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Expression of adhesion molecules by sphingosine 1-phosphate and histamine in endothelial cells

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Abstract

We investigated the effects of sphingosine 1-phosphate and histamine on the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, and their signaling pathways in human umbilical vein endothelial cells. Sphingosine 1-phosphate increased the mRNA and protein level of VCAM-1, and the mRNAs of E-selectin and ICAM-1. The effects of sphingosine 1-phosphate were inhibited by the pertussis toxin and the respective inhibitors (10 μM 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) for phosphoinositide-specific phospholipase C; 10 μM 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) for p38 mitogen-activated protein kinase (MAPK); 1 μM 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976) for the α form of protein kinase C (PKC-α)), but not by a PKC-δ inhibitor (1 μM rottlerin). Histamine, which alone showed no effect, enhanced the sphingosine 1-phosphate-induced expressions via histamine H_1 receptor. The histamine response decreased by U73122 and rottlerin, but not by SB203580 and Gö6976. The effects of sphingosine 1-phosphate with and without histamine were abolished by the higher concentrations of PKC inhibitors and in the PKC-depleted cells. Sphingosine 1-phosphate and histamine alone stimulated phosphorylation of p38 MAPK in a phosphoinositide-specific phospholipase C-dependent but not in a PKCs-independent manner. These findings suggest that sphingosine 1-phosphate-induced expression of adhesion molecules was mediated by phosphoinositide-specific phospholipase C and preferentially by PKC-α and p38 MAPK, and the histamine response was mediated by phosphoinositide-specific phospholipase C and PKC-δ in human umbilical vein endothelial cells.

Keywords: Sphingosine 1-phosphate; Histamine; Protein kinase C; p38 MAPK; Adhesion molecule; Endothelial cell; (Human)

1. Introduction

Cell adhesion molecules play an important role in allergy and inflammation. Activated endothelial cells transiently express adhesion molecules involved in the trafficking of circulating immune cells across the endothelial cell barrier into underlying tissues (Ley and Tadder, 1995; Luscinskas and Gimbrone, 1996). Expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin was induced on endothelial cells by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins (Ley and Tadder, 1995). Sphingosine 1-phosphate, a bioactive sphingolipid metabolite, has been implicated in the regulation of various cell functions, and many actions by sphingosine 1-phosphate are likely to be due to activation of endothelial differentiation gene (EDG) family of receptors (Pyne and Pyne, 2000; Toman and Spiegel, 2002). Stimulation of endothelial cells with sphingosine 1-phosphate induces the cell survival pathway, DNA synthesis, chemotactic motility, cytoskeletal rearrangement and tube formation, etc. (Panetti, 2002; Tamama and Okajima, 2002). EDG-1, the prototypical sphingosine 1-phosphate receptor, was originally cloned as an early response gene from human umbilical vein endothelial cells (Hla and Maciag, 1990), and EDG-1 was shown to be abundantly expressed, whereas EDG-3 was expressed at a lower level in human umbilical vein endothelial cells (Lee et al., 1999b). Masamune et al. (1996) reported that addition of 5 μM sphingosine 1-phosphate did not stimulate E-selectin expression in human umbilical vein endothelial cells. In contrast, several studies showed that

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sphingosine 1-phosphate at μM concentrations induced the expression of VCAM-1 and E-selectin in human umbilical vein endothelial cells (Xia et al., 1998) and in human aortic endothelial cells (Rizza et al., 1999). Although the involvement of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) and p38 MAPK on the cytokines-induced expression of adhesion molecules was proposed (Read et al., 1997; Xia et al., 1998; Rahman et al., 2001), the exact role of MAPKs and other kinases on sphingosine 1-phosphate-induced expression of adhesion molecules, including whether sphingosine 1-phosphate induces the expression or not, has not been well established in endothelial cells.

Protein kinase C (PKC) is a multigene family of serine/threonine kinases mediating various intracellular signals (Nishizuka, 1995). PKC isoforms are classified into three groups, conventional PKC (cPKC-α, -β and -γ), novel PKC (nPKC-δ, -ε, -μ, -θ, and -η) and atypical PKC (aPKC-ζ and -λ/ι), and the tissue distribution of PKC isoforms varies considerably. Endothelial cells expresses the PKC-α, -δ, -ε, -θ, -η, and -ζ isoforms, and PKC-β1 and PKC-λ/ι are undetectable (Rahman et al., 2001; Gliki et al., 2001). Although it was reported that various PKC isoforms are involved in the stimulants-induced expression of adhesion molecules in endothelial cells such as human umbilical vein endothelial cells (May et al., 1996; Tamaru and Narumi, 1999; Rahman et al., 1999, 2000, 2001, 2002; Minami et al., 2003), the role of PKC isoforms appeared to be dependent on the types of receptors and/or cells. The activation of phorbol 12-myristate 13-acetate (PMA)-sensitive PKCs was not involved in sphingosine 1-phosphate-induced chemotaxis in human umbilical vein endothelial cells (Lee et al., 2000). The role of PKC isoforms in the sphingosine 1-phosphate-induced expression of adhesion molecules remains unclear in human umbilical vein endothelial cells.

The inflammatory mediator histamine increases endothelial permeability, and induces adhesion of leukocytes and platelets (Saito et al., 1996; Hu et al., 2002). Histamine alone did not modulate the expression of VCAM-1, E-selectin and ICAM-1 in human umbilical vein endothelial cells, although histamine induced an expression of P-selectin (Jeannin et al., 1994). Histamine via histamine H_1 receptors enhanced the TNF-α- and interleukin-4-induced expression of adhesion molecules such as VACM-1 in human umbilical vein endothelial cells (Miki et al., 1996; Saito et al., 1996). However, the signal transduction pathways regulating expression of adhesion molecules by histamine have not been well understood, and the effect of histamine on the sphingosine 1-phosphate-induced response was not determined. In the present study, we investigated the effects of sphingosine 1-phosphate and histamine on expression of adhesion molecules in human umbilical vein endothelial cells. In addition, the signaling pathways such as phosphoinositide-specific phospholipase C, MAPKs and PKCs involved in sphingosine 1-phosphate and histamine responses were examined using pharmacological methods.

2. Materials and methods

2.1. Chemicals

The following materials and chemicals were obtained from the indicated suppliers: D-*erythro*-sphingosine 1-phosphate and anthra[1,9-cd]pyrazol-6(2H)-one (SP600125) were from Biomol (Plymouth Meeting, PA, USA); histamine dihydrochloride, famotidine, wortmannin, rottlerin, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), PMA and N^G-nitro-L-arginine methyl ester were from Sigma (St. Louis, MO, USA); 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) was from Cayman (Ann Arbor, MI, USA); calphostin C, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976), 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X), (*E*)3-[(4-metylphenyl)sulfonyl]-2-propenenitrile (BAY117082) and 2′-amino-3′-methoxylfavone (PD98059) were from Calbiochem (La Jolla, CA, USA). Diphenhydramine hydrochloride was from Wako (Osaka, Japan).

2.2. Cell culture

Human umbilical vein endothelial cells, purchased from Dainippon Pharm. (Osaka, Japan), were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 [HAM] (1:1) supplemented with 10% fetal calf serum, 10 ng/ml of recombinant human basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), penicillin G (67 units/ml), and streptomycin sulfate (100 μg/ml), at 37 °C under an atmosphere of 5% CO_2 and 95% air. The endothelial cells were used between passages 2 and 6 for all experiments. The cells were replaced in the collagen-coated 60-mm or 100-mm dishes (Iwaki, Tokyo, Japan), and were used when sub-confluent (70–80%). To measure the expression of adhesion molecules, the endothelial cells were cultured with MCDB-131 medium (Sigma) supplemented with 0.5% fetal calf serum without basic fibroblast growth factor in the presence of the indicated agents.

2.3. Analysis of mRNA levels of adhesion molecules

Total RNA was isolated with TRIzol reagent (Sigma), and total RNA (5 μg) was used for reverse transcription (RT) to generate cDNA. RT cDNA (2.5 μg) was then used for the polymerase chain reaction (PCR) amplification. The primers for VCAM-1 were: sense 5′-ACCCTCCCAGGCACACACAG-3′ and antisense 5′-GTAAGTCTATCTCCAGCCTGTC-3′ (expected product 535 bp); for ICAM-1 they were: sense 5′-CAGTGACCATCTACAGCTTTCCGG-3′ and antisense 5′-GCTGCTACCACAGTGATGATGACAA-3′ (556 bp); for E-selectin they were: sense 5′-GATGTGGGCATGTGGAATGATG-3′ and antisense 5′-AGGTACACT-

GAAGGCTCTGG-3′ (502 bp); for β-actin they were: sense 5′-TGCCCATCTATGAGGGTTACG-3′ and antisense 5′-TAGAAGCATTTGCGGTGCACG-3′ (650 bp). The number of cycles selected for each primer pair was found to produce a linear relation between the input cDNA and the resulting PCR products. The PCR products were analyzed by electrophoresis on 1.5% agarose gel and visualized under UV light with ethidium bromide. Computer imaging analysis (NIH Image 1.57) was used to quantify the density of each band and to obtain semiquantitative information on the relative changes in mRNA levels. β-Actin mRNA was used as an internal control and the rations of RT-PCR products of adhesion molecules to β-actin were determined. A quantitative real-time RT-PCR analysis showed similar results in a typical experiment (data not shown).

2.4. Western blotting

Cell lysates from human umbilical vein endothelial cells were loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinyldifluoride membrane. The blocked membranes were then incubated with the respective antibody. The antibodies were obtained from the indicated suppliers: anti-VCAM-1 antibody (C-19, sc-1504), anti-PKC-α antibody (sc-208) and anti-PKC-δ antibody (sc-937) were from Santa Cruz Biotech (Santa Cruz, CL, USA), the anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody (No9211) and anti-p38 MAPK antibody (No9212) were from Cell Signaling Tech. (Beverly, MA, USA). The immunoreactive bands were visualized using chemiluminescent reagent as recommended by Amersham Pharm. Biotechnology. The signals of the bands were quantitated using a densitometer.

2.5. Data analysis and statistics

Values are presented as percentage ± S.E.M. of control. Statistical comparisons between multiple groups were performed using one-way analysis of variance followed by the Bonferroni test. P values at <0.05 were considered significant.

3. Results

3.1. Sphingosine 1-phosphate-stimulated expression of adhesion molecules and its enhancement by histamine H_1 receptor activation in human umbilical vein endothelial cells

We examined the effect of sphingosine 1-phosphate on the mRNA levels of adhesion molecules in human umbilical vein endothelial cells using RT-PCR analysis (Fig. 1, Panel A). Addition of sphingosine 1-phosphate increased VCAM-1 and E-selectin mRNA levels in the endothelial cells at 4 h after the addition. The effect of sphingosine 1-phosphate was concentration-dependent and maximal at 10 μM (Fig. 1B), as described previously (Xia et al., 1998). Sphingosine 1-phosphate also increased the ICAM-1 mRNA level. The increases in the mRNA levels of the three adhesion molecules were observed at 2 h, and the maximal responses were obtained at 4 h after the addition of

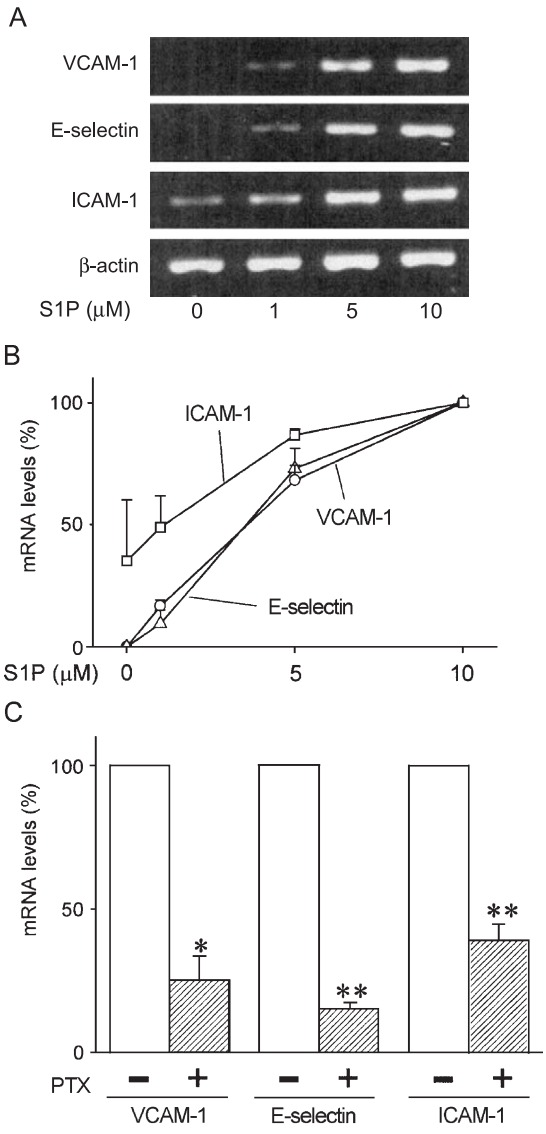

Fig. 1. Increase in mRNA levels of adhesion molecules induced by sphingosine 1-phosphate in human umbilical vein endothelial cells. Cells were cultured with the indicated concentrations of sphingosine 1-phosphate (S1P) for 4 h. Total RNA was isolated from the respective cells and used for RT-PCR analysis using specific primers. A typical and representative response is shown in Panel A. Quantitative analyses of the ratios of RT-PCR products of VCAM-1 (○), E-selectin (△) and ICAM-1 (□) to β-action are shown in Panel B. The values were calculated as percentages of the expression of the respective adhesion molecules in the 10 μM sphingosine 1-phosphate-stimulated endothelial cells. The expression of adhesion molecules induced by 10 μM sphingosine 1-phosphate in the pertussis toxin (PTX)-treated cells is shown in Panel C. Values are means ± S.E.M. for three independent experiments. *$P<0.05$, **$P<0.01$, significantly different from the control cells.

10 μM sphingosine 1-phosphate. The mRNA levels of VCAM-1 and E-selectin decreased to the basal level at 8 h after the sphingosine 1-phosphate addition, but the level of ICAM-1 at 8 h remained at the high level similar to that at 4 h (data not shown). The expression of three adhesion molecules induced by 10 μM sphingosine 1-phosphate was significantly inhibited in the human umbilical vein endothelial cells treated with 200 ng/ml of pertussis toxin (Fig. 1C), as reported in human aortic endothelial cells (Rizza et al., 1999).

The addition of 10 μM sphingosine 1-phosphate for 4 h increased the intensities of a major band (100 kDa) and a minor band (about 90 kDa) that reacted with the anti-VCAM-1 antibody (Fig. 2A). Coaddition of 10 μM histamine for 4 h enhanced the increase in the protein and mRNA level of VCAM-1 induced by 10 μM sphingosine 1-phosphate. Histamine also enhanced sphingosine 1-phosphate-induced increases in E-selectin and ICAM-1 mRNA levels (Fig. 2B). Histamine alone showed no or limited effects on the level of VCAM-1 protein and the mRNAs levels of the three adhesion molecules. The stimulatory effects induced by 10 μM histamine on the sphingosine 1-phosphate response were almost completely inhibited by 10 μM diphenhydramine (an antagonist of histamine H_1 receptors) but not by 10 μM famotidine (an antagonist of histamine H_2 receptors) (Fig. 3). The antagonists alone showed no effect on the expression or on the sphingosine 1-phosphate response without histamine.

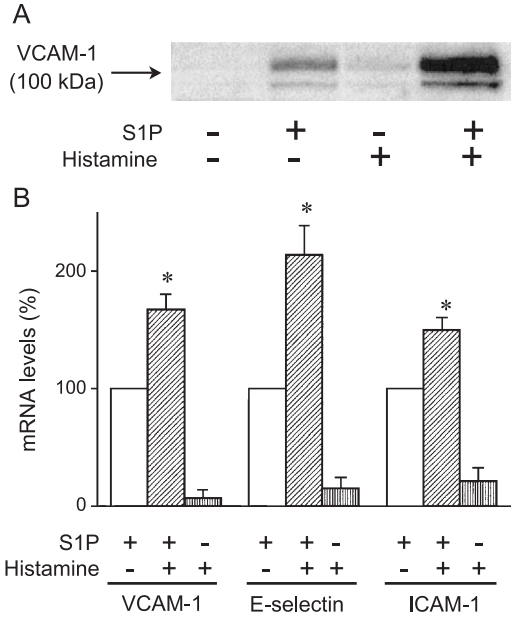

Fig. 2. Enhancement of sphingosine 1-phosphate-induced expression of adhesion molecules by histamine. Cells were cultured with 10 μM sphingosine 1-phosphate (S1P) with and without 10 μM histamine for 4 h. Panel A shows the VCAM-1 protein, which cross-reacted with the anti-VCAM-1 antibody. Panel B shows quantitative data of the ratios of RT-PCR products of VCAM-1, E-selectin and ICAM-1 to β-actin. Values are means ± S.E.M. for three independent experiments. *$P<0.05$ significantly different from the value induced by 10 μM sphingosine 1-phosphate alone.

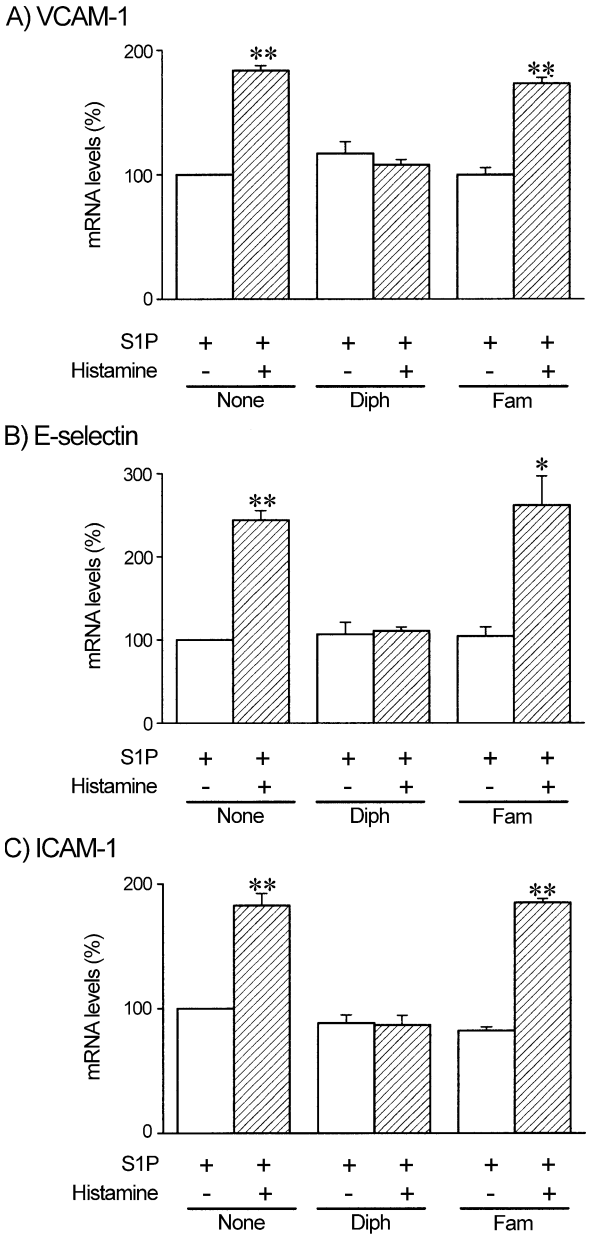

Fig. 3. Inhibitory effect of diphenhydramine, but not famotidine, on the histamine response. Cells were cultured with vehicle, 10 μM diphenhydramine (Diph) or 10 μM famotidine (Fam) for 10 min, and then the cells were cultured for 4 h with 10 μM sphingosine 1-phosphate (S1P) with or without 10 μM histamine. Total RNA was isolated from the respective cells and used for RT-PCR analysis. Values are means ± S.E.M. for three independent experiments. *$P<0.05$, **$P<0.01$ significantly different from the value induced by 10 μM sphingosine 1-phosphate alone.

3.2. Involvement of phosphoinositide-specific phospholipase C and p38 MAPK on sphingosine 1-phosphate- and histamine-induced expression of adhesion molecules

Next we investigated the signal transduction pathways involved in sphingosine 1-phosphate and histamine responses in human umbilical vein endothelial cells pharmacologically using several inhibitors (Fig. 4 and Table 1).

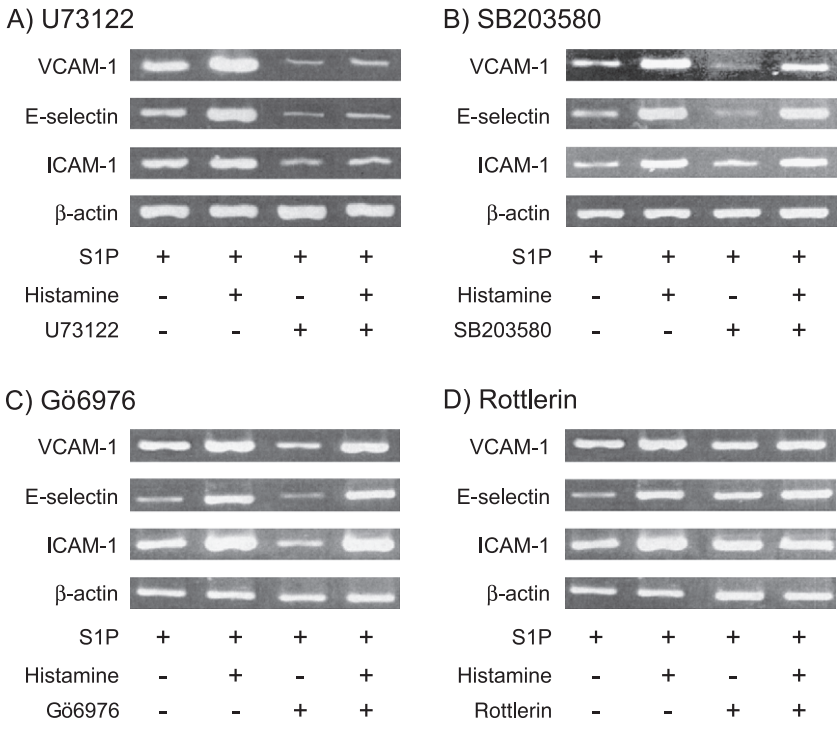

Fig. 4. Effects of U73122, SB203580, Gö6976 and rottlerin on sphingosine 1-phosphate- and histamine-induced expression of VCAM-1, E-selectin and ICAM-1. Cells were cultured with vehicle, 10 μM U73122 (Panel A), 10 μM SB203580 (Panel B), 1 μM Gö6976 (Panel C) or 1 μM rottlerin (Panel D) for 10 min, and the cells were cultured for 4 h with 10 μM sphingosine 1-phosphate (S1P) with or without 10 μM histamine. Total RNA was isolated from the respective cells and used for RT-PCR analysis. A typical and representative response is shown ($n=3$). The results of the quantitative analyses of the ratios of the RT-PCR products of the adhesion molecules to β-actin are shown in Table 1.

Treatment with 10 μM U73122 (a selective inhibitor of phosphoinositide-specific phospholipase C, Rhee, 2001), which inhibited sphingosine 1-phosphate-induced chemotaxis in human umbilical vein endothelial cells (Lee et al., 2000), significantly inhibited the increases in mRNA levels of VCAM-1, E-selectin and ICAM-1 induced by 10 μM sphingosine 1-phosphate with and without 10 μM histamine (Fig. 4A). Treatment with 10 μM SB203580 (a selective inhibitor of p38 MAPK), which inhibited sphingosine 1-phosphate-induced p38 MAPK activation almost completely (Lee et al., 1999a), significantly inhibited 10 μM sphingosine 1-phosphate-induced increases in mRNAs levels of VCAM-1 and E-selectin, but not ICAM-1 (Fig. 4B). In the presence of 10 μM histamine, however, the treatment with SB203580 exhibited marginal effects on VCAM-1 and E-selectin expression induced by 10 μM sphingosine 1-phosphate; the hold-stimulations of VCAM-1 and E-selectin expression induced by histamine in the SB203580-treated cells were in 3.0–3.8-fold, which were higher compared with the values (1.7–2.2-fold) in the control cells without SB203580. Treatment with 2 μM SB203580 markedly inhibited the sphingosine 1-phosphate-induced expression of VCAM-1 mRNA, but did not inhibit the histamine response (data not shown). Treatment with other selective

Table 1
Effects of U73122, SB203580, Gö6976 or rottlerin on sphingosine 1-phosphate- and histamine-induced expression of adhesion molecules in human umbilical vein endothelial cells

Additions	VCAM-1		E-selectin		ICAM-1	
	S1P	S1P+histamine	S1P	S1P+histamine	S1P	S1P+histamine
None	100	170.4±7.0[a] (1.7)	100	226.5±13.2[a] (2.2)	100	185.6±13.5[a] (1.8)
U73122	6.5±3.8[b]	33.5±12.6	14.3±5.7[b]	59.4±12.7[a]	23.5±14.9[b]	60.9±7.6
SB203580	39.7±14.4[b]	121.2±19.0[a] (3.0)	52.7±12.3[b]	203.2±23.8[a] (3.8)	92.1±11.8	151.8±24.4 (1.6)
Gö6976	42.1±8.1[b]	111.3±8.8[a] (2.6)	40.1±7.2[b]	186.8±20.2[a] (4.6)	65.7±23.5	147.9±7.5[a] (2.2)
Rottlerin	85.0±5.2	89.9±3.9 (1.0)	183.6±23.5[b]	218.1±25.2 (1.1)	118.3±5.9	135.5±8.2 (1.1)

The results of the quantitative analysis of the ratios of the RT-PCR products of the adhesion molecules to β-actin are shown. Fold-stimulation induced by 10 μM histamine is shown in parentheses. Values are means±S.E.M. for three to four independent experiments.

[a] $P<0.05$, significantly different from the value without histamine.

[b] $P<0.05$, significantly different from the value induced by sphingosine 1-phosphate (S1P) alone.

inhibitors (20 μM PD98059 for the ERK pathway, 20 μM SP600125 for c-*Jun* N-terminal kinases, 100 nM wortmannin for phosphoinositide 3-kinase, and 1 mM N^G-nitro-L-arginine methyl ester for nitric oxide synthase) did not modulate the 10 μM sphingosine 1-phosphate-induced expression of adhesion molecules with and without 10 μM histamine.

3.3. Different involvement of PKC-α and PKC-δ on sphingosine 1-phosphate- and histamine-induced expression of adhesion molecules

In endothelial cells, PKC-α and PKC-δ isoforms play role in various cell functions such as proliferation and prostacyclin production (Haller et al., 1996; Xia et al., 1996; Gliki et al., 2001). We also confirmed the existence of PKC-α and PKC-δ in the human umbilical vein endothelial cells by Western blotting by the respective specific antibody (data not shown). Gö6976 was reported to inhibit cPKC isoforms such as PKC-α with an IC_{50} of < 10 nM, but not to inhibit PKC-δ (Martiny-Baron et al., 1993). Treatment with 1 μM Gö6976 significantly inhibited sphingosine 1-phosphate-induced increases in VCAM-1 and E-selectin mRNA levels and slightly inhibited the ICAM-1 mRNA level (Fig. 4C and Table 1). In the presence of 10 μM histamine, however, the treatment with 1 μM Gö6976 exhibited little and/or marginal effects on the sphingosine 1-phosphate-induced expression of these three molecules. The expression of E-selectin and ICAM-1 induced by sphingosine 1-phosphate plus histamine in the Gö6976-treated cells was similar to that in the control cells without Gö6976. Although the expression of VCAM-1 induced by sphingosine 1-phosphate plus histamine decreased in the Gö6976-treated cells (111% of control), the expression was still high as that in the sphingosine 1-phosphate-induced response in the con-

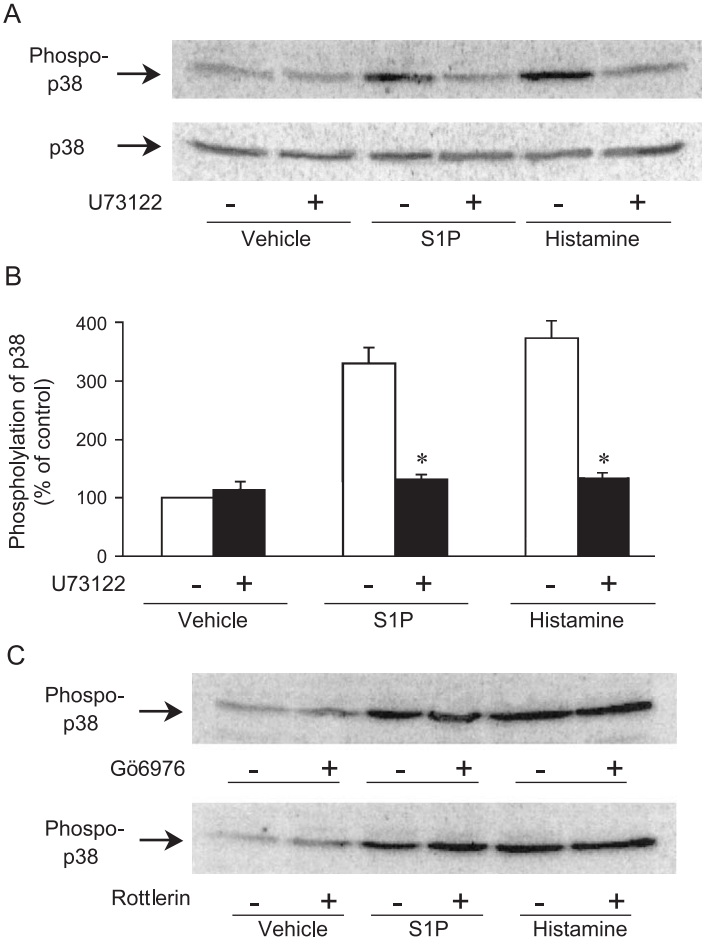

Fig. 5. Phosphoinositide-specific phospholipase C-dependent activation of p38 MAPK induced by sphingosine 1-phosphate and histamine, and no involvement of PKCs on p38 MAPK activation. Cells were cultured with vehicle or 10 μM U73122 for 10 min, and then the cells were stimulated with 10 μM sphingosine 1-phosphate (S1P) or 10 μM histamine. The cell lysates were analyzed by Western blotting using the respective antibodies. A typical and representative response is shown in Panel A. The results of the quantitative analyses of the phosphorylation of p38 MAPK are shown in Panel B. Values are calculated as percentages of the density in the control cells without stimulants, and are means ± S.E.M. for three independent experiments. In Panel C, cells were cultured with 1 μM Gö6976 or 1 μM rottlerin for 10 min, and then the cells were stimulated with 10 μM sphingosine 1-phosphate or 10 μM histamine. A typical and representative response is shown ($n = 3$). *P<0.05, significantly different from the value without U73122.

trol cells. The hold-stimulations by 10 μM histamine on the sphingosine 1-phosphate response in the Gö6976-treated cells were about 2.2–4.6-fold, which were higher than those in the control cells. Treatment with calphostin C, a classical inhibitor of cPKCs/nPKCs, at 10 nM showed similar results; sphingosine 1-phosphate-induced expression of E-selectin in the calphostin C-treated cells decreased to 51 ± 3% ($n = 3$, $P < 0.05$) of that in the control cells, but sphingosine 1-phosphate plus histamine-induced expression of E-selectin in the calphostin C-treated cells was 186 ± 12% ($n = 3$), which was similar to the value (226 ± 13%) in the vehicle-treated cells. The simultaneous treatment with 10 μM SB203580 and 1 μM Gö6976 did not show an additive inhibitory effect on sphingosine 1-phosphate-induced expression of VCAM-1 and E-selectin; the values were 56.2 ± 7.2% and 43.3 ± 4.2% ($n = 3$), respectively.

Next we investigated the effect of rottlerin, a selective inhibitor of PKC-δ (Gschwendt et al., 1994; Lu et al., 1998; Ghelli et al., 2002). Rahman et al. (2001) reported that treatments of human umbilical vein endothelial cells with 5 and 10 μM rottlerin prevented thrombin-induced PKC-δ activity markedly and almost completely, respectively. In our preliminary experiments, treatment of human umbilical vein endothelial cells with 1 μM rottlerin slightly (about 20%) inhibited the PKC-δ activity (data not shown). Treatment with 1 μM rottlerin did not inhibit increases in VCAM-1 and ICAM-1 mRNA levels induced by 10 μM sphingosine 1-phosphate (Fig. 4D). However, the stimulatory effects by 10 μM histamine on sphingosine 1-phosphate-induced VCAM-1 and ICAM-1 expression were significantly inhibited in the rottlerin-treated cells. The expression of E-selectin induced by 10 μM sphingosine 1-phosphate was enhanced in the rottlerin-treated cells, although the reasons are not clear at present. The treatment with rottlerin without stimulants showed no effect on the expression of the three adhesion molecules.

PKC inhibitors at high concentrations were not specific for the PKC isoforms. Treatment with 5 μM rottlerin markedly inhibited the 10 μM sphingosine 1-phosphate-induced increase in the mRNAs levels of the three adhesion molecules; for example, the VCAM-1 mRNA levels in the absence and presence of 10 μM histamine were 18 ± 4% and 27 ± 18% of the control, respectively ($n = 3$). Treatment with 10 μM Gö6976, 100 nM calphostin C or 10 μM GF109203X, which were known to inhibit both cPKCs and nPKCs at high concentrations, almost completely inhibited the 10 μM sphingosine 1-phosphate-induced expression of VCAM-1 and E-selectin with or without 10 μM histamine (data not shown). In the human umbilical vein endothelial cells treated with 200 nM PMA for 24 h, which was reported to delete protein levels of cPKCs and nPKCs and to abolish the PMA response on ICAM-1 expression in endothelial cells (Tamaru and Narumi, 1999; Rahman et al., 1999, 2000), the expression of these three adhesion molecules induced by 10 μM sphingosine 1-phosphate with and without 10 μM histamine were almost completely abolished (data not shown).

3.4. Inhibitory effect of U73122, but not GÖ6976 and rottlerin, on phosphorylation of p38 MAPK induced by sphingosine 1-phosphate and histamine

The addition of 10 μM sphingosine 1-phosphate and 10 μM histamine alone caused phosphorylation of p38 MAPK in human umbilical vein endothelial cells (Fig. 5). The sphingosine 1-phosphate response was abolished in the pertussis toxin-treated endothelial cells (data not shown), as reported previously (Lee et al., 1999a; Kimura et al., 2000). Histamine-induced phosphorylation of p38 MAPK was slightly (20–40%) inhibited by pertussis toxin. Although the phosphorylation of p38 MAPK induced by sphingosine 1-phosphate and histamine were abolished in the 10 μM U73122-treated cells (Fig. 5B), the responses were not inhibited in the 1 μM Gö6976- and 1 μM rottlerin-treated cells (Fig. 5C). Treatment with 10 μM Gö6976 and 5 μM rottlerin did not inhibit the phosphorylation of p38 MAPK induced by sphingosine 1-phosphate and histamine (data not shown, $n = 2$).

3.5. NF-κB-dependent expression of adhesion molecules induced by sphingosine 1-phosphate and histamine

Treatment with 5 μM BAY117082 (an inhibitor of nuclear factor-κB (NF-κB)) selectively suppressed NF-κB activation in cultured vascular endothelial cells (Ohkita et al., 2002). Treatment with 5 μM BAY117082 completely inhibited the increases in the mRNA levels of VCAM-1 and E-selectin induced by 10 μM S1P with and without 10 μM histamine (Fig. 6). The expressions of ICAM-1 mRNA induced by sphingosine 1-phosphate with and without histamine were also inhibited by BAY117082.

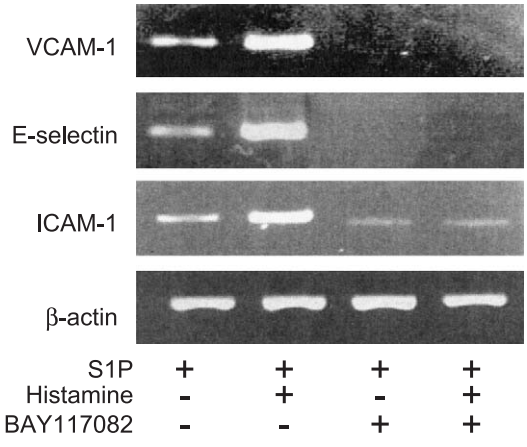

Fig. 6. Effect of BAY117082 on sphingosine 1-phosphate- and histamine-induced expression of adhesion molecules. Cells were cultured with vehicle or 5 μM BAY117082 for 1 h, and then the cells were cultured with 10 μM sphingosine 1-phosphate (S1P) with or without 10 μM histamine for 4 h. Total RNA was isolated from the respective cells and used for RT-PCR analysis. A typical and representative response is shown ($n = 3$).

4. Discussion

4.1. PKC-dependent expression of VCAM-1, E-selectin and ICAM-1 by sphingosine 1-phosphate and histamine H_1 receptor activation in human umbilical vein endothelial cells

We confirmed that sphingosine 1-phosphate increased expression of VCAM-1 and E-selectin in human umbilical vein endothelial cells, as previously reported (Xia et al., 1998). In addition, we found that sphingosine 1-phosphate stimulated ICAM-1 expression. The expression of three adhesion molecules induced by sphingosine 1-phosphate decreased markedly in the pertussis toxin-, U73122 (phosphoinositide-specific phospholipase C inhibitor)-, SB203580 (p38 MAPK inhibitor)- and 1 μM Gö6976 (PKC-α inhibitor)-treated cells, respectively. Since EDG-1 couples with the pertussis toxin-sensitive G proteins and the activation of phospholipase C (Pyne and Pyne, 2000; Panetti, 2002), the sphingosine 1-phosphate responses (p38 MAPK activation and expression of adhesion molecules) appeared to be mediated via receptors probably EDG-1 in endothelial cells. Activation of histamine H_1 receptors, which showed no effect alone, enhanced the sphingosine 1-phosphate-induced expression of adhesion molecules about 2-fold in endothelial cells. The stimulatory effect of histamine H_1 receptor activation was inhibited by U73122 and 1 μM rottlerin (PKC-δ inhibitor), but not by SB203580 or Gö6976.

cPKCs and/or nPKCs were implicated in the expression of adhesion molecules in endothelial cells (May et al., 1996; Tamaru and Narumi, 1999; Kim et al., 2001; Minami et al., 2003). In the present study, the sphingosine 1-phosphate-induced expressions of VCAM-1, E-selection and ICAM-1 were markedly inhibited by 1 μM Gö6976 and by a low concentration of calphostin C (10 nM), but not by 1 μM rottlerin. Rottlerin at 1 μM inhibited the histamine response without changing the sphingosine 1-phosphate response, and the stimulatory effect of histamine (about 2–4-fold stimulation) on sphingosine 1-phosphate-induced expression of the molecules was observed in the presence of 1 μM Gö6976. In endothelial cells such as human umbilical vein endothelial cells, the TNF-α- and interleukin-1β-induced expression of the adhesion molecules were not regulated by cPKCs and nPKCs (Tamaru and Narumi, 1999; Rahman et al., 1999, 2000). Rahman et al. (1999, 2000) reported the involvement of PMA-insensitive PKC-ζ on the expression of ICAM-1 by TNF-α, and Minami et al. (2003) reported thrombin-induced expression of VCAM-1 was regulated by PKC-ζ. In the case of sphingosine 1-phosphate and/or histamine, however, aPKCs such as PKC-ζ did not appear to regulate the expression of adhesion molecules in human umbilical vein endothelial cells, because the response was almost completely inhibited by the tested cPKCs/nPKCs inhibitors at higher concentrations and in the cPKCs/nPKCs-depleted cells. These findings suggest (1) the cPKCs and nPKCs, but not aPKCs, regulate the expression of adhesion molecules induced by sphingosine 1-phosphate and histamine, and (2) the sphingosine 1-phosphate and histamine response were preferentially mediated by PKC-α and PKC-δ, respectively.

4.2. Role of p38 MAPK on the expression of adhesion molecules in human umbilical vein endothelial cells

p38 MAPK is involved in the expression of adhesion molecules induced by several stimulants in the endothelial cells (Read et al., 1997; Rahman et al., 2001). In the present study, treatment with SB203580 significantly inhibited sphingosine 1-phosphate-induced expression of adhesion molecules in human umbilical vein endothelial cells. Activation of p38 MAPK does not appear to be a sufficient pathway for the expression of adhesion molecules in endothelial cells, although the activation is necessary. Firstly, histamine stimulated phosphorylation of p38 MAPK very similar to sphingosine 1-phosphate, although histamine alone did not induce the expression of the adhesion molecules. Secondly, the hold-stimulation by histamine on the sphingosine 1-phosphate response in the presence of SB203580 was similar to that in the absence of SB203580. In addition, the inhibitory effect by SB203580 (10 μM) on the sphingosine 1-phosphate-induced expression of VCAM-1 and the E-selectin response was partial. It has been well established that NF-κB regulates the expression of VCAM-1, E-selectin and ICAM-1 in endothelial cells including human umbilical vein endothelial cells (De Martin et al., 2000; Kokura et al., 2002). In the present study, inhibition of NF-κB by BAY117082 almost completely abolished the expression of the molecules induced by sphingosine 1-phosphate with and without histamine. p38 MAPK was reported to increase NF-κB activity by the transactivation machinery (Berghe et al., 1998). In human umbilical vein endothelial cells, inhibition of p38 MAPK decreased the thrombin-induced NF-κB activity and the expression of ICAM-1 without the DNA binding activity of NF-κB (Rahman et al., 2001). The present and previous findings suggest that the p38 MAPK pathway may act as a necessary cooperative, not a sufficient, mechanism to regulate the expression of adhesion molecules in human umbilical vein endothelial cells. The involvement of nuclear factor of activated T cells (NFAT) on the expression of adhesion molecules was reported in endothelial cells (Jeannin et al., 1994). Thus, other nuclear factors also may be regulated by p38 MAPK activation in a cooperative manner with NF-κB.

4.3. Role of PKC-δ on histamine-induced expression of adhesion molecules

In the present study, the stimulatory effect of histamine H_1 receptor activation on the expression of adhesion molecules was inhibited by a PKC-δ inhibitor (1 μM rottlerin). Histamine H_1 receptor mainly couples with pertussis toxin-insensitive G proteins such as G_q in the endothelial cells (Hill et al., 1997; Muraki and Imaizumi,

2001), and the phosphorylation of p38 MAPK via phosphoinositide specific-phospholipase C activation induced by histamine was not significantly inhibited by pertussis toxin in human umbilical vein endothelial cells. It was reported that thrombin-induced ICAM-1 expression (Rahman et al., 2001, 2002) and VCAM-1 expression (Minami et al., 2003) were mediated by PKC-δ in human umbilical vein endothelial cells. Thus, G_q- and PKC-δ-dependent processes appear to be involved in the regulation of adhesion molecules induced by histamine H_1 receptor activation in human umbilical vein endothelial cells. Rahman et al. (2001) suggested that thrombin-induced ICAM-1 expression was mediated by p38 MAPK via PKC-δ activation, because 5 μM rottlerin inhibited thrombin-induced phosphorylation of p38 MAPK in human umbilical vein endothelial cells. In addition, they showed that the transfection of the activated form of $G_{q\alpha}$ stimulated the NF-κB activity via PKC-δ activation (Rahman et al., 2002). In the present study, however, activation of p38 MAPK induced by histamine was a downstream event after phosphoinositide-specific phospholipase C activation, but not after activation of PKC-α and PKC-δ. The stimulatory effect of histamine on sphingosine 1-phosphate-induced expression of adhesion molecules was marked even in the cells treated with SB203580. The PKC-δ-dependent pathway induced by histamine H_1 receptor activation enhances sphingosine 1-phosphate-induced expression of adhesion molecules independently from the p38 MAPK pathway in phosphoinositide-specific phospholipase C.

4.4. Summary and problems

In the present study, we showed that sphingosine 1-phosphate-induced expression of adhesion molecules was mediated by phosphoinositide-specific phospholipase C and preferentially by PKC-α and p38 MAPK, and the histamine response was mediated by phosphoinositide-specific phospholipase C and PKC-δ in human umbilical vein endothelial cells by using the respective inhibitors. Although the chemical inhibitors are extremely useful in helping to clear the physiological roles of the enzymes, we should be careful about the selectivity of the tested inhibitors. Davies et al. (2000) showed that the specificity of inhibitors cannot be assessed simply by studying their effects on cell responses. The identification using the molecular biological techniques such as overexpression of dominant-negative protein kinases will be an important next step.

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