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Possible participation of intracellular platelet-activating factor in tumor necrosis factor-α production by rat peritoneal macrophages

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Abstract

Stimulation of rat peritoneal macrophages by thapsigargin (46.1 nM) increased levels of tumor necrosis factor-α and prostaglandin E_2 in the conditioned medium. Platelet-activating factor (PAF) was not detected in the conditioned medium, but the level of cell-associated PAF was increased transiently by thapsigargin. The PAF receptor antagonists such as E6123 ((*S*)-(+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno [3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine), L-652,731 (2,5-bis(3,4,5-trimethoxyphenyl) tetrahydrofuran) and CV-6209 (2-[*N*-acetyl-*N*-(2-methoxy-3-octadecyl-carbamoyloxy propoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride) inhibited thapsigargin-induced production of tumor necrosis factor-α. The cyclooxygenase inhibitor indomethacin inhibited prostaglandin E_2 production, and further enhanced thapsigargin-induced tumor necrosis factor-α production in parallel with further increase in cell-associated PAF production. The enhancement of tumor necrosis factor-α production induced by thapsigargin plus indomethacin was also inhibited by E6123, L-652,731 and CV-6209. However, exogenously added PAF up to 100 nM did not stimulate production of tumor necrosis factor-α. The level of tumor necrosis factor-α mRNA was increased by thapsigargin, but was lowered by the PAF receptor antagonist E6123, suggesting that the inhibition of tumor necrosis factor-α production by the PAF receptor antagonist is induced at the level of mRNA for tumor necrosis factor-α. These findings suggested that concurrently produced cell-associated PAF in thapsigargin-stimulated macrophages up-regulates production of tumor necrosis factor-α by acting as an intracellular signaling molecule and the PAF receptor antagonists might penetrate into the cells and antagonize the action of intracellular PAF.

Keywords: Platelet-activating factor; Tumor necrosis factor-α; Prostaglandin E_2; Thapsigargin; E6123; L-652,731; CV-6209; SK&F 98625; Indomethacin

1. Introduction

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Demopoulos et al., 1979; Prescott et al., 1990) has been implicated as a potent lipid mediator of inflammation and allergy. PAF is produced by various inflammatory cells such as macrophages (Drapier et al., 1983), neutrophils (Lynch et al., 1979) and vascular endothelial cells (Camussi et al., 1983; Prescott et al., 1984). In inflammatory cells, PAF is thought to be synthesized via the remodeling pathway (Albert and Snyder, 1983), in which phospholipase A_2, CoA-independent transacylase (Tessner et al., 1990), and acetyl-CoA:lyso-PAF acetyltransferase (Ninio et al., 1982) are involved. When PAF is produced via the remodeling pathway, arachidonic acid is released and metabolized to prostaglandins and leukotrienes by cyclooxygenase and lipoxygenase, respectively.

We previously reported that in rat peritoneal macrophages, stimulation by the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin (Thastrup et al., 1987) or the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Nishizuka, 1992) enhances the release of arachidonic acid from membrane phospholipids and the production of prostaglandin E_2 (Ohuchi et al., 1987, 1988) and cell-associated PAF (Watanabe et al., 1992). Furthermore, we reported (Watanabe et al., 1995; Yamada et al., 1996) that the simultaneously produced prostaglandin E_2 down-regulates the production of cell-associated PAF, and that the cyclooxygenase inhibitors such as indomethacin, naproxen and ibuprofen further enhances cell-associated PAF production by inhibiting prostaglandin E_2 production in thapsigargin- or TPA-stimulated macrophages. How-

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ever, in such conditions, levels of PAF in the conditioned medium were below the limits of detection (< 1.0 pmol/ml) (Watanabe et al., 1995; Yamada et al., 1996). Therefore, in rat peritoneal macrophages, it was suggested that PAF produced by thapsigargin or TPA remains inside the cells as a cell-associated form and is not excreted outside the cells. It is well-known that exogenously added PAF exerts its pharmacological effects through specific cell surface receptors (Bito et al., 1994), but functions of cell-associated PAF have not been clarified.

Tumor necrosis factor-α is produced by monocytes and macrophages and contributes to several inflammatory and autoimmune diseases. It is reported that the gene regulation and protein secretion of tumor necrosis factor-α are under the control of several inflammatory mediators, among which prostaglandin E_2 has attracted particular attention. For example, the level of tumor necrosis factor-α mRNA and the secretion of tumor necrosis factor-α in stimulated macrophages and monocytes were lowered by the addition of prostaglandin E_2. In addition, inhibition of prostaglandin E_2 production by the cyclooxygenase inhibitor indomethacin enhances the induction of tumor necrosis factor-α production (Hart et al., 1995; Kunkel et al., 1995; Spatafora et al., 1995). From these reports and our previous findings (Watanabe et al., 1995; Yamada et al., 1996), we hypothesized that the production of tumor necrosis factor-α in stimulated macrophages is up-regulated by the concurrently produced cell-associated PAF. The present study was intended to clarify roles of cell-associated PAF in the regulation of tumor necrosis factor-α production, using the PAF receptor antagonists, the CoA-independent transacylase inhibitor, and the cyclooxygenase inhibitor.

2. Materials and methods

2.1. Preparation of rat peritoneal macrophages

Male Sprague–Dawley strain rats, specific pathogen-free, and weighing 300–350 g (Charles River Japan, Kanagawa, Japan) were used. The rats were treated in accordance with procedures approved by the Animal Ethics Committee in the Faculty of Pharmaceutical Sciences, Tohoku University, Japan. Rat peritoneal macrophages were harvested 4 days after intraperitoneal injection of a solution of soluble starch (Wako, Osaka, Japan) and bacto peptone (Difco Laboratories, Detroit, MI, USA), 5% (w/v) each at a dose of 5 ml per 100 g body weight (Ohuchi et al., 1985).

2.2. Macrophage culture

The peritoneal cells were suspended in Eagle's minimal essential medium (Nissui, Tokyo, Japan) containing 10% (v/v) calf serum (Flow Laboratories, North Ryde, N.S.W., Australia), penicillin G potassium (Meiji Seika, Tokyo, Japan) (18 μg/ml) and streptomycin sulfate (Meiji Seika) (50 μg/ml) at a density of 1.5×10^6 cells per ml of the medium. A total of 4 ml of the cell suspension was poured into each 60 mm plastic tissue culture dish and the dishes were incubated for 2 h at 37°C. The cells were then washed three times with medium to remove non-adherent cells (Ohuchi et al., 1985). The adherent cells were incubated for 20 h at 37°C in 4 ml of medium containing 10% (v/v) calf serum, washed three times with medium containing no calf serum and used for the following experiments.

2.3. Incubation of macrophages with drugs

After three washes with medium containing no calf serum, the cells were incubated for the periods indicated at 37°C in 4 ml of medium containing no calf serum in the presence or absence of drugs. When the effects of exogenously added PAF were determined, the cells were incubated in medium containing 0.25% (w/v) bovine serum albumin (essentially fatty acid-free, Sigma, St. Louis, MO, USA). Drugs used were the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin (LC Services, Woburn, MA, USA), the protein kinase C activator TPA, the cyclooxygenase inhibitor indomethacin, prostaglandin E_2, PAF (a mixture of C_{16} and C_{18} forms, 1:1, w/w) (Sigma), the CoA-independent transacylase inhibitor SK&F 98625 (diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate) (Chilton et al., 1995), the PAF antagonists E6123 ((*S*)-(+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]-thieno [3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine) (Tsunoda et al., 1990), L-652,731 (2,5-bis(3,4,5-trimethoxyphenyl tetrahydrofuran) (Hwang et al., 1985), and CV6209 (2-[*N*-acetyl-*N*-(2-methoxy-3-octadecyl-carbamoyloxy propoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride) (Terashita et al., 1987). Drugs were dissolved in ethanol. An aliquot of each solution was added to medium, and the final concentration of the vehicle in medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle. After incubation, the conditioned medium was collected to measure the concentration of tumor necrosis factor-α, PAF and prostaglandin E_2. Contents of PAF in the cells were also determined.

2.4. Viability assay

The viability of the cells was examined in each set of experiments by a procedure using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) (Mosmann, 1983; Tada et al., 1986), which is based on the ability of mitochondrial succinate dehydrogenase to cleave MTT to the blue compound formazan. The cells were incubated for the periods indicated in 4 ml of medium containing drugs. Then, 400 μl of MTT solution in phos-

phate-buffered saline (5 mg/ml, pH 7.4) was added to each dish, and cells were further incubated for 4 h at 37°C. After 1 ml of 0.04 N HCl solution in isopropanol was added, the cells were sonicated using a Handy Sonic Disrupter (UR-20P, Tomy, Tokyo, Japan) at 10% maximum power for 3 s and the resultant colored product was read on a Microplate Reader (Bio-Rad, Richmond, CA, USA) at 570 nm. Treatment with drugs at concentrations described in this paper showed no significant changes in cell viability.

2.5. Measurement of the concentration of tumor necrosis factor-α and prostaglandin E_2

The conditioned medium was centrifuged at 1500 × *g* and 4°C for 5 min. Tumor necrosis factor-α concentrations in the supernatant fraction were immunoassayed using a commercially available kit (Cytoscreen Rat Tumor Necrosis Factor-α ELISA Kit, Biosource Int., Camarillo, CA, USA). The procedure for immunoassay is described in the instruction manual accompanying the kit. Prostaglandin E_2 concentrations in the supernatant fraction were radioimmunoassayed (Ohuchi et al., 1985). Prostaglandin E_2 antiserum was purchased from PerSeptive Diagnostics, Cambridge, MA, USA.

2.6. Measurement of the content of PAF

After partial purification of PAF from the total lipids fraction of the cells or from the conditioned medium using an immunoaffinity mini-column (Watanabe et al., 1992), PAF contents were measured using a commercially available kit (Platelet Activating Factor [^{125}I] RIA Kit, DuPont NEN Research Products, Boston, MA, USA) as described previously (Watanabe et al., 1992).

2.7. Semiquantitation of tumor necrosis factor-α mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Peritoneal macrophages were incubated for 4 h at 37°C in 4 ml of medium containing drugs. After incubation, total RNA was prepared from each sample by acid guanidinium–phenol–chloroform extraction (Chomczynski and Sacchi, 1987), and the yield of RNA extracted was determined by spectrophotometry. A total of 1 μg of RNA from each sample was reverse transcribed at 37°C for 1 h in 20 μl of the buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) containing 5 μM of random hexamer oligonucleotides (Gibco, Gaithersburg, MD, USA), 200 U of the reverse transcriptase from moloney murine leukemia virus (Gibco), 0.5 mM deoxyribonucleotide triphosphates (dNTP, Pharmacia Biotech, Uppsala, Sweden) and 10 mM dithiothreitol. The third and the fourth exons of rat tumor necrosis factor-α gene were amplified by polymerase chain reaction (PCR), using specific primers described by Noiri et al. (1994); (sense) 5′-GGATCATCTTCTCAAAACT-CG-3′ and (antisense) 5′-TCACAGGAGCAATGACTC-CAAA-3′, which amplify a 419 base pair (bp) tumor necrosis factor-α fragment. PCR was performed for 30 cycles in 50 μl of the PCR buffer (2.5 mM Tris–HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$) containing 5 μM of the reverse transcribed RNA solution, 0.25 μM of each primer, 170 μM dNTP and 1.25 U Taq polymerase (Takara Shuzo, Shiga, Japan) using a thermal cycler (GeneAmp™ PCR System 2400, Perkin Elmer Cetus, Norwalk, CT, USA). Each cycle consisted of 1 min denaturation at 95°C, 2 min annealing at 58°C, and 2 min extension at 72°C.

The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (a housekeeping gene) was used as an internal standard gene. Because the internal standard gene was also amplified, relative levels of tumor necrosis factor-α mRNA were quantified. PCR primers for rat GAPDH were described by Robbins and McKinney (1992); primers used were (sense) 5′-TGATGACATCAAGAAGGTGGT-GAAG-3′, and (antisense) 5′-TCCTTGGAGGCCATGT-AGGCC-3′, which amplify a 240 bp GAPDH fragment. PCR was performed for 27 cycles; 30 s denaturation at 94°C, 1 min annealing at 57°C, and 2 min extension at 72°C. Other conditions were the same as for tumor necrosis factor-α. After PCR, 10 μl of the reaction mixture was loaded onto a 1.5% agarose mini-gel, and the PCR products were visualized by ethidium bromide staining after electrophoresis. The levels of mRNA for tumor necrosis factor-α and GAPDH were quantified by scanning densitometry, and the ratio of the tumor necrosis factor-α mRNA density vs. the GAPDH mRNA density in each point was calculated.

2.8. Statistical significance

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

3. Results

3.1. Effects of thapsigargin on tumor necrosis factor-α production

Time-course changes of tumor necrosis factor-α production by rat peritoneal macrophages in the presence or absence of thapsigargin (30 ng/ml, 46.1 nM) were examined. In the absence of thapsigargin, tumor necrosis factor-α concentrations in the conditioned medium were very low at 2 to 20 h, while in thapsigargin-stimulated macrophages, tumor necrosis factor-α concentrations in the conditioned medium were increased time-dependently with a significant increase observed at 4, 8, and 20 h (Fig. 1). When examined at 8 h, production of tumor necrosis factor-α

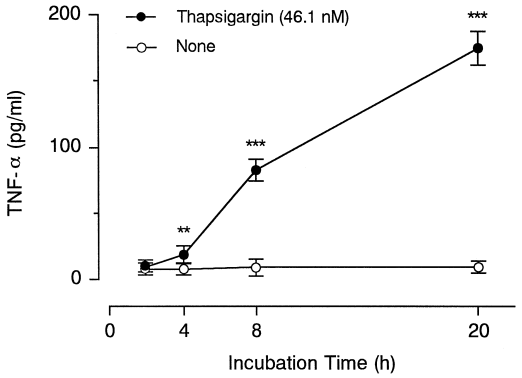

Fig. 1. Time-course changes of the effect of thapsigargin on tumor necrosis factor-α production. Rat peritoneal macrophages (6×10^6 cells) were incubated for the periods indicated at 37°C in 4 ml of medium containing no calf serum in the presence or absence of thapsigargin (30 ng/ml, 46.1 nM). Tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium are shown. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding control. The experiment was repeated twice with similar results.

was increased dose-dependently by thapsigargin at concentrations of 4.61 to 46 l.1 nM (data not shown). Therefore, in the following experiments, macrophages were incubated for 8 h in medium containing 46.1 nM of thapsigargin in the presence or absence of drugs, and determined tumor necrosis factor-α concentrations in the conditioned medium.

3.2. Effects of thapsigargin on the production of PAF and prostaglandin E_2

Effects of thapsigargin (46.1 nM) on the production of PAF and prostaglandin E_2 were examined. Thapsigargin

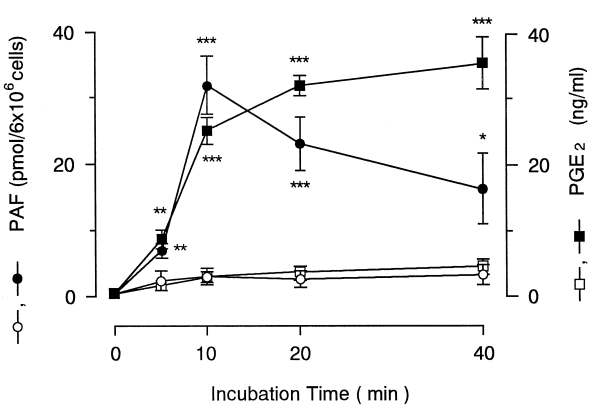

Fig. 2. Time-course changes of the effect of thapsigargin on production of PAF and prostaglandin E_2. Rat peritoneal macrophages (6×10^6 cells) were incubated for the periods indicated at 37°C in 4 ml of medium containing no calf serum in the presence (closed symbols) or absence (open symbols) of thapsigargin (30 ng/ml, 46.1 nM). Contents of cell-associated PAF (●, ○), and prostaglandin E_2 (PGE_2) concentrations in the conditioned medium (■, □) are shown. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding control. The experiment was repeated three times with similar results.

increased the accumulation of cell-associated PAF, but the effect was transient; the levels reached a maximum 10 min after incubation, and declined gradually till 40 min (Fig. 2). Without thapsigargin treatment, the level of cell-associated PAF at 10 min was very low (1.0 ± 0.1 pmol/6×10^6 cells, means ± S.E.M. from four samples). The level of PAF in the conditioned medium at 5 to 40 min was below the limits of detection (< 1.0 pmol/ml) in both thapsigargin-stimulated and non-stimulated macrophages. Prostaglandin E_2 concentrations in the conditioned medium of

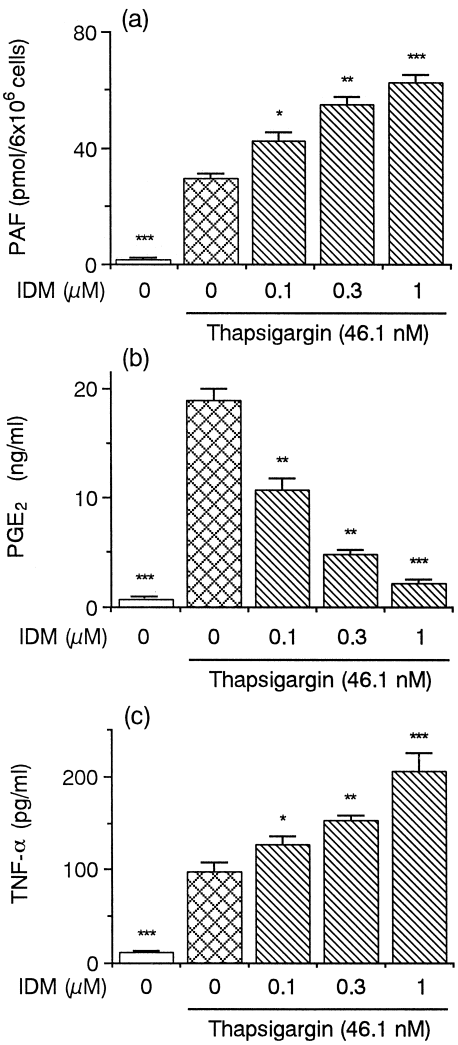

Fig. 3. Effects of various concentrations of indomethacin on thapsigargin-induced production of PAF, prostaglandin E_2 and tumor necrosis factor-α. Rat peritoneal macrophages (6×10^6 cells) were incubated for 10 min at 37°C in 4 ml of medium containing no calf serum in the presence of thapsigargin (30 ng/ml, 46.1 nM) and the indicated concentrations of indomethacin (IDM). Contents of cell-associated PAF (a) and concentrations of prostaglandin E_2 (PGE_2) in the conditioned medium (b) were determined. Another set of macrophages was incubated for 8 h at 37°C, and tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium (c) were determined. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin alone. The experiment was repeated three times with similar results.

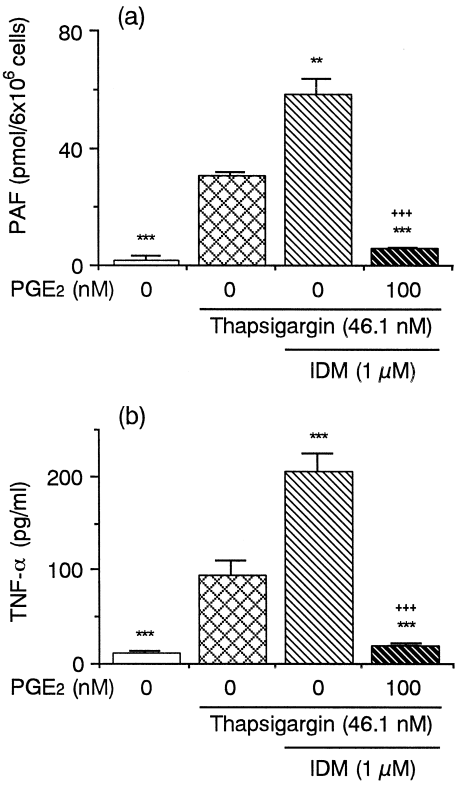

Fig. 4. Effects of indomethacin on thapsigargin-induced production of PAF and tumor necrosis factor-α. Rat peritoneal macrophages (6×10^6 cells) were incubated for 10 min at 37°C in 4 ml of medium containing no calf serum in the presence or absence of thapsigargin (30 ng/ml, 46.1 nM), indomethacin (IDM) (1 μM) and prostaglandin E_2 (PGE_2) (100 nM), and contents of cell-associated PAF were determined (a). Another set of macrophages were incubated for 8 h at 37°C, and tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium were determined (b). Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin alone; $^{+++}P < 0.001$ vs. corresponding control. The experiment was repeated three times with similar results.

macrophages were also increased by thapsigargin treatment, and the increase was time dependent (Fig. 2). In the following experiments, the level of cell-associated PAF and prostaglandin E_2 concentrations in the conditioned medium were determined 10 min after incubation.

3.3. Effects of indomethacin on thapsigargin-induced tumor necrosis factor-α production

To clarify roles of concurrently produced prostaglandin E_2 in thapsigargin-induced tumor necrosis factor-α production, the effects of the cyclooxygenase inhibitor indomethacin were examined. Treatment with indomethacin (0.1–1 μM) inhibited thapsigargin (46.1 nM)-induced prostaglandin E_2 production at 10 min (Fig. 3b), but further enhanced thapsigargin-induced production of cell-associated PAF at 10 min (Fig. 3a) and tumor necrosis factor-α at 8 h (Fig. 3c) in a concentration-dependent manner. In the absence of thapsigargin, indomethacin did not increase tumor necrosis factor-α production at concentrations of 0.1 to 1 μM (data not shown). Whereas, the addition of prostaglandin E_2 (100 nM) inhibited the indomethacin (1 μM) plus thapsigargin (46.1 nM)-induced tumor necrosis factor-α production at 8 h (Fig. 4b) in parallel with the inhibition of cell-associated PAF production at 10 min (Fig. 4a). These findings seemed to suggest that tumor necrosis factor-α production in thapsigargin-stimulated macrophages is down-regulated by the concurrently produced prostaglandin E_2, but is up-regulated by the concurrently produced cell-associated PAF.

3.4. Effects of the PAF receptor antagonists on thapsigargin-induced and thapsigargin plus indomethacin-induced tumor necrosis factor-α production

To clarify roles of concurrently produced cell-associated PAF in tumor necrosis factor-α production, the ef-

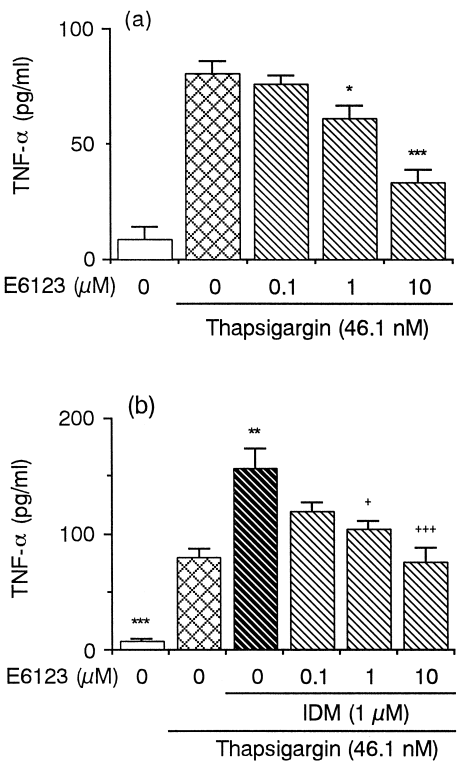

Fig. 5. Effects of various concentrations of E6123 on thapsigargin- and thapsigargin plus indomethacin-induced tumor necrosis factor-α production. Rat peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 8 h in 4 ml of medium containing no calf serum in the presence of thapsigargin (30 ng/ml, 46.1 nM), indomethacin (IDM) (1 μM) and the indicated concentrations of E6123. Tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium at 8 h are shown. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin alone (a and b), and $^{+}P < 0.05$, $^{+++}P < 0.001$ vs. thapsigargin plus IDM (b). The experiment was repeated twice with similar results.

fects of several PAF receptor antagonists were examined. It was demonstrated that tumor necrosis factor-α production induced by thapsigargin (46.1 nM) at 8 h was partially inhibited by E6123 at 10 μM (Fig. 5a), L-652,731 at 1 and 10 μM (Fig. 6a), and CV-6209 at 1 μM (Fig. 7a). The tumor necrosis factor-α production at 8 h induced by thapsigargin (46.1 nM) plus indomethacin (1 μM) was also inhibited partially by E6123 at 10 μM (Fig. 5b), L-652,731 at 1 and 10 μM (Fig. 6b), and CV-6209 at 1 μM (Fig. 7b). These findings suggested that concurrently produced PAF partially participates in thapsigargin-induced tumor necrosis factor-α production.

3.5. Effects of exogenously added PAF on tumor necrosis factor-α production

To obtain further insight into the mechanism of tumor necrosis factor-α production by concurrently produced cell-associated PAF, the effects of exogenously added PAF on tumor necrosis factor-α production in the presence or absence of thapsigargin were examined. Incubation of macrophages for 8 h in medium containing 0.1 to 10 nM of PAF did not stimulate tumor necrosis factor-α production (data not shown). PAF at a concentration of 100 nM also did not stimulate tumor necrosis factor-α production (Fig. 8). Furthermore, in the presence of thapsigargin (46.1 nM), exogenous PAF (100 nM) did not enhance thapsigargin-induced tumor necrosis factor-α production (Fig. 8). In addition, in the presence of thapsigargin (46.1 nM) and SK&F 98625 (10 μM) where PAF production was inhibited (Fig. 8, inset), exogenously added PAF (100 nM) did not restore the SK&F 98625-induced suppression of tumor necrosis factor-α production at 8 h (Fig. 8). These findings suggested that cell-associated PAF produced by thapsigargin treatment does not act to produce tumor necrosis factor-α by an autocrine mechanism. Cell-associated PAF might play significant roles as an intracellular signaling molecule in tumor necrosis factor-α production.

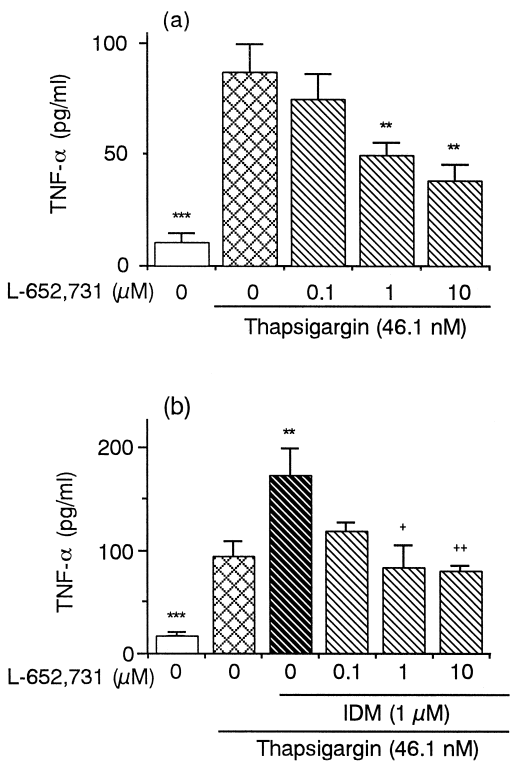

Fig. 6. Effects of various concentrations of L-652,731 on thapsigargin-induced tumor necrosis factor-α production. Rat peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 8 h in 4 ml of medium containing no calf serum in the presence of thapsigargin (30 ng/ml, 46.1 nM), indomethacin (IDM) (1 μM) and the indicated concentrations of L-652,731. Tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium at 8 h are shown. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin alone (a and b), and $^{+}P < 0.05$, $^{++}P < 0.01$ vs. thapsigargin plus IDM (b). The experiment was repeated twice with similar results.

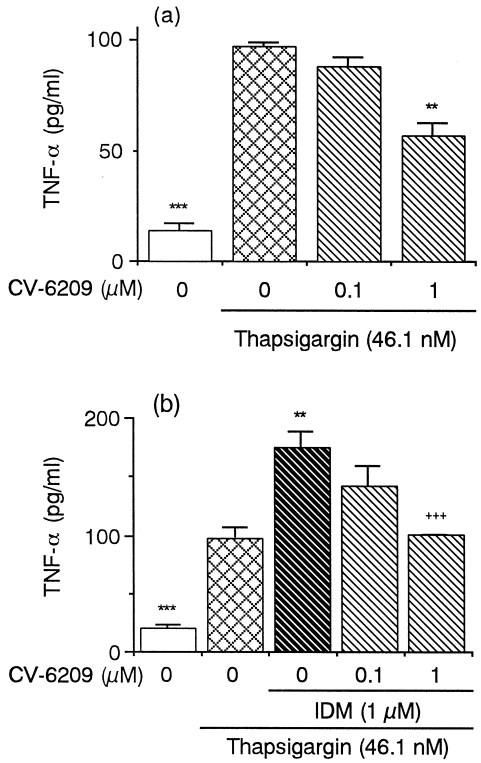

Fig. 7. Effects of various concentrations of CV-6209 on thapsigargin-induced tumor necrosis factor-α production. Rat peritoneal macrophages (6×10^6 cells) were incubated at 37°C for 8 h in 4 ml of medium containing no calf serum in the presence of thapsigargin (30 ng/ml, 46.1 nM), indomethacin (IDM) (1 μM) and the indicated concentrations of CV-6209. Tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium at 8 h are shown. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. thapsigargin alone (a and b), $^{+++}P < 0.001$ vs. thapsigargin plus IDM (b). The experiment was repeated twice with similar results.

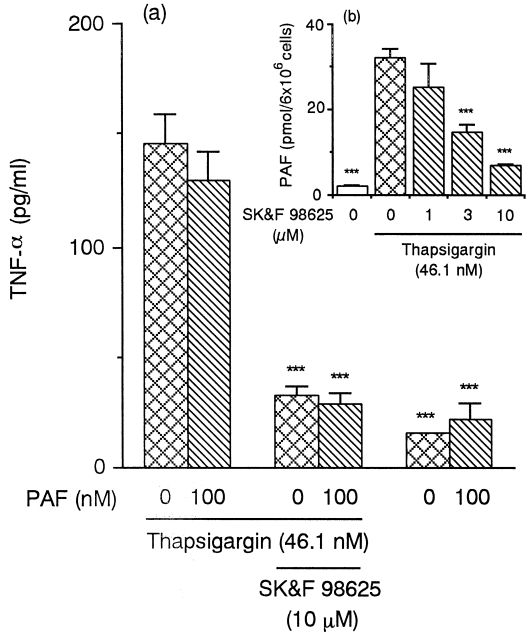

Fig. 8. Effects of exogenously added PAF on tumor necrosis factor-α production. Rat peritoneal macrophages (6×10^6 cells) were incubated for 8 h at 37°C in 4 ml of medium containing 0.25% (w/v) bovine serum albumin in the presence or absence of thapsigargin (30 ng/ml, 46.1 nM), SK&F 98625 (10 μM) and PAF (100 nM). Tumor necrosis factor-α (TNF-α) concentrations in the conditioned medium at 8 h were determined. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{***}P < 0.001$ vs. corresponding thapsigargin control. The experiment was repeated twice with similar results. Inset: Effects of various concentrations of SK&F 98625 on thapsigargin (46.1 nM)-induced production of cell-associated PAF. Levels of cell-associated PAF were determined as described in the legend to Fig. 3. Values are the means from four samples with S.E.M. shown by vertical bars. Statistical significance: $^{***}P < 0.001$ vs. thapsigargin alone.

3.6. Effects of E6123, indomethacin and SK&F 98625 on the level of tumor necrosis factor-α mRNA in thapsigargin-stimulated macrophages

Incubation of macrophages in medium containing thapsigargin (46.1 nM) increased the accumulation of mRNA for tumor necrosis factor-α at 4 h, but did not affect the level of GAPDH mRNA (Fig. 9a and b). The thapsigargin-induced increase in the level of tumor necrosis factor-α mRNA at 4 h was lowered by 3 μM of SK&F 98625 (Fig. 9a); at which concentration, SK&F 98625 suppressed the production of PAF and tumor necrosis factor-α (Fig. 8). In contrast, thapsigargin-induced increase in the level of tumor necrosis factor-α mRNA was further increased by indomethacin (1 μM) (Fig. 9a). In addition, treatment with the PAF receptor antagonist E6123 (10 μM) suppressed both the thapsigargin-induced and the thapsigargin plus indomethacin-induced increase in the level of tumor necrosis factor-α mRNA at 4 h, but the level of GAPDH mRNA was not affected by treatment with thapsigargin, SK&F 98625, indomethacin and E6123 (Fig. 9a and b). These findings suggested that the concurrently produced cell-associated PAF up-regulates the level of tumor necrosis factor-α mRNA in thapsigargin-stimulated macrophages. It was also suggested that the inhibition by SK&F 98625 or by the PAF receptor antagonist E6123 of tumor necrosis factor-α production is induced by lowering the level of mRNA for tumor necrosis factor-α. The same effects were observed when other PAF receptor antagonists L-652,731 and CV-6209 each at 10 μM were used (data not shown).

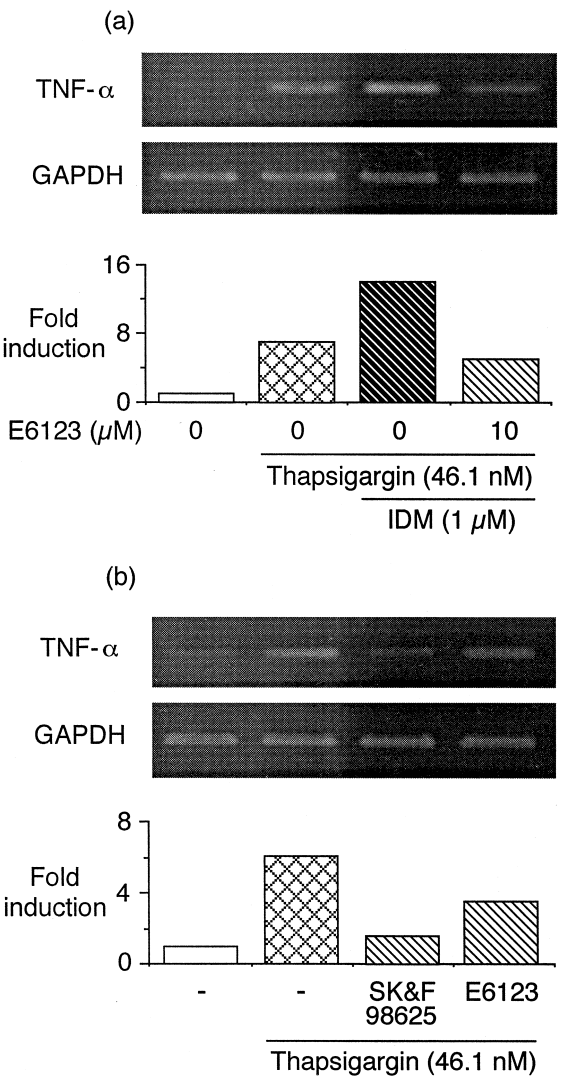

Fig. 9. Effects of indomethacin, SK&F 98625 and E6123 on the levels of mRNA of tumor necrosis factor-α and GAPDH in thapsigargin-stimulated macrophages. Rat peritoneal macrophages (6×10^6 cells) were incubated for 4 h at 37°C in 4 ml of medium containing no calf serum in the presence or absence of thapsigargin (30 ng/ml, 46.1 nM), indomethacin (IDM) (1 μM), SK&F 98625 (3 μM) and E6123 (10 μM). Total RNA was extracted and RT-PCR for tumor necrosis factor-α (TNF-α) mRNA and GAPDH mRNA was performed as described in Section 2. The ratio of TNF-α mRNA density to GAPDH mRNA density is shown in the lower panel. The ratio in the non-treated group is set to 1.0. The experiment was repeated twice with similar results.

4. Discussion

Stimulation of rat peritoneal macrophages by thapsigargin induced production of cell-associated PAF (presumably cytosolic PAF and membrane-bound PAF), prostaglandin E_2 and tumor necrosis factor-α (Figs. 1 and 2). Although production of cell-associated PAF was transient, it was possible to speculate that concurrently produced cell-associated PAF and prostaglandin E_2 play significant role in tumor necrosis factor-α production in thapsigargin-stimulated macrophages. As to the participation of prostaglandin E_2 in tumor necrosis factor-α production, it is reported that the enhancement by indomethacin of tumor necrosis factor-α production in macrophages and monocytes is due to the inhibition of prostaglandin E_2 production (Hart et al., 1995; Kunkel et al., 1995; Spatafora et al., 1995). The present study also demonstrated that the inhibition of prostaglandin E_2 production by indomethacin increases tumor necrosis factor-α production in thapsigargin-stimulated macrophages (Figs. 5 and 6).

We previously reported (Watanabe et al., 1995; Yamada et al., 1996) that the concurrently produced prostaglandin E_2 down-regulates the thapsigargin- or TPA-induced production of cell-associated PAF and that the cyclooxygenase inhibitors such as indomethacin, ibuprofen and naproxen further enhance cell-associated PAF production by inhibiting prostaglandin E_2 production. In these conditions, PAF produced by macrophages was not released from the cells, but remained to be cell-associated PAF because the levels of PAF in the conditioned medium were less than the limits of detection (< 1.0 pmol/ml) (Watanabe et al., 1995; Yamada et al., 1996). Ammit and O'Neill (1997) described that the failure to detect PAF released from endothelial cells is due to the method of PAF extraction from the conditioned medium. In the presence of albumin in the medium, the released PAF is bound to albumin in a protected form, thus, becomes unextractable PAF by organic solvent. However, in the present study, we did not use the medium containing albumin when the cells were stimulated by thapsigargin. Therefore, it was strongly suggested that PAF is not released into the medium by thapsigargin treatment, and PAF synthesized by thapsigargin treatment remained associated with the cells. Taken together, we hypothesized that concurrently produced cell-associated PAF directly up-regulates production of tumor necrosis factor-α in thapsigargin-stimulated macrophages. This notion was supported by the following three findings obtained in the present study. Firstly, SK&F 98625, a CoA-independent transacylase inhibitor, inhibited thapsigargin-induced production of cell-associated PAF and tumor necrosis factor-α production (Fig. 8 and Yamada et al., 1998). Secondly, tumor necrosis factor-α production induced by thapsigargin, or by thapsigargin plus indomethacin was inhibited by treatment with the PAF receptor antagonists such as E6123 (an analog of triazolobenzodiazepine), L-652,731 (an analog of tetrahydrofuran), or CV-6209 (an analog of phospholipid) (Figs. 5–7). And thirdly, exogenously added PAF did not stimulate tumor necrosis factor-α production (Fig. 8). Because exogenously added PAF (100 nM) stimulated prostaglandin E_2 production in rat peritoneal macrophages (data not shown), no effect by exogenously added PAF on tumor necrosis factor-α production is not due to an inactive PAF preparation.

As to the role of cell-associated PAF, Stewart et al. (1989) speculated that PAF acts as an intracellular messenger for eicosanoid production in guinea-pig peritoneal macrophages. Müller and Nigam (1992) also suggested that PAF produced in human neutrophils reacts with intracellular PAF binding sites to activate phospholipase A_2. In addition, distinct PAF binding sites in plasma membranes and in microsomal membranes were identified in rat cerebral cortex (Marcheselli et al., 1990). Therefore, in thapsigargin-stimulated macrophages, cell-associated PAF might bind to intracellular binding sites and induce signal transduction to stimulate production of tumor necrosis factor-α. In this respect, we are studying to clarify mechanisms of action of cell-associated PAF.

As to the action of exogenously added PAF, it is reported that the binding of exogenous PAF to its cell surface receptor stimulates gene expression of c-*fos* and c-*jun* in rabbit corneal epithelial cells (Bazan et al., 1993), and induces activation of nuclear factor κB (NF-κB) in stably transfected Chinese hamster ovary cells expressing PAF receptors (Kravchenko et al., 1995). Tumor necrosis factor-α gene includes sequences similar to the NF-κB enhancers, the cyclic AMP-responsive element, and the c-Jun/AP-1 binding site (Economou et al., 1989; Kruys et al., 1992; Newell et al., 1994). Therefore, it is likely that exogenous PAF induces gene expression of tumor necrosis factor-α. However, in the present study, exogenously added PAF did not induce tumor necrosis factor-α production at concentrations up to 100 nM in non-stimulated macrophages and thapsigargin-stimulated macrophages (Fig. 8). Furthermore, the addition of PAF did not restore the SK&F 98625-induced suppression of tumor necrosis factor-α production (Fig. 8). Because macrophages were incubated in a medium containing no calf serum, it is hardly possible that exogenously added PAF is metabolized to lyso-form of PAF, a biologically inactive form. Therefore, it was strongly suggested that concurrently produced cell-associated PAF does not act by an autocrine mechanism. Cell-associated PAF might act as an intracellular signaling molecule for the induction of tumor necrosis factor-α production.

As shown in Figs. 5–7, PAF receptor antagonists inhibited thapsigargin- and thapsigargin plus indomethacin-induced tumor necrosis factor-α production. But the concentrations of these antagonists required for the inhibition of tumor necrosis factor-α production (1 to 10 μM) were higher than those required for the inhibition against exogenous PAF (Watanabe et al., 1994). Furthermore, the order

of potency of these PAF receptor antagonists to inhibit the pharmacological activity of exogenous PAF is as follows: E6123 > L-652,731 ≫ CV-6209. However, as shown in Figs. 5–7, inhibition by CV-6209 of tumor necrosis factor-α production was induced at almost the same concentration ranges of other PAF receptor antagonists. The difference of the order of potency might be due to the difference of penetration of the cell membrane by each antagonist. These findings suggested the possibility that the target of these PAF receptor antagonists for the inhibition of tumor necrosis factor-α production is not the cell surface receptor of PAF.

The CoA-independent transacylase inhibitor SK&F 98625 inhibited production of PAF (Fig. 8) and prostaglandin E_2 in thapsigargin-stimulated macrophages (Yamada et al., 1998), as reported by Winkler et al. (1995) in A23187-stimulated human neutrophils. But we found that SK&F 98625 inhibits thapsigargin-induced tumor necrosis factor-α production, and suggested that thapsigargin-induced tumor necrosis factor-α production is up-regulated by concurrently produced cell-associated PAF. Thapsigargin-stimulated rat peritoneal macrophages produce arachidonate metabolites in the following order: prostaglandin E_2 > 6-keto-prostaglandin $F_{1\alpha}$ (a stable metabolite of prostaglandin I_2) > prostaglandin $F_{2\alpha}$ ≫ thromboxane B_2 (a stable metabolite of thromboxane A_2) (unpublished observations). Recently, Caughey et al. (1997) reported that thromboxane A_2 stimulates tumor necrosis factor-α production by human monocytes. Therefore, it is possible that SK&F 98625 inhibits tumor necrosis factor-α production by inhibiting thromboxane A_2 production, because the inhibition by SK&F 98625 of tumor necrosis factor-α production (Fig. 8 and Yamada et al., 1998) was much more prominent than that of the PAF receptor antagonists (Figs. 5–7). However, in our assay system, because indomethacin (1 μM) did not affect the inhibitory effect of SK&F 98625 (10 μM) on thapsigargin (46.1 nM)-induced TNF-α production at 8 h, participation of TXA_2 in TNF-α production was suggested to be very low.

The increase in tumor necrosis factor-α production by indomethacin paralleled the increase in the levels of cell-associated PAF (Fig. 3). The inhibition of cyclooxygenase by indomethacin might lead to the increased production of leukotrienes. Therefore, the possibility remains that the enhancement of tumor necrosis factor-α production by indomethacin is mediated not only by cell-associated PAF but also by certain lipoxygenase products.

The thapsigargin-induced increase in the levels of tumor necrosis factor-α mRNA was lowered by the PAF receptor antagonist E6123 (Fig. 9). Therefore, the suppression of thapsigargin-induced tumor necrosis factor-α production by E6123 might be induced at mRNA levels. Usually, cytokine mRNA is accumulated after stimulation by stabilization of the message linked to Shaw–Kamen sequences in the 3′-untranslated sequence (Jacob and Tashman, 1993). At present, it is unclear whether the cell-associated PAF stimulates tumor necrosis factor-α production at the transcriptional levels or at the post-transcriptional level. Further investigations are necessary to clarify the mechanism of up-regulation by cell-associated PAF in thapsigargin-stimulated production of tumor necrosis factor-α. It is also necessary to clarify whether cell-associated PAF produced by physiological stimuli also plays a significant role in tumor promoter-α production.

Finally, our findings suggest that concurrently produced cell-associated PAF up-regulates thapsigargin-induced tumor necrosis factor-α production. It does not act by an autocrine mechanism, but may act as an intracellular signaling molecule for tumor necrosis factor-α production. The PAF receptor antagonist might penetrate into the cells, thus compete with concurrently produced cell-associated PAF to inhibit thapsigargin-induced tumor necrosis factor-α production.

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