Tumor Necrosis Factor α Induces Endothelial Galactosyl Transferase Activity and Verocytotoxin Receptors. Role of Specific Tumor Necrosis Factor Receptors and Protein Kinase C

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Infections with verocytotoxin (VT) producing *Escherichia coli* have been strongly implicated in the epidemic form of hemolytic uremic syndrome (HUS). Endothelial damage plays a central role in the pathogenesis of HUS. In vitro studies have shown that VT can damage endothelial cells after interaction with its cellular receptor globotriaosylceramide ($GbOse_3cer$). Cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) can potentiate the toxic effect of VT by inducing a protein-synthesis dependent increase in VT receptors on endothelial cells. In this study, the mechanisms underlying the increase in endothelial VT receptors induced by TNFα were studied in more detail. To investigate which proteins were involved in this induction, endothelial cells were incubated with and without TNFα in the presence of ^{14}C-galactose or ^{14}C-glucose. Thin-layer chromatography (TLC) analysis of the glycolipid extracts of these cells demonstrated a markedly enhanced incorporation of ^{14}C-galactose in $GbOse_3cer$ and other galactose-containing glycolipids, suggesting that TNFα enhanced galactosyltransferase activity. To examine the role of the two recently cloned TNF-receptors (TNFR-p75 and TNFR-p55) in the TNFα-induced increase in $GbOse_3cer$ in human endothelial cells, cells were incubated with TNFα, the TNFR-p55 selective R32W-S86T-TNFα-mutant, or the TNFR-p75 selective D143N-A145R-TNFα-mutant. The effect of TNFα activation, determined by binding-experiments with ^{125}I-VT-1, could be largely, but not completely mimicked by R32W-S86T-TNFα. Although incubation of cells with D143N-A145R-TNFα did not show an increase in VT-1 binding, the monoclonal antibody utr-1, which prevents binding to TNFR-p75, decreased the TNFα-induced VT-1 binding. Activation of protein kinase C (PKC) by phorbol ester increases the expression of VT-1 receptors; this effect was prevented by the PKC inhibitor Ro31-8220 and by homologous desensitization by pretreatment with phorbol ester. In contrast, the presence of the protein kinase inhibitor Ro31-8220 or desensitization of PKC activity reduced the TNFα-induced increase in VT-1 receptors maximally by 50% and 24%, respectively. Comparable reductions in overall protein synthesis and the synthesis of E-selectin and plasminogen activator inhibitor-1 (PAI-1) were observed. This suggests an effect on general protein synthesis rather than a specific effect of PKC in the signal transduction pathway, by which TNFα induces VT-1 receptors. Our results indicate that TNFα can increase the VT-1 receptors on endothelial cells by inducing galactosyl-transferase activity, that this action of TNFα mainly occurs via the TNFR-p55; and that PKC activation increases expression of VT-1 receptors by a separate mechanism that acts additively to the TNFα-induced increase in VT-1 receptors.

THE EPIDEMIC FORM of the hemolytic uremic syndrome (HUS) is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. It is mostly seen in young children and has a prodromal phase of acute, often bloody, gastroenteritis.[1] Since the beginning of the 1980s, it has become clear that verocytotoxin (VT)- or Shiga-like toxin-producing *Escherichia coli* infections are the main cause of this form of HUS.[2] A family of three VTs has been described: verocytotoxin-1 (VT-1) or shiga-like toxin I (SLTI), verocytotoxin-2 (VT-2) or shiga-like toxin II (SLTII) and verocytotoxin-2 variant (VT-2c).[3] Although the exact pathogenesis is still unknown, endothelial cell damage, predominantly seen in the glomeruli in the kidney, is believed to play a central role.[1] Several in vitro studies have shown that purified VT can damage the endothelium.[4-7] The functional VT receptor, the glycosphingolipid globotriaosylceramide ($GbOse_3cer$), plays a crucial role in endothelial cell damage.[8] This receptor has been found in the human kidney[9] and on cultured endothelial cells.[10] Recently, we have reported that inflammatory mediators, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), can potentiate the toxic effect of VT-1 to human endothelial cells by inducing an increase in the $GbOse_3cer$ synthesis in these cells.[7] Inflammatory mediators are produced and released by monocytes[11] and mesangial cells in vitro,[12,13] and may play a local role in the kidney. A recent report from Harel et al[14] demonstrated that SLTI can specifically induce TNFα activity in mouse kidney. Increased production of the cytokines TNFα, IL-1β, and IL-6 can also be found in the media of cultured human monocytes after stimulation with VT-1.[15] In this report, we extend our observations and show that TNFα and IL-1 induce specific galactosyl-transferase(s), which is (are) necessary for the synthesis of $GbOse_3cer$ in the endothelial cells.

Recently, two TNF receptors (TNFRs) have been identified and cloned, a 55-kD receptor TNFR-p55 and a 75-kD receptor TNFR-p75.[16] Both receptors are present on human endothelial cells.[17,18] The TNFR-p55 has been shown to be involved in the signal transduction of TNFα during the induction of several products of endothelial cells, such as E-selectin and VCAM-1.[17] The involvement of both TNF receptors in the TNFα-induced increase in $GbOse_3cer$ synthesis and the role of protein kinase C (PKC) in the induction of VT receptors in human endothelial cells were studied.

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Submitted May 4, 1994; accepted October 4, 1994.

Supported by a grant from the Dutch Kidney Foundation, Grant No. C90.1021.

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0006-4971/95/8503-0011$3.00/0

MATERIALS AND METHODS

Materials. Purified VT-1 was prepared in the laboratory of Dr M.A. Karmali (Hospital for Sick Children, Toronto, Canada) (1.2 mg protein/mL; CD_{50} vero-cells: titer 10^{-8} to 10^{-9}).[19] Endotoxin content of the VT-1 preparation was less than 0.05 EU/mL by Limulus amoebocyte lysate assay (E-Toxic; Sigma Chemicals, St Louis, MO) at a detection level of 0.05 to 0.10 EU/mL. M199 medium supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al.[20] Human serum was obtained from a local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was from GIBCO (Grand Island, NY), and it was heat-inactivated before use (56°C for 30 minutes). Heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands). Penicillin/streptomycin was from Boehringer Mannheim (Mannheim, Germany). Human fibronectin was a gift from J.A. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Pyrogen-free human serum albumin (HSA) was purchased from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Human recombinant TNFα was a gift from Jan Tavernier (Biogent, Ghent, Belgium). The TNFα preparation contained 2.45×10^7 U/mg protein and less than 40 ng lipopolysaccharide (LPS) per mg protein. Human recombinant IL-1β was a gift from S. Gillis (Immunex, Seattle, WA); it had a specific activity of 10^8 U/mg. The mutant R32W-S86T-TNFα, which selectively binds and activates the human TNFR-p55, and does not react with the human TNFR-p75, and the mutant D143N-A145R-TNFα, which specifically recognizes the TNFR-p75, were previously reported.[21] The antagonistic monoclonal antibody utr-1, specific for the TNFR-p75, and the agonistic monoclonal antibody htr-9, specific for the TNFR-p55 were previously reported.[22] The specific PKC-inhibitor C3 (Ro-31-8220)[23] was a gift from Dr G. Lawton (Hoffmann La Roche, Welwyn Garden City, UK). The inhibitors H-7 and HA-1004 were purchased from Seikagaku, Tokyo, Japan). Phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), and Tween 20 were obtained from Sigma Chemical Co. $Na^{125}I$-iodine, ^{14}C-glucose (50 to 60 mCi/mmol) and ^{14}C-galactose (50.3 mCi/mmol) were purchased from Amersham (Amersham, UK). Iodo-gen iodination reagent was obtained from Pierce (Rockford, IL). Chloroform, methanol, and hexane were obtained from Merck (Darmstadt, Germany). Plastic coated silica gel F1500 thin-layer chromatography (TLC) plates were from Schleicher and Schüll (Dassel, Germany). Polyisobutylmethacrylate was obtained from PolySciences (Washington, MD). A standard mixture of pure neutral glycosphingolipids containing Galβ1-1Ceramide(Cer) (CMH), Galβ1-4Glcβ1-1Cer (CDH), Galα1-4Galβ1-4Glcβ1-1Cer (GbOse$_3$cer), GalNacβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (GbOse$_4$cer) and GalNacα1-3GalNacβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (Forssmann pentasaccharide) was from Biocarb (Lund, Sweden). X-OMAT x-ray film was from Eastman Kodak (Rochester, NY).

Cell culture. Endothelial cells from human umbilical vein (HUVEC) and from human foreskin were isolated by collagenase treatment, cultured, and characterized as previously described.[24-27] The endothelial cells were seeded in fibronectin-coated 10-cm^2 wells and cultured in M199 medium supplemented with 20 mmol/L HEPES (pH 7.4), 10% (vol/vol) human serum (HS), 10% (vol/vol) heat-inactivated newborn calf serum, 2 mmol/L L-glutamine, 5 U/mL heparin, and 150 μg/mL crude preparation of endothelial growth factor under 5% CO_2 and 95% air at 37°C. When the cells reached confluency, they were detached with trypsin/EDTA and seeded in 2-cm^2 fibronectin coated dishes with a split ratio of 1:3. HUVEC in the experiments were used after one to three passages. Human foreskin microvascular endothelial cells were used after four to six passages. The medium was renewed every 2 or 3 days.

Binding of ^{125}I-VT-1 to human endothelial cells. VT-1 was radiolabeled with $Na^{125}I$ according to the Iodogen procedure.[28] Five preparations of purified VT-1 were iodinated to specific activities ranging from 16.2 μCi/μg to 26.8 μCi/μg of protein. All preparations gave similar results. For the experiment, confluent HUVEC, cultured in 2-cm^2 wells, were incubated for indicated times with medium M199 to which the appropriate concentration of the test compounds were added. The inhibitory antibody utr-1 and the PKC-inhibitor Ro31-8220 were added 1 hour before the start of the experiment. The binding assay was performed as follows: after the incubation period at 37°C with the indicated compounds, the endothelial cell cultures in 24-well plates were washed with M199 medium plus 0.1% HSA (wt/vol). Subsequently, the cells were incubated for 3 hours with 1.0 nmol/L ^{125}I-VT1 in M199 plus 0.1% HSA (wt/vol) at 0°C. After the incubation, the supernatant fluid was aspirated, the cells were washed five times with M199 plus 0.1% HSA, and total cell protein was solubilized in 400 μL 0.5 mol/L sodium hydroxide at room temperature. Radioactivity of the endothelial cells was measured in a gamma-counter. Nonspecific binding was determined by assay of ^{125}I-VT-1 binding in the presence of 50-fold excess of unlabeled VT-1. Cellular specific binding was determined by subtracting the nonspecific binding from the cellular binding of ^{125}I-VT-1 determined in the absence of unlabeled VT-1.

Extraction of glycolipids. Confluent endothelial cell cultures were incubated for 24 hours with or without 20 ng/mL TNFα or 0.5 ng/mL IL-1β. Six hours after the start of the incubation, 0.5 μCi/mL ^{14}C-glucose or ^{14}C-galactose was added to the media. Subsequently, after the 24 hours incubation period, the glycolipids were extracted as described by Lingwood et al.[8] In short, the cells were trypsinized, harvested with ice-cold phosphate-buffered saline (PBS), and spun down by a 3-minute centrifugation (3,000 rpm) at 4°C. The pellet was washed three times with PBS. The pellet was finally suspended in PBS, and 20 vol of chloroform/methanol (2:1, vol/vol) was added. Cell debris was removed by filtration through glass-wool. One volume of water was added to obtain phase separation. The lower phase was dried and incubated at 37°C for 2 hours in 1 mL 0.4 mol/L KOH in ethanol; 2 vol of chloroform was added and the mixture was partioned against 2 vol of water. The lower phase was separated and frozen until TLC studies were performed.

Thin-layer chromatography. The lower phase from the extraction above was dried and resuspended in chloroform/methanol (2:1, vol/vol). Samples were separated on a silica gel TLC plate using chloroform:methanol:water (65:25:4, vol/vol/vol). Standard neutral glycosphingolipids, 2 μg of each glycolipid, together with an equal volume of unlabeled glycolipid cell-extract, were run on the same TLC and afterwards stained by orcinol-spray. After separation, the plate was air-dried, and exposed to X-OMAT x-ray film. After developing the film, the plate was soaked three times for 1 minute in 0.01% polyisobutylmethacrylate in hexane and air-dried, followed by overnight incubation in PBS supplemented with 1% BSA and 0.05% Tween 20. Subsequently, the plate was incubated with 50 mL VT-1 solution (15 nmol/L unlabeled, 1.5 nmol/L ^{125}I-VT-1 in PBS supplemented with 1% BSA and 0.05% Tween 20) for 4 hours at 4°C. The plate was extensively washed with 0.05% Tween 20 and 1% BSA in PBS, air-dried, and exposed to X-OMAT x-ray film.

Assays. Levels of PAI-1 antigen in endothelial cell-conditioned medium were assayed by ELISA (IMULYSE PAI-1) obtained from Biopool (Umea, Sweden), according to the manufacturer's description.

The presence of E-selectin was determined by cell enzyme-linked immunosorbent assay (ELISA) in triplicate wells with HUVEC cultured in fibronectin-coated 96-multiwell dishes. After a 5-hour incu-

bation with 20 ng/mL TNFα (inhibitors added 1 hour before addition of TNFα), the amount of E-selectin was determined on fixed cells by using an anti-E-selectin monoclonal antibody Ena-2[29] (gift of Jet Leeuwenberg, Maastricht, The Netherlands) and a rat peroxidase-labeled antimouse immunoglobulin G (IgG). After development of the assay, the optical density was recorded.

Statistics. Experiments were done with at least three different cultures of HUVEC, unless otherwise mentioned. Data are given as mean ± SEM. Statistical analysis was performed with the Wilcoxon test. Statistical significance was accepted for $P < .05$.

RESULTS

TNFα increases the activities of glycolipid galactosyl transferases. Our earlier report showed that the incubation of human endothelial cells with the cytokines TNFα or IL-1β causes a protein synthesis-dependent increase in the number of VT-1 receptor molecules, the glycosphingolipid globotriaosylceramide ($GbOse_3cer$, Galα1-4Galβ1-4Glcβ1-1Cer).[7] The biosynthesis of glycosphingolipids occurs via sequential transfer of sugar moieties from nucleotide sugar donors to ceramide. Specific glucosyl- and galactosyltransferases are involved in this process. To investigate whether the increase in $GbOse_3cer$ molecules by the cytokines TNFα or IL-1β is caused by the induction of glucosyl- or galactosyltransferases, HUVEC were incubated with or without the cytokines TNFα or IL-1β together with ^{14}C-glucose or ^{14}C-galactose for a period of 24 hours. After the incubation-period, the ^{14}C-labeled neutral glycosphingolipids were extracted and separated on TLC. In Fig 1, it is shown that the incorporation of ^{14}C-galactose in $GbOse_3cer$ and other neutral glycosphingolipids is markedly enhanced after incubation of the cells with TNFα and, to a less extent, with IL-1β (lanes b through d). The presence of $GbOse_3cer$ was confirmed by the standard sample of neutral glycosphingolipids (lane a) and by incubation of the TLC with ^{125}I-VT-1, which specifically binds to $GbOse_3cer$ (lanes e through g). The increase in the incorporation of ^{14}C-galactose in $GbOse_3cer$ is parallelled by an increase in ^{125}I-VT-1 binding to the $GbOse_3cer$ position on the TLC. A similar TNFα-induced increase in ^{14}C-galactose incorporation in $GbOse_3cer$ and other neutral glycosphingolipids was also observed in human foreskin microvascular endothelial cells (not shown). Subsequent experiments, in which the incorporation of ^{14}C-

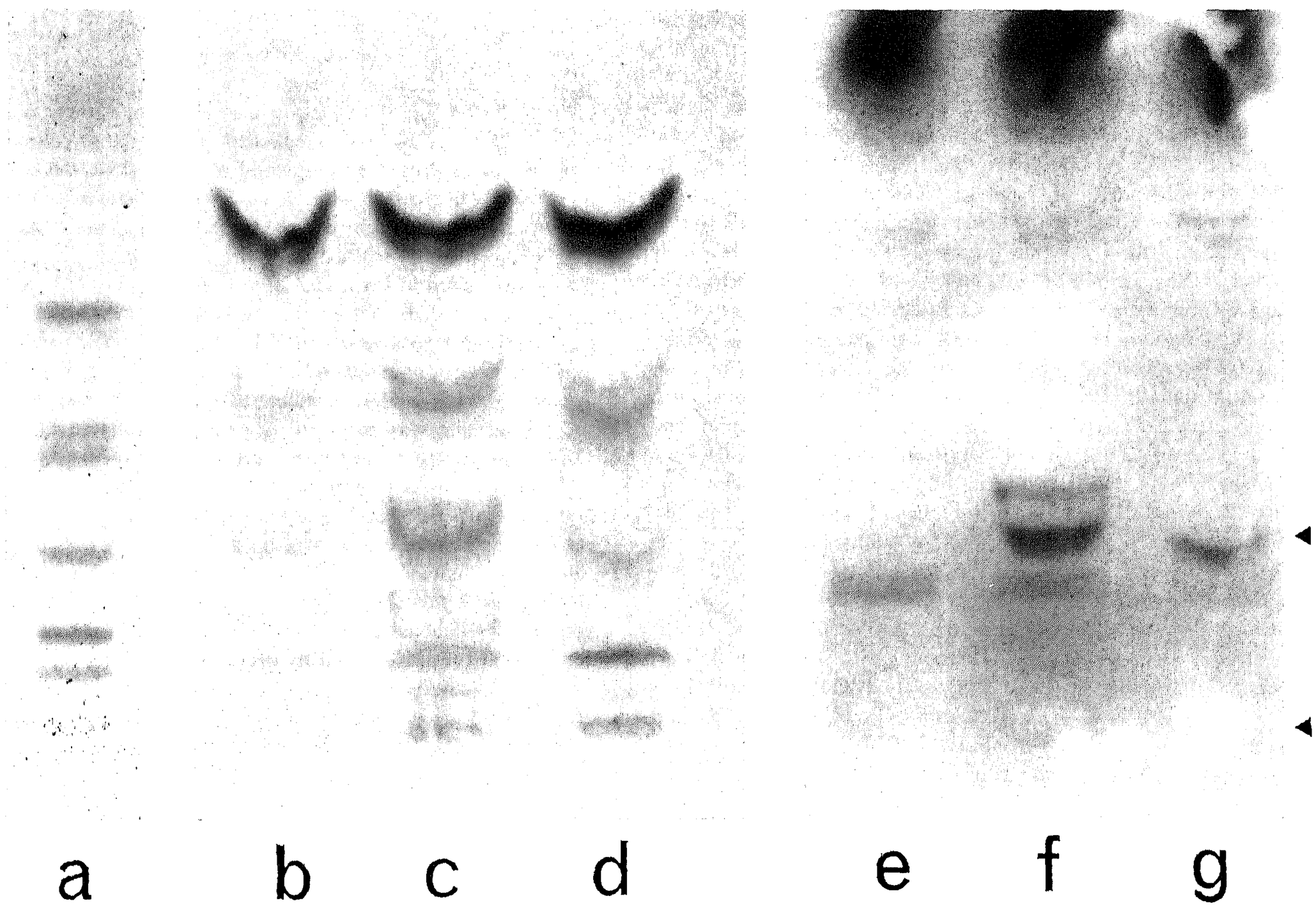

Fig 1. Increase of galactose-containing glycolipids extracted from 1.6×10^6 HUVEC. Confluent HUVEC were incubated for 24 hours with or without 20 ng/mL TNFα or 0.5 ng/mL IL-1β. Six hours after addition of TNFα, 0.5 μCi/mL ^{14}C-galactose was added to the medium. After the incubation-period, glycolipids of 1.6×10^6 cells were extracted and separated by TLC. Lane a, standard neutral glycosphingolipids, 2 μg of each glycolipid, visualized by orcinol-spray. Lanes b through d, autoradiograms of ^{14}C-galactose containing endothelial glycosphingolipids. Separated glycolipid-extracts of 1.6×10^6 cells treated with no inflammatory mediator (lane b), with 20 ng/mL TNFα (lane c), and with 50 U/mL IL-1β (lane d). Lanes e through g, the same TLC was assayed for ^{125}I-VT-1 binding. Autoradiographs of ^{125}I-VT-1 binding to glycolipid-extracts of control cells (lane e), treated with 20 ng/mL TNFα (lane f), or with 50 U/mL IL-1β (lane g). 1, galactosylceramide; 2, lactosylceramide; 3, globotriaosylceramide ($GbOse_3cer$); 4, globotetraosylceramide; 5, Forssman pentasaccharide; and 6, origin of the lane.

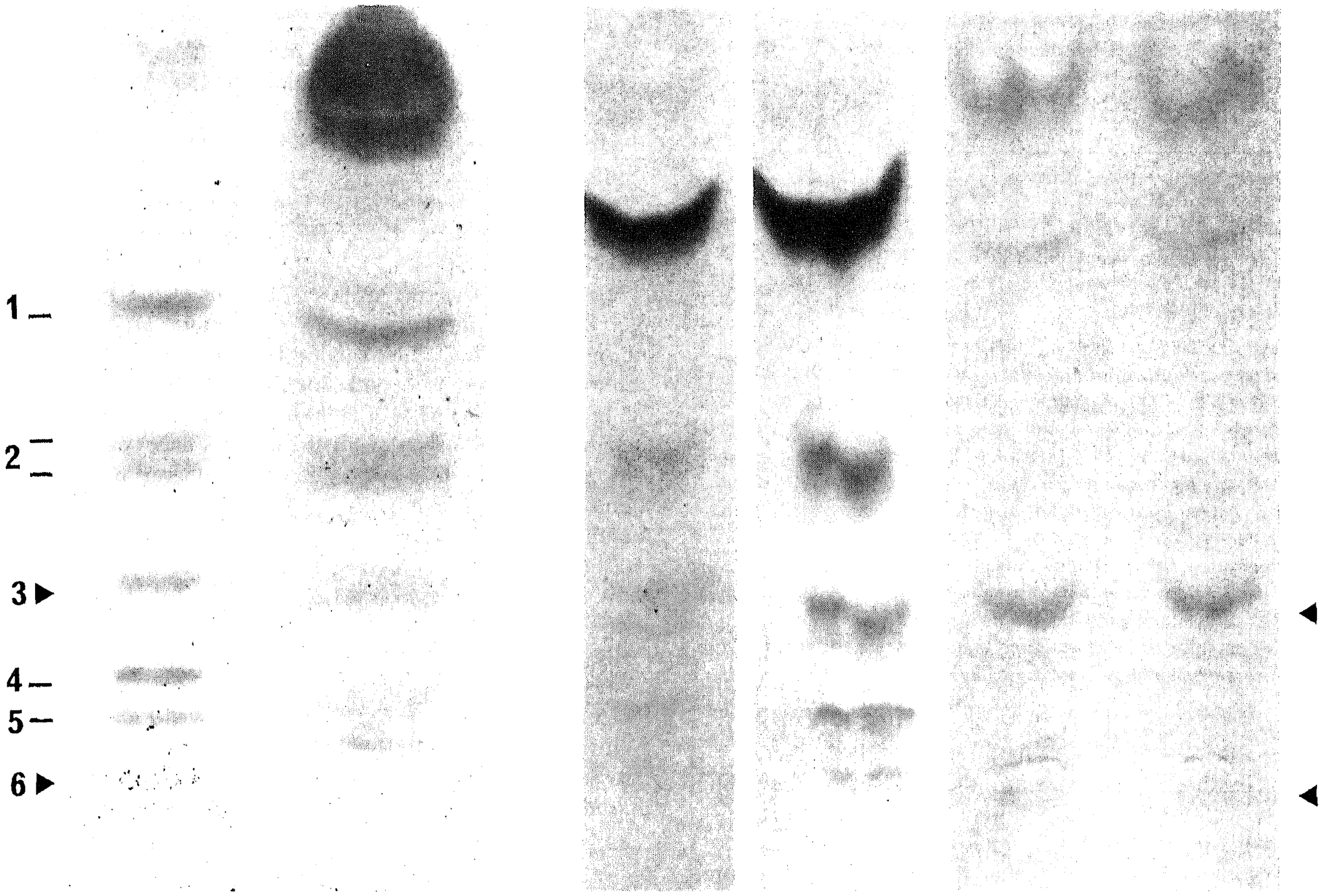

Fig 2. Autoradiogram of ^{14}C-glucose- or ^{14}C-galactose-containing glycolipid-extracts separated on TLC. Confluent HUVEC treated for 24 hours without or with 20 ng/mL TNFα with ^{14}C-glucose or ^{14}C-galactose. Glycolipids of 1.6 × 10^6 cells were extracted and separated on TLC. Lane a, standard mixture of 2 μg of each neutral glycosphingolipids visualized by orcinol-spray. Lane b, standard mixture of neutral glycosphingolipids mixed with unlabeled glycosphingolipid cell-extract, visualized with orcinol spray. ^{14}C-galactose containing glycolipid-extract of control cells (lane c) and TNFα-treated cells (lane d). ^{14}C-glucose containing glycolipid-extract of control cells (lane e), ^{14}C-glucose containing glycolipid-extract of TNFα-treated cells (lane f), 1, galactosylceramide; 2, lactosylceramide; 3, globotriaosylceramide (GbOse$_3$cer); 4, globotetraosylceramide; 5, Forssman pentasaccharide; and 6, origin of the lane.

glucose and ^{14}C-galactose were compared, demonstrated that incorporation of ^{14}C-glucose in glycosphingolipids in the TNFα-treated HUVEC were comparable to that in control cells (Fig 2).

Involvement of the two TNF receptors in the TNFα-mediated increase in VT receptors. To investigate which of the recently cloned TNF receptors, TNFR-p55 and TNFR-p75, is involved in the induction of GbOse$_3$cer in human endothelial cells by TNFα, the binding of ^{125}I-VT-1 was determined after incubation of HUVEC with wild-type TNFα, the mutant R32W-S86T-TNFα, which recognizes and stimulates TNFR-p55 only, or the mutant D143N-A145R-TNFα, which specifically interacts with the TNFR-p75. With concentrations up to 20 ng/mL, R32W-S86T-TNFα induced a concentration-dependent increase in VT-1 binding, whereas D143N-A145R-TNFα had no effect (Table 1, Fig 3A-C). Thus, the sole stimulation of the TNFR-p55 is sufficient to induce VT-1 receptor synthesis in endothelial cells. This was confirmed with the agonistic monoclonal antibody htr-9, that specifically activates the TNFR-p55 (Table 1). However, when the effects of wild-type TNFα and R32W-S86T-TNFα were compared, the mutant reached 77% ± 5% of the effect of the wild-type TNFα (mean ± SEM, $P < .05$; paired data of seven cultures, incubated for 24 hours with 20 ng/mL of both TNFα forms). Furthermore, it was observed in several time course experiments that, at low concentrations of TNFα and its mutant (0.2 ng/mL), the initial increase of VT-1 receptors induced by the R32W-S86T-TNFα was detectable 1 or 2 hours later than that by wild-type TNFα. These observations suggest that wild-type TNFα provides a signal additional to stimulation of the TNFR-p55, probably via the TNFR-p75. This may occur by activation of the TNFR-p55 via TNFR-p75-mediated signal transduction, or by concentration of TNFα molecules on the cell surface by the TNFR-

Table 1. Effect of TNF-Receptor Agonists on VT-1 Binding to Human Endothelial Cells

Addition	^{125}I-VT-1 Binding (fmol/10^5 cells)	
	9-h Incubation	24-h Incubation
Control	2.3 ± 0.8 (9)	1.7 ± 0.6 (10)
Wild-type TNFα (20 ng/mL)	7.7 ± 2.6 (9)*	23.2 ± 5.8 (10)†
R32W-S86T-TNFα (20 ng/mL)	5.8 ± 3.8 (5)*	19.7 ± 7.2 (7)*
D143N-A145R-TNFα (20 ng/mL)	2.4 ± 0.2 (3)‡	2.2 ± 0.1 (4)‡
D143N-A145R-TNFα (200 ng/mL)	2.2 ± 0.1 (2)‡	2.0 ± 0.1 (3)‡
MoAb htr-9 (10 μg/mL)	ND	8.5 ± 1.6 (3)

Specific binding of 1 nmol/L ^{125}I-VT to confluent HUVEC was determined after a 9- or 24-hour incubation with wild-type TNFα, the mutant R32W-S86T-TNFα, which only activates TNFR-p55, or the mutant D143N-A145R-TNFα, which only activates TNFR-p75, or with the monoclonal antibody htr-9 (MoAb htr-9), which activates the TNFR-p55. Data are expressed as the mean ± SEM of the number of independent experiments indicated in parentheses. Statistically significant difference was evaluated by the Wilcoxon test for paired data.

Abbreviation: ND, not determined.

* $P < .05$ compared with control cells.

† $P < .01$ compared with control cells.

‡ The control data of these experiments were normalized to the mean control value to aid comparability.

p75, so that the binding to the TNFR-p55 is facilitated by ligand passing.[30] When we added the TNFR-p75-stimulating mutant with the TNFR-p55-stimulating mutant to the cells, no further increase in VT-1 binding was observed as compared with the TNFR-p55 mutant alone (Fig 3D). Therefore, TNFR-p75-mediated enhancement of the TNFR-p55 activity is unlikely. Similarly, when the TNFR-p75-binding mutant D143N-A145R-TNFα was added simultaneously with wild-type TNFα, no reduction of the VT-1 binding was observed (not shown). However, when the cells were preincubated with the monoclonal antibody utr-1, that blocks the TNFR-p75, a reduction in the effect of TNFα on VT-1 binding was observed after 9 hours of incubation (Table 2). After 24 hours of incubation, this effect was still observed at moderate concentrations of TNFα (2 ng/mL), but not at high concentrations (20 ng/mL TNFα) (not shown). This is compatible with a ligand passing effect, which vanishes at saturating TNF concentrations. The utr-1 monoclonal antibody had no effect on the ability of the TNFR-p55-stimulating R32W-S86T-TNFα to induce VT-1 receptors (Table 2). This makes an aspecific effect of the utr-1 antibody unlikely.

Involvement of PKC in the induction of VT-1 receptors. The PKC activator phorbol myristate acetate (PMA) and the PKC inhibitors Ro31-8220 and H-7 were used to study the role of PKC in the induction of the VT-1 receptor $GbOse_3cer$. Stimulation of PKC by PMA enhanced the specific binding of VT-1 to a moderate extent in comparison with TNFα (Fig 4A). In six different HUVEC cultures 10 nmol/L PMA stimulated specific ^{125}I-VT-1 binding 4 ± 1-fold, while 20 ng/mL TNFα induced a 16 ± 3-fold increase in the same cells (mean ± SEM, 24-hour incubation). When HUVEC were incubated with PMA and TNFα or R32W-S86T-TNFα simultaneously, the increase of VT-1 receptors was considerably larger than that obtained by TNFα or R32W-S86T-TNFα alone, and the induction occurred faster than after stimulation by TNFα, R32W-S86T-TNFα, or PMA alone (Fig 4A). No additional increase in specific VT-1 binding was seen when PMA was given with D143N-A145R-TNFα, as compared with PMA alone (not shown). For comparison, the production of PAI-1 was assayed in the conditioned medium of the same cells. As expected, PMA did not alter PAI-1 production; addition of 10 nmol/L PMA had a slight stimulatory effect on the TNFα-induced increase in PAI-1 production (Fig 4B).

To evaluate the role of PKC activity in the PMA- and TNFα-induced increases in VT-1 receptors, HUVEC were preincubated for 20 hours with 10^{-6} mol/L PMA, washed three times and subsequently incubated for another 24 hours with 10 nmol/L PMA or 2 ng/mL TNFα. Whereas PMA enhanced the induction of VT-1 receptors under control conditions, its effect disappeared after homologous desensitization (Fig 5). Desensitization of PKC activity by PMA pretreatment reduced TNFα-induced increase in VT-1 receptors only by 24% ± 9%. A similar decrease (22% ± 11%) was seen in TNFα-increased PAI-1 synthesis of these cells (302 ± 19 *v* 392 ± 18 ng PAI-1/24 h/10^5 cells in TNFα-stimulated cells pretreated with 10^{-6} mol/L PMA or control medium, respectively, three independent cultures).

Comparable results were obtained with PKC inhibitors. While the inhibitor Ro31-8220 at 3 μmol/L completely suppressed the effect of PMA on VT-1 binding (Fig 6, inset), it reduced the induction of VT-1 receptors by the single

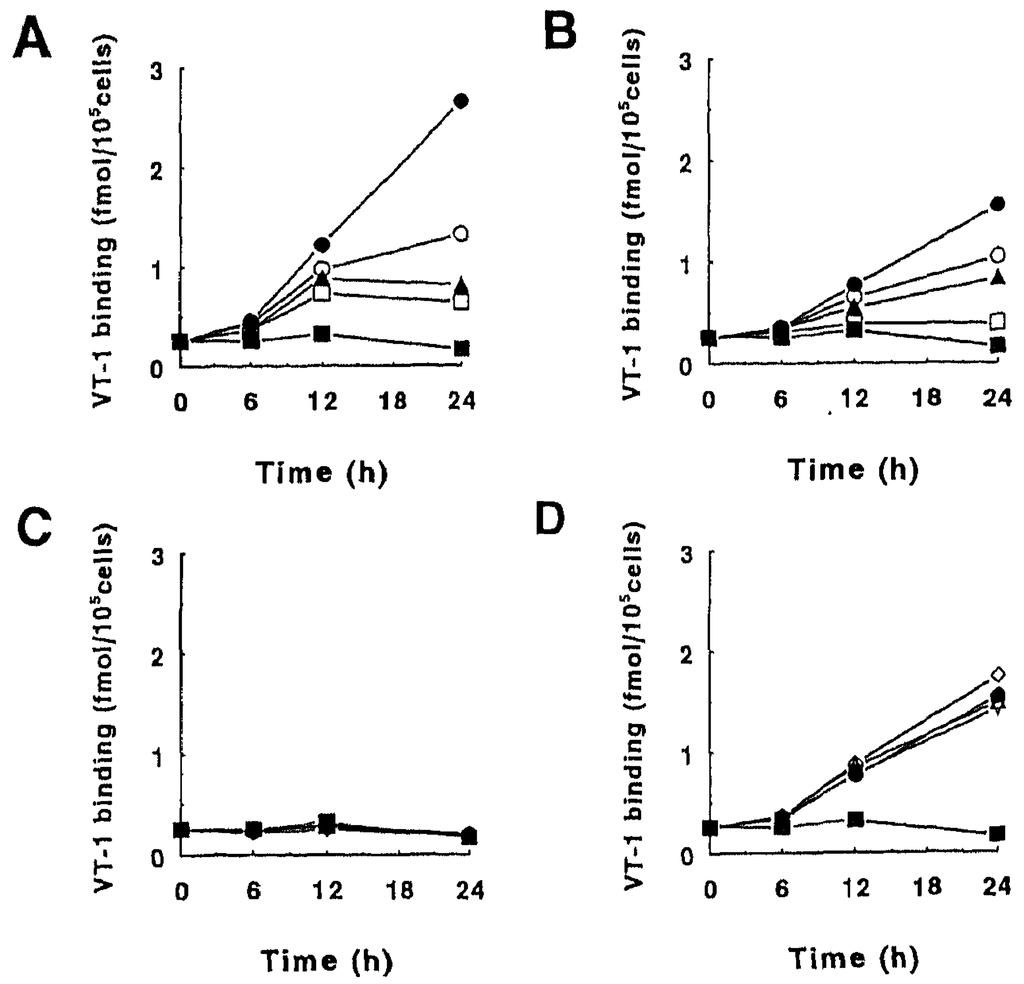

Fig 3. Effect of various concentrations of wild-type TNFα (A), the TNFR-p55-specific mutant R32W-S86T-TNFα (B), and the TNFR-p75-specific mutant D143N-A145R-TNFα (C) on the specific ^{125}I-VT-binding to HUVEC. Cells were incubated with a low concentration range wild-type TNFα or TNFα mutant for 6, 12, or 24 hours, respectively (■ control cells, □ 0.2 ng/mL, ▲ 0.4 ng/mL, ○ 0.8 ng/mL, ● 2 ng/mL). Concentrations of 20 or 200 ng/mL D143N-A145R-TNFα showed the same binding as 2 ng/mL D143N-A145R-TNFα. (D) ^{125}I-VT binding to endothelial cells incubated for 6, 12, or 24 hours with 2 ng/mL R32W-S86T-TNFα (●) together with D143N-A145R-TNFα at 0.4 ng/mL (△), 4 ng/mL (◇), or 20 ng/mL (▽); (■ control cells).

Table 2. Effect of the TNFR-p75 Blocking Antibody utr-1 on the TNFα-Induced Increase of VT-1 Receptors

Addition	^{125}I-VT-1 Binding (fmol/10^5 cells) Culture 1	Culture 2	Culture 3	% Effect utr-1 (mean ± SEM)
None	0.6	0.4	6.5	100
utr-1 (10 μg/mL)	0.6	0.5	6.7	108 ± 6
TNFα (2 ng/mL)	3.2	3.1	19.4	100
TNFα (2 ng/mL) + utr-1 (10 μg/mL)	1.6	2.2	15.4	66 ± 9
TNFα (20 ng/mL)	4.9	4.7	26.1	100
TNFα (20 ng/mL) + utr-1 (10 μg/mL)	2.7	3.6	22.6	73 ± 9
R32W-S86T-TNFα (20 ng/mL)	2.6	2.9	20.9	100
R32W-S86T-TNFα (20 ng/mL) + utr-1 (10 μg/mL)	2.3	2.6	21.5	93 ± 5

Effect of the antagonistic monoclonal antibody utr-1, specific for TNFR-p75 on the ^{125}I-VT-1 binding to three different cultures of confluent HUVEC. Cells were treated for 9 hours with TNFα or R32W-S86T-TNFα in the absence or presence of the antibody utr-1. The antibody utr-1 was added to the cells 1 hour before addition of TNFα or its mutant. After the 9-hour incubation period, the media above the cells were removed and 1 nmol/L ^{125}I-VT-1 was added to the cells as described in Materials and Methods. Data are also expressed as the percentage toxin binding as compared with their counterparts, which were not incubated with utr-1 (mean ± SEM for the three experiments).

addition of TNFα or R32W-S86T-TNFα by 48% ± 9% (Figs 6 and 7A). Incubation of the cells with another PKC inhibitor, H-7 (30 μmol/L), gave the same results as obtained with Ro31-8220, whereas a structural homologue of H-7, HA-1004 (30 μmol/L), which has a similar protein kinase A-inhibiting capacity as H-7, but much less PKC-inhibiting activity, was inactive in this respect (Fig 7A). However, Ro-31-8220 (3 μMol/L) and H-7 (30 μmol/L), but not HA-1004 (30 μmol/L), inhibited TNFα-induced expression of PAI-1 and E-selectin, a protein of which the TNFα induction is not dependent on PKC activity,[31,32] to a comparable extent (Fig 7B,C). Furthermore, the reduction in TNFα-induced VT-1 receptors by these inhibitors was paralleled by a comparable reduction in overall protein synthesis, as estimated from the incorporation of ^{35}S-methionine in proteins (Fig 7D). In the absence of TNFα, these inhibitors affected protein synthesis by less than 10% (not shown).

These findings indicate that activation of PKC underlies the stimulation of VT-1 receptors by PMA. They suggest that PKC is not directly involved in the TNFα-dependent signaling pathway causing induction of VT-1 receptors, but can contribute via another pathway additionally to the effect of TNFα on the expression of VT-1 receptors.

DISCUSSION

The inflammatory mediators TNFα and IL-1 increase the toxicity of VT and the closely related shiga toxin for human endothelial cells.[5-7] Previously, we have demonstrated that TNFα and IL-1 increase the number of VT-1 receptors known to be globotriaosyl-ceramide ($GbOse_3cer$) on endothelial cells, and that protein synthesis was necessary for this induction.[7] Here, we have demonstrated that the TNFα-induced increase in VT-1 receptors is due to an increase in galactosyl-transferase activity in the endothelial cell. This induction occurs predominantly via the TNFR-p55 by a mechanism distinct from the increase of VT-1 receptors by PKC activation.

The kidney contains a relatively high amount of glycosphingolipids.[8,33] $GbOse_3cer$ is, in particular, found in the

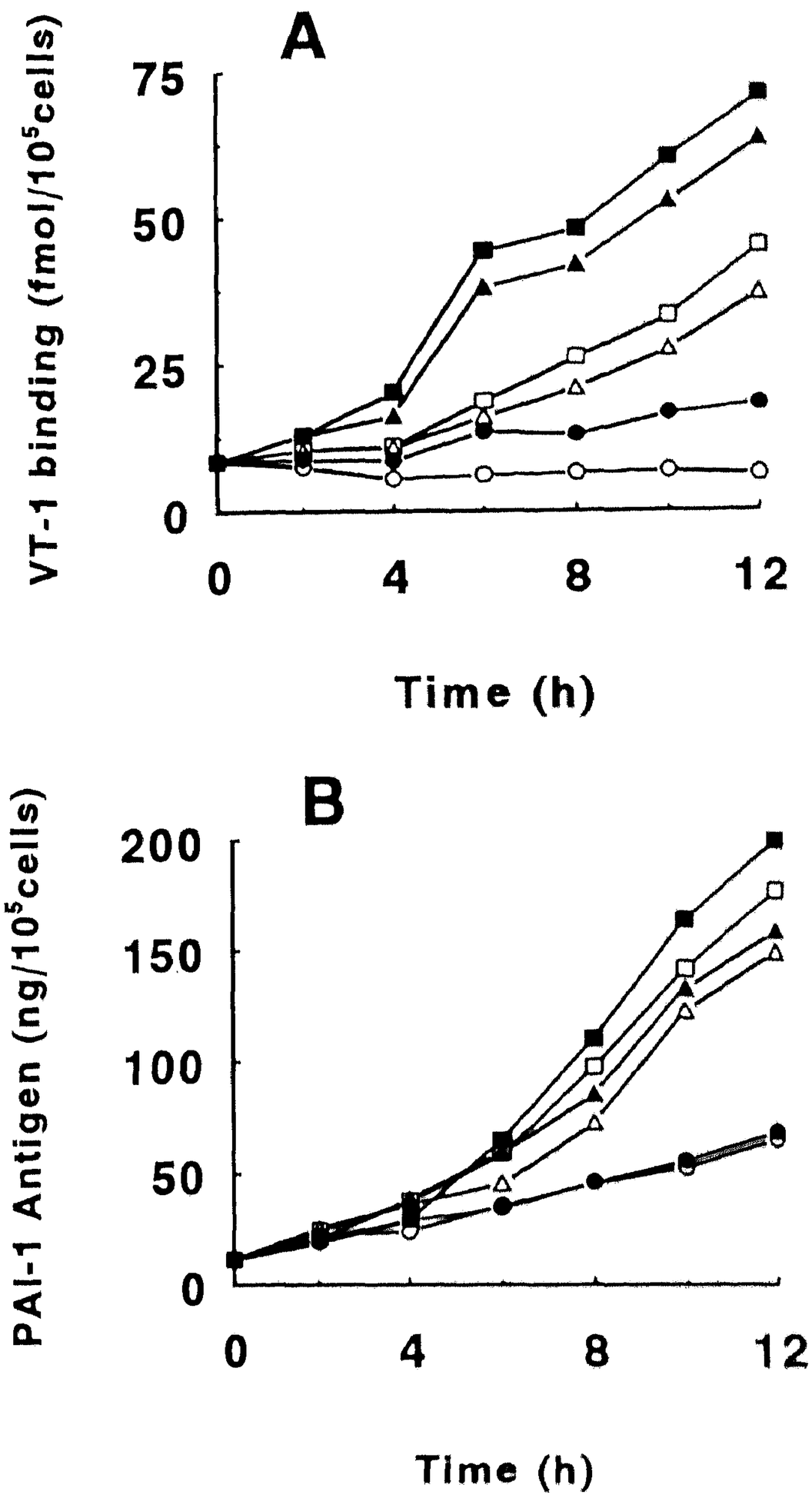

Fig 4. Specific binding of ^{125}I-VT-1 (A) and production of PAI-1 antigen (B) by confluent human endothelial cells incubated with 10 nmol/L phorbol ester PMA (closed symbols) or in its absence (open symbols). HUVEC were simultaneously incubated with 20 ng/mL TNFα (■, □) or 20 ng/ml R32W-S86T-TNFα (▲, △) for the indicated time intervals. The control cells are indicated with circles. No difference in ^{125}I-VT-1 binding and PAI-1 production was observed when D143W-A145R-TNFα was incubated together with 10 nmol/L PMA as compared with PMA alone (data not shown).

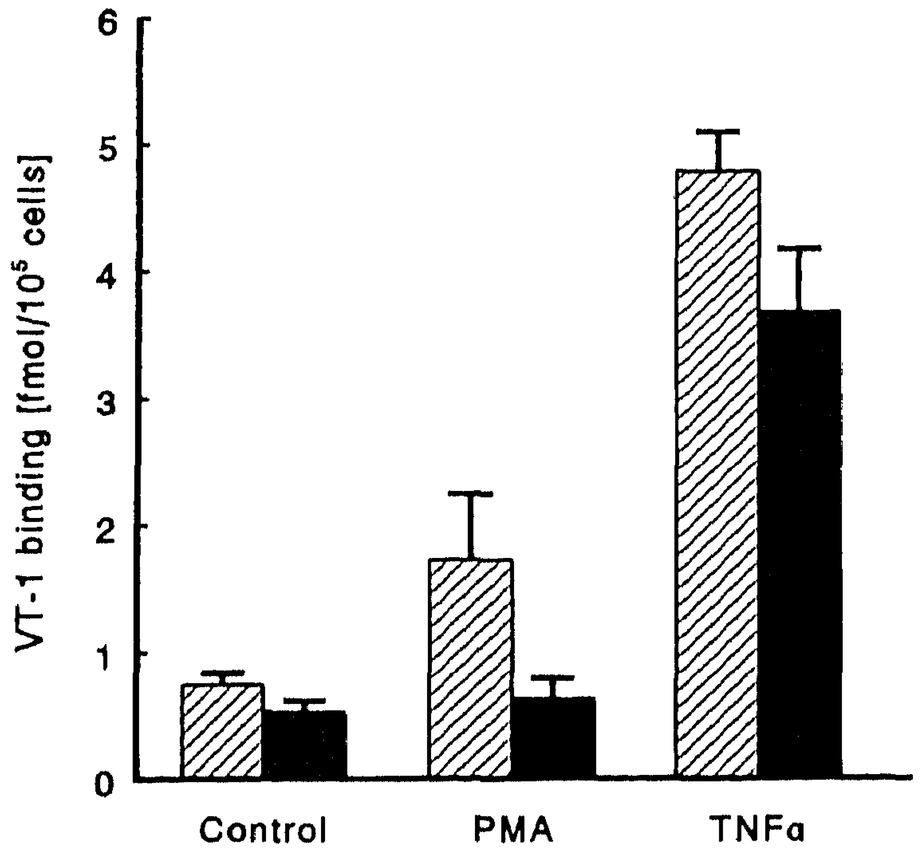

Fig 5. Effect of PKC desensitization on the induction of VT-1 receptors by TNFα and phorbol ester PMA. HUVEC were preincubated for 20 hours in culture medium supplemented with 10^{-6} mol/L PMA (■) or in the same medium without PMA (▨). Subsequently, the cells were washed three times and incubated for 24 hours in culture medium supplemented with 20 ng/mL TNFα, 10 nmol/L PMA or without addition (control), after which specific binding of ^{125}I-VT-1 to the cells was determined. The data are the mean ± SEM of three different HUVEC cultures.

tubular epithelial cells.[33,34] It is also encountered in the glomeruli of children younger than 2 years old,[35] but the cellular distribution in the glomeruli has not yet been resolved. In the glomeruli of kidneys of adults and children older than two years, no significant expression of $GbOse_3cer$ was found.[35] This suggests a developmental shift in the synthesis of glycosphingolipids in glomerular cells. In cultured cells, glycosphingolipids play a role in cell growth and cell differentiation,[33] but little is known about the physiologic role of these glycosphingolipids in various cell types of the intact kidney. Bacterial exotoxins use specific glycolipids as receptors to enter eukaryotic cells where they interfere with the metabolism of the cell.[36,37] In the case of VT, the toxicity is primarily caused by inhibition of the interaction of elongation factor-1 with the ribosome, which results in a complete inhibition of protein synthesis.[38] Previous studies have demonstrated that the sensitivity of endothelial cells for the toxin is determined by the number of toxin receptors, ie, $GbOse_3cer$,[5-7] and that the number of the VT-1 receptors is markedly increased after exposure of endothelial cells to inflammatory mediators.[7] We have suggested that local generation of inflammatory mediators may increase the sensitivity of the kidney and, in severe cases of HUS, that of the endothelium of other organs to VT.

Our present data demonstrate that TNFα, and also IL-1β, induce an enhanced production of neutral galactose-containing glycolipids by an increase in galactosyl-transferase activity. This explains the increase in $GbOse_3cer$ molecules found on TNFα-stimulated endothelial cells.[5-7] To our knowledge, this is the first report indicating an inductive effect of inflammatory mediators TNFα and IL-1 on the synthesis of neutral cellular glycolipids. A confirmation of the induction of galactosyl-transferase(s) at the mRNA level is not yet possible, because ceramide glycosyl-transferases have not been cloned, with the exception of a brain-specific ceramide uridine-5′-diphosphate (UDP)-galactosyl transferase, which was reported very recently.[39] The physiologic meaning of the induction of galactosyl-transferase(s) in inflammation is not known. On the basis of sequence homologies of verotoxins and the α-interferon receptor, Lingwood et al[40] has suggested that $GbOse_3cer$ may act as an accessory molecule for the α-interferon receptor. Hence, the altered synthesis of glycosphingolipids may play a role in the modulation of the inflammatory process. In this respect, it is of interest to note that another inflammatory mediator, γ-interferon shifts the cellular distribution of glycosphingolipids towards the surface of endothelial cells.[41]

TNFα acts on cells via two receptors, TNFR-p55 and TNFR-p75, to which it binds with similar affinity.[16] Both receptors are expressed on unstimulated HUVEC,[17,18] but this study shows that activation of TNFR-p55 by TNFα is sufficient for the induction of $GbOse_3cer$ in human endothelial cells. This finding corresponds well with the TNFα-induced expression in endothelial cells of E-selectin, VCAM-1, ICAM-1, interleukin-8, interleukin-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are under TNFR-p55 control.[17,18,42] However, the TNFR-p55 selective mutant was always slightly less potent

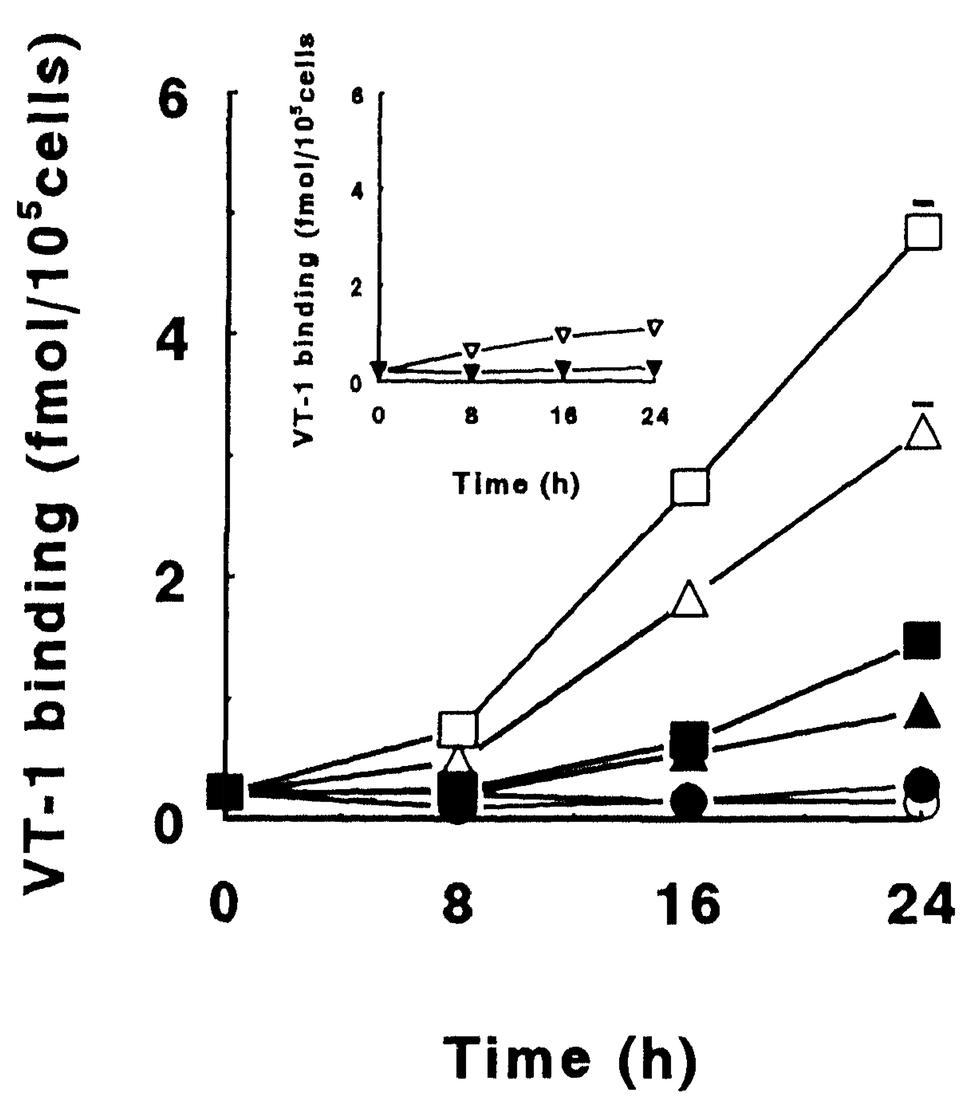

Fig 6. Specific binding of ^{125}I-VT-1 to HUVEC incubated for 8, 16, or 24 hours with 20 ng/mL TNFα (□), 20 ng/mL R32W-S86T-TNFα (△) or without addition (○). The PKC inhibitor Ro31-8220 (3 μmol/L) was added 1 hour before the start of the experiment to the cells and remained present during the incubation period (closed symbols). Results are the mean ± SEM of three independent experiments. Inset: Specific binding of ^{125}I-VT-1 to confluent HUVEC treated with 10 nmol/L PMA in the presence of 3 μmol/L Ro31-8220 (▼) or without inhibitor (▽).

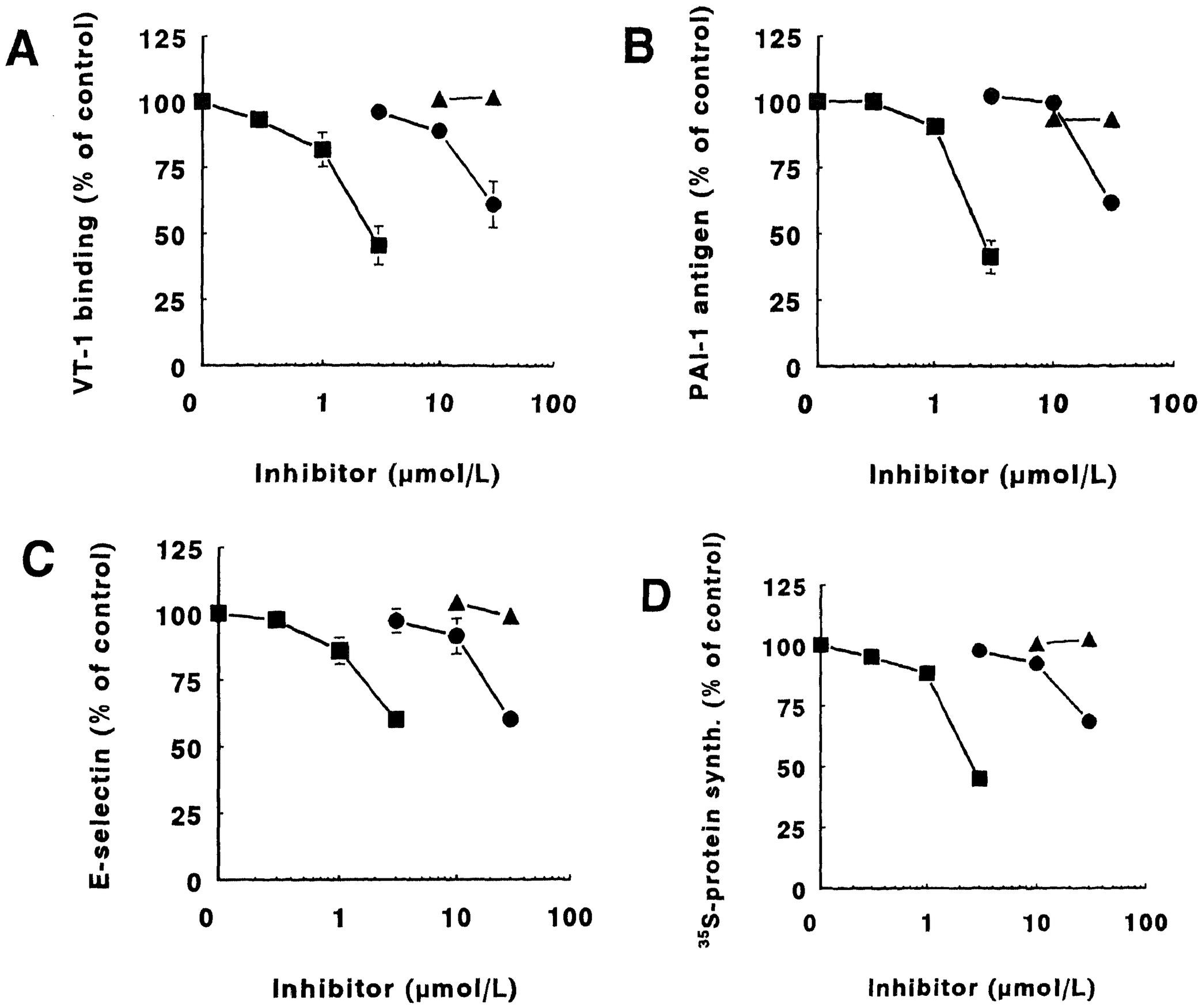

Fig 7. (A) Concentration dependency of the inhibition of TNFα-induced ^{125}I-VT-1 binding to HUVEC by PKC inhibitors. HUVEC were preincubated for 1 hour with various concentrations of Ro31-8220 (■), H-7 (●), or HA-1004 (▲) and incubated for 24 hours in the presence of these inhibitors and 20 ng/mL TNFα. Subsequently, the binding of 1 nmol/mL ^{125}I-VT-1 was determined. (B) Production of PAI-1 antigen by the same cells during the 24-hour incubation with inhibitors. (C) Expression of E-selectin by HUVEC after 5 hours exposure to 20 ng/mL TNFα and the indicated inhibitors. E-selectin was assayed by cell ELISA as described in Materials and Methods. (D) Incorporation of ^{35}S-methionine in 10% TCA-precipitable proteins[7] during a 24-hour incubation with TNFα and the various inhibitors, indicated in (A). The values represent the mean ± SEM of three to five independent HUVEC cultures.

than the wild-type TNFα. While activation of TNFR-p75 by D143N-A145R-TNFα mutant had no effect on $GbOse_3cer$ synthesis, blocking of TNFR-p75 by the monoclonal antibody utr-1 reduced the TNFα-induced increase in VT-1 receptors, in particular, at low TNFα concentrations and at early time points. Because simultaneous stimulation of both TNF receptor types by two TNFα mutants did not enhance VT-1 receptor expression more than obtained by stimulation of the TNFR-p55, it is unlikely that an intracellular signal generated via the TNFR-p75 enhanced TNFR-p55 activity or TNFR-p55-mediated signals. Our findings are consistent with the hypothesis of Tartaglia et al,[30] who proposed that TNFR-p75 can concentrate the TNFα molecules at the cell-surface, thereby facilitating the TNFα molecule to be passed on to the TNFR-p55. Similar observations have been made regarding the TNFα-induced expression of α2-integrins[17] and the TNFα-induced synthesis of E-selectin.[18]

A complex cascade of signal transducing events, including activation of the nuclear transcription factor NF-κB is probably involved in the induction of various proteins by TNFα in endothelial cells.[43] PKC activity has been reported to be needed for the induction of some TNFα-induced proteins. The TNFα-induced synthesis of urokinase[44] and the adhesion molecule VCAM-1[31] can be reduced by inhibitors of PKC, whereas these inhibitors do not affect the TNFα-induced synthesis of E-selectin,[31,32] ICAM-1,[32] and PAI-1.[44,45] Our data indicate that the TNFα-induced increase of the synthesis of VT-1 receptors does not require PKC activity. This con-

clusion is based on the observations that heterologous desensitisation by PMA did not specifically reduce the TNFα-dependent increase in VT-1 binding, whereas homologous desensitization entirely prevented an increase of VT-1 receptors by PMA. In the presence of TNFα, the PKC inhibitors Ro31-8220 and H-7, but not the structural analogue HA-1004, reduced VT-1 receptor expression to a similar extent as that of E-selectin and PAI-1, probably as the result of a generally reduced protein synthesis. This suggests that under our experimental conditions PKC activity can become a limiting factor in protein synthesis in endothelial cells exposed to TNFα.

Activation of PKC by itself causes a moderate increase in VT-1 receptors, and as such, adds to the TNFα-induced increase in VT-1 receptors. It is not yet known whether the effect of PMA on VT-1 binding is caused by an increased insertion of $GbOse_3cer$-containing caveolae in the plasma membrane after activation of PKC,[46] or by an increased synthesis of VT-1 receptors, similar to what happens after activation of endothelial cells by TNFα. In the latter case, the induction of galactosyl transferase activity, which underlies the increase in VT-1 receptors, behaves similarly as the induction of E-selectin, which can also be induced by PKC activation by a pathway that is different from the TNFα-induced expression.[31,32]

ACKNOWLEDGMENT

We thank Dr M.A. Karmali (Department of Microbiology, Hospital for Sick Children, Toronto, Canada) for providing us with purified VT-1, and Drs J. Leeuwenberg and W. Buurman for providing monoclonal antibodies against E-selectin.

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