

Protein Kinase C-Activated Oxidant Generation in Endothelial Cells Signals Intercellular Adhesion Molecule-1 Gene Transcription

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ABSTRACT

We tested the hypothesis that activation of protein kinase C (PKC) and generation of oxidants are critical sequential signals mediating tumor necrosis factor (TNF)- α -induced activation of nuclear factor- κ B (NF- κ B) and transcription of the intercellular adhesion molecule (ICAM)-1 gene. Stimulation of human pulmonary artery endothelial (HPAE) cells with TNF- α (100 U/ml) induced the activation of PKC and, subsequently, generation of oxidants. Pretreatment with calphostin C, a specific PKC inhibitor, prevented oxidant generation after TNF- α stimulation, indicating that PKC activation mediated the production of oxidants in HPAE cells. In contrast, pretreatment of HPAE cells with *N*-acetylcysteine, an antioxidant and a precursor of glutathione, failed to prevent PKC activation, indicating that PKC activation was not secondary to the oxidant production. These

findings suggest that oxidant generation in endothelial cells occurs downstream of PKC activation. However, both PKC activation and oxidant generation were necessary for ICAM-1 mRNA expression because the pretreatment of HPAE cells with either calphostin C or *N*-acetylcysteine inhibited the TNF- α -induced activation of NF- κ B and prevented the activation of ICAM-1 promoter. Prolonged exposure of HPAE cells to the phorbol ester, phorbol-12-myristate-13-acetate, which is known to deplete all except atypical PKC isozymes, failed to prevent TNF- α -induced ICAM-1 mRNA expression. We conclude that TNF- α -induced oxidant generation secondary to the activation of a phorbol ester-insensitive PKC isozyme signals the activation NF- κ B and ICAM-1 gene transcription.

Transendothelial trafficking of leukocytes during inflammation requires the expression of adhesion molecules on the endothelial cell surface and their corresponding receptors on the surface of leukocytes (Springer, 1994). The release of inflammatory mediators [e.g., cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β] activates the rapid expression of intercellular adhesion molecule-1 (ICAM-1; CD54) on the surface of endothelial cells (Wertheimer et al., 1992; Rahman et al., 1996). The interaction of ICAM-1 with its counterreceptors, the CD11a/CD18 and CD11b/CD18 integrins on the leukocyte plasmalemma, is a requirement for stable polymorphonuclear leukocyte adhesion to endothelial cells and transendothelial migration of polymorphonuclear leukocytes (Smith et al., 1988, 1989). Studies of human ICAM-1 promoter suggest that TNF- α -induced transcription of ICAM-1 gene in endothelial cells

critically depends on the activation of the transcription factor nuclear factor- κ B (NF- κ B) (Hou et al., 1994; Ledebur and Parks, 1995; Rahman et al., 1996). NF- κ B, a heterodimer of 50 kDa (p50) and 65 kDa (p65) subunits, exists in the cytoplasm in an inactive form bound to the inhibitory protein I- κ B (I κ B) through p65 (Beg and Baldwin, 1993). The treatment of cells with TNF- α leads to the activation of I κ B kinases α and β (DiDonato et al., 1997; Zandi et al., 1997), which phosphorylate serine residues 32 and 36 of I κ B α and serine residues 19 and 23 of I κ B β , respectively, and which in turn target them for rapid polyubiquitination followed by degradation through 26S proteasome (Brown et al., 1995; Traenckner et al., 1995; Chen et al., 1996). An alternative mechanism of NF- κ B activation, independent of proteolytic degradation of I κ B, involves the tyrosine phosphorylation of I κ B (Imbert et al., 1996). The activated NF- κ B dimer then translocates to the nucleus, where it binds to DNA and regulates transcription of genes such as ICAM-1 (Baldwin, 1996; Barnes and Karin, 1997). Protein kinase C (PKC) isozymes are serine/threonine kinases mediating intracellular signaling

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ABBREVIATIONS: ICAM-1, intercellular adhesion molecule-1; C-DCDHF-DA, 5- (and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate bis(acetoxy-methyl) ester; PMA, phorbol-12-myristate-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; HPAE, human pulmonary artery endothelial; LUC, luciferase; NF- κ B, nuclear factor- κ B; EGM, endothelial cell growth medium; PMSF, phenylmethylsulfonyl fluoride; SSC, standard saline citrate.

(Nishizuka, 1992). Based on their structure and requirement for activation, PKC isozymes can be divided into three groups: 1) conventional (cPKCs) α , β I, β II, and γ require negatively charged phospholipids, diacylglycerol or phorbol ester, and calcium for optimal activation (Hug and Sarre, 1993; Jaken, 1996); 2) novel (nPKCs) δ , ϵ , θ , μ , and η /L (mouse/human) require negatively charged phospholipids or diacylglycerol or phorbol ester but no calcium (Hug and Sarre, 1993; Johannes et al., 1994; Jaken, 1996); and 3) atypical form (aPKCs) ζ and λ/ι (mouse/human) do not require calcium, diacylglycerol, or phorbol esters but require only negatively charged phospholipids (Jaken, 1996). Of these, α , β , δ , ϵ , η , θ , and ζ isozymes have been identified in endothelial cells (Bussolino et al., 1994; Kizbai et al., 1995). Studies based on phorbol-12-myristate-13-acetate (PMA)-mediated depletion of PKC and inhibitors such as staurosporine concluded that TNF- α -induced ICAM-1 expression is independent of PKC activation (Richie et al., 1991; Myers et al., 1992). Because endothelial cells express atypical PKC isozymes such as PKC ζ in abundance (Kizbai et al., 1995) and because PKC ζ is staurosporine insensitive (Seynaeve et al., 1994) and is not depleted by phorbol esters (Wooten et al., 1994; Hofmann, 1997), atypical PKC isozymes may be important in signaling TNF- α -induced ICAM-1 expression. In the present study, we addressed the role of PKC activation in mediating oxidant generation and ICAM-1 gene transcription in human pulmonary artery endothelial (HPAE) cells. The data suggest that TNF- α -induced oxidant generation secondary to activation of a phorbol ester-insensitive PKC isozyme signals the activation of NF- κ B and ICAM-1 gene transcription in endothelial cells.

Materials and Methods

Cell Culture. HPAE cells were obtained from Clonetics (La Jolla, CA) and grown on gelatin-coated flasks or plates in endothelial cell growth medium (EGM) containing 10% fetal calf serum and 3.0 mg/ml endothelium-derived growth factor from bovine brain extract protein. Human recombinant TNF- α with a specific activity of 2.3×10^7 was purchased from Promega Corp. (Madison, WI). All experiments, except where indicated, were conducted using cells under the 10th passage. Confluent HPAE cells were washed twice with serum- and phenol red-free Dulbecco's modified Eagle's medium containing 20 mM HEPES or EGM containing 2% fetal calf serum and then incubated in the same medium with the indicated concentrations of *N*-acetylcysteine or calphostin C for 0.5 h before the addition of TNF- α . TNF- α was added directly to the medium for the times and at concentrations indicated in each experiment. *N*-Acetylcysteine was neutralized to pH 7.0 before use.

PKC Activity. Endothelial cells were washed twice with ice-cold calcium- and magnesium-free PBS. Extraction buffer A [50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 0.35 w/v β -mercaptoethanol, 50 mg/ml phenylmethylsulfonyl fluoride (PMSF)] was added, and cells were scraped and lysed by four cycles of freeze-thawing. All subsequent steps were carried out at 4°C. The resulting lysates were centrifuged at 39,000 rpm for 1 h at 4°C, and the supernatants were collected and designated the cytosolic fraction. The remaining pellets were resuspended in extraction buffer B (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 0.3% Triton X-100, 0.35 w/v β -mercaptoethanol, 50 mg/ml PMSF) and homogenized with a small pestle. These lysates were microfuged at 4°C, and the supernatants were designated the soluble membrane fraction. PKC kinase activity was determined by measuring phosphorylation of the PKC-specific peptide (RKRTLRL) substrate based on

the conserved region of the epidermal growth factor receptor using the PKC assay system from Amersham Life Sciences, Inc. (Arlington Heights, IL). Extracts were quantified for protein concentration using a Bio-Rad protein determination kit (Bio-Rad Laboratories, Hercules, CA).

Northern Analysis. Total RNA was isolated from HPAE cells with TRIzol (GIBCO BRL, Grand Island, NY) according to manufacturer's recommendations. Quantification and purity of RNA were assessed by A_{260}/A_{280} absorption, and an aliquot of RNA (20–30 mg) from samples with ratio of more than 1.6 was fractionated using a 1% agarose formaldehyde gel. The RNA was transferred to Duralose-UV nitrocellulose membrane (Stratagene, La Jolla, CA) and covalently linked by ultraviolet irradiation using a Stratalinker UV crosslinker (Stratagene). Human ICAM-1 (0.96-kb *SalI/PstI* fragment) (Staunton et al., 1988) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.1-kb *PstI* fragment) were labeled with [32 P]dCTP using the random primer kit (Stratagene), and hybridization was carried out as we described previously (Roebuck et al., 1995). Briefly, the blots were prehybridized for 30 min at 68°C in QuikHyb solution (Stratagene) and hybridized for 2 h at 68°C with random primed 32 P-labeled probes. After hybridization, the blots were washed twice for 30 min at room temperature in $2\times$ standard saline citrate (SSC) with 0.1% SDS followed by two washes for 30 min each at 60°C in $0.1\times$ SSC with 0.1% SDS. Autoradiography was performed with an intensifying screen at -70°C for 12 to 24 h. The signal intensities were quantified by scanning the autoradiograms with a laser densitometer (Howtek, Hudson, NH) linked to a computer analysis system (PDI, Huntington Station, NY). The nitrocellulose membrane was soaked for stripping the probe with boiled water or $0.1\times$ SSC with 0.1% SDS.

Detection of Oxidant Generation. Confluent HPAE monolayers were stimulated for 1 h with TNF- α (100 U/ml) in EGM containing 2% serum as described above. Cells were washed twice with EGM (2% serum) and stained for 20 min with 2.5 mM 5- (and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate bis(acetoxy-methyl) ester (C-DCDHF-DA; Molecular Probes, Eugene, OR) in EGM (with 2% serum) as described previously (Rahman et al., 1998). Cultures were viewed with fluorescence microscopy and photographed. Quantitative fluorescence was imaged using a Nikon Diaphot 200 Microscope (Nikon Corp., Garden City, NJ) and Image Pro Plus software (Media Cybernetics Inc., Silver Spring, MD). Each sample was independently stained so the samples were exposed to the dye for the same time. The dye solution was freshly prepared in prewarmed EGM (with 2% serum) for each sample. After staining for 20 min at 37°C, samples were rinsed twice with EGM (with 2% serum) containing no dye and scanned on the fluorescence microscope. Samples were epilluminated by a 150-W mercury lamp and viewed with fluorescein filters (B2E cube). Fields were observed at $20\times$ N.A. 0.4 and were acquired with a CCD imaging array (Photometrics Inc., Tucson, AZ) under computer control with 1-s integration time. Illumination caused increased fluorescence because of oxidation of the dye; therefore, each field was exposed to light for exactly the same time. The image size for scanning was 768 horizontal \times 468 vertical. The average relative fluorescence intensity for every cell in each field was determined using Image Proplus software (Media Cybernetics Inc.).

Nuclear Extract Preparation HPAE cells were pretreated for 0.5 h without or with *N*-acetylcysteine (30 mM) or calphostin C (25 nM) and then left untreated or stimulated for 1 h with TNF- α (100 U/ml). Cells were washed twice with ice-cold Tris-buffered saline and resuspended in 400 μ l of buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF]. After 15 min, Nonidet P-40 was added to a final concentration of 0.6%. Nuclei were pelleted and resuspended in 50 μ l of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 30 min at 4°C, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. The protein concentration of the

extract was measured using a Bio-Rad protein determination kit (Bio-Rad Laboratories).

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays were performed as described previously (Rahman et al., 1998). Briefly, nuclear extract (10 μ g) was incubated with 1 mg of poly(dI/dC) in a binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10% glycerol in a 20-ml final volume) for 15 min at room temperature. Then, end-labeled double-stranded oligonucleotides containing the NF- κ B binding site of the ICAM-1 promoter (30,000 cpm each) were added in the absence or presence of 25- or 100-fold molar excess cold competitor, and the reaction mixtures were incubated for 15 min at room temperature. The DNA/protein complexes were resolved on 4.5 to 5% native polyacrylamide gels in low-ionic-strength buffer (0.25 \times Tris-borate-EDTA) for 2 to 2.5 h at 130 V. Oligonucleotides used for the gel shift analysis were as follows: ICAM-1 NF- κ B, 5'-AGCTTGGAAATTCGAGCTG-3'; mut-ICAM-1 NF- κ B, 5'-AGCTTccAAATTCC GGAGCTG-3'; Ig κ B 5'-AGT-TGAGGGGACTTTCAGGC-3'; and Oct-1 DNA, 5'-AATTGCAT-GCCTGCAGGTCGACTCTAGAGGATCCATGCAAATGGATCCC-GGGTACCGAGCTC-3'. The oligonucleotide designated as ICAM-1 NF- κ B represents a 21-bp sequence of ICAM-1 promoter encompassing NF- κ B binding site located 183 bp upstream of transcription initiation site (Hou et al., 1994). The oligonucleotide mut-ICAM-1 NF- κ B is the same as ICAM-1 NF- κ B except that it has 2-bp mutations in the NF- κ B site. The Ig κ B oligonucleotide contains the consensus NF- κ B binding site present in the Ig gene. The oligonucleotide Oct-1 (which contains binding site for Oct-1) was used as a negative control in the competition experiments. Sequence motifs within the oligonucleotides are underlined and the mutations are shown in lowercase.

Reporter Gene Constructs, Endothelial Cell Transfections, and Luciferase Assay. The ICAM-1 firefly luciferase (LUC) plasmids containing wild-type and mutated NF- κ B sites have been described (Hou et al., 1994). The wild-type and NF- κ B mutated versions of ICAM-1 LUC constructs were provided by Dr. Z. Cao (Tularik Inc., San Francisco, CA). Cells under the fifth passage were plated onto six-well Primaria culture dishes 18 to 24 h before transfection. Transfections were performed with Lipofectine (GIBCO BRL) as described previously (Rahman et al., 1998). Briefly, reporter DNA (1 mg) was mixed with 2 ml of Lipofectine in 200 ml of Opti-MEM (GIBCO BRL). We used the plasmid pSVbgal (0.2 mg) containing the β -galactosidase gene driven by the constitutively active SV40 promoter to normalize the transfection efficiencies. Because in the initial experiments we did not observe any significant difference in transfection efficiencies, we did not cotransfect pSVbgal construct in the later experiments. After a 30-min incubation, Opti-MEM (800 ml) was added, and the mixture was applied onto the cells that had been washed twice with Opti-MEM. Three hours later, the medium was changed to EGM containing 10% serum, and the cells were harvested 24 to 48 h after transfection. Using this protocol, we achieved a transient transfection efficiency of $15 \pm 3\%$ (mean \pm S.D.; $n = 3$) for HPAE cells. Recombinant TNF- α was used at a concentration of 100 U/ml for 12 to 18 h before harvesting the cells. Cell extract was prepared and assayed for luciferase activity using Promega Biotech assay system and for β -galactosidase activity using the Tropix assay system (Tropix, Bedford, MA). Luciferase activity was normalized to a value per microgram of protein extract and expressed as fold increase above basal level ICAM-1LUC expression. The protein content was determined using a Bio-Rad protein determination kit (Bio-Rad Laboratories).

Transfection of HPAE Cells with Oligonucleotides. The phosphorothioate oligonucleotides to PKC α , sense (TGA AAC TCA CCA GCG AGA AC) and antisense (GTT CTC GCT GGT GAG TTT CA), have been described previously (Dean et al., 1994) and are targeted to the 3'-untranslated region of PKC α mRNA. HPAE cells were grown on 100-mm dishes to 50% confluence. Transfections were performed with Lipofectine (GIBCO BRL) as described. Briefly, 800 μ l of Opti-MEM containing 20 μ l of Lipofectine was mixed with 800

μ l of Opti-MEM containing antisense or sense oligonucleotides and incubated for 30 min at room temperature. This oligonucleotide/Lipofectine complex solution was added to 6.4 ml of Opti-MEM, and the final oligonucleotide concentration was 0.25 μ M. The diluted complex solution was then applied onto the cells that had been washed twice with Opti-MEM. Three hours later, the medium was changed to EGM containing 10% serum, and the cells were incubated for an additional 36 to 40 h. Total RNA was isolated, and ICAM-mRNA expression was determined by Northern blot analysis as described above.

Results

TNF- α Activates PKC in HPAE Cells. We determined the enzymatic activity of PKC after TNF- α stimulation of HPAE cells. TNF- α induced a 2.5-fold activation of PKC in the cytosolic fraction in a time-dependent manner, with maximum response within 5 min followed by an $\sim 80\%$ decrease from the maximum value at 15 min, which was sustained for 30 min (Fig. 1). TNF- α did not significantly alter the membrane PKC activity (Fig. 1). In contrast, PMA alone (at a concentration of 80 nM for 15 min) produced a ~ 3 -fold increase in membrane activity (data not shown). Pretreatment of HPAE monolayers with the antioxidant *N*-acetylcysteine failed to prevent TNF- α -induced PKC activation (Fig. 1).

Calphostin C Prevents TNF- α -Induced ICAM-1 mRNA Expression. We used calphostin C to determine the role of PKC activation in mediating the TNF- α -induced transcription of ICAM-1 gene. Calphostin C pretreatment of HPAE cells prevented TNF- α induction of ICAM-1 transcription in a dose-dependent manner (Fig. 2). At a lower concen-

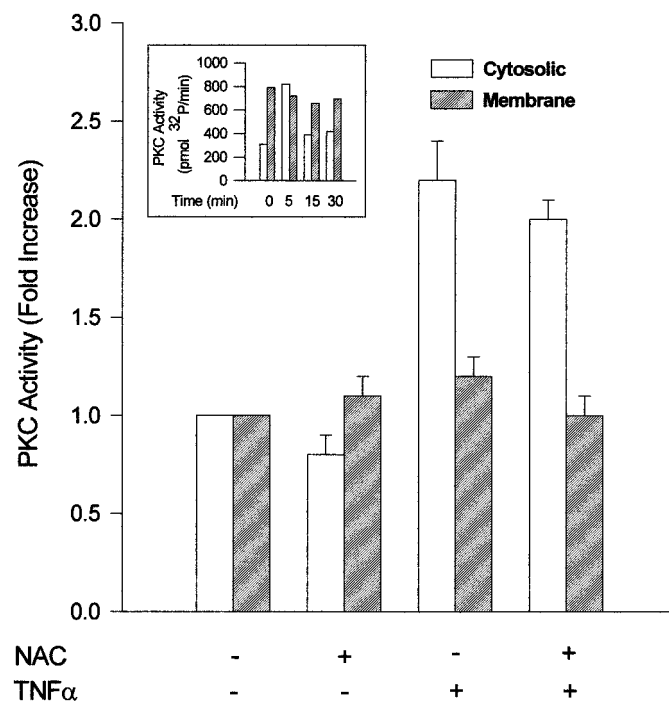


Fig. 1. TNF- α induces PKC activation in endothelial cells. Confluent HPAE monolayers were treated with TNF- α (100 U/ml) for the indicated time periods (inset) or pretreated with *N*-acetylcysteine (NAC; 30 mM) for 0.5 h before stimulation with TNF- α for 5 min. Monolayers then were washed, and cytosolic and membrane fractions were prepared and used for PKC assay as described in *Materials and Methods*. PKC activity normalized per milligram of protein is expressed as pmol ³²P transferred to the substrate/min (pmol ³²P/min) (inset) or as fold increase relative to the PKC activity in untreated control cells.

tration (5 nM), calphostin C prevented >80% of the response, but at a higher concentration (25 nM), calphostin C fully abrogated ICAM-1 mRNA expression (Fig. 2A). We also determined the effects of tyrosine kinase inhibitor genistein on TNF- α -induced ICAM-1 mRNA expression to compare with the effects of calphostin C. Pretreatment of HPAE cells with 5, 25, and 100 μ M genistein failed to prevent TNF- α -induced ICAM-1 gene transcription (Fig. 2B).

In addition to a PKC- and tyrosine kinase-dependent pathway (Folgueira et al., 1996; Imbert et al., 1996), the results of recent studies suggest a TNF- α -signaling pathway involving sphingomyelin metabolism and stimulation of a ceramide-dependent pathway leading to NF- κ B activation (Schutze et al., 1992; Kolesnick and Golde, 1994). To determine the possible role of this pathway in mediating the TNF- α -induced response in endothelial cells, we evaluated the ability of ceramide to induce ICAM-1 mRNA expression. The exposure of HPAE cells to a membrane-permeable form of ceramide, C₂-ceramide, did not increase either basal or TNF- α -induced ICAM-1 transcript (Fig. 2C). However, at a concentration of 100 μ M, ceramide slightly reduced TNF- α -induced ICAM-1 expression, which may be ascribed to toxic effects of ceramide at this concentration. These data indicate that activation of PKC is a critical signal mediating TNF- α -induced ICAM-1 transcription in endothelial cells.

Phorbol Ester-Induced PKC Depletion Fails to Prevent TNF- α -Induced ICAM-1 mRNA Expression. To de-

termine whether PKC isozyme involved in TNF- α signaling was phorbol ester sensitive, we studied the effects of PKC depletion by PMA on the TNF- α -induced ICAM-1 mRNA expression. HPAE cells were pretreated with 500 nM PMA for 24 h followed by stimulation with TNF- α (100 U/ml) or PMA (100 nM) for 3 h and then compared the results with those for untreated cells. Figure 3 demonstrates that pretreatment with PMA for 24 h prevented ICAM-1 mRNA induction elicited by the subsequent stimulation with PMA; however, PMA treatment did not inhibit the TNF- α -induced ICAM-1 mRNA expression. We also determined the effects of a PKC β inhibitor, LY333531, and antisense oligonucleotide to PKC α on TNF- α -induced ICAM-1 mRNA expression. Pretreatment with LY333531 did not modify the cellular response to TNF- α (data not shown). Transfection of antisense oligonucleotide to PKC α (GTT CTC GCT GGT TTT CA), which has been reported to prevent PMA-induced ICAM-1 mRNA expression (Dean et al., 1994), failed to reduce TNF- α -induced ICAM-1 transcript (Fig. 4). These data together with the results in Fig. 2A suggest that a phorbol ester-insensitive and a calphostin C-sensitive PKC isozyme (i.e., an atypical PKC isozyme) is responsible for the TNF- α -induced ICAM-1 transcription.

TNF- α -Induced Oxidant Generation Is Secondary to PKC Activation. We used the redox-sensitive dye C-DCDHF-DA to determine oxidant production in HPAE cells. We determined whether PKC activation was required for the oxidant generation by pretreating confluent HPAE cells with calphostin C followed by stimulation with TNF- α . The cells [loaded for 20 min with 2.5 mM C-DCDHF-DA in EGM (with 2% serum) and containing no TNF- α] were examined for their initial fluorescence intensity by viewing under a fluorescence microscope. Control cells showed a low intensity of fluorescence. In contrast, cells treated with TNF- α (100 U/ml) for 1 h showed markedly increased fluorescence (Fig. 5A). In control experiments, the antioxidant *N*-acetylcysteine prevented TNF- α -induced increase in fluorescence (Rahman et al., 1998). The preincubation of cells for 5 min with 25 nM calphostin C activated by exposure to light prevented TNF- α -induced increase in fluorescence (Fig. 5A), whereas unactivated calphostin C had no effect (data not shown). These results indicate that the effect of calphostin C in inhibiting TNF- α -induced oxidant generation was not secondary to direct scavenging of oxidants by calphostin C. Figure 5B shows the results of the above experiment in which fluorescence imaging was used to quantify the relative fluo-

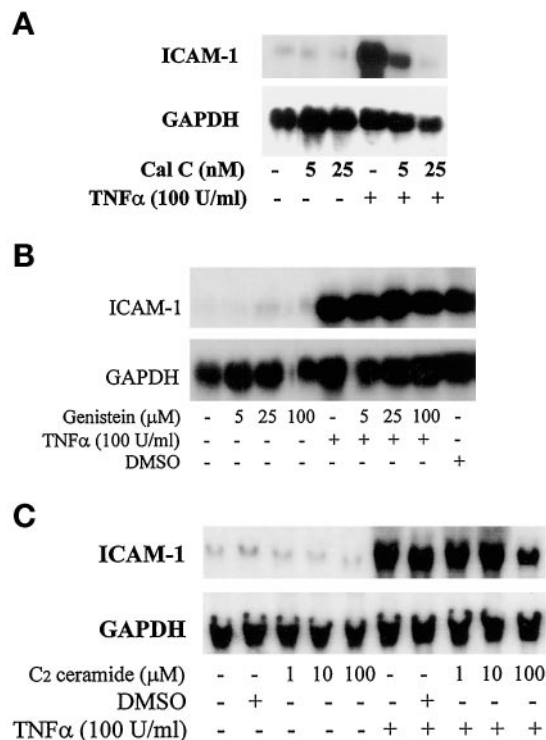


Fig. 2. Calphostin C prevents TNF- α -induced ICAM-1 mRNA expression. Confluent HPAE monolayers were pretreated for 0.5 h with (A) 5 and 25 nM concentrations of calphostin C (Cal C), (B) 5, 25, and 100 μ M genistein, or (C) 1, 10, and 100 μ M C₂-ceramide, followed by stimulation with or without TNF- α (100 U/ml) for a period of 2 h. Total RNA was isolated and analyzed by Northern hybridizations with a human ICAM-1 or rat GAPDH cDNAs, which hybridize to a 3.3- or 1.3-kb mRNA transcripts, respectively. GAPDH was a constitutively expressed gene that is not induced by TNF- α was used to normalize the loading of the gel. A representative experiment of two performed is shown.

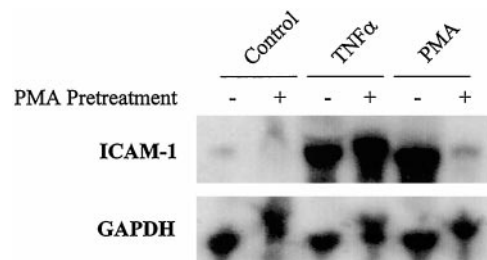


Fig. 3. PMA-mediated down-regulation of PKC does not prevent TNF- α -induced ICAM-1 mRNA expression. Confluent HPAE monolayers were pretreated with (+) or without (-) PMA (500 nM in 10% fetal bovine serum/EGM) for 24 h and subsequently incubated with TNF- α (100 U/ml) or PMA (100 nM) for 3 h. ICAM-1 and GAPDH mRNA expression was determined by Northern blotting as described in *Materials and Methods* (representative of two separate experiments).

rescence intensity. The total relative fluorescence for each image was divided into four classes of brightness, where brightness class 1 represents the area of each cell with the lowest brightness intensity, and class 4 represents the area of each cell with the highest brightness intensity. Figure 5B shows that the control cells exhibit fluorescence in brightness classes 1 and 2 and that treatment with TNF- α causes a shift to higher brightness classes, with maximum fluorescence occurring in brightness class 3. Pretreatment with calphostin C before TNF- α stimulation reduces the generation of ROS, as demonstrated by a decrease in the number of cells exhibiting fluorescence in brightness classes 3 and 4 and an increase in the number of cells exhibiting fluorescence in brightness class 2 relative to TNF- α alone. Thus, these results indicate that TNF- α stimulation of HPAE cells activates oxidant generation via a PKC-dependent mechanism.

Oxidant Generation Signals ICAM-1 mRNA Expression We determined the effects of *N*-acetylcysteine on TNF-

α -induced ICAM-1 mRNA expression to address whether oxidant generation was involved in TNF- α -induced ICAM-1 gene transcription. The preincubation of HPAE monolayers with *N*-acetylcysteine (5–30 mM) for 0.5 h caused a dose-dependent decrease in the TNF- α -induced ICAM-1 mRNA expression (Fig. 6, A and B). The lowest concentration of *N*-acetylcysteine (5 mM) had an $\sim 10\%$ inhibitory effect on TNF- α -induced ICAM-1 transcript. Monolayers exposed to 15 mM *N*-acetylcysteine showed a significant decrease ($\sim 35\%$) in TNF- α -induced ICAM-1 mRNA expression, and 30 mM *N*-acetylcysteine prevented the ICAM-1 mRNA expression (Fig. 6, A and B). Figure 6C shows that 30 mM *N*-acetylcysteine prevented the expression of ICAM-1 mRNA at all time points tested.

Activation of ICAM-1 Promoter Requires Oxidant Generation and NF- κ B Activation. We determined the function of the NF- κ B site in TNF- α -induced transcriptional activity of ICAM-1 promoter by transfecting HPAE cells with expression plasmids containing LUC reporter gene driven by wild-type (ICAM-1 LUC) and NF- κ B mutant versions (ICAM-1 NF- κ B_m LUC) of ICAM-1 promoter. As shown in Fig. 7, TNF- α treatment caused ~ 4 -fold increase in the ICAM-1 promoter activity. TNF- α did not induce the LUC activity directed by the ICAM-1 NF- κ B_m (NF- κ B mutant version of ICAM-1 promoter) (Fig. 7). *N*-Acetylcysteine inhibited the TNF- α -induced transcriptional activation of ICAM-1 LUC construct (Fig. 8), indicating that oxidant-dependent activation of NF- κ B regulates the TNF- α -induced activation of ICAM-1 promoter.

PKC-Dependent Oxidant Generation Activates NF- κ B. We performed electrophoretic mobility shift assays using a 21-bp oligonucleotide (designated as ICAM-1 NF- κ B) con-

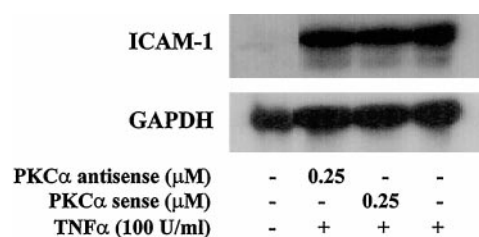


Fig. 4. Antisense oligonucleotides to PKC α fails to prevent TNF- α -induced ICAM-1 mRNA expression. HPAE cells were transfected with sense and antisense oligonucleotides to PKC α as described in *Materials and Methods*. After 36 to 40 h, cells were treated for 2 h with TNF- α (10 U/ml). ICAM-1 and GAPDH mRNA expression was determined by Northern blotting as described in *Materials and Methods* (representative of two separate experiments).

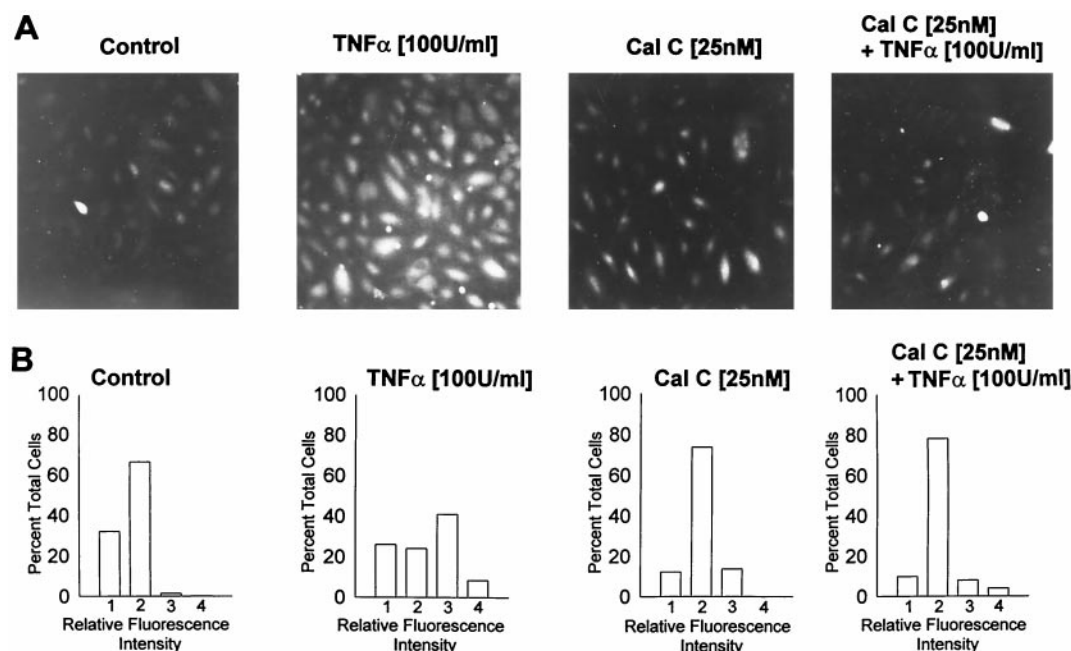


Fig. 5. Calphostin C inhibits TNF- α -induced oxidant production in endothelial cells. Confluent HPAE monolayers were pretreated for 5 min with or without calphostin C (Cal C; 25 nM), followed by stimulation with or without TNF- α (100 U/ml). After 1 h, cells were washed and then stained with C-DCDHF-DA (2.5 mM) for 20 min and analyzed by fluorescence microscopy as described in *Materials and Methods*. A, fluorescent images of representative control, calphostin C-treated, TNF- α -treated, and calphostin C-treated before TNF- α stimulation of HPAE cells (representative of four separate experiments). B, relative fluorescent intensities for each condition in A were determined, compiled, and partitioned into four brightness classes (1 through 4) with class 1 representing the lowest fluorescence intensity and class 4 representing the highest fluorescence intensity. The relative fluorescence intensity for cells stimulated with TNF- α was markedly shifted to the higher fluorescence intensity classes compared with control cells. Pretreatment with calphostin C prevented the TNF- α -induced shift to the higher fluorescence intensity classes.

taining the κ B site present in the ICAM-1 promoter to determine the regulatory function of PKC-dependent oxidant generation in mediating the TNF- α activation of NF- κ B. Nuclear extracts prepared from TNF- α -stimulated HPAE cells contained activities that bound to the NF- κ B binding site in the ICAM-1 promoter (Fig. 9). When visualized by gel electrophoresis, these DNA binding activities resolved into two closely migrating bands [i.e., bands corresponding to p65 homodimers (slow migrating band) and a heterodimeric mix-

ture of p65 and p50 (fast migrating band)] (Hou et al., 1994). These DNA binding activities were competed by a specific oligonucleotide probe yet remained intact when challenged with either an irrelevant probe or an oligonucleotide bearing

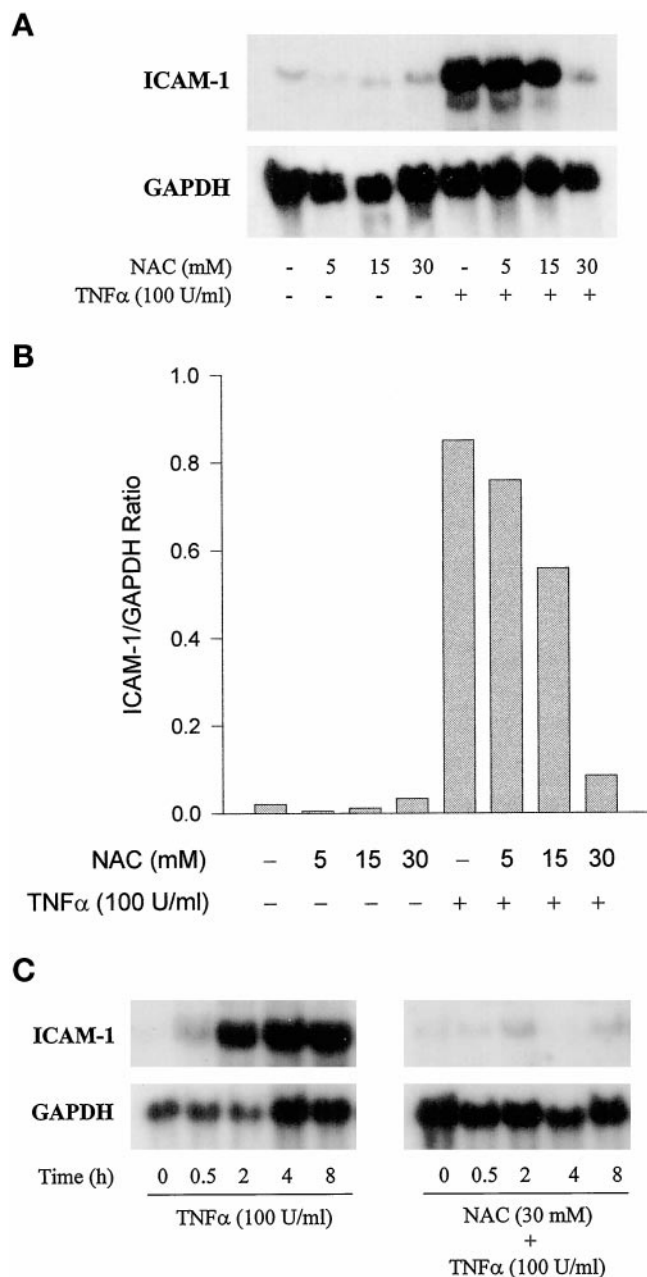


Fig. 6. N-Acetylcysteine prevents TNF- α induction of ICAM-1 transcript. Confluent HPAE monolayers were pretreated for 0.5 h (A) with 5, 15, and 30 mM or (C) with or without 30 mM N-acetylcysteine (NAC), followed by stimulation with or without TNF- α (100 U/ml) for a period of (A) 2 h or (C) 0, 0.5, 2, 4, and 8 h in the continuous presence of N-acetylcysteine. ICAM-1 and GAPDH mRNA expression was determined by Northern blotting as described in *Materials and Methods*. A and C, autoradiograms. B, bar graph representing the dose-dependent effects of N-acetylcysteine on the relative intensities of ICAM-1 mRNA signals. A representative experiment of two performed is shown.

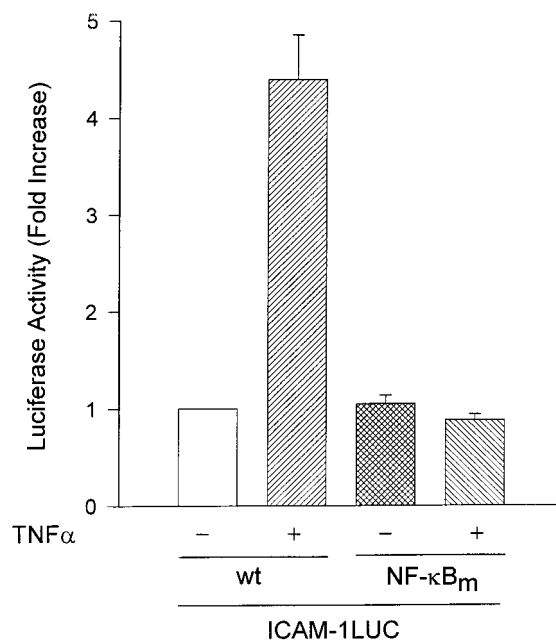


Fig. 7. NF- κ B-dependent activation of ICAM-1 promoter by TNF- α . HPAE cells were transfected with wild-type (wt) or NF- κ B mutated (NF- κ B_m) versions of ICAM-1 luciferase gene construct (ICAM-1 LUC) as described in *Materials and Methods*. After 12- to 18-h treatment with TNF- α (100 U/ml), cytoplasmic extracts were prepared and luciferase activity was determined. Luciferase activity is expressed as fold induction above basal level ICAM-1 LUC expression. Data are mean \pm S.E. ($n = 4$) for each condition.

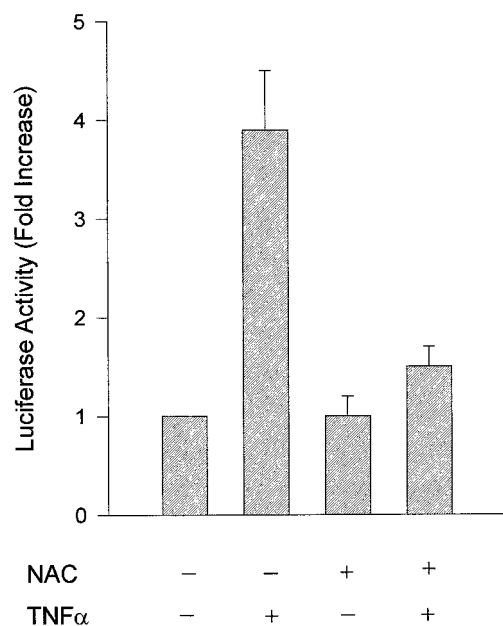


Fig. 8. N-Acetylcysteine inhibits TNF- α -induced activation of ICAM-1 promoter. HPAE cells were transfected with ICAM-1 LUC construct as described in *Materials and Methods*. Cells were stimulated with TNF- α (100 U/ml) without or after pretreatment of N-acetylcysteine (NAC; 30 mM, 0.5 h) for 12 to 15 h before harvesting the cells. Cytoplasmic extracts were prepared, and luciferase activity was determined. Luciferase activity is expressed as fold increase above basal level ICAM-1 LUC expression. Data are mean \pm S.E. ($n = 4$ to 6 for each condition).

mutations in the NF- κ B binding sites (the same mutation that interfered with TNF- α -mediated induction of LUC activity driven by ICAM-1 promoter in transfected HP4E cells) (Fig. 9). The pretreatment of cells for 0.5 h with calphostin C or *N*-acetylcysteine prevented the TNF- α -induced activation of NF- κ B activity (Figs. 10A and 11). In addition to calphostin C ($IC_{50} = 50$ nM), a relatively broad-spectrum inhibitor of PKC isozymes, we used LY333531, a specific inhibitor of PKC β ($IC_{50} = 6$ nM) (Ishii et al., 1996) to determine the role of PKC β isoform in mediating TNF- α -induced activation of NF- κ B. LY333531 failed to prevent the TNF- α -induced activation of NF- κ B (Fig. 10B), indicating that the effects attributed to PKC activation were not the result of cPKC β isozyme.

Discussion

The present study indicates that 1) TNF- α induces PKC activation in endothelial cells, resulting in the generation of oxidants; and 2) the generated oxidants signal the induction of NF- κ B activity and the gene encoding ICAM-1. Both calphostin C, an inhibitor of PKC activation, and *N*-acetylcysteine, an antioxidant and a precursor of glutathione, prevented the TNF- α -induced 1) oxidant generation, 2) NF- κ B activation, and 3) ICAM-1 mRNA expression in endothelial cells. PKC activation after TNF- α stimulation was upstream of the oxidant generation because the inhibition of PKC by calphostin C prevented the oxidant generation. We ruled out oxidant generation itself as being responsible for PKC activation because *N*-acetylcysteine failed to prevent the PKC

activation induced by TNF- α . Moreover, TNF- α was shown to activate PKC within ~ 5 min of adding the cytokine, whereas oxidant production was noted after the 10-min point (data not shown), indicating that the PKC activation and oxidant generation are sequential steps in the induction of ICAM-1 gene expression.

The inhibitory effects of both *N*-acetylcysteine and calphostin C on ICAM-1 mRNA expression (although occurring in different steps of the activation process) are the result of down-regulation of the transcriptional activity of ICAM-1 gene and not secondary to stimulation of the rate of ICAM-1 mRNA degradation. This conclusion is based on a number of

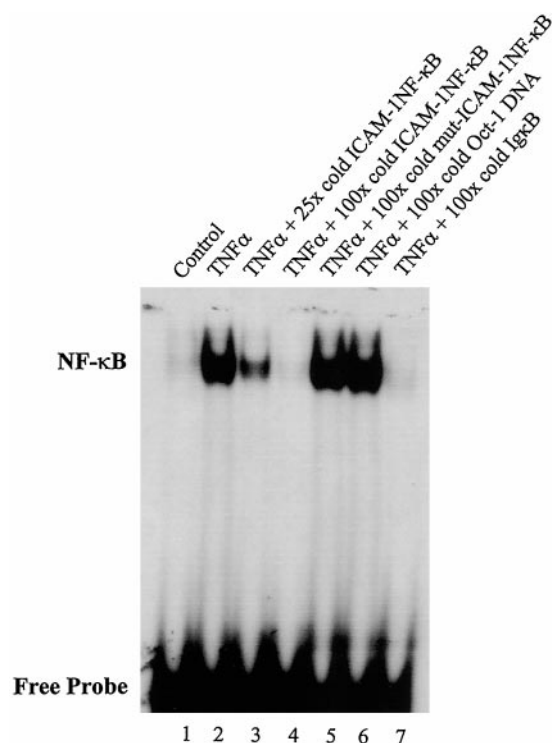


Fig. 9. TNF- α induction of NF- κ B activity. Gel mobility shift assays were performed as described in *Materials and Methods*. Nuclear extracts were prepared from HP4E cells treated for 1 h with TNF- α (100 U/ml) or left untreated and incubated in the absence (lane 2) or presence of indicated molar excess of cold wild-type (lanes 3 and 4) or mut-ICAM-1 NF- κ B (lane 5), Oct-1 DNA (lane 6), or wild-type Ig κ B consensus DNA (lane 7) before the addition of radiolabeled wild-type ICAM-1 NF- κ B probe (representative of two separate experiments).

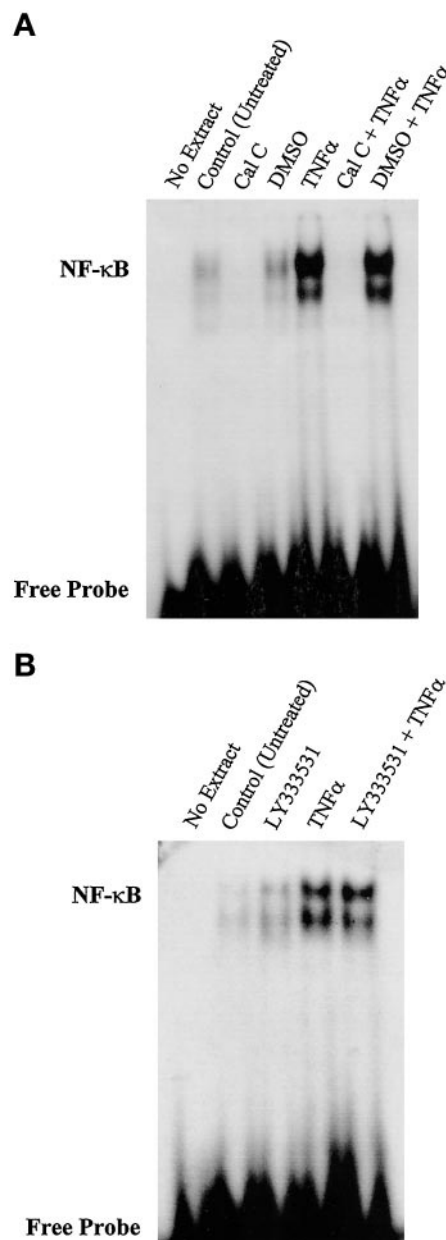


Fig. 10. Calphostin C prevents TNF- α -induced NF- κ B binding to ICAM-1 promoter. Gel mobility shift assays were performed as described in *Materials and Methods*. HP4E cells were pretreated with (A) calphostin C (Cal C, 25 nM) or (B) LY333531 (10 nM) for 0.5 h and then challenged with TNF- α (100 U/ml). After 1 h, nuclear extracts were prepared, and their DNA binding activities were determined using radiolabeled wild-type ICAM-1 NF- κ B probe (representative of two separate experiments).

observations. Pretreatment with 30 mM *N*-acetylcysteine or 25 nM calphostin C, each of which prevented the TNF- α -induced ICAM-1 mRNA expression, did not interfere with the constitutive expression of ICAM-1 mRNA in control cells (Figs. 2A and 6A). *N*-Acetylcysteine and calphostin C also did not prevent transcription of genes expressed constitutively and independently of NF- κ B activation, such as GAPDH (Figs. 2A and 6A) and Cu/Zn superoxide dismutase (data not shown). The independent effects of calphostin C or *N*-acetylcysteine in preventing the activation of NF- κ B in gel shift assays is a further indication that both agents inhibited the activation of ICAM-1 at the transcriptional level. In transfection studies, we also showed that the pretreatment of HPAE cells with *N*-acetylcysteine prevented the activation of ICAM-1 promoter by TNF- α . Finally, we observed a strong correlation between the suppression of mRNA expression and the inhibition of promoter activity of the ICAM-1 gene (maximal inhibition ~90%) in HPAE cells (compares Figs. 6B and 8).

The present study demonstrates a novel mechanism of ICAM-1 expression involving the activation of PKC, which in turn mediates the generation of oxidants. The oxidants generated by the PKC activation signal NF- κ B activation and the resultant ICAM-1 expression. The mechanism of this PKC-dependent oxidant generation in endothelial cells is unclear. Studies have shown that PKC activation can induce the release of oxidants secondary to phosphorylation of a component of NADPH oxidase (Badwey et al., 1989). The failure of inhibition of PKC activation by staurosporine (Richie et al., 1991) and depletion of PKC (Richie et al., 1991; Myers et al., 1992) to prevent TNF- α -induced activation of ICAM-1 may be attributed to the isozyme of PKC mediating the oxidant generation (Hofmann, 1997). Because endothelial cells express multiple isotypes, including α , β , and ζ

(Bussolino et al., 1994; Krizbai et al., 1995) and because PKC ζ is staurosporine insensitive (Seynaeve et al., 1994) and is not depleted by phorbol esters (Wooten et al., 1994; Hofmann, 1997), an atypical PKC isozyme may regulate the TNF- α -activated oxidant generation that signals ICAM-1 expression.

We showed that phorbol ester-induced PKC depletion, which does not affect the atypical PKC isozymes (Wooten et al., 1994), failed to prevent the TNF- α -induced ICAM-1 mRNA expression. Moreover, pretreatment with a PKC β inhibitor, LY333531, or transfection of antisense oligonucleotide to PKC α also failed to prevent TNF- α -induced ICAM-1 transcription in HPAE cells. In contrast calphostin C, which inhibits all PKC isozymes (Larivee et al., 1994), prevented the response. These results are consistent with the idea of an atypical PKC isozyme as being critical in the activation of oxidant generation and the resultant ICAM-1 mRNA expression.

Additional evidence suggesting the involvement of an atypical PKC isozyme comes from the observations that TNF- α increased the PKC activity only in the cytosolic fraction (Fig. 1) unlike phorbol esters and diacylglycerol and diacylglycerol [which are activators of conventional and novel PKC isoforms that increase PKC activity in the membrane fraction (Nagpala et al., 1996; Hofmann, 1997)]. Wooten et al. (1994) showed that nerve growth factor induced an increase in the cytoplasmic PKC ζ in PC12 cells in which PKC was depleted by phorbol esters, suggesting that the atypical PKC isozyme can be activated independently of the other isozymes. Rzymkiewicz et al. (1996) studied the effects of IL-1 β on *cox II* expression in mesengial cells and shed light on the differential effects of PKC inhibitors. Staurosporine potentiated the effect of IL-1 β on *cox II* mRNA expression, whereas calphostin C prevented mRNA expression. Depletion of PKC with phorbol esters did not modify the IL-1 β response (Rzymkiewicz et al., 1996). Furthermore, staurosporine did not inhibit IL-1 β -stimulated binding of a nucleoprotein to κ B motif as determined by gel shift assays. In contrast, calphostin C inhibited the binding event in a dose-dependent manner (Rzymkiewicz et al., 1996), which is consistent with the observation that PKC ζ can lead to the phosphorylation of I κ B (Lozano et al., 1994) and thus to NF- κ B activation in monocytes (Folgueira et al., 1996).

Because *N*-acetylcysteine can function as a precursor for glutathione (γ -glutamylcysteinyl-glycine), the inhibitory effect of *N*-acetylcysteine suggests a role of glutathione in regulating TNF- α -induced ICAM-1 expression. An increase in reduced/oxidized (glutathione/oxidized glutathione) ratio maintains a reducing intracellular environment; therefore, fluctuations in glutathione/GSSG coupled under conditions of oxidative stress may regulate ICAM-1 transcription through a redox-sensitive mechanism. We showed that TNF- α induced a decrease in glutathione, which increased the sensitivity of bovine pulmonary microvascular endothelial cells to H₂O₂ (Ishii et al., 1992). Aoki et al. (1996) showed that *N*-acetylcysteine prevented hyperoxia-induced ICAM-1 expression by increasing the glutathione content of human umbilical vein endothelial and HPAE cells consistent with an important role of glutathione/GSSG coupled in the regulation of ICAM-1 expression.

In summary, this study indicates that TNF- α -induced activation of phorbol ester-insensitive PKC in endothelial cells

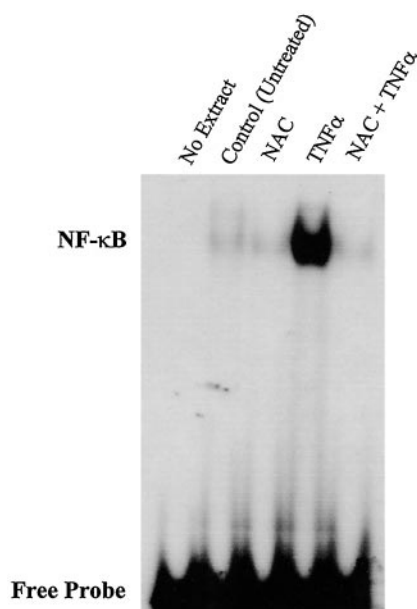


Fig. 11. *N*-Acetylcysteine prevents TNF- α -induced NF- κ B binding to ICAM-1 promoter. Gel mobility shift assays were performed as described in *Materials and Methods*. HPAE cells were pretreated with *N*-acetylcysteine (NAC; 30 mM) for 0.5 h and then challenged with TNF- α (100 U/ml). After 1 h, nuclear extracts were prepared, and their DNA binding activities were determined using radiolabeled wild-type ICAM-1 NF- κ B probe. A representative experiment of two performed is shown.

stimulates oxidant generation, which in turn signals the activation of NF- κ B and transcription of the ICAM-1 gene. The results point to pharmacological inhibition of atypical PKC activity as a novel strategy for preventing TNF- α -induced activation of NF- κ B and ICAM-1 gene transcription.

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