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Tumor necrosis factor-α-induced release of plasminogen activator inhibitor-1 from human umbilical vein endothelial cells: involvement of intracellular ceramide signaling event

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Abstract

We have investigated the biochemical mechanism of tumor necrosis factor (TNF)-α-induced release of plasminogen activator inhibitor-1 (PAI-1) from human umbilical vein endothelial cells (HUVEC). Treatment of HUVEC with TNF-α for 3 h resulted in a 2.8-fold increase in the PAI-1 release compared with control. The increase in PAI-1 release was accompanied by a 133% increase in the intracellular acidic sphingomyelinase (SMase) activity. High-performance liquid chromatographic (HPLC) analysis revealed that the intracellular ceramide levels increased to 126% of the control ($P < 0.05$), but the contents of membranous ceramide remained unaltered. We have previously shown that a cell-permeable ceramide analog, *N*-acetylsphingosine (C_2-ceramide) enhances the PAI-1 release from HUVEC. Here, *N*-acetylsphinganine (C_2-dihydroceramide) was found to specifically suppress both C_2-ceramide- and TNF-α-induced increase in PAI-1 release from HUVEC without affecting the control PAI-1 release. Treatment of HUVEC with staphylococcal SMase that may mimic the activation of the membranous neutral SMase also increased the PAI-1 release. The increase in PAI-1 release by this mechanism was suppressed by a cyclooxygenase inhibitor, aspirin, whereas the inhibitor did not affect TNF-α-induced increase in PAI-1 release. Taken together, these findings suggest that TNF-α prominently utilizes the lysosomal acidic SMase-ceramide signaling pathway in the induction of PAI-1 release from HUVEC.

Keywords: Tumor necrosis factor-α; Plasminogen activator inhibitor-1; Sphingomyelinase; Ceramide; Vascular endothelial cell

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) plays an important role in the regulation of fibrinolysis by inactivating rapidly both tissue-type and urokinase-type plasminogen activators [1,2]. Suppression of fibrinolysis through the elevated levels of plasma PAI-1 has been frequently found in coronary artery dis-

Abbreviations: HUVEC, human umbilical vein endothelial cells; SMase, sphingomyelinase; PAI-1, plasminogen activator inhibitor-1; C_2-dihydroceramide, *N*-acetylsphinganine; C_2-ceramide, *N*-acetylsphingosine; TNF-α, tumor necrosis factor-α; SM, sphingomyelin; HPLC, high-performance liquid chromatography; PA, plasminogen activator; NF-κB, nuclear factor κB; HNP, 2-hexadecanoylamino-4-nitrophenylphosphorylcholine; RNase A, ribonuclease A; NAP, (+)-6-methoxy-α-methyl-2 naphthaleneacetic acid; DCC, *N*,*N*′-dicyclohexylcarbodiimidde; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; CAPK, ceramide-activated protein kinase; MAPK, mitogen-activated protein kinase

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ease patients [3,4]. The PAI-1 production by vascular endothelial cells is increased by a number of cellular stimuli including endotoxin [5], thrombin [6], lipoprotein(a) [7], interleukin-1 [8] and tumor necrosis factor-α (TNF-α) [9,10]. The concentration of plasma PAI-1 is dramatically increased during endotoxemia. Endotoxin can induce the release of TNF-α from vascular endothelium. TNF-α thereafter induces tissue factor activity in the endothelial cells [11] which leads to the generation of a PAI-1 stimulator, thrombin [6], suggesting its key roles in the PAI-1-related thromboembolic diseases.

TNF-α is known to interact with two distinct cell surface receptors of 55 and 75 kDa, respectively, that transmit signals down to the cytoplasm and nucleus to initiate profound alterations of the transcriptional programs [12]. Recently, several reports implicated the lipid messenger ceramide in various important pathways of TNF-α action [13]. In U937 cells, TNF-α, through binding to the 55 kDa receptor, rapidly activates two distinct types of sphingomyelinases (SMases), a cell membrane-bound neutral SMase and a lysosomal acidic SMase. Ceramide generated by the neutral SMase directs the activation of proline-directed serine/threonine protein kinases and phospholipase A_2. In contrast, the acidic SMase triggers the activation of NF-κB through the generated ceramide [14].

We have previously shown that cultured human umbilical vein endothelial cells (HUVEC) increase the release of PAI-1 by being treated with staphylococcal SMase or cell-permeable ceramide analogs, such as *N*-acetylsphingosine (C_2-ceramide) [15]. These results suggested a possible involvement of ceramide in the induction of PAI-1 release. The present study was undertaken to investigate whether TNF-α utilizes ceramide as a signaling molecule for the induction of PAI-1 release. The results demonstrate that ceramide, generated in endosomal/lysosomal compartments, but not in cell membranes, may play a prominent role in the TNF-α signaling.

2. Materials and methods

2.1. Materials

The following materials were commercially obtained: C_2-ceramide, *Staphylococcus aureus* SMase, 2-hexadecanoylamino-4-nitrophenylphosphorylcholine (HNP), ribonuclease A (RNase A) and proteinase K from Sigma; recombinant human TNF-α (1.0×10^8 units/mg protein) from Boehringer Mannheim; C_2-dihydroceramide from Biomol, Plymouth Meeting, PA; aspirin from Wako, Osaka, Japan; (+)-6-methoxy-α-methyl-2 naphthaleneacetic acid (NAP), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) from Aldrich; aspirin from Wako, Osaka, Japan; 500 bp DNA ladder from Takara Shuzo, Shiga, Japan.

2.2. HUVEC culture

HUVEC (passage 2) and the culture medium EGM-MV were purchased from Sanko Junyaku, Tokyo, Japan. The cells (6×10^5 cells) were plated into a 175-cm^2 tissue culture flask (Falcon) and grown to near confluency in EGM-MV (modified MCDB 131 medium that was supplemented with 5% fetal bovine serum, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin B and 12 μg/ml bovine brain extract) at 37°C in a humidified 5% CO_2 atmosphere. The medium was renewed every 2 days. Subcultures were obtained by treating the HUVEC cultures with 0.25% trypsin/0.01% EDTA (JRH, Lenexa, KS) at 37°C for 5 min. All experiments were performed with HUVEC in the 6th to 8th passages.

2.3. Treatment of HUVEC with various agents

The passaged cells were seeded at a density of 2.5×10^3 cells/cm^2 onto collagen-coated 6-well tissue culture plate in EGM-MV and allowed to reach confluency. After removal of the conditioned media, the HUVEC monolayers were rinsed twice with 2 ml of Dulbecco's modified Eagle's medium (DMEM). The HUVEC monolayers were then treated at 37°C for 30 min with 3 ml of DMEM. After removal of DMEM, incubation followed at 37°C in 3 ml of DMEM supplemented with or without the indicated agent(s). Ceramide analogs were dissolved in ethanol, and the other agents were in phosphate-buffered saline (PBS). During treatment with these agents, control HUVEC were treated with an identical volume of the vehicle. At definite times, 100 μl of the con-

ditioned media was withdrawn and subjected to the assay of PAI-1 antigen.

2.4. Assay of PAI-1 antigen

Antigen levels of PAI-1 in the conditioned media were determined with an ELISA kit, Imulyse PAI-1 (Biopool, Umeä, Sweden), according to the manufacturer's instructions.

2.5. Determination of cellular ceramide

Confluent cultures of HUVEC in 175-cm^2 tissue culture flasks were rinsed with DMEM and incubated at 37°C for 30 min in 20 ml of DMEM. After removal of the conditioned media, incubation followed at 37°C for 3 h in 20 ml of DMEM supplemented with or without TNF-α (200 U/ml). Each cell culture was detached by treatment with 10 ml of 0.05% trypsin/0.53 mM EDTA (Gibco-BRL), and pelleted by centrifugation. The pellet was washed three times with 10 ml of cold PBS, and centrifuged again. The washed cells (5×10^6) were resuspended in 220 μl of cold PBS/0.25 M sucrose, transferred into a microtube, and disrupted by sonication. After the cell lysate was centrifuged at $800\times g$ for 10 min, the supernatant (cytosol plus organelle fraction) was withdrawn. The pellet was washed three times with 1 ml of cold PBS, centrifuged, and suspended in 220 μl of PBS (cell membrane fraction). The following lipid extraction and ceramide determination in each fraction were carried out according to the method of Previati et al. [16]. Briefly, each fraction (200 μl) was mixed with 4 ml of chloroform/methanol (2:1) and extracted for 30 min. After the addition of 1 ml of water, the sample was vortexed and centrifuged. The lower phase was collected and evaporated to dryness under a nitrogen stream. The residue was dissolved in 100 μl of chloroform, and the solution was reacted at −20°C for 3 h with 100 mM NAP, 100 mM DCC and 100 mM DMAP (10 μl each). After evaporation of the solvent under a nitrogen stream, the residue was suspended in 15 μl of chloroform. The suspension was mixed with 2 ml of hexane and centrifuged. The supernatant was vigorously mixed with 5 ml of methanol/water (4:1) and centrifuged. A part (1 ml) of the upper phase was collected and filtered through a 0.45-μm membrane filter. The HPLC system consisted of a Model 600 solvent delivery system (Waters), a Model 474 fluorescence detector (Waters) and a Model 805 data station (Waters). A 20-μl portion of the filtrate was injected into an Econosphere CN 5U column (4.6×250 mm, Alltech). The derivatized ceramide was separated from by-products with 3% 2-propanol in *n*-hexane as the mobile phase. The flow rate was 2.0 ml/min, and the effluent was monitored fluorometrically at the excitation wavelength of 230 nm and emission wavelength of 352 nm.

2.6. Determination of acidic SMase activity

The acidic SMase activity in HUVEC was determined photometrically with HNP as a substrate [17]. The fractions of cell membrane and cytosol plus organelles derived from TNF-α-treated or untreated HUVEC were used as enzyme sources. Briefly, the assay mixture contained the following components in a total volume of 100 μl: 15 mM HNP, 250 mM sodium acetate buffer (pH 5.6) containing 0.05% Tween 80 and 50 μl enzyme preparation. The enzyme reaction was carried out at 37°C and terminated with 400 μl of 100 mM glycine buffer (pH 10.5) plus 700 μl ethanol. The tube was vortexed and centrifuged at $2000\times g$ for 10 min. The absorbance of the supernatant solution was determined at 410 nm.

2.7. Assay of cell death and cell lysis

The lysis of HUVEC was measured with a commercial cytotoxicity detection kit (Boehringer Mannheim). This assay method is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant.

2.8. Analysis of DNA fragmentation by agarose gel electrophoresis

HUVEC (1×10^6 cells) were harvested and pelleted by centrifugation at 400×g for 5 min. The pellet was washed with cold PBS and resuspended in 20 μl of a lysis buffer, 50 mM Tris-HCl buffer (pH 7.8)/10 mM EDTA/0.5% sodium *N*-lauroylsarcosinate. To the suspension was added 1 μl of RNase A (10 mg/ml in the lysis buffer) and incubation followed at 50°C

for 30 min. The reaction mixture was mixed with 1 μl of proteinase K (10 mg/ml in the lysis buffer) and incubated again at 50°C for 60 min. After centrifugation, the nuclei sample was electrophoresed at 100 V on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. DNA was visualized under UV light and photographed.

2.9. Statistical analysis of data

All values are expressed as the means ± S.D., and the significant levels between groups were assessed by Student's *t* test.

3. Results

3.1. TNF-α-induced increase in PAI-1 release from HUVEC is accompanied by an increase in intracellular ceramide content and acidic SMase activity

We first examined the effect of TNF-α on PAI-1 release from HUVEC. TNF-α increased the PAI-1 release in a dose-dependent manner, and the maximal levels of PAI-1 (35.3 ± 1.00 ng/ml, a 2.8-fold increase compared with control) were observed at a 3-h incubation with 200 U/ml TNF-α. The leakage of LDH activity from the TNF-α-treated HUVEC was not different from the control level, suggesting that the cytokine-inducible cellular lysis can be negligible (data not shown). Next, to determine whether TNF-α could alter the cellular ceramide levels, confluent cultures of HUVEC were treated for 3 h in DMEM supplemented with or without TNF-α (200 U/ml) and then fractionated into cell membranes and cytosol plus organelles, respectively (see Section 2). As shown in Fig. 1A, TNF-α increased the net content of ceramide in the cytosol plus organelle fraction to 126% of the control (639 ± 18. 3 ng vs. 803 ± 64.7 ng, $P < 0.05$). However, this cytokine did not alter the membranous ceramide content, suggesting little or no involvement of the activation of membranous neutral SMase. On the other hand, there was a 33% increase ($P < 0.01$) in the net activity of acidic SMase (HNP hydrolysis at pH 5.6) present in the cytosol plus organelles derived from TNF-α-treated HUVEC (Fig. 1B). This result implies the possibility that

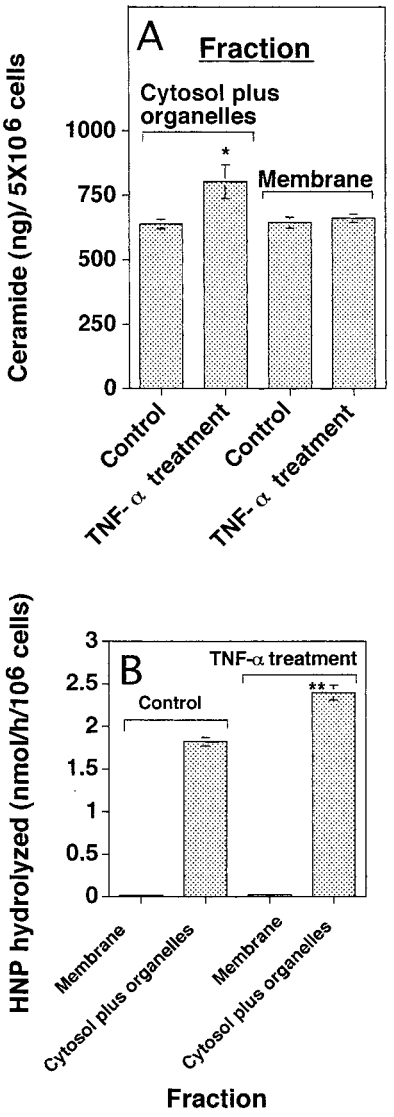

Fig. 1. Effect of TNF-α on the intracellular ceramide levels (A) and acidic SMase activity (B) in HUVEC. Cultured HUVEC were stimulated with TNF-α (200 U/ml) at 37°C for 3 h, and then the cells were harvested and fractionated as described in Section 2. Ceramide levels were determined by a HPLC method. SMase activity was determined with HNP as the substrate. Each bar represents the mean ± S.D. of triplicate experiments. *$P < 0.05$; **$P < 0.01$, compared to control.

TNF-α may prominently activate the acidic SMase located in endosomal/lysosomal compartments.

3.2. C_2-dihydroceramide suppresses both C_2-ceramide- and TNF-α-induced increases in PAI-1 release from HUVEC

Olivera et al. [18] previously demonstrated that

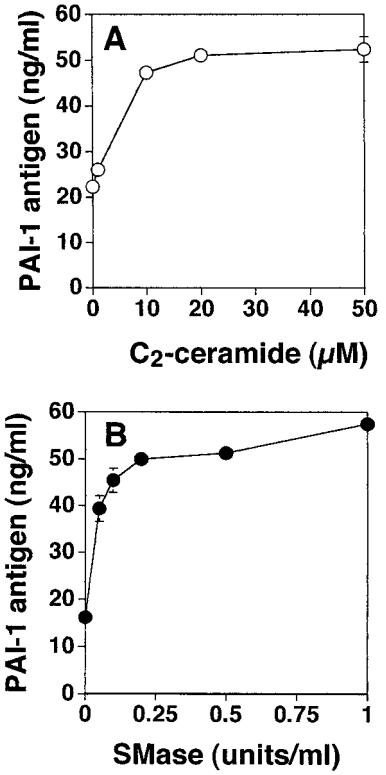

Fig. 2. Concentration-dependent effect of C_2-ceramide (A) or staphylococcal SMase (B) on the release of PAI-1 antigen from cultured HUVEC. Confluent cultures of HUVEC (1×10^6 cells) were incubated at 37°C for 3 h in DMEM supplemented with the indicated concentrations of C_2-ceramide or SMase. Each bar represents the mean ± S.D. of triplicate experiments.

staphylococcal SMase or cell-permeable ceramide analogs, such as C_2-ceramide, stimulated the proliferation of Swiss 3T3 cells and suggested that ceramide was involved in the cellular proliferation induced by various growth factors. By using the same experimental techniques, we also suggested the involvement of ceramide in the induction of PAI-1 release from HUVEC [15]. Fig. 2A and B show the concentration-dependent effects of C_2-ceramide and SMase, respectively, on the release of PAI-1 from cultured HUVEC. By a 3-h incubation of HUVEC with 10 μM C_2-ceramide, the PAI-1 antigen level rose from 22.3 ± 0.25 to 47.3 ± 1.57 ng/ml (a 2.1-fold increase). The treatment of HUVEC for 3 h with 0.2 U/ml SMase caused a nearly maximal effect on the PAI-1 release, and the increased PAI-1 antigen levels were 3.1-fold the control level (16.2 ± 0.95 ng/ml). However, the leakage of LDH activity from the HUVEC, treated for 3 h with 10 μM C_2-ceramide or 0.2 U/ml SMase, was similar to that of control (data not shown). We also examined the effect of C_2-ceramide on the internucleosomal DNA fragmentation in HUVEC to know whether the C_2-ceramide-induced PAI-1 release was due to the results from its leakage followed by apoptosis. The cells were incubated for 3–24 h in DMEM supplemented with or without C_2-ceramide. The genomic DNA of HUVEC was not affected by a 3-h incubation with either 10 or 20 μM C_2-ceramide, although at a 24-h incubation the DNA fragmentation was more clearly observed in C_2-ceramide-treated HUVECs (data not shown). Therefore, these findings indicate that the increase in PAI-1 release at 3 h is not due to the results from cellular lysis or apoptosis in HUVEC by the agent, suggesting the primary role of C_2-ceramide as a lipid messenger for the PAI-1 production.

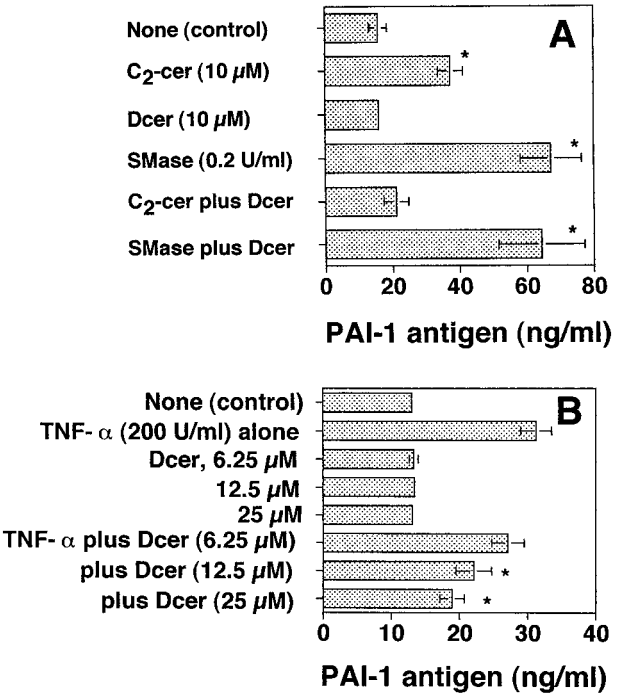

Fig. 3. (A) Effect of C_2-dihydroceramide on the C_2-ceramide (or SMase)-induced increase in PAI-1 release. Confluent cultures of HUVEC (1×10^6 cells) were incubated at 37°C for 3 h in DMEM supplemented with or without the indicated concentrations of agents. (B) Effect of C_2-dihydroceramide on the TNF-α-induced increase in PAI-1 release. Cultured HUVEC were stimulated with TNF-α (200 U/ml) at 37°C for 3 h in the presence or absence of the indicated concentrations of C_2-dihydroceramide. Each bar represents the mean ± S.D. of triplicate experiments. *$P<0.05$, compared to control (or TNF-α alone). C_2-cer, C_2-ceramide; Dcer, C_2-dihydroceramide.

During the experiments that have examined the concentration-dependent effects of C_2-ceramide and SMase on the PAI-1 release (Fig. 2A,B), we found that 20 μM C_2-ceramide and SMase (0.2 U/ml) in combination could synergistically increase the PAI-1 release from 51.1 ± 1.64 to 72 ± 2.76 ng/ml (data not shown). This finding suggested a possibility that ceramide generated by staphylococcal SMase at plasma membrane and C_2-ceramide incorporated into the cells might utilize different signal transduction pathways. To determine this possibility, experiments were undertaken to examine the effect of C_2-dihydroceramide on the C_2-ceramide- or SMase-induced PAI-1 release from HUVEC, since C_2-dihydroceramide differs from C_2-ceramide only in that it lacks the 4,5 *trans* double bond and has no C_2-ceramide-like activity. As shown in Fig. 3A, C_2-dihydroceramide effectively suppressed the C_2-ceramide-induced increase in PAI-1 release to near the control levels (control, 15.8 ± 2.63 ng/ml vs. C_2-ceramide plus C_2-dihydroceramide, 21.1 ± 3.65 ng/ml). However, the PAI-1 release was not affected by this compound alone (15.9 ± 1.13 ng/ml). C_2-dihydroceramide had little or no effect on the SMase-induced increase in PAI-1 release (SMase alone, 67.2 ± 9.13 ng/ml vs. SMase plus C_2-dihydroceramide, 64.4 ± 14.8 ng/ml). We next examined the effect of C_2-dihydroceramide on TNF-α-induced increase in PAI-1 release (Fig. 3B). C_2-dihydroceramide at 6.5–25 μM significantly suppressed the TNF-α-induced PAI-1 release in a concentration-dependent manner (TNF-α alone, 31.3 ± 2.29 ng/ml vs. TNF-α plus 25 μM C_2-dihydroceramide, 18.9 ± 1.78 ng/ml, $P < 0.05$). Higher concentrations of C_2-dihydroceramide (50 μM) did not cause a further increase in the suppressive effect (data not shown). This finding indicates that C_2-dihydroceramide competes with the incorporated C_2-ceramide for the induction of PAI-1 and additionally that it could also compete with the intracellular ceramide generated by acidic SMase after induction by TNF-α (Figs. 1B and 2A).

To further confirm this possibility, we next attempted to use a cyclooxygenase inhibitor, aspirin, for the inhibition of TNF-α-induced PAI-1 release from HUVEC. Treatment of HUVEC with staphylococcal SMase that mimics the action of neutral SMase may hydrolyze the membrane SM to ceramide and phosphocholine. The membrane ceramide

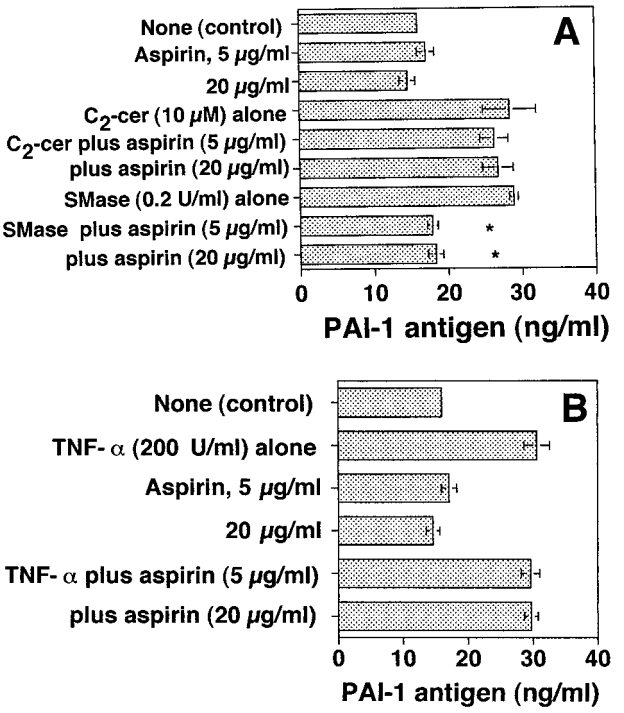

Fig. 4. (A) Effect of aspirin on the C_2-ceramide (or SMase)-induced increase in PAI-1 release. Confluent cultures of HUVEC (1×10^6 cells) were incubated at 37°C for 3 h in DMEM supplemented with or without the indicated concentrations of agents. (B) Effect of aspirin on the TNF-α-induced increase in PAI-1 release. Cultured HUVEC were stimulated with TNF-α (200 U/ml) at 37°C for 3 h in the presence or absence of the indicated concentrations of aspirin. Each bar represents the mean ± S.D. of triplicate experiments. *$P < 0.05$, compared to SMase alone. C_2-cer, C_2-ceramide.

signal transduction pathway may direct the activation of proline-directed serine/threonine protein kinases and phospholipase A_2 [14]. The activation of phospholipase A_2 could generate prostaglandins via activation of cyclooxygenase. As shown in Fig. 4A, C_2-ceramide-induced increase in PAI-1 release was not affected by the clinically useful concentrations of aspirin (28.4 ± 3.59 ng/ml vs. 26.8 ± 2.06 ng/ml). However, the presence of 5 or 20 μg/ml aspirin decreased SMase-induced PAI-1 release to the control levels (control, 16.0 ± 0.51 ng/ml vs. SMase plus 20 μg/ml aspirin, 17.9 ± 0.72 ng/ml). Additionally, 5 or 20 μg/ml aspirin had no effect on the TNF-α-induced PAI-1 release from HUVEC (Fig. 4B). These results indicate that the cyclooxygenase inhibitor aspirin specifically suppresses staphylococcal SMase-induced increase in PAI-1 release from HUVEC, but neither TNF-α- nor C_2-ceramide-induced increase is

affected, suggesting a possible role of intracellular ceramide in the TNF-α signaling.

4. Discussion

In the present work, we indicate the possibility that the lysosomally generated ceramide-mediated signal transduction pathway(s) is involved in TNF-α-induced increase in PAI-1 release from HUVEC. This conclusion is based on the following observations. First, addition of TNF-α prominently increased the intracellular ceramide levels in HUVEC, but did not alter the membranous ceramide content (Fig. 1A). Second, the increase in PAI-1 release was accompanied by a significant activation of the intracellular acidic SMase (Fig. 1B). Third, C_2-dihydroceramide, a derivative of C_2-ceramide, suppressed the increase in PAI-1 release induced not only by the cell-permeable ceramide analog (Fig. 3A), but also by TNF-α (Fig. 3B). C_2-dihydroceramide failed to suppress the increase in PAI-1 release induced by staphylococcal SMase (Fig. 3A), while aspirin was found to selectively suppress the increase in PAI-1 release induced by the SMase, but not by TNF-α (Fig. 4A,B).

However, the increase in the intracellular ceramide levels observed after TNF-α stimulation was significant, but very small (26% increase). The kinetics of ceramide formation in response to TNF-α are very different in different studies. TNF-α appears to induce prolonged and persistent accumulation of ceramide that occurs over a period of several hours, but serum withdrawal from culture medium also affects the ceramide accumulation [19]. In this study, the exchange of EGM-MV for serum-free DMEM may have resulted in an increase in the ceramide levels of control cells.

When considering the role of ceramide in the signal transduction, the most important thing is that where, but not how much, the intracellular lipid mediator was generated. The increased ceramide is probably due to the hydrolysis of SM by lysosomal acidic SMase (TNF-α induced a 33% increase in activity). The lysosomally generated ceramide might induce a signal for the production of PAI-1. However, Slowik et al. [20] reported that the treatment of HUVEC with TNF-α did not alter the intracellular ceramide and SM levels, but activated NF-κB. This suggests that TNF-α does not activate the SMase-ceramide signaling pathway in HUVEC. One of the reasons by which these contradicting results occurred may be due to the difference in culture conditions. Although we cultivated HUVEC in MCDB 131 substituted with 5% serum and supplements, such as epidermal growth factor, hydrocortisone, and bovine brain extract prior to the experiments with serum-free DMEM, they used a medium 199 supplemented with 20% serum. Since there is a report that describes acute changes in intracellular ceramide levels caused by changes in culture conditions [21], preactivation of HUVEC with different supplements could account for the different results. Additionally, we used a higher concentration of TNF-α (200 U/ml) in our experiments as compared to 100 U/ml used by Slowik et al. [20]. The difference in dose of TNF-α does not account for the different results, since 100 U/ml of TNF-α induced PAI-1 to near the maximal level in our experiments (a 2.5-fold increase, data not shown).

A 3-h treatment of HUVEC with 10 μM C_2-ceramide induced a 2.1-fold increase in PAI-1 release (Fig. 2A). However, TNF-α increased PAI-1 release by 2.8-fold in spite of a small increase in the intracellular ceramide levels (approx. 200 ng/5×10^5 cells). This discrepancy may be explained as follows. First, the added C_2-ceramide does not seem to be perfectly incorporated into the cells. Second, since the alkyl chain length of C_2-ceramide is extremely short, it may be unable to act as fully as the intracellular ceramides. Additionally, 10 μM C_2-ceramide and TNF-α (200 U/ml) in combination did not affect the sole effect of TNF-α on PAI-1 production (data not shown), suggesting that both inducers utilize the same signal transduction pathway.

Endogenous dihydroceramide is rapidly converted to ceramide by the desaturase(s) present in the site of microsomes [22]. In our system, however, C_2-dihydroceramide still had the suppressive effect over 6 h (data not shown), suggesting that it was hardly converted to C_2-ceramide. Michel et al. [23] have clearly demonstrated the in vitro conversion of dihydroceramide to ceramide, using rat liver microsomes. They have shown that the desaturase activity is influenced by the alkyl chain length of the sphingoid base (in the order $C_{18} > C_{12} > C_8$). This finding allowed us to

speculate that the acetyl group of C_2-dihydroceramide might be too short for the binding to dihydroceramide desaturase(s) in HUVEC. Ceramide generated by SM breakdown at the site of plasma membrane is proposed to select major signaling pathways such as phospholipase A_2, and proline-directed serine/threonine-specific protein kinases that include CAPK and MAPK [14]. The activation of phospholipase A_2 may couple to cyclooxygenase activation that is capable of generating prostaglandins. We showed that aspirin, a specific inhibitor of cyclooxygenase, effectively suppressed the induction of HUVEC PAI-1 release by staphylococcal SMase, but neither by C_2-ceramide nor by TNF-α (Fig. 4A,4B). This suggests that the cyclooxygenase has a key role in mediating the staphylococcal SMase (plasma membrane-associated neutral SMase)-induced signaling cascade for PAI-1 release. After induction by TNF-α, failure to determine the increase in membranous ceramide level suggests little or no involvement of the activation of neutral SMase in HUVEC. If the so-called 55 kDa TNF-α receptor contributes to the PAI-1 induction, it might simultaneously trigger the neutral SMase-ceramide signaling pathway [14]. In this regard, Marathe et al. [24] have reported that HUVEC is a rich and regulatable source of secretory SMase and that when HUVEC are incubated with interleukin-1β or interferon-γ, the secretion of SMase is increased 2–3-fold compared with the control. Interleukin-1β is known to promote the release of PAI-1 from HUVEC [8]. It is not known why interferon-γ does not induce the PAI-1 release in spite of its close relation with the SM cycle. They also suggested that the secretory SMase was a gene product of the lysosomal acidic SMase. In the present work, we did not determine the secretion of SMase from HUVEC. However, these findings imply that SMase(s) arising from the acidic SMase gene may play prominent roles in the cytokine-associated ceramide signaling events in HUVEC. Thus, the lysosomal/endosomal SMase-ceramide signaling pathway appears to have a prominent role in the mechanism of TNF-α-induced PAI-1 release from HUVEC, although there is evidence against the involvement of lysosomal acidic SMase in TNF-α-induced signaling in human fibroblasts [25,26].

One could speculate on the pathophysiological relationship between TNF-α-induced activation of lysosomal SMase and PAI-1 release in vascular endothelial cells. Products of the acidic SMase gene may be implicated not only in ceramide-mediated cellular differentiation or apoptosis, but also in early atherogenic processes. The secreted SMase may hydrolyze lipoprotein SM to lipoprotein ceramide that leads to the protein aggregation [27] and retention onto arterially derived matrix [28]. The aggregation may lead to induction of macrophage cholesteryl ester accumulation [27,28]. On the other hand, intracellular ceramide generated by lysosomal acidic SMase could induce PAI-1 release. The secreted PAI-1 is accumulated in the extracellular matrix [29], where it may protect matrix components from proteolysis by limiting plasmin generation. In spite of the pathological role of plasma PAI-1 in coronary artery disease [3,4], the PAI-1 function may be important to preserve the integrity of the endothelium elements. Therefore, to understand further the atherogenic processes, studies addressing the roles of PAI-1 pathophysiologically deposited into vascular endothelium and the regulatory mechanism of ceramide-induced PAI-1 will hereafter be needed. The action of C_2-dihydroceramide presented here give us a hint that may lead to improved methods for controlling PAI-1 that increases the risk for atherosclerosis and thrombosis.

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