Protein kinase C-ζ mediates TNF-α-induced ICAM-1 gene transcription in endothelial cells

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Rahman, Arshad, Khandaker N. Anwar, and Asrar B. Malik. Protein kinase Cζ (PKCζ) isoforms in mediating tumor necrosis factor-α (TNF-α)-induced oxidant generation in endothelial cells, a requirement for nuclear factor-κB (NF-κB) activation and intercellular adhesion molecule-1 (ICAM-1) gene transcription. We showed that expression of the dominant negative mutant of PKCζ also induced the TNF-α-induced NF-κB binding to the ICAM-1 promoter and the resultant ICAM-1 gene transcription. We showed that expression of the dominant negative mutant of PKCζ prevented the TNF-α-induced ICAM-1 promoter activity, whereas overexpression of the wild-type PKCζ augmented the response. These data imply a critical role for the PKCζ isozyme in regulating TNF-α-induced oxidant generation and in signaling the activation of NF-κB and ICAM-1 transcription in endothelial cells.

protein kinase C isoforms; oxidants; nuclear factor-κB; intercellular adhesion molecule-1; endothelium

THE BASIS OF STABLE POLYMPHONUCLEAR LEUKOCYTE (PMN) ADHESION TO THE VASCULAR ENDOTHELIAL CELLS INVOLVES THE EXPRESSION OF INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) ON THE ENDOTHELIAL CELL SURFACE (36, 37). ICAM-1 MEDIATES FIRM ADHESION OF PMN TO THE VASCULAR ENDOTHELIAL BY SERVING AS A COUNTER-RECEPTOR FOR LEUKOCYTE β2-INTEGRINS, CD11a/CD18 AND CD11b/CD18. ALTHOUGH ICAM-1 IS EXPRESSED BASALLY IN ENDO THELIAL CELLS, ITS EXPRESSION IS MARKEDLY INDUCED BY THE INFLAMMATORY CYTOKINES SUCH AS TUMOR NECROSIS FACTOR-α (TNF-α) THROUGH ACTIVATION OF THE TRANSCRIPTION FACTOR NUCLEAR FACTOR-κB (NF-κB) (14, 20).

The NF-κB/Rel family of transcription factors is composed of the transcriptionally active p65/Rel A (25, 30), c-Rel (42), and Rel B (31) and transcriptionally silent p50/NF-κB1 (12, 17), and p52/NF-κB2 (5, 32). All NF-κB proteins exist as an inactive dimer in the cytoplasm bound to the inhibitory proteins of the IkB family, IkBα, IkBβ, IkBγ, p100, p105, and IkBe (3, 41). TNF-α stimulation of cells results in the phosphorylation, and subsequent ubiquitination-dependent degradation of IkBα by the proteasome 26S (6, 7, 40). This allows the NF-κB dimer to migrate to the nucleus, where it activates transcription of the ICAM-1 gene. We have shown the critical involvement of protein kinase C (PKC), a family of serine/threonine kinases, in the activation of NF-κB and the transcription of ICAM-1 (29); however, the specific PKC isozyme(s) responsible for this effect in endothelial cells has not been identified. PKC isoforms have differential cellular distributions, substrate specificities, and activator responsiveness are classified into three groups: conventional (cPKCs; α, β1, β2, and γ), novel (nPKCs; δ, ε, μ, θ, and γ/L (mouse/human)), and atypical (aPKCs; ζ, and η/λ (mouse/human)) (13, 16). cPKCs are Ca2+-dependent and are activated by diacylglycerol and phorbol esters, whereas neither nPKCs nor aPKCs require Ca2+ for activation (15, 16). The nPKCs are activated by diacylglycerol and phorbol esters, whereas aPKCs are irreversible to both diacylglycerol and phorbol esters (16).

We showed that stimulation of human pulmonary artery endothelial (HPAE) cells with TNF-α resulted in activation of PKC and oxidant generation (29). We also showed that PKC activation and oxidant generation were both necessary for NF-κB activation and ICAM-1 expression, since the inhibition of PKC by calphostin C and scavenging of oxidants by antioxidants prevented the TNF-α-induced NF-κB activation and ICAM-1 gene transcription (29). These results demonstrated that PKC-α-induced oxidant generation in endothelial cells occurred downstream of PKC activation (29). In the present study, we have extended these observations by showing that PKC-ζ, the aPKC isozyme in endothelial cells, is critically involved in the mechanism of TNF-α-induced oxidant generation and thereby in the signaling of activation of NF-κB and ICAM-1 gene transcription.

METHODS

Cell culture. HPAE cells, obtained from Clonetics (La Jolla, CA), were grown on gelatin-coated flasks or plates in endothelial cell growth medium (EGM) containing 10% FCS and...
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3.0 mg/ml of endothelial-derived growth factor from bovine brain extract protein. Human recombinant TNF-α with a specific activity of 2.3 x 10^4 was purchased from Promega (Madison, WI). All experiments were made in cells under the 10th passage except where indicated otherwise.

**Northern analysis.** Total RNA was isolated from HPAE cells with an RNaseasy kit (Qiagen, Chatsworth, CA) according to manufacturer’s recommendations. Quantification and purity of RNA were assessed by A260/A280 absorption, and an aliquot of RNA (20 μg) from samples with a ratio above 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 (UV) irradiation using a Stratalinker UV crosslinker (Stratagene, La Jolla, CA) and covalently linked by UV irradiation using a Stratalinker UV crosslinker (Stratagene). Human ICAM-1 (0.96-kb Sal I-to-Pst I fragment) (39) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.1-kb Sal I fragment) (30) were fractionated using a 1% agarose formaldehyde gel. The blots were prehybridized for 30 min at 68°C in QuikHyb hybridization buffer (Stratagene), and hybridization was carried out as described (38). Briefly, the blots were prehybridized for 30 min at 68°C in QuikHyb hybridization buffer (Stratagene), and hybridization was carried out as described (28). Briefly, the blots were washed 2 x for 30 min at room temperature in 2 x SSC with 0.1% SDS followed by two washes for 15 min each at 60°C in 0.1 x SSC with 0.1% SDS. Autoradiography was performed with an intensifying screen at −70°C for 12–24 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 0.1x SSC with 0.1% SDS.

**Detection of oxidant generation.** Oxidant generation in HPAE cells was measured as described (27). Briefly, confluent HPAE monolayers were stimulated for 1 h with TNF-α (100 U/ml) in EGCM containing 2% serum as described above. Cells were washed 2 x with EGM (2% serum) and stained for 20 min with 1 μM 5(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate bis(acetoxymethyl) ester (C-DCFH-DA; Molecular Probes, Eugene, OR) in EGM (with 2% serum). Cultures were viewed with fluorescence microscopy and photographed. Fluorescence was imaged using a Nikon Diaphot 200 microscope (Nikon, Garden City, NJ), and the results were quantified using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

**Nuclear extract preparation.** Nuclear protein extracts were prepared, and an electrophoretic mobility shift assay (EMSA) was performed as described (29). After treatments, cells were washed twice with ice-cold Tris-buffered saline (TBS) and resuspended in 400 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). After 15 min, Nonidet P-40 (NP-40) was added to a final concentration of 0.6%. Nuclei were pelleted and resuspended in 50 μl of buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). After 30 min at 4°C, lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. Protein concentration of the extract kit (Bio-Rad, Hercules, CA).

**Electrophoretic mobility shift assay.** EMSAs were performed as described (29). Briefly, 10 μg of nuclear extract was incubated with 1 μg of poly(dI-dC) in a binding buffer [10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10% glycerol (20 μl final volume)] for 15 min at room temperature. Then, end-labeled double-stranded oligonucleotides containing the NF-κB site of ICAM-1 promoter (30,000 cpm each) were added, and the reaction mixtures were incubated for 15 min at room temperature. The DNA-protein complexes were resolved in 5% native polyacrylamide gel electrophoresis in low ionic strength buffer (0.25 x Tris-borate-EDTA). The oligonucleotide used for the gel shift analysis was ICAM-1 NF-κB 5'-AGCTTGGAAATTCCGGAGCTG-3'. This represents a 21-bp sequence of ICAM-1 promoter encompassing the NF-κB binding site located 183 bp upstream of the transcription initiation site (14); this sequence motif within the oligonucleotide is underlined.

**Reporter gene constructs, endothelial cell transfections, and luciferase assay.** ICAM-1 promoter-firefly luciferase (LUC) plasmids containing wild-type (ICAM-LUC) and mutated NF-κB site (ICAM-1NF-κBmut-LUC) were provided by Dr. Zhaodan Cao (Tularik, San Francisco, CA) (14). The pcDNA3 vector harboring tagged wild-type and dominant negative forms of Xenopus laevis PKC-ζ were gifts of Dr. J. Moscat (Universidad Autonoma, Madrid, Spain). The PKC-ζ mutant (4) is a kinase-deficient PKC-ζ generated by a substitution of lysine-275 for tryptophan and thus lacks a functional catalytic domain. The plasmid pNF-κB-LUC containing five copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was purchased from Stratagene. The expression vector pcDNA3 containing tagged dominant negative form of PKC-α, -δ, and -ε isoforms (38) was provided by Dr. I. B. Weinstein (Columbia University, New York, NY). Transfections were performed with Superfect (Qiagen) as described (28), with slight modifications. Briefly, reporter DNA (1 μg) was mixed with 5 μl of Superfect in 100 μl serum-free EGM (Clonetech). We used 0.2 μg pTKRLUC plasmid (Promega) containing Renilla luciferase gene driven by the constitutively active thymidine kinase promoter to normalize the transfection efficiencies. Because we did not observe any significant difference in transfection efficiencies in initial experiments, we did not cotransfect the pTKRLUC construct in the later experiments. After a 5- to 10-min incubation at room temperature, 0.6 ml EGM containing 10% FCS was added, and the mixture was applied onto the cells that had been washed once with PBS. Three hours later, the medium was changed to EGM containing 10% FCS, and the cells were grown to confluence.

Using this protocol, we achieved a transient transfection efficiency of 20 ± 2% (mean ± SD; n = 3) for HPAE cells. To determine transfection efficiency, HPAE cells were transfected with an expression plasmid pGreen Lantern-1 containing green fluorescence protein (GFP) gene (GIBCO-BRL; Life Technologies). Transfected cells were subjected to fluorescence-activated cell sorting (FACS) analysis for GFP expression to determine transfection efficiency. In some experiments, we used the DEAE-dextran method (22) with slight modifications. Briefly, 5 μg DNA were mixed with 50 μg/ml DEAE-dextran in serum-free EGM, and the mixture was added onto cells that had been washed once with PBS. Three hours later, the medium was changed to EGM containing 10% FCS, and the cells were grown to confluence.

We used Trypan blue (Sigma Chemical, St. Louis, MO) exclusion assay to evaluate cell viability following transfection. Cells were washed gently with 2 x PBS and trypsinized, and the cells were resuspended and washed with EGM containing 10% FCS. The cell suspension (10 μl) was mixed with 10 μl of 1 x Trypan blue solution, and 10 μl of the resulting
mixture were loaded onto a hemocytometer. Results showed that >95% of the cells were viable.

Transfection of HPAE cells with oligonucleotides. Phosphorothioate oligonucleotides to PKC-ζ sense (ATG CCC AGC AGG ACC) and anti-sense (GGT CCT GCT GGG CAT) have been described elsewhere (10); both are targeted to translation initiation codon of PKC-ζ mRNA. Phosphorothioate antisense oligonucleotides to PKC-α (GGT CTC GCT GGG GAG TTT CA) are directed to the 3’-untranslated region of PKC-α mRNA (8). HPAE cells were grown in 100-mm dishes to 50% confluence. Transfections of oligonucleotides were performed with Lipofectin (GIBCO-BRL) as described (29).

Western blot analysis. Confluent HPAE cells grown in six-well plates were stimulated for the indicated time periods and harvested in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 75 mM NaCl, 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, supplemented with 50 μg/ml PMSF, 2 mM sodium orthovanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1.25 mM NaF, and 1 mM sodium pyrophosphate). Each cell lysate (8–10 μg) was denatured by boiling for 5 min before being loaded onto 12.5% SDS-polyacrylamide gel. Gels were run at 110 mV for 1.5 h at room temperature. Proteins were transferred to Immobilon membranes (Millipore, Bedford, MA) in blotting buffer (25 mM Tris base, 192 mM glycine, and 10% methanol) at 400 mA for 1.5 h at 4°C. Membranes were blocked for 1 h with 5% (wt/vol) nonfat dry milk solution in TBST (10 mM Tris base, 150 mM NaCl, and 0.05% Tween 20) before incubating the membrane for 1 h with rabbit polyclonal anti-human IκBα or IκBβ (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000. Membranes were washed three times with TBST and incubated for another hour with goat anti-rabbit horseradish peroxidase-linked IgG (Amersham, Arlington Heights, IL) diluted 1:5,000. After another three washes, antibody-labeled protein was detected by enhanced chemiluminescence (ECL kit, Amersham) according to manufacturer’s recommendations.

RESULTS

PKC isozymes present in HPAE cells. We first identified the PKC isozymes present in HPAE cells and determined their sensitivities to phorbol ester. Western blot analysis showed that PKC-α, -δ, -ε, and -ζ isozymes were abundantly expressed (Fig. 1), whereas PKC-βI and PKC-γ were not detectable (data not shown). PKC-δ appeared as a doublet corresponding to 78- and 76-kDa bands, consistent with previous observations in other cell types (26). Exposure of HPAE cells to phorbol ester (500 nM for 24 h) resulted in depletion of PKC-α, -ε, and -ζ isoforms. PKC-α was the most sensitive to phorbol ester, whereas residual levels of PKC-ε and PKC-ζ (76 kDa) remained detectable (Fig. 1). In contrast, phorbol ester treatment failed to deplete the aPKC isozyme, PKC-ζ (Fig. 1).

We also determined the effects of an antisense oligonucleotide directed against the translation initiation codon of PKC-ζ mRNA (10, 11). PKC-ζ expression was analyzed by Western blotting following the transfection of the sense and antisense phosphorothioate oligonucleotides in HPAE cells. Antisense oligonucleotide concentration of 0.25 μM inhibited PKC-ζ expression (Fig. 2); thus this concentration was used in subsequent studies for analysis of the role of PKC-ζ in ICAM-1 transcription. In contrast, the antisense oligonucleotide concentration of 1 μM failed to prevent PKC-ζ expression (Fig. 2). The failure of higher concentrations (1 μM) of oligonucleotides to inhibit expression of PKC-ζ is consistent with the reported effect of PKC-β antisense oligonucleotide (18). One explanation may be that the increasing oligonucleotide/Lipofectin ratio interferes with the complex formation and hence oligonucleotide transfection efficiency; however, increasing the incubation time or Lipofectin concentration did not succeed because of a loss of cell viability. Sense oligonucleotide had no significant effect on PKC-ζ expression (Fig. 2). In another control experiment, antisense oligonucleotide to PKC-ζ failed to prevent synthesis of PKC-δ (Fig. 2), indicating specificity of the antisense oligonucleotide.

Inhibition of PKC-ζ expression prevents TNF-α-induced oxidant generation. We determined the effects of the phorbol ester-induced depletion of CPKC and nPKC isozymes and the inhibition of PKC-ζ expression by antisense oligonucleotide on the TNF-α-induced oxidant production.
dant generation. Studies were made in cells stimulated with TNF-α for 1 h to allow maximum oxidant production. Control cells exhibited low fluorescence intensity, whereas stimulation of HPAE cells with TNF-α resulted in markedly increased fluorescence (Fig. 3). We observed oxidant production as early as 5 min after TNF-α challenge of HPAE cells (data not shown). Depletion of cPKC and nPKC isozymes by the phorbol ester treatment failed to prevent the TNF-α-induced oxidant generation (Fig. 3). However, antisense oligonucleotide to PKC-ζ markedly reduced the oxidant generation, whereas sense oligonucleotides had little effect (Fig. 4, A and B). In another control experiment, the antisense oligonucleotide to PKC-α failed to prevent TNF-α-induced oxidant generation in endothelial cells (Fig. 4, C and D).

Effects of depletion of cPKC and nPKC isozymes and PKC inhibitors on TNF-α-induced IkBα degradation. We next evaluated the role of PKC isozymes in mediating IkBα degradation, a requirement for NF-κB activation (6, 7, 40). TNF-α stimulation of endothelial cells induced IkBα degradation, whereas it had no effect on IkBβ (Figs. 5 and 6). Depletion of cPKC and nPKC isozymes failed to prevent the TNF-α-induced IkBα degradation, and expectedly it inhibited IkBα degradation in response to the phorbol ester stimulation (Fig. 5). Moreover, pretreatment of HPAE cells with calphostin C, the broad spectrum inhibitor of PKC isozymes (19), prevented the TNF-α-induced IkBα degradation, whereas staurosporine, which inhibits cPKC and nPKC but not aPKC isozymes (23, 35), failed to prevent the TNF-α response (Fig. 6). Taken together, these results suggest that the TNF-α-induced IkBα degradation in HPAE cells is independent of cPKC and nPKC isozymes and support the involvement of the aPKC isozyme.

Inhibition of PKC-ζ synthesis prevents TNF-α-induced NF-κB binding to ICAM-1 promoter. We performed the EMSA to determine the role of the aPKC isozyme, PKC-ζ, in the mechanism of TNF-α-induced NF-κB binding to the ICAM-1 promoter. Depletion of cPKC and nPKC isozymes prevented NF-κB binding to

Fig. 3. Effects of phorbol ester-induced depletion of cPKC and nPKC isozymes on oxidant generation induced by tumor necrosis factor-α (TNF-α) in HPAE cells. Confluent HPAE monolayers were treated without (−) or with (+) PMA (500 nM in 10% FBS/EGM) for 24 h. Cells were stimulated with TNF-α (100 U/ml) for 1 h to yield maximum reactive oxygen species (ROS) production. Cells were washed and then stained with 5(andra)-carboxy-2,7'-dichlorodihydrofluorescein (C-DCDHF-DA, 1 μM) for 20 min and analyzed by fluorescence microscopy as described in METHODS. A: fluorescent images of representative control or TNF-α-stimulated cells without (−) or after (+) PMA pretreatment (results are representative of 3 separate experiments). B: relative fluorescent intensities for each condition in A were determined, compiled, and partitioned into 4 brightness classes (1–4), 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 shifted to the higher fluorescence intensity classes compared with control cells. Pretreatment with PMA failed to prevent the TNF-α-induced shift to the higher fluorescence intensity classes. Results are representative of 3 experiments.
ICAM-1 promoter in response to stimulation of HPAE cells with the phorbol ester (Fig. 7). However, the depletion of these isozymes failed to prevent TNF-α-induced NF-κB binding to the ICAM-1 promoter (Fig. 7). In contrast, inhibition of PKC-ζ expression by the antisense oligonucleotide prevented the TNF-α-induced NF-κB binding to the ICAM-1 promoter (Fig. 8). In a control experiment, inhibition of PKC-α expres-
Inhibition of PKC-\(\zeta\) prevents TNF-\(\alpha\)-induced NF-\(\kappa\)B activity, ICAM-1 promoter activation and mRNA expression. We evaluated the role of PKC-\(\zeta\) in mediating NF-\(\kappa\)B activity by cotransfecting a plasmid, pNF-\(\kappa\)B-LUC, containing five copies of consensus NF-\(\kappa\)B sequence from Ig gene linked to a minimal adenovirus E1B promoter-luciferase reporter gene with constructs encoding dominant negative forms of PKC-\(\alpha\) (PKC-\(\alpha^{\text{mut}}\)), -\(\epsilon\) (PKC-\(\epsilon^{\text{mut}}\)), -\(\delta\) (PKC-\(\delta^{\text{mut}}\)), or -\(\zeta\) (PKC-\(\zeta^{\text{mut}}\)), isozyme in endothelial cells. As shown in Fig. 9A, expression of only PKC-\(\zeta^{\text{mut}}\) prevented the TNF-\(\alpha\)-induced NF-\(\kappa\)B activity, whereas PKC-\(\alpha^{\text{mut}}\), -\(\epsilon^{\text{mut}}\), -\(\delta^{\text{mut}}\), or -\(\zeta^{\text{mut}}\) failed to prevent the response.

We next determined the effects of PKC-\(\zeta\) on NF-\(\kappa\)B-dependent ICAM-1 promoter activity. We cotransfected the wild-type (ICAM-1LUC) or NF-\(\kappa\)B mutant (ICAM-1NF-\(\kappa\)B\(_{\text{mut}}\)-LUC) versions of the ICAM-1 promoter-luciferase reporter gene construct with the PKC-\(\zeta\)wt or PKC-\(\zeta^{\text{mut}}\) in HPAE cells and then determined luciferase activity. As shown in Fig. 9B, expression of PKC-\(\zeta^{\text{mut}}\) prevented the TNF-\(\alpha\)-induced ICAM-1 promoter activity, whereas overexpression of PKC-\(\zeta^{\text{wt}}\) significantly augmented the TNF-\(\alpha\) response. These data indicate the critical role of PKC-\(\zeta\) in activating the ICAM-1 promoter. Mutation of the downstream NF-\(\kappa\)B site prevented the TNF-\(\alpha\)-induced ICAM-1 promoter

Fig. 6. Differential effects of calphostin C and staurosporine on TNF-\(\alpha\)-induced I\(\kappa\)B\(_\alpha\) degradation. HPAE cells were pretreated with calphostin C or staurosporine for 15 min before stimulation with TNF-\(\alpha\) (100 U/ml). Total cell lysate (10 \(\mu\)g) was separated by 12.5% SDS-PAGE and immunoblotted for I\(\kappa\)B\(_\alpha\) and I\(\kappa\)B\(_\beta\). Results are representative of 2 experiments.

Fig. 5. Phorbol ester-induced depletion of conventional PKC (cPKC) and novel PKC (nPKC) isozymes fails to prevent TNF-\(\alpha\)-induced I\(\kappa\)B\(_\alpha\) degradation. Confluent HPAE monolayers were treated without (–) or with (+) PMA (500 nM in 10% FBS/EGM) for 24 h followed by stimulation for 20–30 min with TNF-\(\alpha\) (100 U/ml) or PMA (100 nM). Total cell lysate (10 \(\mu\)g/lane) was separated by 12.5% SDS-PAGE and immunoblotted for I\(\kappa\)B\(_\alpha\) and I\(\kappa\)B\(_\beta\). Results are representative of 2 experiments.

Fig. 7. Effects of phorbol ester-induced depletion of cPKC and nPKC isozymes on TNF-\(\alpha\)-induced nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) binding to intercellular adhesion molecule-1 (ICAM-1) promoter. Confluent HPAE monolayers were treated without (–) or with (+) PMA (500 nM in 10% FBS/EGM) for 24 h and subsequently incubated for 1 h with TNF-\(\alpha\) (100 U/ml) or PMA (100 nM). Nuclear extracts were prepared and assayed for NF-\(\kappa\)B binding activity by electrophoretic mobility shift assay (EMSA) using radiolabeled oligonucleotide containing the ICAM-1-\(\kappa\)B site. Results are representative of 2 experiments.
activation (Fig. 9B, inset). Moreover, the expression of PKC-\(\zeta\)mut or overexpression of PKC-\(\zeta\)wt failed to modify the TNF-\(\alpha\) response (Fig. 9B, inset). These data indicate that PKC-\(\zeta\) activates ICAM-1 promoter through an NF-\(\kappa\)B-dependent pathway. To further address the role of PKC-\(\zeta\) in the mechanism of the response, we showed that the antisense oligonucleotide to PKC-\(\zeta\) prevented TNF-\(\alpha\)-induced ICAM-1 mRNA expression (by \(-50\%\)), whereas the sense oligonucleotide had no effect (Fig. 10).

**DISCUSSION**

The PKC isozymes are structurally related proteins having different cofactor and substrate specificities...
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(15, 24). On the basis of their ability to phosphorylate effector proteins at serine and threonine residues, they can signal diverse responses in a cell- and stimulus-specific manner (18, 21). In a previous study, we showed that PKC activation induces the generation of oxidants in endothelial cells, which in turn mediates the activation of NF-κB and transcription of ICAM-1 gene (29). In the present study, we have identified the atypical endothelial PKC isozyme, PKC-ζ, in transducing the TNF-α-induced oxidant generation and the downstream activation of NF-κB and ICAM-1 transcription.

We used the approaches described below to address the contributions of PKC-α, -δ, -ε, and -ζ isozymes, which are abundantly expressed in HPAE cells, in mediating the TNF-α-activated responses. To address the role of PKC-α, -δ, and -ε isozymes in mediating oxidant production following TNF-α challenge, we depleted these isozymes by exposing HPAE cells to phorbol ester for 24 h in the standard manner. To study the role of the phorbol ester-insensitive aPKC isozyme, PKC-ζ, we used the antisense oligonucleotide, which inhibits synthesis by binding to the translation initiation codon of PKC-ζ mRNA (10, 11). We showed that depletion of cPKCs and nPKCs failed to prevent TNF-α-induced oxidant production in endothelial cells, whereas the inhibition of PKC-ζ synthesis by the antisense oligonucleotide prevented the oxidant production. Thus the results indicate an important role of the aPKC isozyme PKC-ζ in activating oxidant generation induced by TNF-α stimulation of endothelial cells. Generation of oxidants following TNF-α stimulation may be mediated by the activation of endothelial NADPH oxidase (1). Components of the NADPH oxidase complex were present in endothelial cells, and stimulation with TNF-α resulted in their translocation and activation (A. Rahman, unpublished results); thus a possible mechanism of oxidant generation may involve PKC-ζ-induced phosphorylation of the p47 subunit of NADPH oxidase, resulting in its translocation to the plasma membrane where it interacts with the cytochrome_555, to form the active NADPH oxidase complex (9).

Because the present results implicate PKC-ζ in the mechanism of TNF-α-induced oxidant production, we next addressed the possibility that the oxidant production mediated by PKC-ζ was responsible for the NF-κB activation and ICAM-1 gene transcription in endothelial cells. We showed that inhibition of PKC-ζ expression using the antisense oligonucleotide prevented both NF-κB activation and ICAM-1 transcription, whereas in control experiments, the sense oligonucleotide had no inhibitory effect. Moreover, expression of the dominant negative form of PKC-ζ prevented TNF-α-induced NF-κB activity, whereas the expression of dominant negative forms of PKC-α, -δ, and -ζ isozymes failed to prevent the response. The expression of the PKC-ζ dominant negative mutant also prevented ICAM-1 promoter activity in response to TNF-α challenge, and overexpression of wild-type PKC-ζ augmented this response. Mutation of the downstream NF-κB binding site on the ICAM-1 promoter prevented the TNF-α-induced ICAM-1 promoter activation. Taken together, these results indicate the essential role of the PKC-ζ isozyme in mediating TNF-α-induced NF-κB activation and ICAM-1 transcription.

In the present study, we also addressed the mechanism of the PKC-ζ-induced activation of NF-κB. The results showed that inhibition of PKC-ζ expression prevented IκB degradation and NF-κB binding to the ICAM-1 promoter induced by TNF-α. These results can be explained on the basis of PKC-ζ-induced oxidant generation promoting the degradation of IκBα and activation of NF-κB (33, 34). Another possibility is that PKC-ζ can directly phosphorylate NF-κB p65, since the dominant negative mutant of PKC-ζ was shown to inhibit NF-κB p65 phosphorylation, resulting in the loss of NF-κB p65 transcriptional activity (2). Hence, PKC-ζ may induce NF-κB activity by 1) activation of the oxidant signaling pathway and downstream induction of NF-κB binding to ICAM-1 promoter and 2) direct phosphorylation of NF-κB p65 resulting in increased transcriptional activity of the bound NF-κB.

In summary, we have shown that 1) phorbol ester-induced depletion of cPKC and nPKC isozymes failed to prevent TNF-α-induced oxidant generation and NF-κB activation; 2) inhibition of PKC-ζ synthesis by the antisense oligonucleotide prevented TNF-α-induced oxidant generation, NF-κB activation, and ICAM-1 gene transcription; 3) expression of the dominant negative mutant form of PKC-ζ inhibited the TNF-α-induced NF-κB activity; and 4) expression of the dominant negative mutant form of PKC-ζ also inhibited the TNF-α-induced ICAM-1 promoter activity, whereas overexpression of wild-type PKC-ζ augmented the response. Thus the aPKC isozyme PKC-ζ plays a critical role in mediating TNF-α-induced oxidant generation, NF-κB activation, and resultant ICAM-1 gene transcription in endothelial cells. The results point to the endothelial cell PKC-ζ as an important target in preventing the proinflammatory effects of TNF-α such as ICAM-1 expression and neutrophil adhesion.

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REFERENCES


