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European Journal of Pharmacology 312 (1996) 367–377

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Thromboxane A_2 receptor blockade suppresses intercellular adhesion molecule-1 expression by stimulated vascular endothelial cells

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Received 11 April 1996; revised 14 June 1996; accepted 18 June 1996

Abstract

Inhibition of the thromboxane A_2-synthesizing enzyme (DP-1904; [±]-6-[1-imidazolylmethyl]-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid hydrochloride hemihydrate) reportedly suppresses intercellular adhesion molecule-1 (ICAM-1) expression on the surface of stimulated vascular endothelial cells (Ishizuka et al., 1994, Eur. J. Pharmacol. 262, 113). In the present study, thromboxane A_2 receptor antagonists suppressed the expression of ICAM-1 on the surface of human vascular endothelial cells that were stimulated by tumor necrosis factor α (TNFα), platelet activating factor (PAF), or U46619 (9,11-dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$). Augmentation of ICAM-1 expression on human vascular endothelial cells stimulated by U46619 was suppressed by protein kinase C inhibitors. Thromboxane A_2 receptor antagonist suppressed U46619 stimulation of protein kinase C activity of a cell membrane fraction. These results indicate that in human vascular endothelial cells, thromboxane A_2, the production and secretion of which is stimulated by TNFα or PAF, binds to the thromboxane A_2 receptors on cell membranes and augments ICAM-1 expression on the cell surfaces mainly through protein kinase C.

Keywords: Vascular endothelial cell, human; Thromboxane A_2 receptor; ICAM-1 (intercellular adhesion molecule-1); Protein kinase C

1. Introduction

Enhanced expression of thromboxane A_2 in human vascular endothelial cells is closely related to the expression of ICAM-1 on the surface of these cells (Ishizuka et al., 1994). In inflammatory kidney diseases, enhanced thromboxane A_2 production has been seen in the kidney and kidney glomeruli (Lianos et al., 1983; Stahl et al., 1987). Intercellular adhesion molecule-1 (ICAM-1) is expressed prominently in various cells at sites of inflammation (Simmons et al., 1988) and its expression by vascular endothelial cells is markedly potentiated by inflammatory cytokines (Dustin et al., 1986; Pober et al., 1986).

It has been suggested that the thromboxane A_2 receptor in platelets is coupled to a guanine nucleotide binding protein (G protein) that activates phospholipase C and protein kinase C (Brass et al., 1987). It was reported recently that the receptor is present in the vascular endothelial cells of a rabbit coronary artery or rat aorta (Kanmura et al., 1987; Hanasaki et al., 1988). Protein kinase C is involved in the interleukin-1- or tumor necrosis factor (TNF)-stimulated expression of ICAM-1 on human vascular endothelial cells (Lane et al., 1990). Inhibition of the thromboxane A_2-synthesizing enzyme suppresses ICAM-1 expression on the surface of TNFα- or platelet activating factor (PAF)-stimulated vascular endothelial cells (Ishizuka et al., 1994). TNFα augments protein kinase C activity, thromboxane A_2 synthesis, and ICAM-1 expression in human vascular endothelial cells (Lane et al., 1990; Ishizuka et al., 1994). However, it is still uncertain whether or not thromboxane A_2 stimulates ICAM-1 expression on human vascular endothelial cells through the action of protein kinase C.

The present study is designed to verify the presence of thromboxane A_2 receptors in human vascular endothelial cells. Given their presence, the subsequent purposes are then: (1) to stimulate the vascular endothelial cells with TNFα or PAF and observe the effect on thromboxane A_2 receptor expression; (2) to evaluate the effect of thromboxane A_2 receptor antagonists on ICAM-1 expression on the surface of human vascular endothelial cells; and (3) to

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PII S0014-2999(96)00478-5

determine whether the intracellular signal transmission that culminates in ICAM-1 expression on the cell surface involves protein kinase A or protein kinase C, following stimulation of thromboxane A_2 synthesis in vascular endothelial cells.

2. Materials and methods

2.1. Reagents

Recombinant human TNFα was provided by Teijin (Tokyo, Japan). Human PAF was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 8-Bromoadenosine 3′:5′-cyclic monophosphate (8-bromo-cAMP), *N*6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cAMP), and phorbol 12-myristate 13-acetate (PMA) were all purchased from Sigma Chemical (St. Louis, MO, USA). H-7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine dihydrochloride), staurosporine, H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), and HA1004 (*N*-[2-guanidinoethyl]-5-isoquinolinesulfonamide hydrochloride) were purchased from Seikagaku Co. (Tokyo, Japan). SQ29,548 (7-[3-[[2-[(phenylamino)-carbonyl]-hydrazino]methyl]-7-axabicyclo[2.2.1]hept-2-yl], [1*S*(1a,2a,(*Z*),3a,4a)]-5-heptenoic acid), an antagonist of thromboxane A_2 receptor, was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). AA-2414 ([±]-7-(3,5,6-trimethyl-1,4-benzoquinon-2-yl)-7-phenylheptanoic acid), an another antagonist of thromboxane A_2 receptor, was provided by Takeda Chemical Industries (Osaka, Japan). U46619 (9,11-dideoxy-9a,11a-epoxymethanoprostagrandin $F_{2\alpha}$), an agonist of thromboxane A_2 receptor, was also purchased from Cayman Chemical Co.

2.2. Vascular endothelial cell cultures

Human vascular endothelial cells, collected from human umbilical cord veins by Jaffe's method (Jaffe et al., 1973), were cultured according to procedures described earlier (Ishizuka et al., 1994). The human vascular endothelial cells used in this experiment were between their 1st and 3rd passages. When cultures reached confluence, the cells were re-plated at a density of 10^6 cells/dish. The dishes were then washed with a phosphate-buffered saline (PBS). To these cells, 2 ml of Medium 199 with or without specific inclusions (100 U/ml TNFα; 10^{-7} M PAF; 10^{-6} M U46619; 10 or 100 μM 8-bromo-cAMP; 10 or 100 μM dibutyryl cAMP; 10^{-7} M PMA) were added for 48 h. In some cases, AA-2414, SQ29,548, H-7, staurosporine, H-89, and HA1004 were added 15 min prior to the treatment with TNFα, PAF, or U46619 (Ritchie et al., 1991). Cell number was assessed in a hemocytometer (Erma, Tokyo, Japan). Cellular viability was assessed by colorimetric assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (Chemicon International, Temecula, CA) (Mosmann, 1983).

2.3. Thromboxane A_2 receptor binding assay

For a binding assay, confluent human vascular endothelial cells were washed twice with PBS and then harvested using PBS containing 0.05% etylenediamine-tetraacetic acid (EDTA). After washing twice with PBS, the cells were suspended with Medium 199 containing 0.1% bovine serum albumin (a binding medium). The binding study was performed by incubating the cells with [^{3}H]SQ29,548 (1.11 Tbq/mmol; NEN Research Products, Boston, MA, USA) in a 0.5 ml binding medium at 24°C for 40 min, unless otherwise stated. Specific binding is defined as the difference between binding in the presence or absence of 10 μM of unlabeled SQ29,548. After incubation, ice-cold saline (3 ml) was added and the reaction mixture was immediately filtered by suction through a glass-microfiber filter which was then washed 4 times with cold saline. The radioactivity was measured in a liquid-scintillation counter (LSC-5100, Aloka, Tokyo, Japan). The receptor site and affinity were analyzed by a Scatchard plot analysis.

2.4. Immunofluorescence staining and analysis

Immunofluorescence staining and analysis procedures were described previously (Ishizuka et al., 1994). Briefly, cells were harvested and washed in PBS containing 1% fetal calf serum. Human γ-globulin (Teijin) (10 mg/ml) was added to cultures to block nonspecific binding, after which 1 μg/ml of mouse anti-ICAM-1 monoclonal antibody (British Biotechnology, Oxon, UK) was added and the cells were incubated at 4°C for 80 min. After washing, they were combined with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (Tago, Burlingame, CA, USA) and allowed to react at 4°C for 80 min. They were quantitatively analyzed with a Cytoron (Ortho Diagnostic Systems, Tokyo, Japan). The results are expressed as the percentage of cells with a fluorescence intensity exceeding the threshold obtained with nonspecific mouse IgG, after adjusting the gating scale of the histogram to keep the percentage of positive cells in the negative control below 1%.

2.5. Assays of protein kinase C activity

Cells were cultured in 60 × 15 mm dishes in Medium 199 containing 20% fetal calf serum. When cultures reached confluence, the medium was replaced with fetal calf serum-free Medium 199 containing 0.1% bovine serum albumin and either TNFα, PAF, or U46619 was added; the cells were incubated further for 1–2 h. After the medium was discarded, the cells were washed with ice-cold PBS. The cells were scraped with a rubber policeman with 1 ml of cold extraction buffer (20 mM Tris-HCl: pH 7.5, containing 250 mM sucrose, 10 mM EGTA, 5 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, and 0.3% v/v β-mercaptoethanol), sonicated for 30 s, and then centrifuged at 100 000 × *g* for 1 h

at 4°C. Supernatants were collected and designated as the cytosolic fraction. Pellets were resonicated in an extraction buffer containing 1% Nonidet P-40 (NP-40) for 20 s. After a 15-min extraction period with occasional vortexing, the detergent-solubilized membranes were again centrifuged at 100 000 × g for 1 h. Supernatants thus obtained were designated as the membrane fraction. Separate aliquots from the supernatant (the cytosolic fraction) and the resonicated pellet (the membrane fraction) were assayed for protein kinase C activity by a protein kinase C enzyme assay kit (Amersham International; Amersham, UK; House et al., 1987). Data are expressed as protein kinase C specific activity (pmol transferred phosphate/min/mg of total protein).

2.6. Assays of protein kinase A activity

When the cells reached confluence, the medium was replaced with fetal calf serum-free Medium 199 containing 0.1% bovine serum albumin, and either TNFα, PAF, or U46619 was added; the cells were incubated further for 1–2 h. The cells were washed three times in ice-cold PBS (pH 7.4) and suspended in a homogenization buffer (5 mM Tris-HCl (pH 7.2), 250 mM sucrose, 1 mM PMSF, 0.1 mM dithiothreitol, 1 mM EGTA). The cells were disrupted by sonication (60 Hz, 2 cycles for 15 s each). Crude homogenates were centrifuged at 750 × *g* for 10 min at 4°C to remove nuclei. The cell pellets were discarded and the supernatants were aliquoted and kept frozen at −70°C until assayed.

Total cellular protein kinase A phosphotransferase activity was quantified by measuring the transfer of [^{32}P]ATP to Kemptide, as described (Roskoski and Roskoski, 1987; Hasler et al., 1992). The reaction mixture contained 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM EGTA, 100 μM ATP, 100 μM kemptide, 50 μM [^{32}P]ATP (222 Tbq/mmol; NEN Research Products), and 10 μM cyclic AMP. Phosphotransferase activity was determined by subtracting cpm in the presence of protein kinase A inhibitor H-89 from cpm in the absence of H-89. Results were expressed as pmol transferred phosphate/min/mg of total protein. The protein content of protein kinase C or A in the dishes was determined by the standard procedures (Lowry et al., 1951; Bradford, 1976), with bovine serum albumin as the standard.

2.7. Determination of protein synthesis in cultured endothelial cells

The effects of AA-2414 or SQ29,548 on protein synthesis in cultured endothelial cells were measured by [^{35}S]methionine incorporation. The cells were plated at a density of 10^6 cells/dish and incubated in 5% CO_2 at 37°C for 24 h. The medium was removed and 1.5 ml/dish of methionine-free medium was added. AA-2414 (10^{-9}, 10^{-8}, or 10^{-7} M), SQ29,548 (10^{-7}, 10^{-6}, or 10^{-5} M) and cycloheximide (10 μg/ml) were added together prior to incubation. [^{35}S]Methionine (37 Tbq/mmol; Amersham International) was then added to cultures at a final concentration of 1.3 Gbq/ml, and the cells were incubated for an

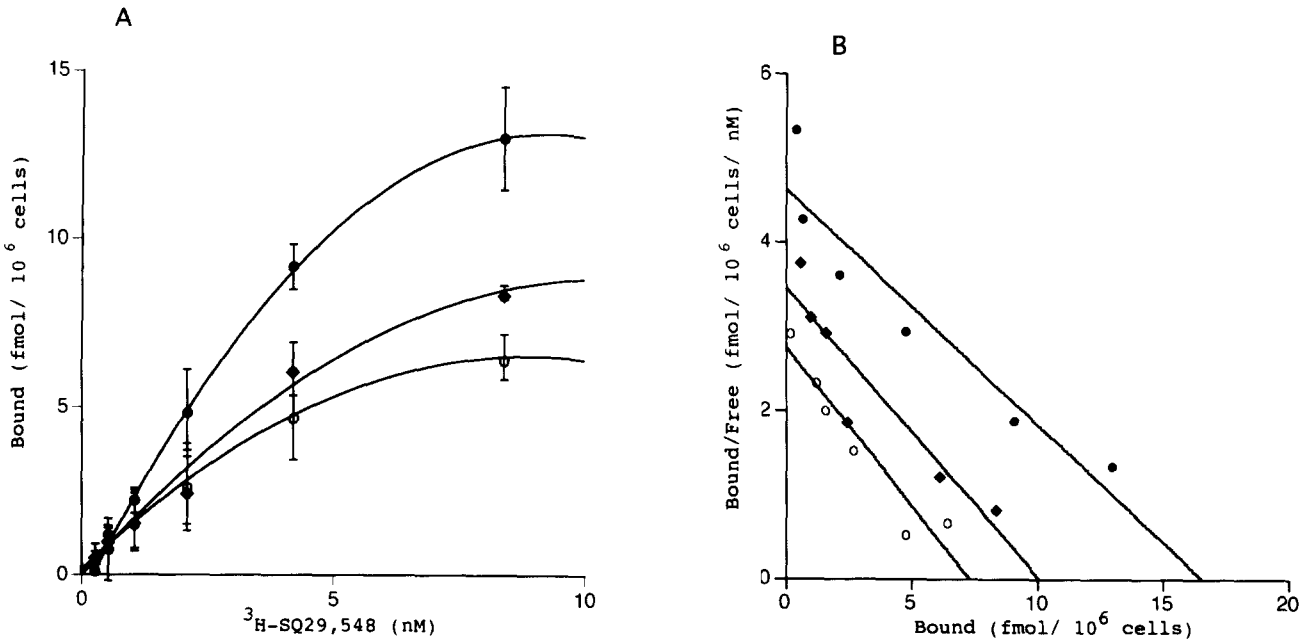
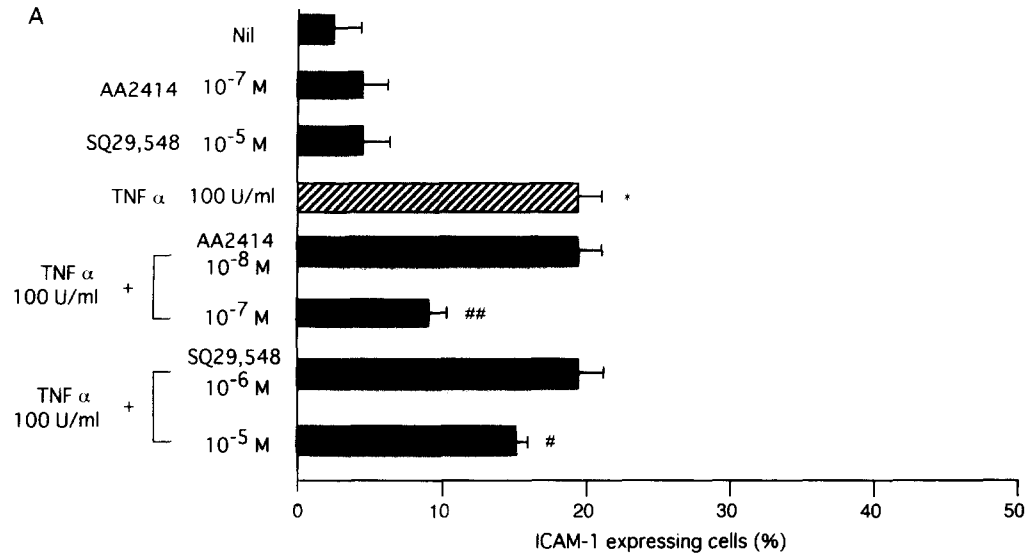


Fig. 1. Saturation binding of [^{3}H]SQ29,548 to human vascular endothelial cells. Monolayers of confluent human vascular endothelial cells (5 × 10^6 cells) were not stimulated (○), or stimulated by 100 U/ml recombinant TNFα (●) or 10^{-7} M PAF (◆) for 1 h and incubated in the presence of increasing concentrations of [^{3}H]SQ29,548 (0.27, 0.53, 1.05, 2.1, 4.2, and 8.4 nM) for 40 min at 24°C. (A) Specific binding of [^{3}H]SQ29,548. (B) Scatchard plot of the specific binding data from (A). Each point represents the mean value of triplicate determinations for three experiments.


A
Nil
AA2414 10^{-7} M
SQ29,548 10^{-5} M
TNF α 100 U/ml
TNF α 100 U/ml + AA2414 10^{-8} M
10^{-7} M
TNF α 100 U/ml + SQ29,548 10^{-6} M
10^{-5} M
*
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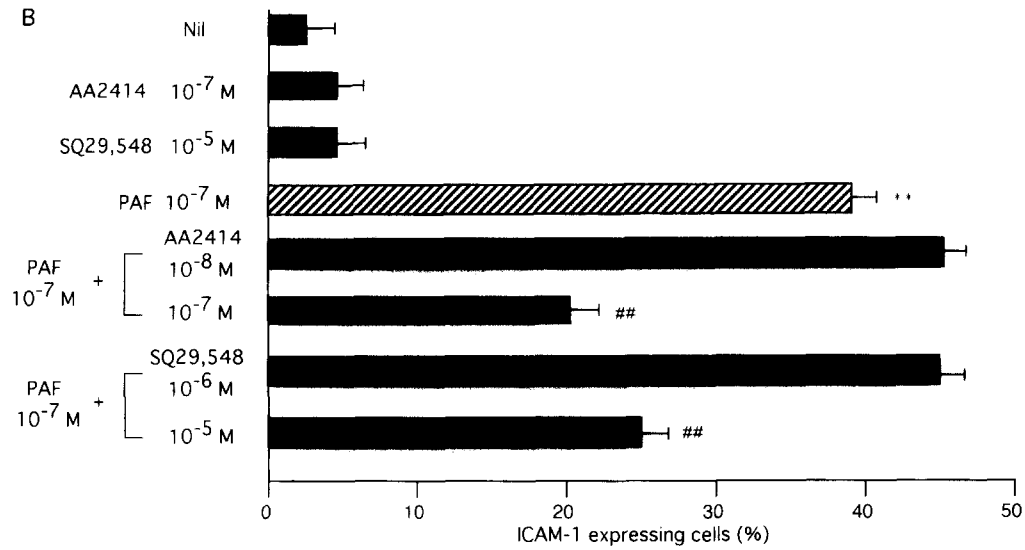
#
0 10 20 30 40 50
ICAM-1 expressing cells (%)


B
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AA2414 10^{-7} M
SQ29,548 10^{-5} M
PAF 10^{-7} M
PAF 10^{-7} M + AA2414 10^{-8} M
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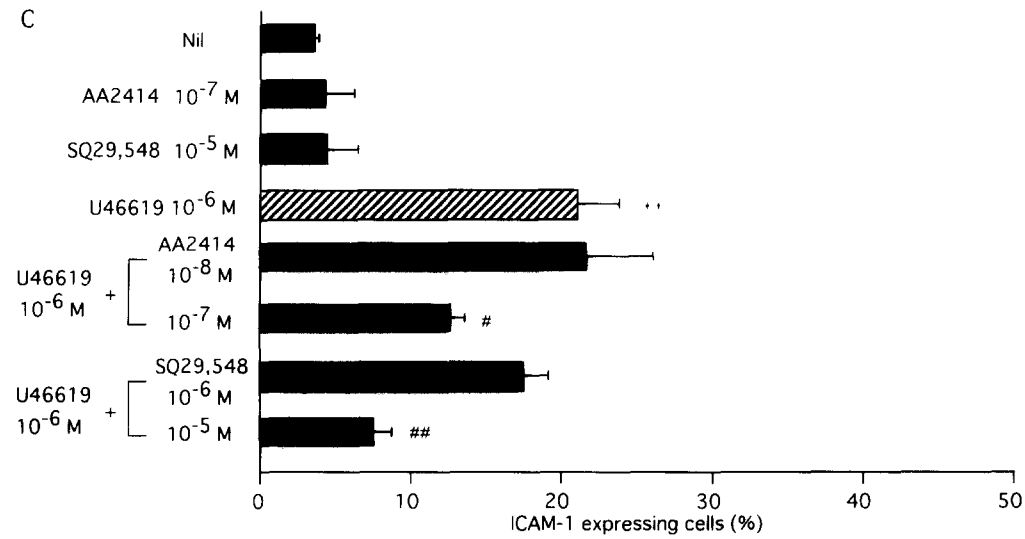
0 10 20 30 40 50
ICAM-1 expressing cells (%)


C
Nil
AA2414 10^{-7} M
SQ29,548 10^{-5} M
U46619 10^{-6} M
U46619 10^{-6} M + AA2414 10^{-8} M
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U46619 10^{-6} M + SQ29,548 10^{-6} M
10^{-5} M
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#
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0 10 20 30 40 50
ICAM-1 expressing cells (%)

Table 1
Parameters describing the specific binding of [^{3}H]SQ29,548 to unstimulated or stimulated human vascular endothelial cells

	K_d (nM)	B_{max} (fmol/10^6 cells)	Thromboxane A_2 receptor concentration (sites/cell)
Nil	2.6 ± 0.6	7.3 ± 0.9	4500 ± 590
PAF 10^{-7} M	2.9 ± 0.6	10.1 ± 0.6 [a]	6300 ± 340 [a]
PAF 10^{-7} M + H-89 20 μM	2.5 ± 0.8	12.6 ± 1.6	6700 ± 360
PAF 10^{-7} M + staurosporine 100 nM	3.1 ± 0.6	10.1 ± 0.6	6700 ± 400
TNFα 100 U/ml	3.6 ± 0.9	16.6 ± 1.4 [b]	10300 ± 900 [b]
TNFα 100 U/ml + H-89 20 μM	3.6 ± 0.1	15.7 ± 1.6	9500 ± 1100
TNFα 100 U/ml + staurosporine 100 nM	3.8 ± 0.9	15.0 ± 1.6	9800 ± 860

These data are means ± S.D. of three separate experiments. [a] $P < 0.05$ as compared to those without stimulation. [b] $P < 0.01$ as compared to those without stimulation.

additional 48 h under these conditions. The cell layers were then solubilized at 4°C with a lysis buffer (containing 0.5% Triton X-100, 0.25% deoxycholic acid, 10 mM EDTA, 1 mM PMSF, and 50 mM Tris-HCl, pH 8.5). Trichloroacetic acid (10%) was added to cell lysates. After a 20 min incubation on ice, the cell lysates were collected on glass-microfiber filters on a vacuum manifold and washed three times with 5% trichloroacetic acid and then once with ethanol. Filters were transferred to a liquid scintillation cocktail (NEN Research Products) and counted with a liquid scintillation analyzer (Aloka).

2.8. Determination of thromboxane A_2 synthesis in cultured endothelial cells

The capacity of the cultured vascular endothelial cells to synthesize thromboxane A_2 was evaluated by determining the concentrations of thromboxane B_2 in the culture supernatant, using the respective ELISA Kits (Cayman Chemical). The eicosanoid that had been linked to acetylcholinesterase in the sample was allowed to react with the rabbit antiserum. Next, the antigen-antibody complex was then allowed to react with the mouse monoclonal anti-rabbit IgG antibody, which was in a solid phase in the wells. A color developed in the substrate solution, and the absorption of light at 412 nm was measured with the EIA reader (SLT Labinstruments, Vienna, Austria). The concentration of thromboxane A_2 was determined by interpolation from a standard curve.

2.9. Statistical analysis

All results are expressed as means ± S.D. Changes with respect to basal values when only two observations were performed were analyzed by a paired or unpaired Student's *t*-test. Comparisons between means of multiple groups were analyzed by one-way analysis of variance and Scheffé's multiple comparisons test. A P value equal to or less than 0.05 was regarded as significant.

3. Results

3.1. Equilibrium binding assay of [^{3}H]SQ29,548 to unstimulated or stimulated human vascular endothelial cells

To establish the presence and determine the number of thromboxane A_2 receptors, and measure the affinity of thromboxane A_2 to the surface of human vascular endothelial cells, a [^{3}H]SQ29,548 binding assay was performed. Binding occurred, as shown in the results of a representative binding experiment (Fig. 1). A plot of specific bound counts indicates that the binding was dose-dependent and saturable (Fig. 1A), and nonspecific binding constituted less than 5% of the total counts. The data of a Scatchard plot (Fig. 1B) were best fit by a single site model. The number of thromboxane A_2 receptor sites (mean ± S.D.) per cell was 4500 ± 590 with an equilibrium dissociation constant (K_d) of 2.6 ± 0.6 nM on unstimulated endothelial cells. It was found that stimulation with TNFα or PAF results in a significant increase in the number of thromboxane A_2 receptors on the surface of human vascular endothelial cells in comparison with unstimulated cells, but there was no significant difference in the binding affinity (Table 1). Prior addition of a protein kinase A inhibitor, H-89, or a protein kinase C inhibitor, staurosporine, could not significantly suppress the augmentation of the number of thromboxane A_2 receptors by TNFα or PAF.

Fig. 2. Effect of AA2414 or SQ29,548 on the expression of ICAM-1 on the surface of human vascular endothelial cells. AA2414 (10^{-8} or 10^{-7} M) or SQ29,548 (10^{-6} or 10^{-5} M) was added to monolayers of confluent human vascular endothelial cells 15 min before the addition of 100 U/ml TNFα (A), 10^{-7} M PAF (B), or 10^{-6} M U46619 (C). Forty-eight hours after the addition, the percentage of ICAM-1 expressing cells was determined as detailed in Materials and methods. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). Results are expressed as the means ± S.D. of three experiments. * $P < 0.05$, ** $P < 0.01$ as compared to those without stimulation. # $P < 0.05$, ## $P < 0.01$ as compared to those stimulated TNFα, PAF, or U46619.

Table 2
Effect of AA-2414 or SQ29,548 on protein synthesis of human vascular endothelial cells

	[^{35}S]methionine counts [a] (dpm/μg protein)
Nil	3138.0 ± 147.0
AA-2414 10^{-9} M	3405.0 ± 240.0
AA-2414 10^{-8} M	3465.0 ± 105.0
AA-2414 10^{-7} M	3150.0 ± 300.0
SQ29,548 10^{-7} M	3216.0 ± 96.0
SQ29,548 10^{-6} M	3552.0 ± 87.0
SQ29,548 10^{-5} M	3282.0 ± 384.0
Cycloheximide 10 μg/ml	387.0 ± 159.0 [b]

[a] Measured as [^{35}S]methionine incorporation, as described in Materials and methods. Monolayers of human vascular endothelial cells in methionine-free medium were incubated with AA-2414 (10^{-9} M, 10^{-8} M, or 10^{-7} M), SQ29,548 (10^{-9} M, 10^{-8} M, or 10^{-7} M), or 10 μg/ml cycloheximide. Controls consisted of monolayers of human vascular endothelial cells in methionine-free medium alone (Nil). The cells were incubated for 48 h. These data are means ± S.D. of three separate experiments. [b] $P < 0.01$ as compared to those without stimulation.

3.2. Effects of thromboxane A_2 receptor antagonists on the expression of ICAM-1 on the surface of human vascular endothelial cells

The doses of AA-2414 or SQ29,548 used in the present study did not affect cell counts or cellular viability in human vascular endothelial cells (data not shown). We then assessed the effect of the doses of AA-2414 or SQ29,548 on total protein synthesis. These concentrations of AA-2414 or SQ29,548 did not affect [^{35}S]methionine incorporation in human vascular endothelial cells (Table 2).

AA-2414 (10^{-9} M, 10^{-8} M, or 10^{-7} M) or SQ29,548 (10^{-7} M, 10^{-6} M, or 10^{-5} M) was added to human vascular endothelial cells prior to the addition of 100 U/ml of TNFα. Forty-eight hours after the addition, the enhancement of TNFα-stimulated ICAM-1 expression was significantly suppressed by 10^{-7} M of AA-2414 or 10^{-5} M of SQ29,548 (Fig. 2A).

AA-2414 (10^{-9} M, 10^{-8} M, or 10^{-7} M) or SQ29,548 (10^{-7} M, 10^{-6} M, or 10^{-5} M) was added to human vascular endothelial cells together with PAF at 10^{-7} M. After 48 h, potentiation of ICAM-1 expression by 10^{-7} M PAF was significantly suppressed by 10^{-7} M AA-2414 or 10^{-5} M SQ29,548 (Fig. 2B).

AA-2414 or SQ29,548 was added to human vascular endothelial cells together with U46619 at 10^{-6} M. After 48 h, potentiation of ICAM-1 expression of 10^{-6} M U46619 was significantly suppressed by 10^{-7} M AA-2414 or 10^{-5} M SQ29,548 (Fig. 2C).

3.3. Effects of TNFα, U46619, cyclic AMP analogues, and protein kinase C activator on the expression of ICAM-1 on the surface of human vascular endothelial cells

The expression of ICAM-1 on the surface of human vascular endothelial cells was 3.4 ± 2.5% without stimulation (Fig. 3). Forty-eight hours after the addition of 100 U/ml TNFα, 10^{-6} M U46619, or PMA, ICAM-1 expression was significantly ($P < 0.01$) intensified. When the human vascular endothelial cells were stimulated with

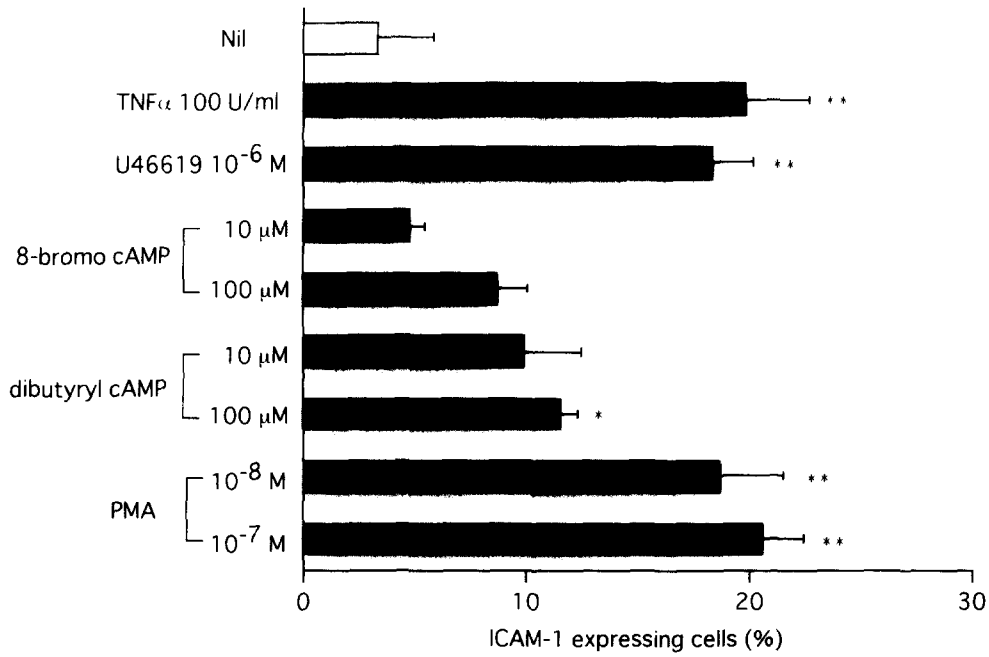

Fig. 3. Effects of cyclic AMP analogues and PMA on the expression of ICAM-1 on the surface of human vascular endothelial cells. Monolayers of confluent human vascular endothelial cells in Medium 199 were not stimulated (Nil), or stimulated by 10 or 100 μM 8-bromo cAMP, by 10 or 100 μM dibutyryl cAMP, or by 10^{-8} or 10^{-7} M PMA and incubated for 48 h. The percentage of ICAM-1-expressing cells was determined as detailed in Materials and methods. Results are expressed as the means ± S.D. of three experiments. ** $P < 0.01$ as compared to those without stimulation (open column).

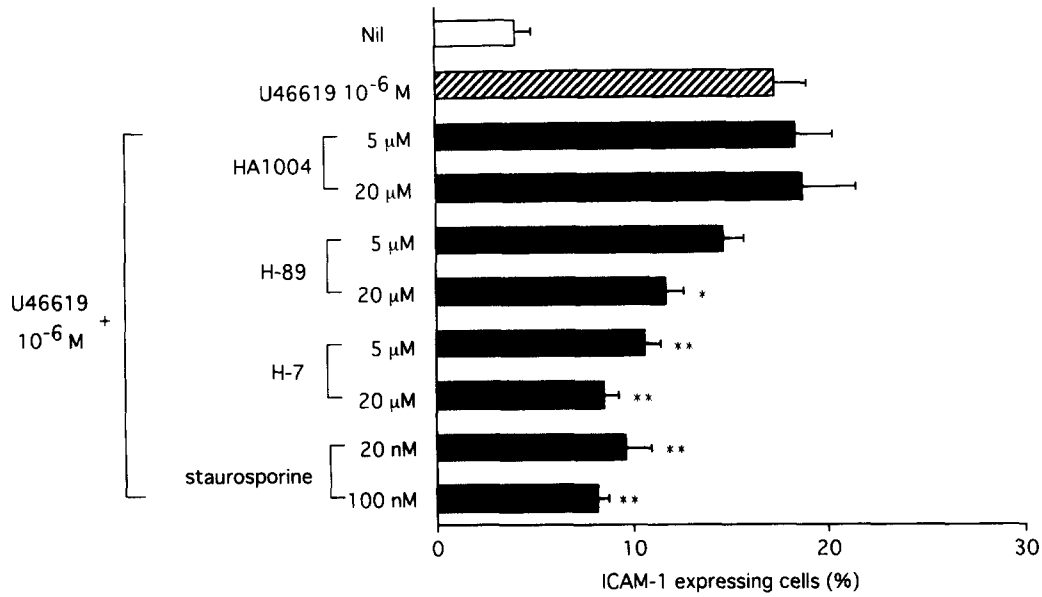

Fig. 4. Effects of HA1004, H-89, H-7, and staurosporine on the expression of ICAM-1 on the surface of human vascular endothelial cells. HA1004 (5 or 20 μM), H-89 (5 or 20 μM), H-7 (5 or 20 μM), or staurosporine (20 or 100 nM) was added to monolayers of confluent human vascular endothelial cells 15 min before the addition of 10^{-6} M U46619. Forty-eight hours after the addition, the percentage of ICAM-1-expressing cells was determined as detailed in Materials and methods. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). Results are expressed as the means ± S.D. of three experiments. * $P < 0.05$, ** $P < 0.01$ as compared to those stimulated by U46619 (hatched column).

8-bromo-cAMP, ICAM-1 expression was not significantly enhanced. However, stimulation with 100 μM dibutyryl cAMP, a potent cAMP analogue, resulted in a significant ($P < 0.05$) enhancement of ICAM-1 expression.

3.4. Effects of protein kinase inhibitors on the expression of ICAM-1 on the surface of human vascular endothelial cells

To determine whether or not the enhancement of ICAM-1 expression by a thromboxane A_2 receptor agonist is dependent on protein kinases, we evaluated the effect of protein kinase inhibitors on the ability of U46619 to augment ICAM-1 expression on human vascular endothelial cells. When H-7 or staurosporine, a protein kinase C inhibitor, was added prior to stimulation with 10^{-6} M U46619, augmentation of the expression of ICAM-1 on the surface of human vascular endothelial cells by U46619 was significantly suppressed. On the other hand, prior addition of HA1004, a protein kinase A inhibitor, did not significantly suppress the augmentation of ICAM-1 by U46619. The addition of H-89, a more potent and selective protein kinase A inhibitor, produced only a slightly suppressive effect (Fig. 4).

3.5. Effects of TNFα, PAF, SQ29,548, and U46619 on protein kinase C activity in human vascular endothelial cells

The protein kinase C activity in the cytosol fraction of human vascular endothelial cells was 345.0 ± 34.9 pmol/min/mg protein (Fig. 5A). The protein kinase C activity in the membrane fraction of the control human vascular endothelial cells was 34.9 ± 2.1 pmol/min/mg protein. Cells treated for 2 h with 100 U/ml TNFα showed a significant ($P < 0.01$) increase in membrane-associated protein kinase C activity. The prior addition of SQ29,548 caused a partial suppression of the protein kinase C activity of the membrane fraction augmented by TNFα ($P < 0.01$). SQ29,548 alone did not affect the protein kinase C activity of the cytoplasmic or membrane fraction.

Cells treated for 2 h with 10^{-7} M PAF showed a significant ($P < 0.01$) increase in membrane-associated protein kinase C activity (Fig. 5B). The prior addition of

Table 3
Effect of TNFα, PAF, H-89, or staurosporine on thromboxane A_2 synthesis of human vascular endothelial cells

	Thromboxane B_2 (pg/ml)
Nil	467.8 ± 22.4
TNFα 100 U/ml	557.4 ± 2.8 [b]
TNFα 100 U/ml + H-89 20 μM	539.4 ± 12.6
TNFα 100 U/ml + staurosporine 100 nM	548.6 ± 16.3
PAF 10^{-7} M	553.7 ± 12.8 [a]
PAF 10^{-7} M + H-89 20 μM	566.0 ± 4.9
PAF 10^{-7} M + staurosporine 100 nM	564.9 ± 5.2

Human vascular endothelial cells in Medium 199 were exposed to the medium alone (Nil) or stimulated by TNFα or PAF and incubated for 48 h. H-89 or staurosporine were added 15 min prior to treatment with TNFα or PAF. The harvested supernatants were assayed for their thromboxane contents by ELISA (as described in Materials and methods). Results are expressed as the means ± S.D. of three experiments. [a] $P < 0.05$, [b] $P < 0.01$ as compared to those without stimulation.

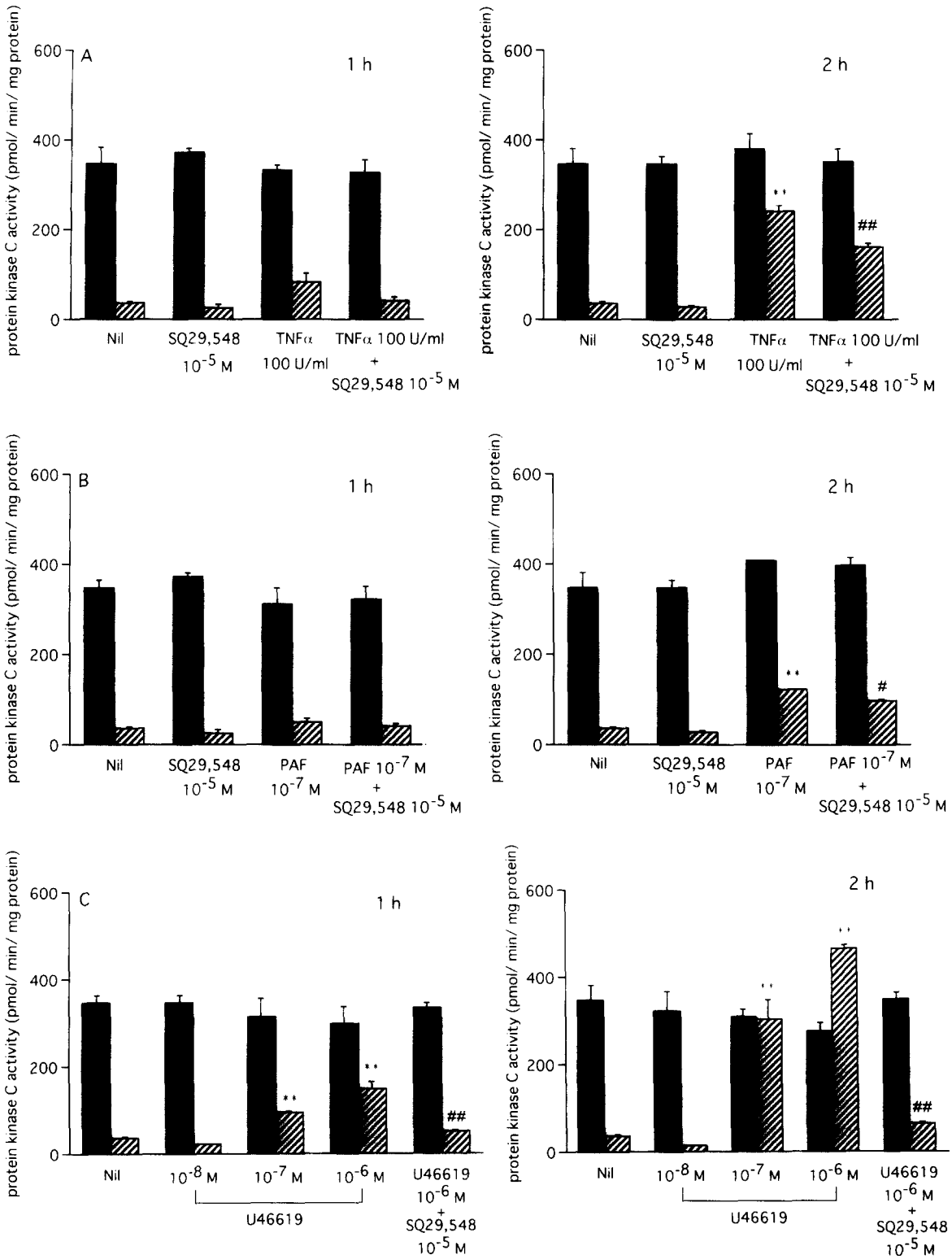

Fig. 5. Effects of TNFα, PAF, SQ29,548, or U46619 on the protein kinase C activities on human vascular endothelial cells. 10^{-5} M SQ29,548 was added to monolayers of confluent human vascular endothelial cells 15 min before the addition of 100 U/ml TNFα (A) or 10^{-7} M PAF (B). Monolayers of confluent human vascular endothelial cells were incubated with U46619 (10^{-7}, 10^{-6}, or 10^{-5} M) (C). One or two hours after the addition, the protein kinase C activity in cytosolic fraction (closed column) or membrane fraction (hatched column) was determined as detailed in Materials and methods. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). Results are expressed as the means ± S.D. of three experiments. ** $P < 0.01$ as compared to those without stimulation. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ as compared to those stimulated by TNFα or PAF.

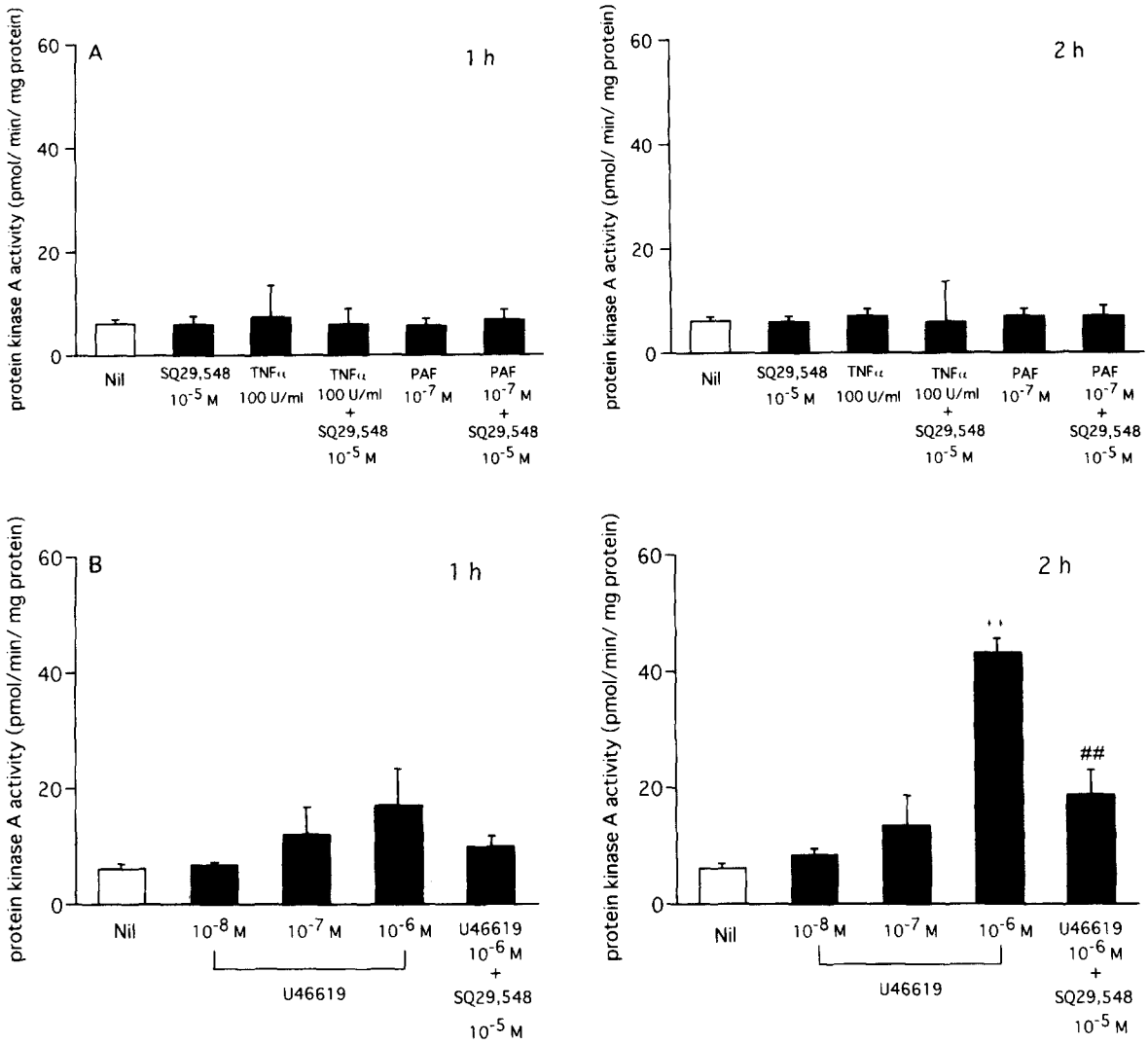

Fig. 6. Effects of TNFα, PAF, SQ29,548, or U46619 on the protein kinase A activities on human vascular endothelial cells. 10^{-5} M SQ29,548 was added to monolayers of confluent human vascular endothelial cells 15 min before the addition of 100 U/ml of TNFα or 10^{-7} M PAF (A). Monolayers of confluent human vascular endothelial cells were incubated with U46619 (10^{-7}, 10^{-6}, or 10^{-5} M) (B). One or two hours after the addition, the protein kinase A activity in human vascular endothelial cells was determined as detailed in Materials and methods. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). Results are expressed as the means ± S.D. of three experiments. ** $P < 0.01$ as compared to those without stimulation.

SQ29,548 resulted in a partial suppression of the protein kinase C activity of the membrane fraction that had been augmented by PAF stimulation ($P < 0.05$).

Stimulation with U46619 resulted in a dose-dependent increase in membrane-associated protein kinase C activity (Fig. 5C). The prior addition of SQ29,548 almost completely suppressed the augmentation of the protein kinase C activity of the membrane fraction by U46619 ($P < 0.01$). In contrast, identical U46619 treatment had no demonstrable effect on cytosol-associated protein kinase C activity.

3.6. Effects of TNFα, PAF, U46619, and SQ29,548 on protein kinase A activity by human vascular endothelial cells

The protein kinase A activity of the control human vascular endothelial cells was 5.89 ± 1.07 pmol/min/mg protein (Fig. 6). Stimulation by TNFα or PAF caused no significant increase in the protein kinase A activity (Fig. 6A). The addition of SQ29,548 prior to TNFα or PAF stimulation did not cause a significant change in the protein kinase A activity. On the other hand, the protein kinase A activity was significantly ($P < 0.01$) augmented 2 h after U46619 stimulation (Fig. 6B). The prior addition of SQ29,548 caused a significant ($P < 0.05$) suppression of the protein kinase A activity augmented by U46619. SQ29,548 alone did not affect the protein kinase A activity.

3.7. Effect of H-89 and staurosporine on thromboxane A_2 synthesis by human vascular endothelial cells

The addition of H-89 or staurosporine and TNFα, in contrast to the addition of TNFα alone, did not result in a

reduction in the quantity of thromboxane B_2 in the culture supernatant of human vascular endothelial cells after 48 h (Table 3). The increase in the quantity of thromboxane B_2 in response to addition of PAF 10^{-7} M was not suppressed by H-89 or staurosporine.

4. Discussion

The binding of SQ29,548, a thromboxane A_2 receptor antagonist, was characterized by high-affinity, specificity, and saturability in the vascular endothelial cells of rat aorta. The ligand binds to a single class of recognition site (Hanasaki et al., 1988). The results of a Scatchard plot analysis in the present study correspond well with a single site model, and the binding affinity obtained in this study (K_d: 2.6 ± 0.6 nM) is close to the binding affinity of thromboxane A_2 receptors in rat aortic endothelial cells. When stimulated with TNFα or PAF, the maximum binding capacity (B_{max}) of human vascular endothelial cell receptor increases (Table 1, Fig. 1).

SQ29,548 is a potent and selective thromboxane A_2 receptor antagonist for rat platelets, vascular endothelial cells, and vascular smooth muscle cells (Ogletree et al., 1984; Hanasaki et al., 1988). AA-2414 is another potent thromboxane A_2 receptor antagonist at both the receptor sites of platelets and vascular beds (Imura et al., 1990). We found that, like a thromboxane A_2-synthesizing enzyme inhibitor (Ishizuka et al., 1994), SQ29,548 and AA-2414 suppress the enhancement of ICAM-1 expression on the surface of human vascular endothelial cells that have been stimulated by TNFα or PAF. This finding indicates that thromboxane A_2, the production of which has been enhanced through stimulation by TNFα or PAF, is secreted into the extracellular space and bound to the thromboxane A_2 receptor on the cell membrane, resulting in augmented expression of ICAM-1 expression on the cell surface.

Thromboxane A_2 receptor antagonists partially suppressed the TNFα- or PAF-enhanced ICAM-1 expression (Fig. 2A,B). The finding suggests the presence of a system that has a direct effect on augmenting ICAM-1 expression (without the participation of thromboxane A_2 receptors) following TNFα or PAF stimulation.

It has been demonstrated that the thromboxane A_2 receptor is linked through a G protein to phospholipase C, which hydrolyzes membrane phosphoinositides, resulting in the release of two intracellular messengers, inositol-1,4,5-triphosphate and diacylglycerol. These release intracellular Ca^{2+} and stimulate protein kinase C (Brass et al., 1987). Following incubation of human platelets with GR32191, an antagonist of the thromboxane A_2 receptor, U46619 failed to stimulate the formation of inositol phosphate or to activate protein kinase C (Takahara et al., 1990). In the present study, the protein kinase C activity of the human vascular endothelial cells increased significantly when stimulated with U46619, a thromboxane A_2 agonist. This finding suggests that, like human platelets, a signal transmission pathway via protein kinase C exists in the human vascular endothelial cells. The protein kinase C activity of human vascular endothelial cells was augmented by stimulation with TNFα and PAF, but administration of SQ29,548 partially suppressed the response. Thus we believe that the human vascular endothelial cells possess both a pathway whereby TNFα or PAF stimulation directly activates protein kinase C from each receptor, and another pathway where thromboxane A_2, which has been produced and secreted from the cells by the aforementioned stimulation, binds with thromboxane A_2 receptors and activates protein kinase C. The latter pathway was exclusively blocked by the thromboxane A_2 receptor antagonist. In contrast, the former pathway was not inhibited by the agent.

The potent protein kinase C inhibitor, H-7, inhibited TNFα-induced enhancement of ICAM-1 expression at both the mRNA and the protein level in a human endothelial cell line EA.hy 926 (Mattila et al., 1992). Cytokine (TNFα, LPS)-induced ICAM-1 expression in human vascular endothelial cells was inhibited by H-7 (Sung et al., 1994). The results of our study also showed that ICAM-1 expression by human vascular endothelial cells was augmented by stimulation with PMA. The exaggerated expression of ICAM-1 on human vascular endothelial cells caused by stimulation with U46619 was significantly inhibited by H-7 or staurosporine. It was postulated, therefore, that ICAM-1 expression is augmented by intracellular signal transmission mainly through the protein kinase C system in the stimulation via the thromboxane A_2 receptor in human vascular endothelial cells.

Stimulation by 100 μM of dibutyryl cAMP slightly enhanced ICAM-1 expression on the surface of human vascular endothelial cells, and U46619 stimulation augmented the protein kinase A activity of the cells. Twenty micromolair of H-89 had a mildly suppressive effect on ICAM-1 expression that was enhanced by U46619 stimulation. It is suggested that U46619 stimulation of human endothelial cells results in an increase in the intracellular cyclic AMP level through enhanced synthesis of prostacyclin (Jeremy et al., 1985; Mizuno-Yagyu et al., 1987). Forskolin treatment, which increases intracellular cyclic AMP, upregulates interleukin-1 induction of ICAM-1 expression by human vascular endothelial cells. These findings suggest a possibility that not only protein kinase C but also protein kinase A is involved to a limited extent in the ICAM-1 expression that has been augmented by U46619 stimulation of human vascular endothelial cells.

In agreement with our earlier study (Ishizuka et al., 1994), the present study shows the presence of thromboxane A_2 receptors on human vascular endothelial cells, and that stimulation of these cells with TNFα or PAF results not only in augmented production of thromboxane A_2 but also in an increase in the number of thromboxane A_2 receptors. We believe that thromboxane A_2 synthesis in-

hibitors and thromboxane A_2 receptor antagonists inhibit the expression of the adhesion molecule in the vascular endothelial cells and prevent exacerbation of inflammation by blocking these responses.

Acknowledgements

We thank Dr S. Horii (Research and Development Division, Pharmaceutical Group, Takeda Chemical Industries, Osaka, Japan) for the gift of AA-2414.

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