Services Co., Woburn, MA, U.S.A.), indomethacin, naproxen, ibuprofen, PGE_2, arachidonic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.), or dibutyryl cyclic AMP (Wako Pure Chemical Ind.) dissolved in ethanol. The final concentration of ethanol in the medium was adjusted to 0.1% (v/v). Control medium contained the same amount of vehicle. After incubation, the conditioned medium was collected to measure the PGE_2 concentrations, and the PAF content of the cells was determined.

Measurement of PGE_2 concentrations

The conditioned medium was centrifuged at 1500 *g* and 4°C for 5 min, and PGE_2 concentrations in the supernatant fraction were radioimmunoassayed (Ohuchi *et al.*, 1985). PGE_2 antiserum was purchased from Seragen Inc., Boston, MA, U.S.A.

Sample preparation for PAF determination

The conditioned medium was removed, then adherent cells were washed three times with ice-cold medium and 2 ml of ice-cold 2% (v/v) acetic acid in methanol was added. To measure recovery, 3.0×10^{-3} μCi of [^{3}H]-PAF (158.7 Ci $mmol^{-1}$, Amersham, Buckinghamshire) was added to each dish. The cells were then scraped off the dish with a rubber policeman and poured into siliconized glass tubes. The dishes were then rinsed three times with 2 ml of the 2% (v/v) acetic acid in methanol, and the washes were collected into the same tube. Total lipids were then extracted by Bligh & Dyer's method (1959) from the acetic acid solution, and reconstituted in 1 ml of 25 mM sodium phosphate buffer (pH 6.9) containing 20% (v/v) methanol (Watanabe *et al.*, 1992). The reconstituted solution was loaded onto an immunoaffinity mini-column for PAF (Watanabe *et al.*, 1992), which was washed with 5 ml of 50% (v/v) methanol in water. PAF was then eluted with 5 ml of methanol. The eluate was evaporated to dryness and the residue was reconstituted in 50 mM sodium citrate buffer (pH 6.3) containing 0.05% (v/v) Tween 20 (Wako Pure Chemical Co.), and a portion of the solution was used for radioimmunoassay. The remainder of the solution was used to measure the radioactivity level of [^{3}H]-PAF, and the recovery was calculated (Watanabe *et al.*, 1992).

Radioimmunoassay of PAF

The level of PAF in the cell fraction was measured by radioimmunoassay (Watanabe *et al.*, 1992), using a commercially available kit (Platelet Activating Factor [^{125}I]-PAF kit, New England Nuclear, Boston, MA, U.S.A.), in which a calibration graph was constructed with cold PAF as a mixture of 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}-PAF) and 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{18}-PAF) (1:1, w/w). The cross-reactivity of the antibody is described in the Instruction Manual accompanying the kit. For example, the % cross-reactivity for the representative compounds is as follows; lyso-PAF (γ-hexadecyl), 0.003; arachidonic acid, 0.002; triglyceride (1,3-disteroyl-2-oleoylglycerol), 0.002; phosphatidylcholine (β,γ-disteroyl), 0.002; and phosphatidylethanolamine (β,γ-disteroyl), 0.03. Further details of the radioimmunoassay procedure are also given in the Instruction Manual.

Statistical significance

Results were analyzed for statistical significance by Dunett's test for multiple comparison and Student's *t* test for unpaired observations.

Results

Stimulation by thapsigargin of PAF accumulation and PGE_2 production

The time-course of PAF and PGE_2 generation in the macrophages incubated in the medium containing thapsigargin (30 $ng\ ml^{-1}$, 46.1 nM) was examined. Accumulation of cell-associated PAF was significantly increased by thapsigargin treatment within 5 min after incubation, and reached a maximum 10 min after incubation (PAF, 30.8 ± 3.5 pmol per 6×10^6 cells, mean ± s.e.mean from 4 samples); the levels then declined gradually till 40 min. Without thapsigargin, the levels of cell-associated PAF were very low (1.2 ± 0.1 pmol per 6×10^6 cells, mean ± s.e.mean from 4 samples). The PAF level in the conditioned medium was below the limits of detection (< 1.0 pmol) at 5 to 30 min in the presence or absence of thapsigargin. Therefore, in the following experiments, the levels of cell-associated PAF were determined.

When the macrophages were incubated in medium containing various concentrations of thapsigargin, levels of cell-associated PAF determined 10 min after incubation were increased in a concentration-dependent manner. A significant increase in PAF accumulation was induced by thapsigargin at 3 $ng\ ml^{-1}$ (4.61 nM) and above, and the effect was maximal at 30 $ng\ ml^{-1}$ (46.1 nM); the level of cell-associated PAF was 26.0 ± 2.0 pmol per 6×10^6 cells (mean ± s.e.mean from 4 samples). No further increase in PAF accumulation was induced when the thapsigargin concentration was increased to 100 $ng\ ml^{-1}$ (154 nM). We used 30 $ng\ ml^{-1}$ (46.1 nM) thapsigargin for the following pharmacological experiments. At this concentration of thapsigargin, the concentrations of PGE_2 in the conditioned medium was increased time-dependently. At

10 min, PGE_2 concentrations in the conditioned medium of thapsigargin-treated macrophages and control macrophages were 25.6 ± 1.6 and 1.2 ± 0.1 ng ml^{-1}, respectively.

The effects of cyclo-oxygenase inhibitors on thapsigargin-induced PAF accumulation and PGE_2 production

To clarify the role of PGE_2 during PAF induction, the effects of indomethacin on thapsigargin-induced PAF accumulation were investigated. As shown in Figure 1, indomethacin inhibited thapsigargin-induced PGE_2 production in a concentration-dependent manner when measured 10 min after incubation. In accordance with the decrease in the PGE_2 level, thapsigargin-induced PAF accumulation was enhanced dose-dependently by indomethacin 10 min after incubation (Figure 1). At 10^{-6} M indomethacin, the PGE_2 levels decreased and those of PAF increased about twofold over that induced by thapsigargin alone. There was no detectable amount of PAF in the conditioned medium of the macrophages incubated with thapsigargin alone or with thapsigargin and indomethacin at 10 min. In the absence of thapsigargin, indomethacin did not enhance PAF accumulation at concentrations of 10^{-7} to 10^{-6} M (data not shown).

The time-course of the stimulant effect of indomethacin on thapsigargin-induced PAF accumulation was then examined. Thapsigargin increased PAF levels for the first 10 min (Figure 2a) in parallel with a rapid increase in that of PGE_2 (Figure 2b). When PGE_2 production was almost completely blocked by indomethacin (10^{-6} M) (Figure 2b), the PAF level at 10 min was increased twofold over that induced by thapsigargin alone. These results suggested that the rapid increase in the PGE_2 level down-regulates PAF accumulation during this period. At 20 min, although PGE_2 production continued to be inhibited by indomethacin, PAF accumulation was not significantly increased. At 40 min, the stimulant effect of indomethacin on PAF accumulation was completely abolished. These results indicated that the stimulant effect of indomethacin is apparent only when PAF accumulation is rapidly increasing.

The effects of other cyclo-oxygenase inhibitors, naproxen and ibuprofen, were also examined. As shown in Figures 3 and 4, both cyclo-oxygenase inhibitors enhanced thapsigargin-stimulated PAF accumulation at 10 min dose-dependently, in accordance with the decrease in PGE_2 production. At 40 min, these cyclo-oxygenase inhibitors did not affect PAF accumulation (data not shown) like indomethacin. These results also suggested that the PGE_2 produced during the first 10 min plays a role in down-regulation of thapsigargin-induced PAF accumulaiton.

The effects of PGE_2 and arachidonic acid on thapsigargin-induced PAF accumulation

To obtain further evidence for this theory, the effects of exogenous PGE_2 on the cyclo-oxygenase inhibitor-induced enhancement of PAF accumulation were examined. As shown in Figure 5, exogenous PGE_2 (35.3 ng ml^{-1}, 10^{-7} M) inhibited the PAF accumulation that was enhnaced by the cyclo-oxygenase inhibitors, indomethacin (0.358 μg ml^{-1}, 10^{-6} M), naproxen (0.230 μg ml^{-1}, 10^{-6} M), or ibuprofen (0.619 μg ml^{-1}, 3×10^{-6} M) in the presence of thapsigargin (30 ng ml^{-1}, 46.1 nM). These cyclo-oxygenase inhibitors at such concentrations prominently suppressed PGE_2 production as shown in Figures 1, 3 and 4. In the presence of 10^{-7} M PGE_2, PAF accumulation was inhibited to levels far below that induced by thapsigargin alone. These results indicated that exogenous PGE_2 inhibits PAF accumulation stimulated by thapsigargin. Therefore, the effects of exogenous PGE_2 on thapsigargin-stimulated PAF accumulation were examined. As shown in Figure 6, the PAF

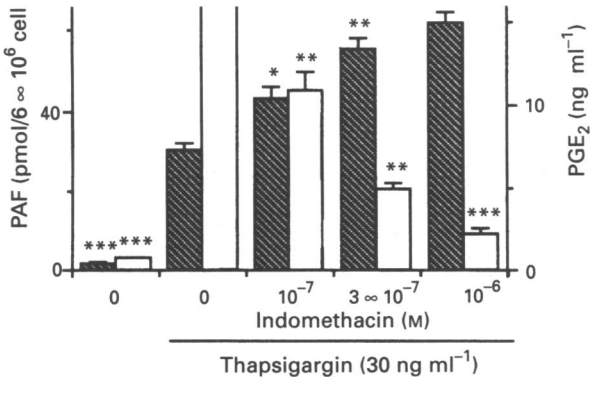

Figure 1 Effects of various concentrations of indomethacin on thapsigargin-induced accumulation of PAF and PGE_2 in rat peritoneal macrophages. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and the indicated concentrations of indomethacin. PAF contents of cells (hatched columns) and PGE_2 concentrations (open columns) in the conditioned medium are shown. Values are the means from four samples with s.e.mean. The results were confirmed by three separate experiments. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin alone.

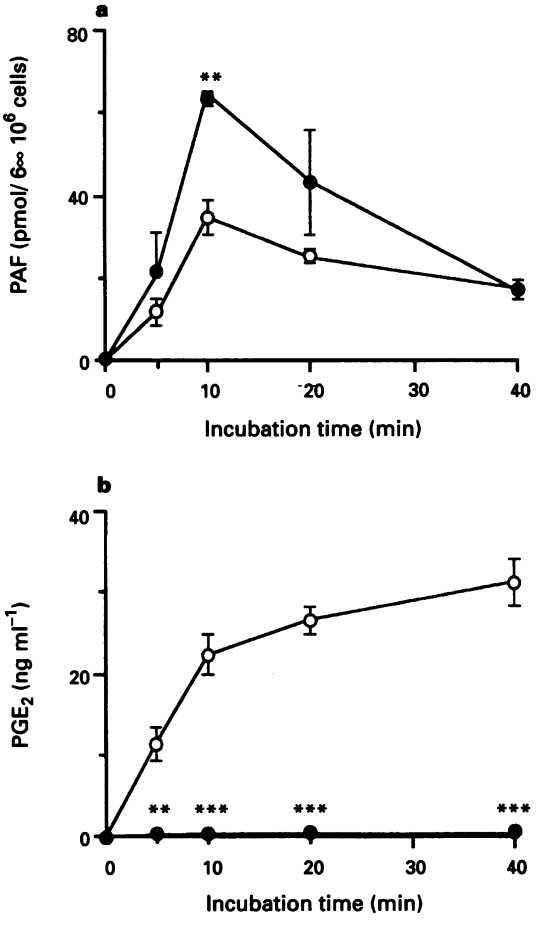

Figure 2 Time-course of indomethacin effect on thapsigargin-induced accumulation of PAF and PGE_2. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for the indicated periods in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) alone (○) or thapsigargin (30 ng ml^{-1}, 46.1 nM) and indomethacin (0.358 μg ml^{-1}, 10^{-6} M) (●). PAF contents of cells (a) and PGE_2 concentrations in the conditioned medium (b) are shown. Values are the means from four samples with s.e.mean. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding thapsigargin control. The results were confirmed by three separate experiments.

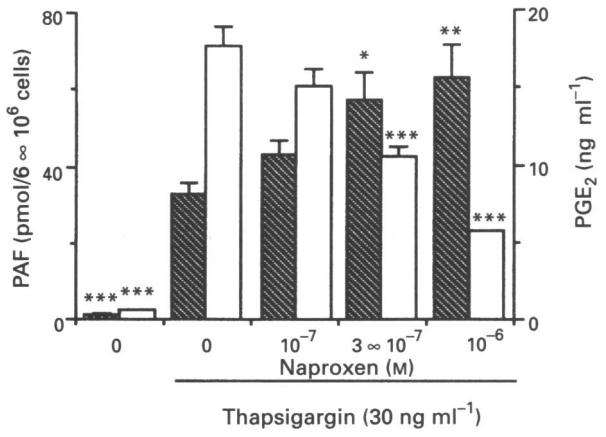

Figure 3 Effects of various concentrations of naproxen on thapsigargin-induced accumulation of PAF and PGE_2 in rat peritoneal macrophages. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and the indicated concentrations of naproxen. PAF contents of cells (hatched columns) and PGE_2 concentrations (open columns) in the conditioned medium are shown. Values are the means from four samples with s.e.mean. Statistical significance: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. thapsigargin alone. The results were confirmed by three separate experiments.

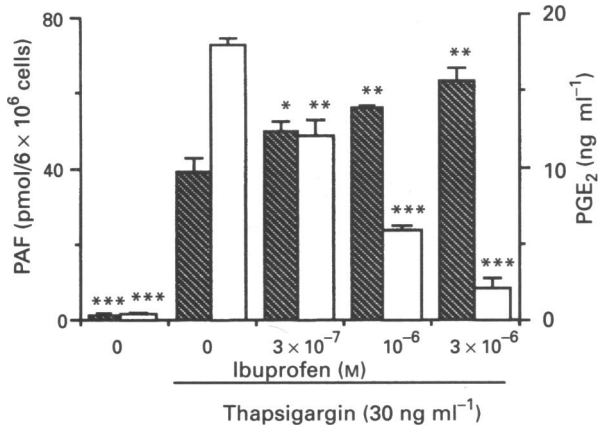

Figure 4 Effects of various concentrations of ibuprofen on thapsigargin-induced accumulation of PAF and PGE_2 in rat peritoneal macrophages. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and the indicated concentrations of ibuprofen. PAF contents of cells (hatched columns) and PGE_2 concentrations (open columns) in the conditioned medium are shown. Values are the means from four samples with s.e.mean. Statistical significance: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. thapsigargin alone. The results were confirmed by three separate experiments.

level induced by thapsigargin at 10 min was reduced by exogenous PGE_2 in a concentration-dependent manner. The inhibition was significant at 10^{-8} and 10^{-7} M PGE_2. These data indicated that PGE_2 down-regulates the PAF accumulation stimulated by thapsigargin. Furthermore, when arachidonic acid was added to the incubation medium, the PAF accumulation induced by thapsigargin at 10 min was decreased at 10^{-6} and 10–5 M (0.305 and 3.05 μg ml^{-1}, respectively), in accordance with the increase in PGE_2 production (Figure 7). These results also indicated that PGE_2 produced by thapsigargin plays a role in down-regulation of PAF accumulation that is stimulated by thapsigargin.

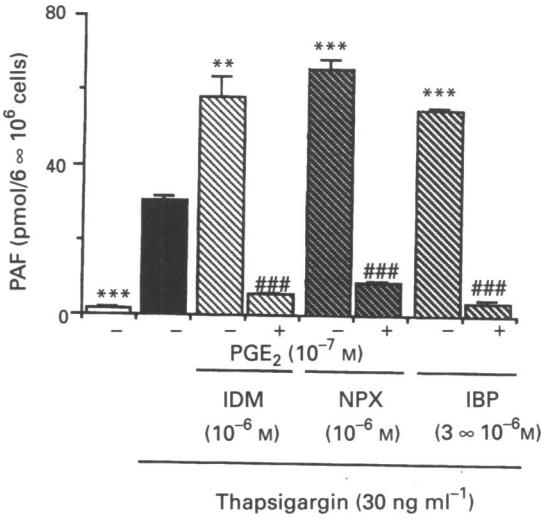

Figure 5 Enhancement of PAF accumulation by cyclo-oxygenase inhibitors and its suppression by PGE_2 in thapsigargin-stimulated rat peritoneal macrophages. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and the indicated concentrations of cyclo-oxygenase inhibitors with (+) or without (−) PGE_2 (35.3 ng ml^{-1}, 10^{-7} M). PAF contents of cells are shown. Values are the means from four samples with s.e.mean. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs. thapsigargin alone; $^{\#\#\#}P<0.001$ vs. corresponding control. IDM, indomethacin; NPX, naproxen; and IBP, ibuprofen.

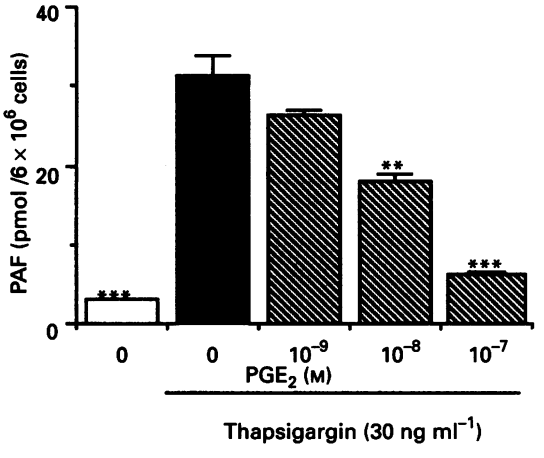

Figure 6 Inhibition of PAF accumulation by PGE_2 in thapsigargin-stimulated rat peritoneal macrophages. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and the indicated concentrations of PGE_2. PAF contents of the cells are shown. Values are the means from four samples with s.e.mean. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs. thapsigargin alone. The results were confirmed by three separate experiments.

The effects of dibutyryl cyclic AMP on PAF accumulation stimulated by thapsigargin alone or by thapsigargin plus indomethacin

To obtain further insight into the mechanism of the inhibition of PAF generation by simultaneously produced PGE_2, the effects of dibutyryl cyclic AMP on PAF accumulation stimulated by thapsigargin alone or by thapsigargin and indomethacin were examined. As shown in Figure 8, PAF accumulation stimulated by thapsigargin in the presence or

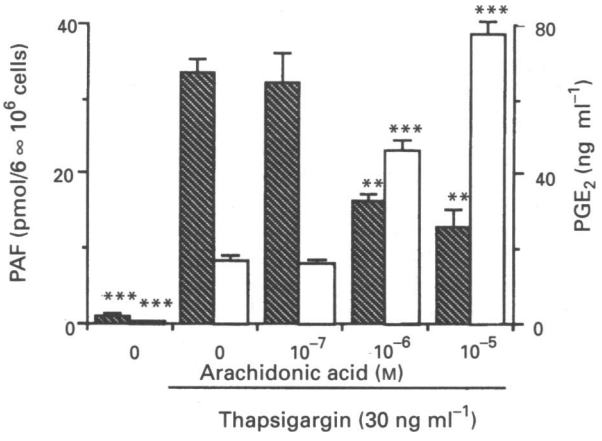

Figure 7 Inhibition of PAF accumulation by arachidonic acid in thapsigargin-stimulated rat peritoneal macrophages. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) and indicated concentrations of arachidonic acid. PAF contents of cells (hatched columns) and PGE_2 concentrations (open columns) in the conditioned medium are shown. Values are the means from four samples with s.e.mean. Statistical significance: $^{**}P < 0.01$, $^{**}P < 0.001$ vs. thapsigargin alone. The results were confirmed by three separate experiments.

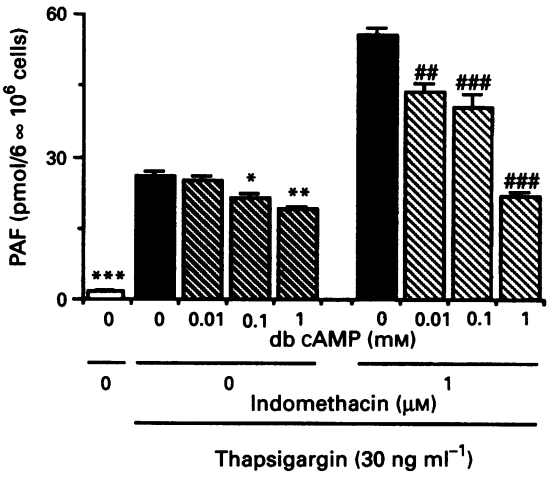

Figure 8 Effects of dibutyryl cyclic AMP on PAF accumulation in rat peritoneal macrophages stimulated by thapsigargin alone and by thapsigargin and indomethacin. Peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 10 min in 4 ml of the medium containing thapsigargin (30 ng ml^{-1}, 46.1 nM) with or without indomethacin (0.358 μg ml^{-1}, 1 μM) and the indicated concentrations of dibutyryl cyclic AMP (db cAMP). PAF contents of cells are shown. Values are the means from four samples with s.e.mean. Statistical significance: in the absence of indomethacin, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin control; in the presence of indomethacin, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. thapsigargin control. The results were confirmed by three separate experiments.

absence of indomethacin was inhibited by dibutyryl cyclic AMP in a concentration-dependent manner. The enhanced PAF accumulation in the presence of indomethacin was much more effectively inhibited by dibutyryl cyclic AMP than that stimulated by thapsigargin alone.

Discussion

Here, we present new information about the role of PGE_2 concurrently produced during thapsigargin-induced PAF accumulation in rat peritoneal macrophages. We reported (Ohuchi *et al.*, 1987; 1988) that thapsigargin at concentrations of 10–100 ng ml^{-1} (15.4–154 nM) stimulates the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages and PGE_2 production. In addition, we demonstrated that thapsigargin at a concentration of 10 ng ml^{-1} (15.4 nM) stimulates the accumulation of cell-associated PAF in rat peritoneal macrophages (Watanabe *et al.*, 1992). Later, Rodriguez *et al.* (1993) also reported that thapsigargin stimulates PAF production in human polymorphonuclear leucocytes. Because thapsigargin stimulates the accumulation of cell-associated PAF and extracellular PGE_2, and both are biologically active lipid mediators, we assumed that the PAF accumulation is regulated by the simultaneously produced PGE_2, or the converse. The results obtained by pharmacological modulation of PGE_2 production raised the idea that PGE_2 produced by thapsigargin plays a role in down-regulation of PAF accumulation. This notion was indicated by the following three observations. Firstly, thapsigargin-stimulated PAF accumulation was enhanced by inhibition of PGE_2 production induced by cyclo-oxygenase inhibitors such as indomethacin, naproxen, and ibuprofen (Figures 1 to 4). Furthermore, the cyclo-oxygenase inhibitor-induced increase in PAF accumulation was suppressed by exogenous PGE_2 (Figure 5). Secondly, the thapsigargin-induced PAF accumulation was also suppressed by exogenous PGE_2 (Figure 6), and thirdly, exogenous arachidonic acid also suppressed the thapsigargin-induced PAF accumulation, in accordance with the increase in PGE_2 production (Figure 7).

As to the effect of cyclo-oxygenase inhibitors on thapsigargin-induced PAF accumulation, a significant enhancement of PAF accumulation by indomethacin was observed only at 10 min (Figure 2). During the 10 min immediately after thapsigargin exposure, PAF accumulation rapidly increased. Thereafter, the levels of PAF in the cells decreased. Therefore, simultaneously produced PGE_2 may inhibit PAF accumulation only when the latter is rapidly increasing. At 20 min, although PGE_2 production continued to be inhibited by indomethacin, PAF accumulation was not stimulated significantly, and at 40 min, the PAF content of the cells decreased to the level of the thapsigargin-treated group. Consequently, the stimulant effect of the cyclo-oxygenase inhibitor on thapsigargin-induced PAF production is transient. However, it should be stressed that although this effect is transient, the increase in the PAF levels induced by the cyclo-oxygenase inhibitors may affect cellular functions in such a way that pharmacological effects other than the widely accepted inhibition of arachidonate metabolite production through the cyclo-oxygenase pathway, are expressed. It is possible that the transient increase in PAF production is a pharmacological characteristic of the cyclo-oxygenase inhibitors. Because no detectable amount of PAF was released into the conditioned medium of the thapsigargin-treated cells, it is suggested that the indomethacin-induced increase of PAF accumulation is not due to the inhibition of the release of PAF into the medium, but is a reflection of the enhanced production of PAF by indomethacin treatment.

The stimulation of PAF accumulation by cyclo-oxygenase inhibitors might be due to inhibition of PGE_2 production, because PGE_2 increases intracellular cyclic AMP levels (Gemsa *et al.*, 1975; Yamamoto & Suzuki, 1987), which inhibits PAF production (Fonteh *et al.*, 1993). The stimulated peritoneal macrophages produced arachidonate metabolites in the following order; PGE_2 > 6-keto-$PGF_{1\alpha}$ > $PGF_{2\alpha}$ > > thromboxane B_2 (data not shown). Therefore, PGI_2 might also participate in increasing the intracellular cyclic AMP level. When the macrophages were incubated in the medium containing dibutyryl cyclic AMP, PAF accumulation stimulated by thapsigargin alone or by thapsigargin and indomethacin was inhibited (Figure 8). PAF accumulation stimulated by thapsigargin and indomethacin was more effectively inhibited by dibutyryl cyclic AMP than that stimulated by thapsigargin alone. Without indomethacin, PGE_2 and PGI_2 are synthesized

by thapsigargin and they may increase intracellular cyclic AMP levels. Therefore, exogenous dibutyryl cyclic AMP might not be able to inhibit PAF accumulation as potently as when production of PGE_2 and PGI_2 are inhibited by indomethacin. Our preliminary experiments revealed that thapsigargin-induced PAF production was significantly inhibited by 10^{-5} M forskolin, an activator of adenylate cyclase (data not shown). This result also indicates that the increase in the intracellular cyclic AMP levels inhibits PAF accumulation.

If cyclo-oxygenase inhibitors directly inhibit the acetylhydrolase that metabolizes PAF to biologically inactive lyso-PAF, PAF levels in the cells should be increased. However, this might not be so because the addition of PGE_2 abrogated the stimulant effect of cyclo-oxygenase inhibitors on PAF accumulation. Further studies are necessary to clarify whether PGE_2 modulates acetylhydrolase activity in the cells.

Without thapsigargin, where PGE_2 production is not stimulated, the cyclo-oxygenase inhibitors at concentrations of 10^{-7} to 3×10^{-6} M did not increase the levels of PAF in the macrophages and in the conditioned medium (data not shown). These results also support our notion that simultaneously produced PGE_2 down-regulates PAF accumulation, because without thapsigargin, little PGE_2 is produced, and at the lower concentrations, PGE_2 could not down-regulate PAF production. Therefore, it is reasonable to conclude that without stimulation, the inhibition of PGE_2 production by cyclo-oxygenase inhibitors does not induce PAF accumulation.

An autoregulation loop is caused by simultaneously produced PGE_2 in the interferon-induced cytotoxic activity in mice macrophages (Schultz *et al.*, 1979), cyclic AMP accumulation by platelet-derived growth factor in 3T3 cells (Rozengurt *et al.*, 1983), and tumour necrosis factor-induced activation of peritoneal macrophages in mice (Lehmann *et al.*, 1988). The data presented here also indicate that PGE_2 is an essential element of an autoregulatory loop that controls PAF accumulation in rat peritoneal macrophages under thapsigargin stimulation. Preliminary experiments have revealed that during stimulation with 12-O-tetradecanoylphorbol 13-acetate (TPA), the cyclo-oxygenase inhibitors enhance PAF accumulation at 10 min in accordance with the inhibition of PGE_2 production (unpublished observations), indicating that PGE_2 controls PAF accumulation under TPA stimulation.

In conclusion, it is strongly suggested that the cyclo-oxygenase inhibitors enhance PAF accumulation by inhibiting PGE_2 production. Because the accumulation of PAF in the cells is the net reflection of synthesis and degradation of PAF, further investigations are necessary to clarify whether the cyclo-oxygenase inhibitors stimulate the synthesis or inhibit the degradation.

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