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Sensitization of Human Umbilical Vein Endothelial Cells to Shiga Toxin: Involvement of Protein Kinase C and NF-κB

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Infection of humans with Shiga toxin-producing *Escherichia coli* O157:H7 and *Shigella dysenteriae* 1 is strongly associated with vascular endothelial cell damage and the development of hemolytic-uremic syndrome. The cytotoxic effect of Shiga toxins on vascular endothelial cells in vitro is enhanced by prior exposure to bacterial lipopolysaccharide (LPS) or either of the host cytokines tumor necrosis factor alpha (TNF) and interleukin-1β (IL-1). The purpose of this study was to examine individual signal transduction components involved in the sensitization of human umbilical vein endothelial cells (HUVEC) to Shiga toxin 1. The results demonstrate that class I and II protein kinase C (PKC) isozymes are required for sensitization of HUVEC to Shiga toxin by phorbol myristate acetate (PMA) or LPS but not by TNF or IL-1. Thus, the specific competitive inhibitor of class I/II PKC, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG), prevented only the action of PMA and LPS on HUVEC. Additional data obtained with ATP binding site inhibitors which affect all PKCs (i.e., classes I, II, and III) suggest that TNF may utilize class III PKC isozymes in the Shiga toxin sensitization of HUVEC. Transcriptional activator NF-κB did not appear to be involved in the sensitization of HUVEC to Shiga toxin by LPS, TNF, IL-1, or PMA. Thus, the specific serine protease inhibitor L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) did not inhibit the sensitization of HUVEC to Shiga toxin by LPS, TNF, IL-1, or PMA despite its ability to inhibit NF-κB activation and the induction of the NF-κB-dependent tissue factor gene by these agents. Finally, all-*trans* retinoic acid partially inhibited the sensitization of HUVEC to Shiga toxin, by unknown mechanisms which also appeared to be independent of NF-κB activation. These results indicate that PKC plays a role in the sensitization of HUVEC to Shiga toxin in response to some, but not all, sensitizing agents. In contrast, NF-κB activation appears not to be involved in the sensitization of HUVEC to Shiga toxin by LPS, TNF, IL-1, or PMA.

Hemolytic-uremic syndrome (HUS) is a vascular disease of humans which is marked by thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure (for reviews, see references 14 and 43). As evidenced in the 1996 epidemic of *Escherichia coli* O157:H7 in Japan, involving more than 9,000 people, HUS develops several days after an initial diarrheal illness caused by Shiga-like toxin 1 (Stx1)- and toxin 2 (Stx2)-producing *E. coli* (14, 43). Stx1- and Stx2-mediated damage to vascular endothelium is believed to cause a procoagulant and prothrombotic state, acting together with bacterial lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF), and interleukin-1β (IL-1), resulting in the occlusion of microvessels with platelets and fibrin (2, 22, 44, 59, 60). Stx's inhibit protein synthesis and are directly cytotoxic to cultured human endothelial cells of several vascular origins, particularly renal glomerular cells (20, 38, 39, 53, 58).

Recent studies have shown that preincubation of human umbilical vein endothelial cells (HUVEC) with LPS or with either of the LPS-elicited cytokines TNF and IL-1 increases the sensitivity of the cells to Stx (19, 27–29, 39, 53, 57, 58). This process is associated with a protein synthesis-dependent increase in biosynthesis of the Stx receptor, the glycolipid globotriaosylceramide, Gb_3 (19, 27–29, 39, 53, 57). The events and/or genes which lead to this upregulation of Gb_3 are not known. It is known, however, that treatment of HUVEC with LPS, TNF, or IL-1 induces many changes in HUVEC physiology, including activation of a signal transduction cascade and the induction of gene expression (6, 7, 37, 40). Actions which appear to require both protein kinase C (PKC) and NF-κB include the induction of vascular cell adhesion molecule-1, of urokinase-type plasminogen activator, and of tissue factor (6, 7, 37, 40). Although transcriptional regulation is very common in eukaryotes, presently there is no direct evidence that induction of Stx receptor by LPS and cytokines occurs at this level in HUVEC.

PKC is actually a group of related enzymes which can be classified according to the signals required for their activation (16). The class I (alpha, beta, and gamma) PKC types differ from the class II (delta, epsilon, eta, and theta) PKC types by the presence of a Ca^{2+} binding site within the class I isoforms. However, both the classical and novel forms are activated by the second messenger diacylglycerol (DAG), a product which is generated by treatment of cells with TNF, and probably by treatment with LPS and IL-1 as well (48). Class III PKC isoforms (zeta and lambda) are not activated by DAG or Ca^{2+}. Nevertheless, the zeta isozyme appears to be activated in some nonendothelial cells by TNF (9, 10). HUVEC express the alpha, beta, epsilon, and zeta forms of PKC (5, 32). The present study examines the potential role of the different PKC isoforms in the induction of Stx sensitivity by LPS, TNF, and IL-1 in HUVEC.

PKC activity, at least in some cells, can lead to the activation of the transcription factor NF-κB (9, 10, 13, 17, 30). NF-κB is a family of proteins including NFKB1 (p105/p50), NFKB2 (p100/p52), RelA (p65), c-Rel, and RelB, which can homo- and heterodimerize to form functionally active NF-κB (15). Inactive NF-κB is localized in the cytoplasm of most unstimu-

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lated cells in complex with its inhibitor, I kappa B (12). Phosphorylation of I kappa B, followed by its degradation, allows the translocation of NF-κB into the nucleus to activate NF-κB-dependent gene transcription (12). TNF, LPS, and IL-1 all activate NF-κB in HUVEC (7, 31, 35, 46). However, this activation of NF-κB in HUVEC appears not to require PKC (7, 35). Thus, another purpose of this study was to examine the role of NF-κB in the sensitization of HUVEC to Stx. The experimental plan of the present study includes pretreatment of HUVEC with specific inhibitors of class I, II, or III PKCs, or of NF-κB activation prior to exposure of the cells to inducers of Stx sensitivity. Further, phorbol myristate acetate (PMA), which interacts with the DAG binding site present in the class I and II PKC isoforms (for a review, see reference 36), was examined for its ability to sensitize HUVEC to Stx. A greater knowledge of the factors involved in the sensitization of HUVEC to Stx may lead to the development of strategies for the prevention of the vascular complications of HUS.

MATERIALS AND METHODS

Reagents. Reagents and their sources are the following: LPS (from *E. coli* O55:B5), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), 3,4-dichloroisocoumarin (DCIC), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), *N*-acetylleucyl-leucylnorleucinal (calpain inhibitor I), pyrrolidine dithiocarbamate (PDTC), *N*-acetyl-L-cysteine (NAC), PMA, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG), 3-isobutyl-1-methylxanthine, dexamethasone, indomethacin, calcium ionophore A23187, all-*trans* retinoic acid, glycerol, HEPES, $MgCl_2$, EDTA, EGTA, cell culture medium, NaCl, KCl, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, neutral red, dithiothreitol (DTT), and heparin (all from Sigma Chemical Co., St. Louis, Mo.); *E. coli* recombinant TNF (Boehringer Mannheim, Indianapolis, Ind.); polyclonal anti-PKC antibodies (Gibco-BRL); monoclonal anti-PKC antibodies (Transduction Laboratories, Lexington, Ky.); IL-1 (NIH Bioresponse modifier program); Triton X-100 (Bio-Rad Laboratories, Hercules, Calif.); bisindolylmaleimide II and Ro31-8220 (Calbiochem); Klenow fragment (New England BioLabs, Beverly, Mass.); [^{32}P]dATP (3000 mCi/mmol) (New England Nuclear, Wilmington, Del.); unlabeled dCTP, dGTP, and dTTP, and poly(dI-dC) (Pharmacia Biotech, Piscataway, N.J.); fetal bovine serum (HyClone Laboratories, Logan, Utah); endothelial cell mitogen (Biomedical Technologies, Stoughton, Mass.); and chromagenic substrate S2222 (KABI Pharmacia, Franklin, Ohio). Proplex T was a generous gift of Baxter Healthcare (Glendale, Calif.), and rabbit polyclonal anti-human tissue factor antibody was kindly provided by W. Kisiel. Stx was purified from lysates of *Shigella dysenteriae* 1 3818-0 as described previously (38) and was the gift of J. E. Brown.

Cell culture. HUVEC were obtained from individual umbilical cords according to previously published methods (28). Cells were grown in RPMI 1640 containing 20% fetal bovine serum, 76 μg of heparin/ml, and 50 μg of endothelial cell mitogen/ml. Cells were maintained and passaged as described previously (28). The cells in these experiments were between passage numbers 4 and 10.

Cell viability assays. HUVEC viability was measured as the uptake of the dye neutral red, as described previously (28). Briefly, neutral red (50 μg/ml) was added to the cells and they were incubated for 1 h (at 37°C). Cells were rinsed with phosphate-buffered saline (PBS). Dye was eluted from the cells by the addition of 100 μl of 0.5 M HCl–35% ethanol to each well. The absorbance of each well was measured in a BioTek ELISA reader at a wavelength of 570 nm and was directly proportional to the number of viable cells in each well.

Tissue factor activity assays. HUVEC were plated into 96-well plates at a density of 12,000 cells per well, were allowed to attach overnight, and were treated as described in the legend for Fig. 4. Following these treatments, medium was removed, and cells were rinsed twice with RPMI 1640 medium which did not contain phenol red (100 μl/well). Tissue factor activity was then measured as the cleavage of the chromogenic substrate S2222 by activated factor X, as described by Surprenant and Zuckerman (51). Briefly, 50 μl of S2222 and 50 μl of a mixture containing coagulation factors VII, IX, X, and II (proplex T) were diluted in RPMI 1640 without phenol red, added to each well, and incubated with the intact monolayers for 2 h (at 37°C). Final concentrations of substrates in the wells were 0.5 factor VII U of proplex T/ml and 250 μg of S2222/ml. Following this incubation, the absorbance of each well was determined in a BioTek ELISA reader at a wavelength of 405 nm. The concentrations of substrates used in this study were demonstrated to be in excess of the amount of tissue factor activity in each well. That is, the color development in this assay continued in a linear fashion until at least 4 h of incubation with substrates, such that the amount of color at 2 h of incubation was directly proportional to the amount of tissue factor activity on the cells. The specificity of this assay for tissue factor was demonstrated by the ability of a rabbit polyclonal anti-human tissue factor immunoglobulin G (IgG), but not normal rabbit IgG, to neutralize color development in this assay.

PKC translocation assay. HUVEC monolayers were preincubated with inducers as described in Results and washed with PBS (pH 7.5). The cells were collected and homogenized at 4°C in buffer A (50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, leupeptin and aprotinin [25 μg of each/ml], 2 mM EDTA, and 1 mM EGTA). The homogenates were centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant was used as the cytosolic fraction. The pellet was suspended in buffer A containing 1% Triton X-100 and held on ice for 30 min. The suspension was then diluted with buffer A, yielding a final concentration of 0.2% Triton X-100, and centrifuged at 100,000 × *g* for 1 h, resulting in the membrane fraction.

For Western blot analysis, each fraction was measured for total protein by a Lowry assay (Bio-Rad). Samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) system and transferred to a nitrocellulose membrane. The membrane was blocked with 1% bovine serum albumin in TBST solution (50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.05% Tween 20) at 20°C. After washing with TBST, the membrane was probed for 1 h with 4.0 μg of anti-PKC antibody/ml in TBST containing nonfat milk. Afterward, the membrane was washed with TBST solution and PKC was detected with a goat anti-rabbit IgG by using the ECL developing system (Amersham) and exposure on X-OMAT film (Kodak).

Electrophoretic mobility shift assay. Nuclear extracts of HUVEC were prepared according to a modification of the procedure of Marui et al. (31). HUVEC were grown to confluence in T-75 flasks, medium was changed, and experimental treatments were conducted as described in the legend for Fig. 3. Following these experimental treatment periods, cells were rinsed with ice-cold PBS (80 g of NaCl/liter, 2 g of KCl/liter, 22.5 g of $Na_2HPO_4 \cdot 7H_2O$/liter, 2 g of KH_2PO_4/liter, and 100 μM TPCK [pH 7.8]). Cells were removed from the flasks with ice-cold EGTA solution (5 mg of HEPES/ml, 9 mg of NaCl/ml, 0.1 mg of EGTA/ml, 5 mg of polyvinylpyrrolidone/ml, and 100 μM TPCK [pH 7.6]) and were collected by centrifugation at 1,000 × *g* (for 5 min at 4°C). Nuclei were obtained by resuspending each pellet in 500 μl of ice-cold buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM DTT, 1.0 mM PMSF, 0.5 μg of leupeptin/ml, 0.5 μg of aprotinin/ml, and 100 μM TPCK [pH 7.9]) containing 0.1% Triton X-100. After a 10-min incubation (at 4°C), nuclei were collected by centrifugation (at 2,040 × *g* for 7 min at 4°C) and were rinsed with 1 ml of ice-cold buffer A/pellet. Pellets were then resuspended in 40 to 50 μl of ice-cold buffer B (20 mM HEPES, 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF, 0.5 μg of leupeptin/ml, 0.5 μg of aprotinin/ml, and 100 μM TPCK [pH 7.9]). A 30-min incubation (at 4°C) was followed by centrifugation at 16,000 × *g* (for 30 min at 4°C). The supernatant containing the nuclear extract was removed, snap-frozen in liquid N_2, and stored at −80°C. The protein concentration of these nuclear extracts was measured with the Bio-Rad protein assay kit by using bovine serum albumin as a standard and was typically 1 to 2 mg/ml.

For the electrophoretic mobility shift assays, two complementary single-stranded oligomers (21 nucleotides each) were synthesized to encompass the NF-κB site from the murine Ig light-chain enhancer region (5′-AGCTTAGAG GGGACTTTCCGAGAGGA-3′) (45). Hybridization of these two oligonucleotides resulted in the formation of two 5′ overhangs (each 5 nucleotides in length) which were then filled in with the Klenow fragment of *E. coli* DNA polymerase I and a mixture of nucleotides consisting of 1.0 mM each unlabeled dCTP, dGTP, and dTTP, and 25 μCi of [α-^{32}P]dATP. Binding reaction mixtures consisted of 10 mM Tris-Cl (pH 7.5), 60 mM NaCl, 1.0 mM DTT, 1.0 mM EDTA, 10% (vol/vol) glycerol, 1 μg of poly(dI-dC), 100,000 to 200,000 cpm of radiolabeled oligonucleotide, and 5 μg of nuclear extract protein. The total volume of each binding reaction mixture was 20 μl. Binding reaction mixtures were incubated for 25 min (at 25°C), were loaded onto 4% polyacrylamide gels, and were electrophoresed at 32 mA (for 1.5 h) under the high-ionic-strength conditions specified by Ausubel et al. (1). Gels were then dried and autoradiographed for approximately 18 h with Kodak X-OMAT AR film.

Glycosphingolipid analysis. HUVEC were grown to confluence and treated with 81 nM PMA or with medium only for 6 h. Cells were harvested, and neutral glycolipids were extracted and separated by high-performance liquid chromatography on a silica column as described previously (39). Galactosyl diglyceride was included as an internal standard.

Statistics. For cytotoxicity and tissue factor activity assays, experimental manipulations were performed in triplicate, and results are presented as means and standard deviations of the replicate samples. Additionally, all experiments reported herein were repeated at least twice, with similar results each time. The probability that we would obtain by chance the differences in cell viability between cells treated with PMA plus Stx and cells treated with PMA plus Stx plus AMG, if no real difference existed, was analyzed by calculating a one-sided *t* statistic and is represented by the value alpha. This probability was also calculated for cells treated with LPS, IL-1, or TNF plus Stx versus cells treated with LPS, IL-1, or TNF plus Stx plus AMG, and for cells treated with TNF plus Stx versus cells treated with TNF plus Stx plus retinoic acid. Normal population distribution and unequal population variances were assumed for these statistical tests.

RESULTS

Effects of PKC activators and inhibitors on the sensitivity of HUVEC to Shiga toxin. The involvement of PKC in the sensitization of HUVEC to Stx was examined. PMA, a known

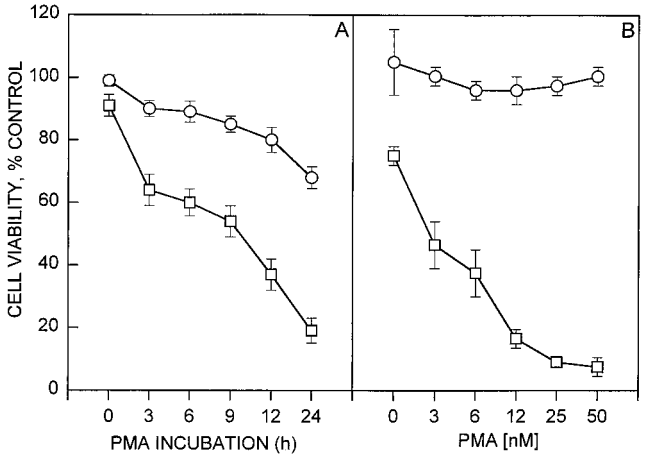

FIG. 1. Effect of the PKC activator PMA on the sensitivity of HUVEC to Stx. HUVEC were placed into 96-well plates and were allowed to attach overnight. (A) Medium was changed, and cells were pretreated with 50 nM PMA at the times indicated. A final concentration of 1 nM Stx (squares) or control medium (circles) was then added, and cells were incubated an additional 24 h. Cell viability was determined by neutral red assay. (B) Conditions were as described for panel A, but with an initial 6-h incubation with the PMA concentrations indicated. Squares, plus Stx; circles, minus Stx. Control untreated cells represent 100% viability. Treatment of HUVEC with Stx alone (no PMA) resulted in 91% cell viability.

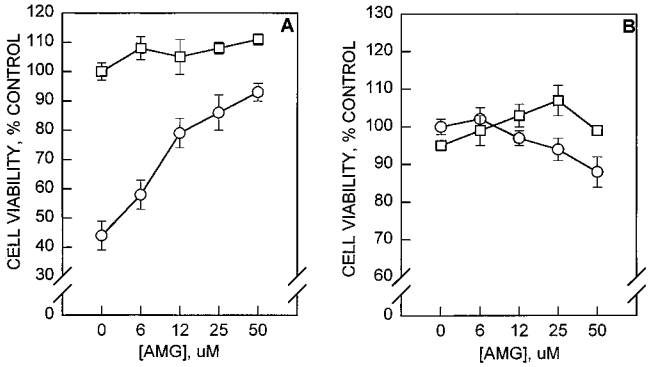

FIG. 2. Effect of the PKC inhibitor AMG on the sensitization of HUVEC to Stx by PMA. HUVEC were placed into 96-well plates and were allowed to attach overnight. Medium was changed, and cells were preincubated for 24 h with 50 μM AMG or with medium only (control). Medium was then replaced with either fresh medium only (control), 50 nM PMA plus AMG (circles), or AMG only (squares), and cells were incubated an additional 6 h. Medium was again replaced, and cells were incubated with (A) or without (B) 1 nM Stx for an additional 24 h. Cell viability was then determined by neutral red assay. Control untreated cells represent 100% viability. The difference in viability between HUVEC treated with PMA plus Stx and HUVEC treated with PMA plus AMG and Stx1 was statistically significant (alpha = 0.05).

activator of PKC, sensitized HUVEC to the cytotoxic action of Stx (Fig. 1). This sensitization process was time dependent (Fig. 1A) and occurred over a concentration range of 3 to 50 nM PMA (Fig. 1B).

Induction by PMA of Stx sensitivity in HUVEC was associated with an increase of nearly fourfold in expression of the Stx receptor, Gb_3 (Table 1). Gb_3 is also increased during induction of Stx sensitivity in HUVEC by LPS, TNF, and IL-1 (19, 27, 28, 39, 53, 57, 58). In the present study, it was noted that PMA caused a general increase in all glycolipids of the Gb_3 pathway (Table 1).

The role of PKC in the Stx sensitivity induction process was further examined by using inhibitors of PKC isozymes. The compound AMG is an analog of DAG which inhibits PKC activation by preventing the binding of DAG or PMA to the DAG-dependent, i.e., class I and II, forms of PKC (24, 50, 56). AMG is specific for PKC and inhibits neither protein kinase A nor calcium/calmodulin-dependent kinase (50). We therefore examined the ability of AMG to inhibit the sensitization of HUVEC to Stx by PMA and by TNF. As expected, AMG inhibited the sensitization of HUVEC to Stx by PMA (Fig. 2). This inhibition occurred over an AMG concentration range of 6 to 50 μM and was almost complete at 50 μM AMG (Fig. 2A). These results were obtained under conditions where treatments of AMG alone or PMA plus AMG were minimally toxic to HUVEC in the absence of Stx (Fig. 2B). These results demonstrated that activation of PKC could sensitize HUVEC to Stx.

It then became necessary to determine whether PKC could be implicated in the LPS-, TNF-, or IL-1-mediated sensitization of HUVEC to Stx. AMG almost completely inhibited the sensitization of HUVEC to Stx in response to LPS (Table 2). This inhibition occurred over the 6 to 50 μM range of AMG concentrations (data not shown), as was observed for the inhibition of PMA. However, AMG only marginally inhibited the sensitization of HUVEC to Stx by TNF and had no effect on IL-1 activity (Table 2). These results suggest that (i) LPS sensitizes HUVEC to Stx through the activation of class I and II PKCs and (ii) TNF and IL-1 do not utilize class I or II PKCs for induction of Stx sensitivity in HUVEC.

The potential use of class III PKCs by TNF was examined further. Because specific inhibitors of class III PKCs have yet to be developed, a slightly different strategy was employed: the use of an agent that inhibits all the PKC isozymes, which would also be expected to affect a class III member, PKC-zeta. Bisin-

TABLE 1. Induction of neutral glycosphingolipid in HUVEC by PMA[a]

Inducer	Glycolipid content (pmol/10^6 cells [fold increase])			
	GC	LC	Gb_3	Gb_4
None	231	77	285	177
PMA (81 nM)	368 (1.6)	401 (5.2)	1,120 (3.9)	475 (2.7)

[a] Confluent HUVEC in T-75 flasks were treated with 81 nM PMA or with medium only for 6 h. The cells were homogenized and analyzed for glucosylceramide (GC), lactosylceramide (LC), Gb_3, and globotetraosylceramide (Gb_4) contents as described in Materials and Methods.

TABLE 2. Summary: AMG inhibition of PMA, IL-1, TNF, and LPS induction of Stx sensitivity in HUVEC[a]

Inducer (concn)	% Cell viability ± SD	
	Without AMG	With AMG
None	100 ± 3	95 ± 3
PMA (50 nM)	45 ± 4*	95 ± 5
PMA (100 nM)	43 ± 5	59 ± 5
LPS (1 μg/ml)	35 ± 2*	97 ± 5
TNF (200 U/ml)	38 ± 2	45 ± 2
IL-1 (100 U/ml)	63 ± 4	66 ± 4

[a] HUVEC were grown in 96-well plates and preincubated with or without 100 μM AMG for 24 h. The medium was changed, and the cells were incubated with inducer plus or minus AMG. The cells were treated with 1 nM Stx for 24 h, followed by the determination of cell viability by neutral red assay. The viabilities of cells treated without Stx, i.e., with 100 μM AMG alone or in combination with 200 U of TNF/ml, 100 U of IL-1/ml, 1 μg of LPS/ml, 50 nM PMA, or 100 nM PMA were greater than 95%. *, statistically significantly different from data for the same inducer with AMG (alpha = 0.05).

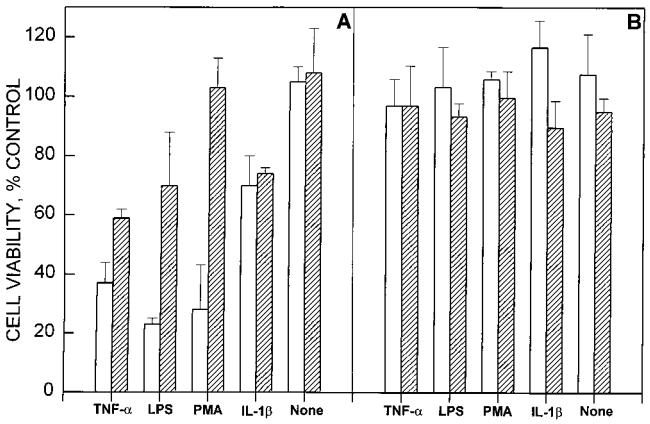

FIG. 3. Effect of the PKC inhibitor Bis II on the sensitization of HUVEC to Stx by different inducers. (A) Cells were preincubated with (diagonally striped bars) or without (open bars) 50 nM Bis II for 30 min at 37°C, followed by a 24-h incubation with the inducers (200 U of TNF/ml, 1 μg of LPS/ml, 50 mM PMA, or 200 U of IL-1/ml). Following a complete change of medium, the cells were incubated with 1 nM Stx for 24 h and subjected to neutral red assay. (B) Cells were treated as for panel A, but without the final Stx incubation. In panel A, values within the TNF data set differed at the 90% confidence level while values within the LPS data set differed at the 95% confidence level.

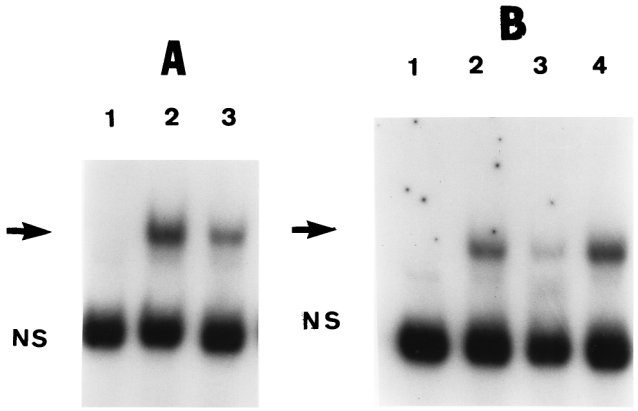

FIG. 4. Effect of experimental agents on the activation of NF-κB in HUVEC. (A) Cells were treated for 1 h with control medium only (lane 1), 200 U of TNF/ml (lane 2), or 160 nM PMA (lane 3). Nuclear extracts were then prepared and analyzed for NF-κB binding activity by electrophoretic mobility shift assay as described in Materials and Methods. (B) Cells were pretreated with either control medium only (lanes 1 and 2), 50 μM TPCK (lane 3), or 10 μM retinoic acid (lane 4). A final concentration of 200 U of TNF/ml (lanes 2 through 4) or control medium was then added and incubated with the cells for an additional hour. Nuclear extracts were then prepared and analyzed for NF-κB binding activity by electrophoretic mobility shift assay. Arrows indicate specific NF-κB binding activity, which could be abolished by a 100-fold excess of unlabeled oligonucleotide. NS denotes nonspecific binding, which was not inhibited by excess unlabeled oligonucleotide.

dolylmaleimide II (Bis II) is a competitive substrate inhibitor for the ATP binding site, a feature of all PKCs. Bis II is approximately 500 times more potent as an inhibitor of PKC than other kinases, such as cyclic AMP-dependent kinase, tyrosine kinase, and calmodulin-dependent kinases. Preincubation of HUVEC with 50 nM Bis II effectively blocked the induction of Stx sensitivity by PMA (a 300% change) and LPS (a 190% change), but Bis II gave less protection (a 50% change) from TNF action (Fig. 3). In each case, these data sets were statistically significant (see the Fig. 3 legend). Consistent with the results described for combinations of IL-1 and AMG, Bis II also was unable to block IL-1 induction of Stx sensitivity in HUVEC. These results, coupled with the AMG data in Table 2, indicate that TNF induction of Stx sensitivity may involve a class III PKC, namely, PKC-zeta. Experiments performed with a Bis II-related inhibitor, Ro31-8220 (180 nM), yielded almost identical results. Other PKC inhibitors, such as calphostin C and staurosporine, were tested for their ability to prevent the sensitization process. However, these agents were cytotoxic to HUVEC at the concentrations required for the inhibition of PKC and were therefore not useful in these experiments.

Translocation of PKC isozymes from the cytosol to the plasma membrane fraction is required for full induction of PKC activity in some cell types. However, HUVEC have an unusually high amount of endogenous membrane-associated PKC (8), and treatment of HUVEC with TNF, or with the other inducers of Stx sensitivity, did not change the intracellular distribution of the PKCs (data not shown). Thus, we were unable to demonstrate directly that TNF requires PKC-zeta for induction of Stx sensitivity.

The role of NF-κB in the sensitization of HUVEC to Stx by LPS, TNF, IL-1, and PMA. LPS, TNF, IL-1, and PMA are all known to induce NF-κB activity in HUVEC (7, 35). We confirmed this result for TNF and PMA (Fig. 4A), and for IL-1 and LPS as well (data not shown). We therefore wished to determine the involvement of NF-κB in the sensitization of HUVEC to Stx. The serine protease inhibitor TPCK inhibits the activation of NF-κB by preventing the degradation of I kappa B (12). Thus, we examined the ability of TPCK to inhibit the sensitization of HUVEC to Stx by IL-1, TNF, LPS, and PMA. TPCK did not prevent the sensitization process in response to any of these agents. Representative results are shown for TNF in Fig. 5. Coincubation of HUVEC with TNF, in the presence or absence of TPCK, resulted in the same degree of sensitization of HUVEC to Stx. In this experiment, the cytotoxic response of the cells to Stx alone was greater than that reported in Fig. 2. This was due to the use of different HUVEC isolates in separate experiments. The differential sensitivity of separate HUVEC isolates to Stx is a stable characteristic which

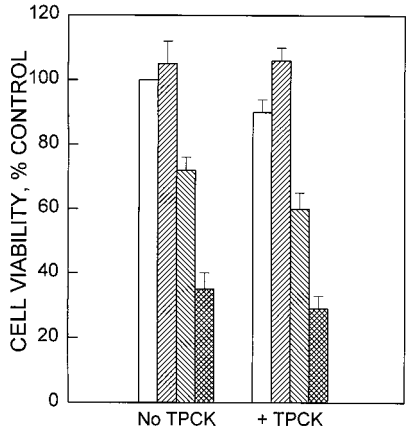

FIG. 5. Effect of the NF-κB inhibitor TPCK on the sensitization of HUVEC to Stx. HUVEC were placed into 96-well plates and allowed to attach overnight. Medium was changed, and cells were pretreated either with medium alone or with medium containing TPCK (40 min). Cells were then incubated for an additional 24 h with control medium or 200 U of TNF/ml plus or minus 50 μM TPCK. Medium was then replaced with fresh medium containing TPCK or DCIC, and the cells were incubated with or without 1 nM Stx for an additional 24 h. Cell viability was determined by neutral red assay. Control untreated cells represent 100% viability. Open bars, no Stx or TNF; left diagonal bars, TNF only; right diagonal bars, Stx only; hatched bars, TNF plus Stx.

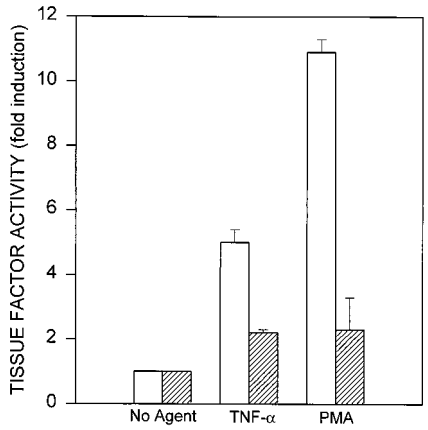

FIG. 6. Effect of the NF-κB inhibitor TPCK and activators on the induction of tissue factor activity in HUVEC. HUVEC were dispensed into 96-well plates and were allowed to attach overnight. Medium was changed, and cells were pretreated (40 min) with or without 50 μM TPCK (diagonally striped bars). TNF (200 U/ml), PMA (81 nM), or control medium was then added to the cells, and they were incubated for 4 h. Tissue factor activity was determined as described in Materials and Methods. Values presented were calculated by dividing the optical density of the treated wells by the optical density of the untreated control wells.

has been reported previously (19, 27, 28). Nevertheless, in repeated experiments using TPCK and different HUVEC isolates, we consistently observed that TPCK inhibited the sensitization of HUVEC to Stx.

To ensure that 50 μM TPCK could effectively inhibit NF-κB activation in HUVEC, nuclear extracts of HUVEC treated with TNF and TPCK were prepared and analyzed directly for NF-κB binding activity by electrophoretic mobility shift assay. With this assay, TPCK inhibited the induction of NF-κB binding activity by TNF (Fig. 4B). Further, this concentration of TPCK effectively inhibited the induction by TNF or PMA of the NF-κB-dependent gene tissue factor in HUVEC (Fig. 6). These results indicate that TPCK clearly, although not completely, inhibited NF-κB activation and NF-κB-dependent gene induction in HUVEC. Thus, the failure of TPCK to inhibit the sensitization of HUVEC to Stx (Fig. 5) was not due to an inability of TPCK to inhibit the induction of NF-κB.

A higher concentration of TPCK (100 μM) inhibited the induction both of NF-κB binding activity and of tissue factor activity in HUVEC to a greater extent than did 50 μM TPCK, but it did not inhibit the sensitization of HUVEC to Stx (data not shown). However, 100 μM TPCK was somewhat cytotoxic to HUVEC over longer incubation periods and in fact appeared to enhance the cytotoxicity of Stx toward the cells in the absence of cytokine treatment (data not shown). Other inhibitors of NF-κB activation, such as the serine protease inhibitors DCIC and BTEE, calpain inhibitor I, and the antioxidants NAC and PDTC were not useful in these studies because they were cytotoxic to HUVEC at the concentrations necessary for inhibition of both NF-κB and tissue factor induction.

In summary, the sensitization of HUVEC to Stx, as well as the activation of NF-κB and induction of tissue factor expression, occurred in the presence of LPS, IL-1, TNF, or PMA (reference 19 and data not shown). Like TNF-mediated sensitization, the LPS-, IL-1-, or PMA-mediated sensitization of HUVEC was not inhibited by TPCK or DCIC, whereas NF-κB activation and induction of tissue factor expression by IL-1, LPS, or PMA were inhibited (Fig. 6 and data not shown).

Involvement of other signal transduction mediators in the sensitization of HUVEC to Stx by TNF. To further understand the signaling events involved in the sensitization of HUVEC to Stx by TNF, we sought to inhibit the sensitization process by using several different types of agents, each affecting different signal transduction mechanisms. Each agent was tested in a range of concentrations that were demonstrated to be biologically active in cell culture studies conducted by others, and these concentrations were nontoxic to HUVEC. The physiological parameters and their modifying agents were as follows: (i) to increase intracellular cyclic AMP, 3-isobutyl-1-methylxanthine (100 μM to 3 mM); (ii) to increase intracellular calcium, ionophore A23187 (50 to 200 nM); (iii) to inhibit cyclooxygenase, indomethacin (0.1 to 10 μM); and (iv) to inhibit phospholipase A_2, dexamethasone (0.1 to 10 μM). All these agents were without effect on the sensitization of HUVEC to Stx by TNF. However, retinoic acid (10 μM) partially inhibited the sensitization of HUVEC to Stx by TNF (Fig. 7). Higher concentrations of retinoic acid did not inhibit the sensitization to Stx to any greater extent than did 10 μM. The ability of retinoic acid to inhibit sensitization of HUVEC to Stx was observed also when LPS or IL-1 was used in place of TNF (data not shown).

Since retinoic acid has been shown to inhibit the induction of the NF-κB-dependent gene tissue factor in HUVEC (18), the ability of retinoic acid to inhibit NF-κB activation in HUVEC was examined. Retinoic acid did not affect the induction of NF-κB binding activity in nuclear extracts of HUVEC treated with TNF (Fig. 4B) or with LPS or IL-1 (data not shown). However, retinoic acid did partially inhibit tissue factor induction by TNF in our experiments (5.2- versus 3.8-fold induction over controls, for cells treated with TNF versus TNF plus retinoic acid, respectively). These results suggest that retinoic acid inhibits sensitization of HUVEC to Stx and tissue factor induction by a mechanism other than inhibiting NF-κB activation.

DISCUSSION

Stx-mediated endothelial cell damage appears to be an important facet of Stx-associated HUS (60). The pathology of

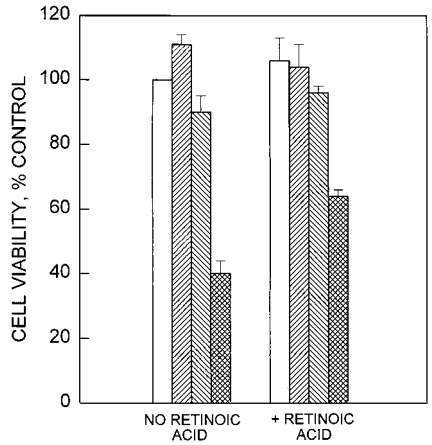

FIG. 7. Effect of retinoic acid on the sensitization of HUVEC to Stx by TNF. HUVEC were dispensed into 96-well plates. Following overnight attachment, medium was changed and cells were treated for 24 h with 200 U of TNF/ml or with control medium, in either the presence or absence of 10 μM retinoic acid. Medium was replaced, and cells were incubated with or without 1 nM Stx for 24 h. Cell viability was determined by neutral red assay. Control untreated cells represent 100% viability. Open bars, no TNF or Stx; left diagonal bars, TNF only; right diagonal bars, Stx only; hatched bars, TNF and Stx. The difference in viability between HUVEC treated with TNF plus Stx and HUVEC treated with TNF plus retinoic acid plus Stx was statistically significant (alpha = 0.05).

HUS includes swollen renal glomerular endothelial cells, as well as occlusion of microvessels with platelets and fibrin (22, 44, 60). During the development of HUS, Stx may work in concert with LPS and the LPS-elicited cytokines TNF and IL-1 to damage vascular endothelial cells, thereby disrupting the normal antithrombotic functions of these cells (19, 28, 29, 58). In support of this hypothesis, circulating LPS has been found in patients with HUS (23). Data obtained with nonrenal endothelial cells may also pertain to the pathophysiology of HUS. LPS, TNF, and IL-1 individually sensitize HUVEC to Stx in a manner which correlates with an increased cellular expression of the Stx receptor, Gb_3 (19, 27–29, 39, 53, 57, 58). Since LPS, TNF, and IL-1 all activate PKC- and NF-κB-dependent activities in HUVEC (7, 31, 45, 46), this study was undertaken to determine whether PKC or NF-κB or both are involved in the sensitization of HUVEC to Stx by these agents.

Activation of PKC by PMA sensitized HUVEC to Stx. This sensitization was completely inhibited by the PKC inhibitor AMG at concentrations which were comparable to those utilized by others to inhibit PKC in HUVEC (5). These results indicate that the PMA-induced sensitization of HUVEC to Stx was indeed mediated through PKC. Further, our results indicate that PMA increased the amount of Stx receptor, the glycolipid Gb_3, in HUVEC. These results corroborate the observations of van de Kar et al. (57), who reported that PMA increased the binding of ^{125}I-labeled Stx1 to HUVEC in a manner which was completely inhibited by the PKC inhibitors H7 and Ro31-8220. It is known that the binding of ^{125}I-labeled Stx1 and Stx2 to HUVEC correlates strongly with the sensitivity of HUVEC to Stx (27), and both toxins bind to the same glycolipid receptor on HUVEC (26). Thus, our current report of an increase in total Gb_3 following exposure of HUVEC to PMA is consistent with the notion that the sensitization of HUVEC to Stx by PMA is mediated by an induction of Stx receptor in HUVEC. These results do not, however, rule out the possibility of a simultaneous and enhanced effect of the inducers on internalization and processing of Stx in HUVEC.

LPS, and to a significant extent TNF also, appeared to sensitize HUVEC to Stx in a manner which was dependent on PKC activation. In contrast, IL-1-induced sensitization of HUVEC to Stx was independent of PKC. That is, inhibition of class I and II PKCs by AMG, an inhibitor of DAG binding, completely inhibited the ability of LPS, but did not significantly affect the ability of TNF or IL-1, to sensitize HUVEC to Stx. Thus, the mechanism by which LPS sensitizes HUVEC to Stx differs from that of TNF and IL-1. This result was somewhat surprising in light of the similar actions of these three agents toward HUVEC in many assay systems, including the sensitization of HUVEC to Stx and the induction of Gb_3 (19, 27–29, 39, 53, 57, 58). Although these experiments do not rule out the use of class III PKCs by TNF and IL-1, others have reported that activities of TNF such as the induction of cellular adhesion molecule expression in HUVEC do not require any of the PKCs (47, 52).

However, that TNF utilizes PKC during the induction of Stx sensitivity in HUVEC was revealed in the present study by use of Bis II, a PKC-specific inhibitor of ATP binding to all PKC isozymes, including class III PKCs. This feature of TNF activity differs from the results of van de Kar et al. (57), who, using a related PKC inhibitor, Ro31-8220 (4), concluded that the TNF-induced increase in Stx receptors on HUVEC did not require PKC activity. This difference appears to be due to the high concentration of Ro31-8220 (3 μM) employed by van de Kar et al. which also inhibited total protein synthesis. In a repeat of this experiment, using 180 nM Ro31-8220, we were able to obtain results virtually identical to those mentioned above with Bis II, without a change in cell protein synthesis. However, the results of the present study and those of van de Kar et al. both indicate that TNF action in these phenomena is not completely dependent on PKC activity. In conclusion, our data suggest that TNF induction of Stx sensitivity in HUVEC occurs, in part, via a class III PKC, most likely PKC-zeta.

In contrast to PKC, NF-κB does not appear to be involved in the sensitization of HUVEC to Stx by any of the agents which sensitized HUVEC to Stx in this study (LPS, TNF, IL-1, or PMA). TPCK, which effectively inhibited NF-κB activation and NF-κB-dependent gene expression in this study, did not prevent the sensitization of HUVEC to Stx by any of the inducers tested (PMA, LPS, TNF, or IL-1). The results presented herein are likely to be true for all forms of NF-κB. That is, NF-κB is actually a family of proteins including NFKB1 (p105/p50), NFKB2 (p100/p52), RelA (p65), c-Rel, and RelB, which can homo- and heterodimerize to form different complexes of functionally active NF-κB (for a review, see reference 15). Nevertheless, our results from the electrophoretic mobility shift assays remained the same when the NF-κB recognition sequence from the human tissue factor gene, which preferentially binds p-65–c-Rel heterodimers (40), was utilized in place of the Ig κ light-chain enhancer sequence, which preferentially recognizes p50-p65 heterodimers (reference 40 and data not shown). Further, TPCK was likely to inhibit the activation of all known forms of NF-κB, since it prevents dissociation of NF-κB from its cytoplasmic inhibitor, I kappa B-alpha (12). Thus, the inability of TPCK to inhibit sensitization of HUVEC to Stx was probably not due to an inability of these agents to inhibit certain forms of NF-κB. While TPCK could conceivably inhibit activities in addition to NF-κB in HUVEC, these results still suggest that NF-κB is not required by HUVEC for the Stx sensitization process.

This study demonstrated that all-*trans* retinoic acid partially inhibited the sensitization of HUVEC to Stx. A greater understanding of the effects of retinoic acid on HUVEC could help define the potential step(s) of the sensitization process which is inhibited by this agent. In the present study, retinoic acid did not inhibit NF-κB activation in HUVEC in response to LPS, TNF, or IL-1. This observation further supports the notion that NF-κB is not required for the sensitization of HUVEC to Stx. Nevertheless, it suggests that some other action of retinoic acid toward HUVEC is responsible for its ability to inhibit the sensitization process. In some cell types, retinoic acid inhibits the action of the transcription factor AP-1 (42, 49), a phenomenon which could explain the ability of retinoic acid to prevent tissue factor induction in HUVEC, as well as the sensitization of HUVEC to Stx. That is, AP-1, in addition to NF-κB, is required for expression of tissue factor by HUVEC (3, 18, 34, 41). The role of AP-1 in the sensitization of HUVEC to Stx is not yet known. Retinoic acid may also act in a steroidal manner to modulate the sensitization of HUVEC to Stx by binding to retinoic acid receptors which are present in HUVEC, thereby translocating to the nucleus to alter gene expression (11, 25, 55).

Certain studies suggest that PKC may mediate some of the effects of retinoic acid on HUVEC (21, 54). That is, some activities of retinoic acid can be inhibited by the PKC inhibitor H7 (21, 54). However, the synergistic action of retinoic acid with PMA (33), as well as the differential ability of retinoic acid versus PMA to synergize with forskolin (21), suggests that PMA works through different molecular mechanisms than does retinoic acid. If retinoic acid does activate PKC, it may involve the zeta form, which is insensitive to PMA and which may display a different intracellular localization and substrate preference from other forms of PKC (16, 32).

In conclusion, the results of this study indicate that PMA and LPS utilize class I and/or class II PKCs and that TNF signals through a class III PKC in the sensitization of endothelial cells to Stx's. Although IL-1 is also an inducer of toxin sensitivity, it appears to function through a PKC-independent mechanism. All four inducers (PMA, LPS, TNF, and IL-1) activate NF-κB in HUVEC, but our data clearly indicate that NF-κB is not required for induction of Stx sensitivity in HUVEC. Thus, different signal transduction pathways are utilized by these agents to achieve a similar end result. Further elucidation of the signal transduction events involved in the sensitization of HUVEC to Stx may help to identify a potential gene(s) and other mechanisms involved in the sensitization process. A greater knowledge of this process may allow insight into the pathogenesis of Stx-associated HUS and may elucidate possible steps at which therapeutic intervention could occur.

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