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D609, a Phosphatidylcholine-Specific Phospholipase C Inhibitor, Blocks Interleukin-1β-Induced Vascular Cell Adhesion Molecule 1 Gene Expression in Human Endothelial Cells

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SUMMARY

At sites of inflammation, endothelial cells play a major role in defining the types of leukocytes that are recruited to a specific area. This is accomplished, at least in part, through the cytokine induction of cell surface adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1). We investigated the role of phosphatidylcholine-specific phospholipase C in the induction of VCAM-1 gene expression by interleukin-1β. D609, a phosphatidylcholine-specific phospholipase C inhibitor, reduced VCAM-1 cell surface expression and VCAM-1 promoter activity in human endothelial cells in a dose-dependent manner. D609 did not affect nuclear translocation of nuclear factor-κB but inhibited nuclear factor-κB-mediated transcription. The results of this study indicate that phosphatidylcholine-specific phospholipase C is required for activation of nuclear factor-κB and cytokine induction of VCAM-1 gene expression in endothelial cells.

During an inflammatory response, leukocyte recruitment to the vascular endothelium is mediated by the interaction of adhesion molecule receptors on endothelial cells and counterreceptors on immune effector cells. Endothelial cells play a major role in defining the repertoire of leukocytes that are recruited to an area of inflammation by expressing specific adhesion molecules in response to various cytokines, including IL-1β and TNF-α (for a review, see Ref. 1). These adhesion molecules include E-selectin, VCAM-1, and ICAM-1.

Cytokine induction of VCAM-1 cell surface expression is regulated at the level of transcription (2, 3). Recently, the promoter region of VCAM-1 was cloned and sequenced and shown to contain putative recognition sequences for a variety of transcription factors, including NF-κB/Rel (4, 5). NF-κB was originally described as a heterodimeric protein composed of 50 kD (p50) and 65 kD (p65) subunits (6–10). In the cytosol, this heterodimer is bound to an inhibitor protein, IκBα (9, 11–13). Stimulation with IL-1β or TNF-α induces a rapid phosphorylation of IκBα and subsequent proteolytic degradation (12, 14–16), which allows NF-κB to translocate to the nucleus and modulate gene expression. Endothelial cells express NF-κB proteins, which control the expression of proinflammatory genes such as VCAM-1 (17, 18).

It has been shown that hydrolysis of phosphatidylcholine through overexpression of a PC-PLC activated NF-κB (19). In addition, cytokine-responsive phospholipase C hydrolyzes phosphatidylcholine to generate the second messenger DAG (20). More recently, TNF-α was shown to activate NF-κB through PC-PLC-induced "acidic" sphingomyelin breakdown, which was inhibited by D609, a selective inhibitor of PC-PLC (21).

In the present study, we investigated the role of PLC in the signaling pathways that result in the activation of NF-κB and the induction of VCAM-1 gene expression in human endothelial cells. We present evidence that inhibition of PC-PLC activity by D609, a PC-PLC-specific inhibitor (21, 22), blocks the induction of VCAM-1 gene expression in response to IL-1β or TNF-α stimulation. This inhibition is also observed when D609 is administered after cytokine stimulation. D609 did not inhibit nuclear translocation of NF-κB but did inhibit NF-κB-mediated transcription. These results indicate that PC-PLC is required for IL-1β induction of VCAM-1 gene expression in human endothelial cells.

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ABBREVIATIONS: IL-1β, interleukin-1β; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule 1; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; PC-PLC, phosphatidylcholine-specific phospholipase C; PLC, phospholipase C; DAG, diacylglycerol; RT-PCR, reverse transcriptase-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; HUVEC, human umbilical vein endothelial cells; PAI-1, plasminogen activator inhibitor type 1; TPCK, *n*-tosyl-Phe-chloromethylketone; RHC, RHC-80267; ET18, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Cell culture. The human endothelial cell line ECV304 was purchased from American Type Culture Collection (Rockville, MD). HUVEC were purchased from Clonetics (San Diego, CA) and used between passages 2–6. ECV304 and HUVEC were maintained in endothelial growth medium (Clonetics).

Chemicals and reagents. All chemical compounds were purchased from BIOMOL (Plymouth Meeting, PA) except TPCK (Sigma Chemical Co., St. Louis, MO). A list of compounds used in this study is presented in Table 1. All DNA oligonucleotides were synthesized with an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA). IL-1β and TNF-α were purchased from R & D Systems (Minneapolis, MN). Compounds were solubilized in dimethylsulfoxide (Sigma).

ELISA. HUVEC (passages 2–6) were plated onto gelatin-coated 96-well tissue culture dishes (CoStar, Cambridge, MA) and incubated until confluent. The media were then replaced by media supplemented with various inhibitors and allowed to incubate for 30 min. The media were removed and replaced by media containing the various inhibitors plus 0.5 ng/ml IL-1β or 0.5 u/ml TNF-α and incubated for 6 hr. Each sample was performed in triplicate. After the 6-hr treatment, the HUVEC were washed once with PBS. Cells were then fixed by incubation in 3.7% formaldehyde/PBS for 5–10 min. Fixed HUVEC were stored at 4° overnight in PBS with 0.02% sodium azide. After the PBS/azide solution was aspirated, fixed cells were incubated at 37° in 1% bovine serum albumin in PBS (dilution/wash buffer) for 60 min before the addition of an anti-VCAM-1 monoclonal antibody 1G11B1 (Monosan, Uden-Holland) diluted 1:1000 or anti-ICAM-1 monoclonal antibody 84H10 (Immunotech, Westbrook, ME) diluted 1:5000. Plates were then incubated at 37° for 1–2 hr. Antibody solutions were aspirated, and the cells were subsequently washed three times before being incubated with a 1:5000 dilution of anti-mouse IgG/horseradish peroxidase conjugate (Promega, Madison, WI) for 60 min at 37°. Conjugate solution was aspirated, and cells were washed three times with wash buffer. TMB/E substrate solution (Chemicon, Temecula, CA) was then applied to the cells. When adequate color had developed, an equal volume of 2 N sulfuric acid was added to stop the reaction. Spectrophotometric readings at wavelength 450 nM were measured with a Molecular Devices (Menlo Park, CA) plate reader. All ELISA experiments were performed at least three times.

RT-PCR analyses. HUVEC were grown in gelatin-coated T25 flasks until confluent. The cells were then treated as described above for the ELISA. After a 6-hr incubation, total RNA was isolated with the use of RNAzolB according to the procedures of the manufacturer (BioTecX, Houston, TX). All RT reactions were performed with the GeneAmp RT-PCR kit according to the instructions of the manufacturer (Perkin Elmer). The PCR primers for β-actin were purchased from Clontech (Palo Alto, CA) and produced an amplified product of 838 base pairs. All PCR reactions were performed according to the instructions of Clontech for their amplimer sets. VCAM-1 primers amplified a product of 1101 base pairs and contained the following sequences: 5′-TTACACATTGATGAAATGGATTCTGT-3′ and 5′-AAGGTGAGAGTTGCATTCTCAGAAAG-3′. The PCR primers for PAI-1 amplified a product of 801 base pairs and contained the following sequences: 5′-CTACTTCAACGGCCAGTGGAAGCATC-3′ and 5′-GAGGCCAAGGTCTTGGAGACAGATCT-3′. The amplified products were electrophoresed in a 3% agarose gel (3:1 GTG NuSieve, FMC, Rockland, ME; Ultrapure agarose, Life Technologies, Gaithersburg, MD). All RT-PCR analyses were performed at least three times.

TABLE 1
Compounds used in this study

Compound	Activity	Reference
ET18	Phosphatidylinositol-specific inhibitor	27
RHC	DAG lipase inhibitor	28
U73122	Nonspecific PLC inhibitor	29, 30
D609	Phosphatidylcholine-specific inhibitor	21, 22
TPCK	Nonspecific serine protease inhibitor	12

Cell viability. Percent viability of HUVEC treated with test compounds was determined with alamarBlue (BioSource International, Camarillo, CA) according to the manufacturer's instructions. HUVEC (passages ≤6) were cultured in gelatin-coated 96-well tissue culture dishes until confluent. Inhibitors were evaluated in triplicate at 200 μM in endothelial growth medium/10% alamarBlue reagent. Fluorimetric readings (excitation, 530 nM; emission, 590 nM) were taken after a 6-hr incubation. Cells treated with inhibitors that showed <80% viability were considered toxic.

Plasmid construction, DNA isolation, and cell transfection. All restriction enzymes were purchased from Stratagene (La Jolla, CA). The neomycin gene was subcloned from pMAMneo (Clontech, Palo Alto, CA) into the *Bam*HI site of pGL2Basic (Promega) to generate pGLneo. Genomic DNA was isolated from HUVEC as described previously (23). The VCAM-1 promoter sequence (4, 5) was amplified from HUVEC DNA with the GeneAmp PCR kit from Perkin Elmer (Norwalk, CT) according to the manufacturer's procedures. The amplification parameters were 30 cycles of 94° for 45 sec, 55° for 45 sec, and 72° for 2 min with a 7-min final elongation step at 72°. An 1811-base pair fragment of the VCAM-1 promoter [−1717 to +94 (4)] was amplified with the use of the following primers: 5′-GGTACCGGCATTTCTCCAATGTTGCAAGCT-3′ and 5′-GGTACCATAGTGGTTCCAAAACCCTTA-3′. The VCAM-1 promoter fragment was cloned into the *Kpn*I site of the pGLneo plasmid to generate pGLneoVCAM-1. Positive clones were confirmed through DNA sequence analysis with Sequenase (U.S. Biochemicals, Cleveland, OH). The plasmid was purified with the use of $CsCl_2$ gradient according to standard procedures (24). Stably transfected cell lines were created as follows: plasmid DNA was transfected into the ECV304 endothelial cell line using Lipofectin (Life Technologies) and selected for resistance to geneticin (Life Technologies) at a concentration of 300 μg/ml. Transient transfections were performed as described previously (25). Plasmid pGL2Promoter was purchased from Promega (Madison, WI), and pCMVβ, which expresses the *Lac*Z gene, was purchased from Clontech. β-Galactosidase levels were determined using the Galacto-Light assay system (Tropix, Bedford, MA) and exhibited <15% variation between samples.

Luciferase assays. Approximately 25,000 cells/well were plated onto a gelatin-coated Falcon 48-well tissue culture plate (Becton-Dickinson, Franklin Lakes, NJ) and incubated overnight. The media were then replaced by media supplemented with inhibitor and allowed to incubate 30 min. The media were removed and replaced by media containing the inhibitor plus 0.5 ng/ml IL-1β and incubated for 6 hr. Each test sample was performed in triplicate. After incubation, the cells were washed with PBS and then lysed with 50 μl of lysis buffer (0.1 M KPO_4, pH 8, 0.1% Triton X-100, 1 mM dithiothreitol, 2 mM EDTA). The lysed cells remained at −20° overnight. After thawing, 5 μl of the lysate was added to 100 μl of luciferase assay buffer (Promega) and immediately analyzed for light production with a Monolight 2010 luminometer (Analytical Luminescence Laboratories, Sorrento Valley, CA). The amount of light emitted (light units) by the cell lysate was measured for 10 sec (experiments were performed at least three times). Protein concentrations varied by <10% between samples.

EMSA. Nuclear extracts were prepared from 5×10^6 HUVEC as described previously (26). Protein concentrations in nuclear extracts were 1–5 mg/ml, as determined with BCA protein assay (Pierce, Rockford IL). Oligonucleotides containing the mouse Igκ site (italicized), 5′-CAGA*GGGACTTTCC*GAGA-3′, were obtained from Operon Technologies, Inc. (Alameda, CA). The tandem NF-κB binding sites (italicized) from the VCAM-1 promoter contained the following sequences: 5′-CTGCCCT*GGGTTTCCCC*TTGAA*GGGATT*-

*TCCC*TCCGCCGGTAC-3′. Oligonucleotides were radiolabeled with [α^{32}P]dCTP (>3000 Ci/mmol) (Amersham) as described (26). Analysis of NF-κB protein binding was performed as follows: nuclear extracts (1–2 μg) were incubated with radiolabeled DNA probes (~10 ng; 1 × 10^6 cpm) for 20 min at room temperature in a 10-μl binding reaction containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.1% Nonidet P-40, and 25 μg/ml poly(dI:dC). Protein.DNA complexes were separated from free DNA probe by electrophoresis through 6% nondenaturing acrylamide gels (Novex, San Diego, CA) in 0.5× Tris/borate/EDTA (1× = 0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA). For antibody supershift experiments, binding reactions were incubated with monospecific antibodies for 20 min before the addition of the radiolabeled oligonucleotide. Anti-p65 and anti-p50 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

We investigated the role of PC-PLC and other phospholipases in the IL-1β induction of VCAM-1 in human endothelial cells by using several phospholipase inhibitors (see Table 1). First, we examined the IL-1β-induced cell surface expression of VCAM-1 in endothelial cells. ELISA were performed on cytokine-treated HUVEC exposed to the various inhibitors. The phosphatidylinositol-specific PLC inhibitor ET18 (27) and the DAG lipase inhibitor RHC (28) did not show any inhibition of cytokine-induced VCAM-1 cell surface expression (Fig. 1, A and B). In addition, the nonspecific PLC inhibitor U73122 (29, 30) did not show any inhibitory effect (data not shown). U73122 was tested at much lower doses due to cytotoxicity that occurred when it was tested at >10 μM (data not shown). In contrast, the PC-PLC specific inhibitor D609 inhibited IL-1β- and TNF-α-induced VCAM-1 cell surface expression in a dose-dependent manner (Fig. 1, A and B). D609 also inhibited the cytokine-induced cell surface expression of ICAM-1, whereas RHC and ET18 failed to inhibit ICAM-1 expression (Fig. 1, C and D). D609 was a less potent inhibitor of ICAM-1 cell surface expression.

The time-dependency of the inhibition was examined by adding D609 before and after cytokine stimulation. D609 abolished cytokine stimulation of VCAM-1 expression when added 30 min before and up to 60 min after stimulation (Fig. 2). Inhibition of cytokine-induced VCAM-1 cell

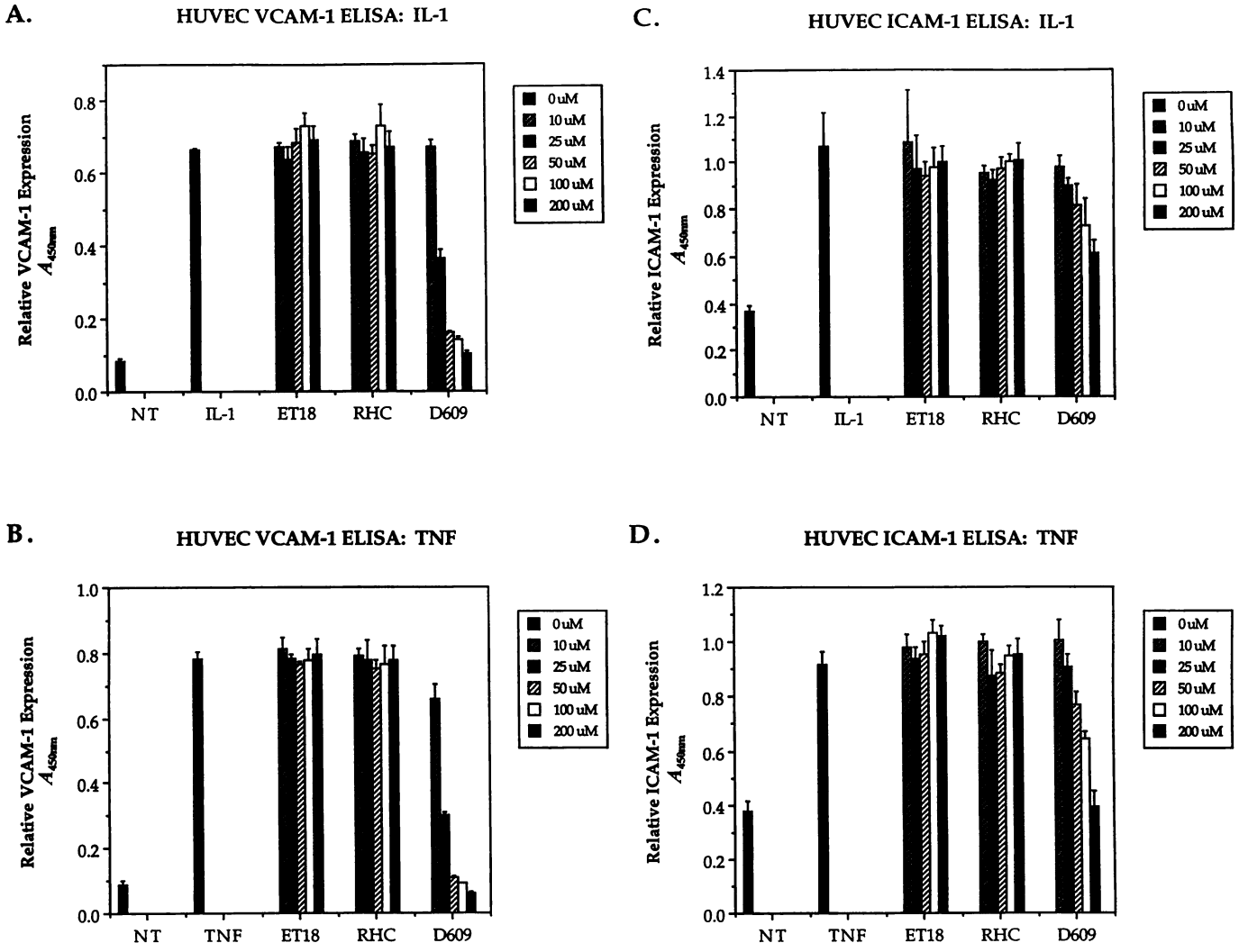

Fig. 1. Inhibition of cytokine-induced cell surface expression of VCAM-1 and ICAM-1. HUVEC monolayers were treated with 10, 50, 100, and 200 μM concentrations of the indicated compounds for 30 min before the addition of 0.5 ng/ml IL-1β or 0.5 u/ml TNF-α for 6 hr. Samples were performed in triplicate. Results are shown as mean ± standard deviation. Each inhibitor was analyzed at least three times. *NT*, no treatment; *IL-1*, 0.5 ng/ml IL-1β only; *TNF*, 0.5 u/ml TNF-α only. All other cells were treated with cytokine and inhibitors.

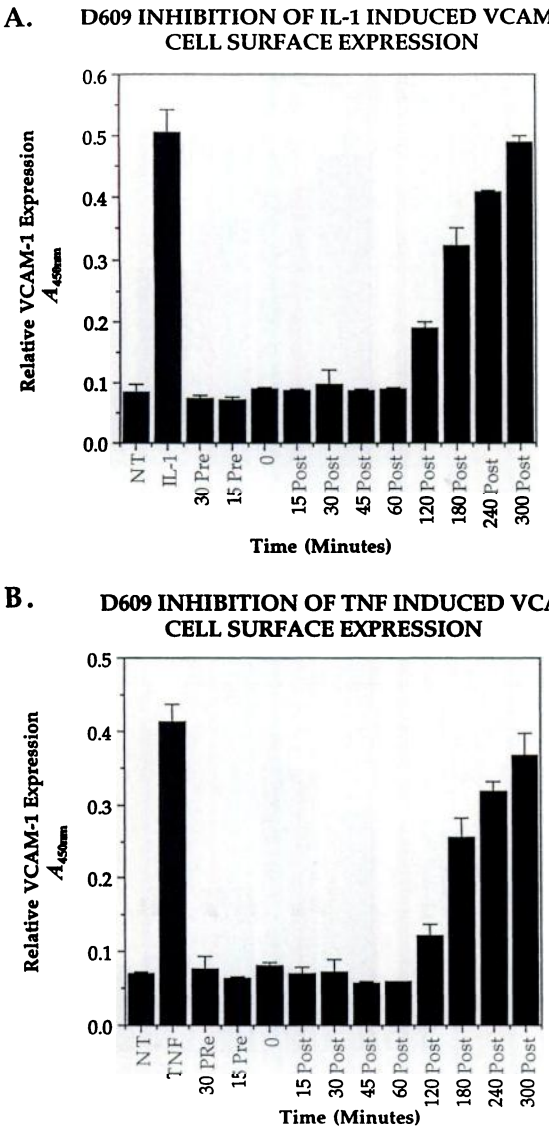

Fig. 2. Time course study of D609 inhibition of cytokine-induced VCAM-1 cell surface expression. Confluent HUVEC monolayers were cultured in a 96-well plate and treated with 200 μM D609 at the indicated times before or after IL-1β (0.5 ng/ml) or TNF-α (0.5 u/ml) stimulation for 6 hr. Cell surface expression of VCAM-1 was detected by ELISA as described in Experimental Procedures. *Pre*, before treatment with D609 before cytokine stimulation; *Post*, after treatment with D609 after cytokine stimulation. A, D609 inhibition of IL-1β-induced VCAM-1 cell surface expression. B, D609 inhibition of TNF-α-induced VCAM-1 cell surface expression.

surface expression was reduced in a time-dependent manner when D609 was added at 120–300 min after stimulation.

Because it has been shown that IL-1β-induced VCAM-1 protein expression is due to an increase in the levels of VCAM-1 mRNA (2, 3), we examined the effects of D609 on VCAM-1 mRNA levels in IL-1β-stimulated and unstimulated HUVEC. We performed RT-PCR on total RNA isolated from these cells. IL-1β induction of VCAM-1 mRNA expression was completely inhibited by treatment with D609 (Fig. 3A). The protease inhibitor TPCK was used as a positive control and also blocked IL-1β induction of VCAM-1 mRNA. D609 did not inhibit all IL-1β-induced genes because it did not demonstrate any inhibitory effects on IL-1β induction of

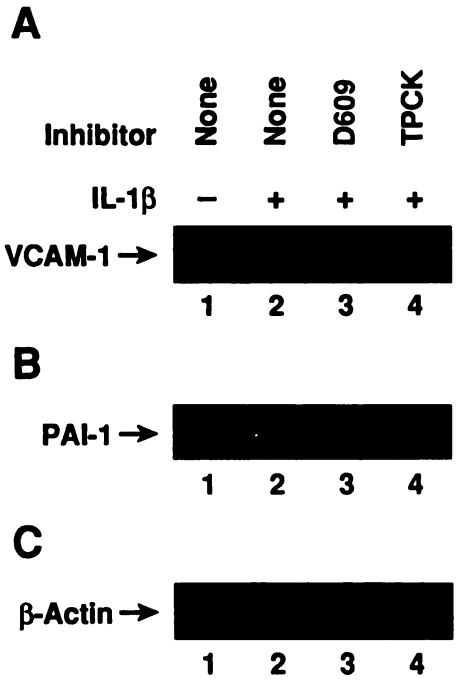

Fig. 3. D609 inhibition of IL-1β-induced VCAM-1 mRNA expression. HUVEC monolayers were treated with D609 (200 μM) or TPCK (50 μM) for 30 min before the addition of 0.5 ng/ml IL-1β for 6 hr. Total RNA was isolated, and 1 μg was used for RT-PCR analysis with VCAM-1-specific primers (A), PAI-1-specific primers (B), or β-actin-specific primers (C). Experiments were repeated three times.

PAI-1 mRNA expression (Fig. 3B). Similar results were observed with TPCK. Previous studies have shown that the PAI-1 gene is induced via an NF-κB-independent pathway (31). Similarly, D609 and TPCK did not show any inhibitory effects on β-actin mRNA expression (Fig. 3C). None of these inhibitors showed any visible effects on the HUVEC monolayer, nor did they demonstrate any cytotoxic effects at the concentrations used in this study as measured with alamarBlue assay (data not shown).

To determine the effects of D609 on VCAM-1 promoter activity, the endothelial cell line ECV304 was stably transfected with a luciferase reporter gene plasmid containing the VCAM-1 promoter (pGLneoVCAM-1). ECV304 cells have been used as a model to analyze the transcriptional regulation of the ICAM-1 gene (32). IL-1β stimulation of ECV304 cells containing pGLneoVCAM-1 induced luciferase expression directed by the VCAM-1 promoter (Fig. 4A). D609 inhibited VCAM-1 promoter activity in a dose-dependent manner, whereas RHC and ET18 had no effect (Fig. 4A). Similarly, IL-1β and TNF-α stimulation of HUVEC transiently transfected with pGLneoVCAM-1 induced luciferase expression, whereas there was no induction of luciferase expression from the promoterless control plasmid pGLneo (Fig. 4B). VCAM-1 promoter activity in HUVEC was inhibited in a dose-dependent manner by D609 (Fig. 4C).

To determine whether D609 treatment inhibited NF-κB-mediated transcription, HUVEC were transiently transfected with a plasmid $p(\kappa B)_4LUC$, which contains four copies of a murine Igκ κB site cloned upstream of a minimal simian virus 40 promoter. IL-1β and TNF-α stimulation of HUVEC transiently transfected with $p(\kappa B)_4LUC$ induced luciferase expression, whereas the parental plasmid, pGL2Promoter, was not affected (Fig. 4B). D609 inhibited IL-1β induction of

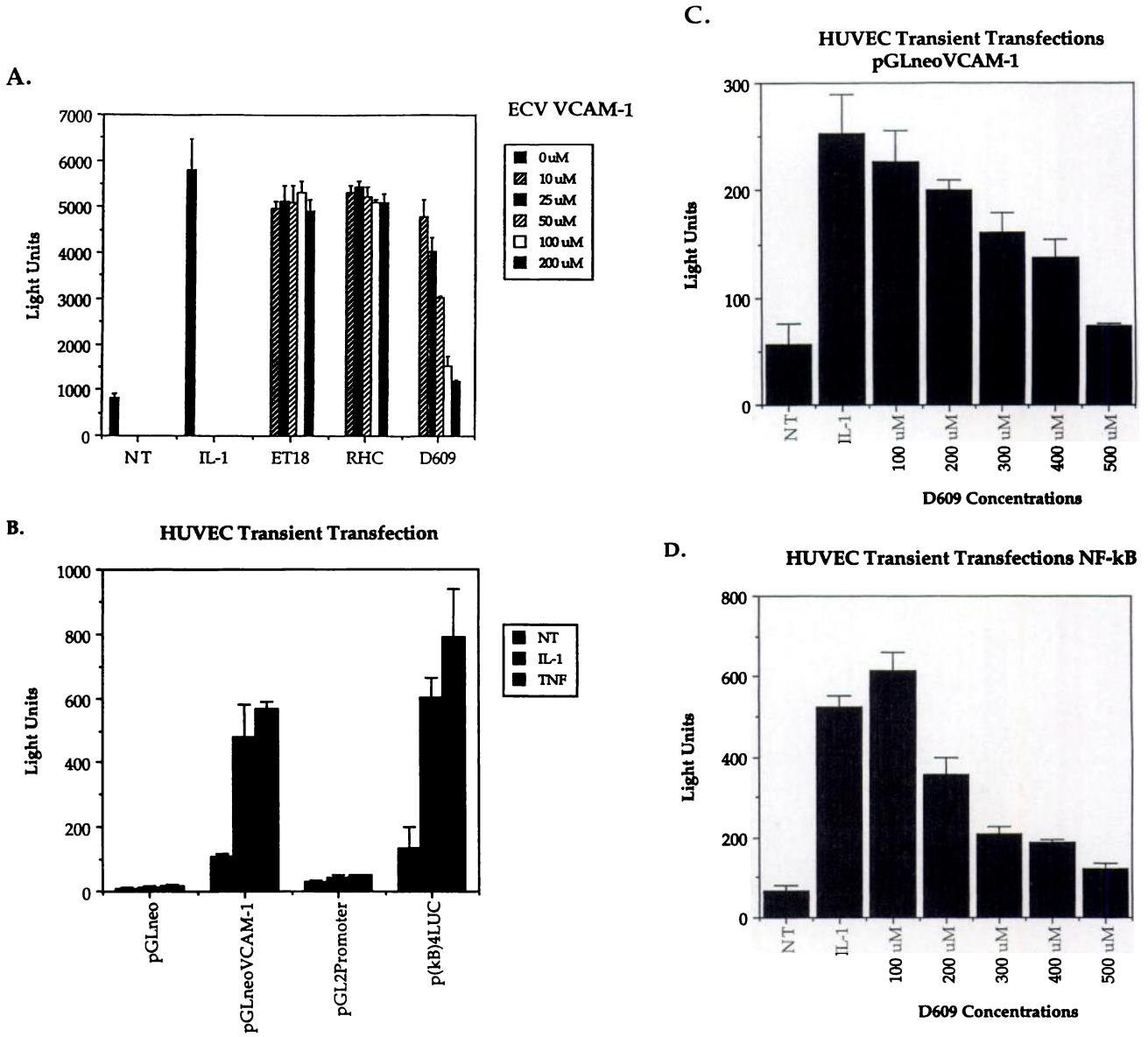

Fig. 4. D609 inhibition of IL-1β-stimulated VCAM-1 promoter activity and NF-κB-mediated transcription. A, ECV304 VCAM-1. ECV304 cells stably transfected with pGLneoVCAM-1 were pretreated with 10, 25, 50, 100, or 200 μM concentrations of the indicated compounds for 30 min before the addition of 0.5 ng/ml IL-1β for 6 hr. Samples were performed in triplicate. Luciferase activity was determined as described in Experimental Procedures. Results are given as mean ± standard deviation. Each inhibitor was analyzed at least three times. *NT*, no treatment; *IL-1*, 0.5 ng/ml IL-1β only. B, Cytokine stimulation of HUVEC transiently transfected with reporter gene plasmids. HUVEC were transiently transfected with 3 μg of the indicated plasmid plus 1 μg of pCMVβ as a control for transfection efficiency. After 18 hr, cells were treated with 0.5 ng/ml IL-1β or 0.5 u/ml TNF-α for 6 hr. Luciferase activity and β-galactosidase activity were determined. Luciferase results are given as mean ± standard deviation. C, D609 inhibition of VCAM-1 promoter activity in HUVEC. HUVEC were transiently transfected with 3 μg of pGLneoVCAM-1 and 1 μg of pCMVβ. After 18 hr, cells were pretreated with the indicated concentrations of D609 for 30 min before the addition of 0.5 ng/ml IL-1β for 6 hr. Luciferase and β-galactosidase activities were determined. Luciferase results are given as mean ± standard deviation. *NT*, no treatment; *IL-1*, 0.5 ng/ml IL-1β. D, D609 inhibition of NF-κB-mediated transcription in HUVEC. HUVEC were transiently transfected with 3 μg of p$(\kappa B)_4$LUC and 1 μg of pCMVβ. After 18 hr, cells were pretreated with indicated concentrations of D609 for 30 min before the addition of 0.5 ng/ml IL-1β for 6 hr. Luciferase and β-galactosidase activities were determined. Luciferase results are given as mean ± standard deviation. *NT*, no treatment; *IL-1*, 0.5 ng/ml IL-1β.

NF-κB-mediated transcription in a dose-dependent manner (Fig. 4D). D609 did not affect luciferase activity (data not shown). In addition, D609 did not affect β-galactosidase expression directed by the cytomegalovirus promoter in pCMVβ, indicating that it did not nonspecifically inhibit gene transcription.

We next investigated whether D609 inhibited the nuclear translocation of NF-κB. Previous studies have shown that activation of NF-κB is required for induction of VCAM-1 gene expression in endothelial cells (4, 5, 17). EMSAs were performed with nuclear extracts from HUVEC exposed to D609 with or without IL-1β stimulation. In unstimulated HUVEC, NF-κB proteins are retained in the cytoplasm, and therefore little or no NF-κB-specific protein/DNA complex is observed with the use of nuclear extracts from unstimulated cells (Fig. 5A). IL-1β stimulation of HUVEC induces nuclear translocation of NF-κB as measured with oligonucleotides containing either the prototypic Igκ κB binding site or the tandem copies

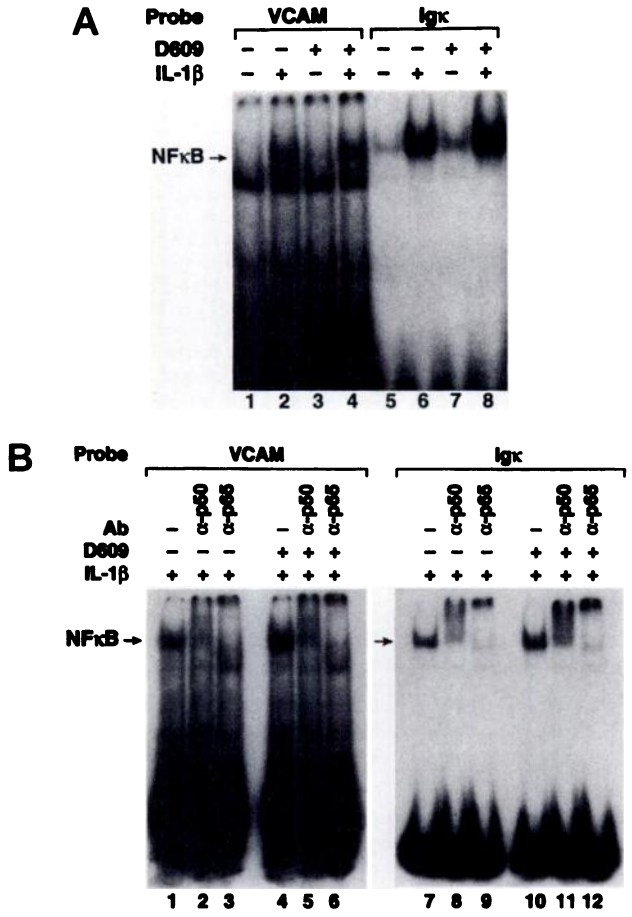

Fig. 5. D609 does not inhibit nuclear translocation of NF-κB in HUVEC. Nuclear extracts were prepared from HUVEC incubated for 1 hr with 1 ng/ml IL-1β with or without a 30-min pretreatment with D609 (200 μM). A, The presence of NF-κB in nuclear extracts was determined through EMSA with radiolabeled oligonucleotides containing the tandem κB sites from the VCAM-1 promoter (*lanes 1–4*) or a prototypic κB site from the murine Igκ gene (*lanes 5–8*). IL-1β stimulation of HUVEC induced the nuclear translocation of NF-κB (*arrow*). B, Protein composition of the NF-κB complex was determined with antibody (*Ab*) supershift experiments. Antibodies were added 20 min before the addition of the radiolabeled probes.

of the κB sites from the VCAM-1 promoter (Fig. 5A). D609 did not inhibit nuclear translocation of NF-κB (Fig. 5A). In contrast, the protease inhibitor TPCK (100 μM) completely blocked the nuclear translocation of NF-κB (data not shown). To exclude the possibility that D609 altered the composition of the nuclear complexes, antibody supershift experiments were performed (Fig. 5B). The NF-κB complexes formed with oligonucleotides containing the VCAM-1 κB sites or the Igκ κB site were both supershifted with anti-p50 and anti-p65 antibodies, indicating that they represented binding of p50/p65 heterodimers as reported previously (33). D609 did not change the composition of these NF-κB complexes (Fig. 5B).

Discussion

In the current study, we investigated the role of PC-PLC and other phospholipases in the IL-1β-stimulated induction of VCAM-1 gene expression in endothelial cells. We demonstrated that PC-PLC is involved in IL-1β and TNF-α signaling in endothelial cells. Furthermore, the specific PC-PLC inhibitor D609 blocked cytokine induction of VCAM-1 gene expression. Our results with other inhibitors suggest that DAG lipase, phosphatidylinositol-specific PLC, and other PLC enzymes are not involved in IL-1β-induced VCAM-1 gene expression in endothelial cells.

The PC-PLC specific inhibitor D609 did not inhibit the general transcriptional machinery of the cell because β-actin mRNA expression was not inhibited. In addition, D609 did not inhibit expression of the cytomegalovirus promoter in transient transfection experiments, indicating that it did not nonspecifically inhibit transcription. D609 did not effect all IL-1β-induced responses within the endothelial cells as it did not show any inhibitory effect on IL-1β induction of PAI-1 mRNA. Previous studies have shown that the PAI-1 gene is regulated by an NF-κB-independent mechanism (31). Our results show that D609 inhibits transcription of the VCAM-1 gene, although we cannot exclude the possibility that D609 may also affect the stability of the VCAM-1 mRNA.

Previous studies suggest that PC-PLC is involved in the IL-1β- and TNF-α-induced activation of NF-κB in a variety of cell types (21, 34–36). The addition of exogenous PC-PLC or overexpression of endogenous PC-PLC but not the addition of exogenous phosphatidylinositol-specific PLC resulted in the activation of NF-κB (19). These results demonstrate that PC-PLC is directly involved in the activation of NF-κB. We present evidence with the use of endothelial cells that D609 does not inhibit IL-1β-induced nuclear translocation of NF-κB or the binding of nuclear p50/p65 heterodimers. However, D609 did inhibit IL-1β-induced NF-κB-mediated transcription in transiently transfected HUVEC, suggesting that D609 inhibits the functional activity of nuclear NF-κB rather than nuclear translocation. In a previous study, D609 inhibited the nuclear translocation of NF-κB (21) in TNF-α-stimulated Jurkat T cells and U937 monocytic cells. Similarly, we have shown that D609 inhibited nuclear translocation of NF-κB in lipopolysaccharide-stimulated THP-1 monocytic cells.[1] Therefore, these differences seem to be due to the use of different cell types.

A variety of agents inhibit cytokine-induced VCAM-1 gene expression in endothelial cells by blocking the nuclear translocation of NF-κB (37, 38). Free radical scavengers, such as pyrrolidine-dithiocarbamate, are potent inhibitors of cytokine-stimulated NF-κB mobilization (38, 39). Aspirin also inhibits VCAM-1 expression by preventing the activation of NF-κB (37). These compounds may inhibit NF-κB release from IκB by blocking the phosphorylation and/or proteolytic degradation of IκBα.

Other studies have also shown inhibition of cytokine-induced expression of E-selectin, ICAM-1, and VCAM-1 in endothelial cells without interfering with NF-κB nuclear translocation (40, 41). It is possible that PC-PLC is required for the activation of other transcription factors that are required for up-regulation of VCAM-1 gene transcription in endothelial cells. Recent studies have shown that an interferon regulatory factor may cooperate with NF-κB in the regulation of VCAM-1 gene induction (33). The interferon regulatory factor, as well as other unidentified transcription factors, may be downstream targets of a signaling cascade in which PC-PLC plays a role. Inhibition of PC-PLC may inhibit their activation and/or their ability to cooperate with NF-κB to activate VCAM-1 gene expression in endothelial cells. Alter-

[1] R. R. Cobb and N. Mackman, unpublished observations.

natively, other proteins required for the functional activity of NF-κB may be modulated by D609 inhibition of PC-PLC. Additional experiments are required to determine whether D609 modulates VCAM-1 gene expression in HUVEC by these or other mechanisms.

In this study, we demonstrated the importance of PC-PLC in gene expression in endothelial cells. The results show that cytokine-induced nuclear translocation of NF-κB is not sufficient for activation of VCAM-1 gene expression in human endothelial cells. We demonstrated that D609 inhibits the functional activity of NF-κB, which may account for D609 inhibition of the cytokine induction of VCAM-1 in human endothelial cells. Because VCAM-1 plays a central role in the pathogenesis of inflammation, PC-PLC may represent a novel anti-inflammatory pharmacological target.

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