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NF-κB Activation Is Required for Human Endothelial Survival during Exposure to Tumor Necrosis Factor-α but Not to Interleukin-1β or Lipopolysaccharide*

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Katsuhiro Zen‡, Aly Karsan§, April Stempien-Otero‡, Esther Yee‡, Joan Tupper‡, Xianwu Li‡, Thomas Eunson‡, Mark A. Kay¶, Christopher B. Wilson‖, Robert K. Winn‡‡§§, and John M. Harlan‡¶¶**

*From the Departments of ‡Medicine, ‖Pediatrics, **Immunology, ‡‡Surgery, and §§Biophysics and Physiology, University of Washington, Seattle, Washington 98195, the ¶Department of Genetics, Stanford University, Palo Alto, California 94305, and the §Department of Pathology, University of British Columbia McDonald Research Laboratories, Vancouver, V6Z 1Y6 British Columbia, Canada*

In the presence of a protein synthesis inhibitor, cycloheximide, tumor necrosis factor-α (TNF-α), interleukin 1-β (IL-1β), or lipopolysaccharide (LPS) induces human umbilical vein endothelial cells (HUVECs) to undergo apoptosis, suggesting that constitutive or inducible cytoprotective pathways are required for cell survival. We studied the correlation between nuclear factor-κB (NF-κB) activation and cell death induced by TNF-α, IL-1β, or LPS. Adenovirus-mediated overexpression of a dominant-negative IκBα (inhibitor of κB) mutant blocked NF-κB activation by gel shift assay and blocked induction of vascular cell adhesion molecule-1 protein by TNF-α, IL-1β, and LPS, a NF-κB-dependent response. In cells overexpressing the IκBα mutant, TNF-α induced cell death, whereas IL-1β or LPS did not. We conclude that cell survival following TNF-α stimulation is NF-κB-dependent but that a constitutive or inducible NF-κB-independent pathway(s) protects IL-1β- or LPS-treated HUVECs from cell death.

The transcription factor nuclear factor-κB (NF-κB)[1] is activated in response to diverse physiological and pathological stimuli and is involved in the regulation of a variety of genes in diverse cell types (1). Human umbilical vein endothelial cell (HUVEC) genes, the expression of which is dependent upon NF-κB, include interleukin (IL)-1β, IL-6, IL-8, E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 (VCAM-1), and tissue factor (2–4). NF-κB activation involves its release from a cytoplasmic inhibitor of NF-κB, (IκB), followed by its translocation to the nucleus, where it binds to cognate sequences in the promoter region of multiple genes. Following stimulation, endogenous IκB is typically degraded in a process involving two distinct steps. First, recently identified IκB kinases phosphorylate Ser-32 and Ser-36 of IκBα (5–10). Second, there is proteolytic degradation of IκB through the ubiquitin-proteasome pathway (11–13).

Recent studies have identified the key intracellular signaling pathways initiated when tumor necrosis factor (TNF)-α binds to its p55 receptor (14–16). TNF-α binding transmits one signal that elicits cell death and another, dependent upon NF-κB activation, that protects against death by induction of cytoprotective genes (16–19). It has been shown that NF-κB is required for cell survival in TNF-α-treated fibroblasts, fibrosarcoma cells, Jurkat cells, porcine endothelial cells (17–20), transforming growth factor-β1-treated B cells (21), Ras-transformed NIH3T3 cells (22), and a protease inhibitor-treated B cells (23).

Previous studies have demonstrated that IL-1β and lipopolysaccharide (LPS) (24), like TNF-α (24, 25), induced HUVEC death when protein synthesis was inhibited by cycloheximide (CHX) or actinomycin D. This finding suggested that inducible or constitutive cytoprotective proteins were required for HUVEC survival during exposure to IL-1β and LPS as well as to TNF-α. The IL-1 signaling pathway is initiated by binding of IL-1 to IL-1receptor type I (IL1-R1), a member of the human Toll/IL-1 receptor family. IL-1β stimulation of cells leads to activation of the serine-threonine IL-1 receptor-associated kinase (IRAK), which is recruited to the IL-1R1 by IL-1 receptor accessory protein and the Toll domain-containing protein MyD88. Phosphorylated IRAK then dissociates from the receptor and interacts with TNF receptor-associated factor (TRAF)-6; IRAK is rapidly degraded by a proteasome-dependent pathway (26, 27). MyD88, a death domain-containing protein, acts as a proximal adapter in the IL-1R signaling complex by independently interacting with IL-1 receptor accessory protein and IRAK (28). TRAF-6 binds to NF-κB-inducing kinase (NIK) (29), thereby linking NIK and, subsequently, IκB kinases (IKKα and IKKβ) to both IL-1- and TNF-α-induced NF-κB activation (8).

Stimulation of cells by LPS involves the plasma protein, LPS-binding protein, membrane-bound or soluble CD14, and a membrane-bound signaling receptor that was unknown until recently (reviewed in Ref. 30). Two groups reported that overexpression of Toll-like receptor 2 (TLR2) conferred LPS responsiveness as assessed by NF-κB activation in human embryonic 293 cells (31, 32). In one of the 293 cell lines examined, overexpression of TLR1 and TLR4 did not confer LPS signaling

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¶¶ To whom correspondence should be addressed: Division of Hematology, Box 357710, University of Washington, Seattle, WA 98195-7710. Tel.: 206-685-7866; Fax: 206-543-3560; E-mail: jharlan@u.washington.edu.

[1] The abbreviations used are: NF-κB, nuclear factor-κB; HUVEC, human umbilical vein endothelial cell; IL, interleukin; VCAM-1, vascular cell adhesion molecule-1; IκB, inhibitor of κB; TNF, tumor necrosis factor; LPS, lipopolysaccharide; CHX, cycloheximide; IRAK, IL-1 receptor-associated protein; TRAF, TNF receptor-associated factor; NIK, NF-κB-inducing kinase; IKK, IκB kinase; FADD, Fas-associated death domain; IκBαM, IkBα mutant; zVAD-fmk, Z-Val-Ala-Asp-fluoromethylketone; PGK, phosphoglycerokinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; mAb, monoclonal antibody; βgal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; FasL, Fas ligand; PI, phosphatidylinositol; Ad, adenovirus.

(31). However, mutations in the TLR4 gene were shown to produce defective LPS signaling in endotoxin-tolerant mouse strains (33, 34). Moreover, TLR4-deficient mice were also found to be hyporesponsive to LPS (35). Also, TLR4 (as well as TLR2) was shown to mediate LPS-induced signal transduction in another 293 cell line (36). Thus, it is clear that TLR4 is essential for LPS signaling *in vivo*, and there is evidence that TLR2 may play a role in some cell types *in vitro*.

The intracellular signaling pathway by which LPS activates NF-κB in 293 cells was blocked by dominant-negative forms of MyD88, TRAF-6, NIK, IKKα, and IKKβ (31). Recently, LPS signaling leading to activation of NF-κB in human dermal microvessel endothelial cells was also shown to signal through MyD88, IRAK, IRAK2, TRAF-6, and NIK (37). Thus, LPS activation uses the many of the same molecules as the IL-1 system. Also, we have recently shown that LPS activation of NF-κB involves tyrosine phosphorylation of a protein(s) leading to degradation of IκBα but not IκBβ in HUVECs (38) and that LPS-induced endothelial cell death is dependent upon FADD, a Fas-associated protein with a death domain (39).

Although the pathways leading to NF-κB activation have now been elucidated, those promoting HUVEC survival following stimulation by IL-1β or LPS remain undefined. To investigate the relationship between NF-κB activation and apoptosis in HUVECs, we determined whether TNF-α, IL-1β, or LPS induced cell death when activation of NF-κB was inhibited by overexpression of a dominant-negative IκBα mutant (IκBαM) using recombinant adenovirus. This study is the first to demonstrate that endothelial cell survival following stimulation by IL-1β and LPS is NF-κB-independent.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HUVECs obtained from collagenase-digested umbilical veins were cultured in RPMI medium with 20% bovine calf serum (Hyclone, Logan, UT), 90 μg/ml heparin (Sigma), and endothelial cell growth factor (50 μg/ml) prepared from bovine hypothalamus. All cells were maintained at 37 °C in 5% CO_2. Recombinant human TNF-α, IL-1β, IL-4, and LPS were purchased from R&D Systems (Minneapolis, MN). Anti-IκBα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Kamiya Biomedical Co. (Seattle, WA). The PI 3-kinase inhibitor LY294002 and the p38 kinase inhibitor SB203580 were obtained from Calbiochem (La Jolla, CA). The neutralizing anti-Fas L monoclonal antibody (mAb) 4H9 was obtained from Immunotech-Coulter Corp. (Miami, FL).

Construction of Recombinant Adenovirus—The IκBα mutant (S32A/S36A) cDNA was kindly provided by Drs. J. DiDonato and M. Karin (University of California, San Diego, CA) (40). This cDNA was inserted into the vector pXCJL1 under the transcriptional control of the phosphoglycerokinase (PGK) promoter, upstream of the bovine growth hormone polyadenylation signal. The E1A- and partially E3-deleted recombinant adenovirus (Ad.PGK-IκBαM) was generated by the recombination of the pXCJL.1-IκBα mutant plasmid and pJM17 in 293 cells as described (41). Recombinant adenovirus expressing a nuclear-targeted reporter enzyme (Ad.RSV-βgal) used as a control adenovirus was generated as described in a previous report (42). Purification of a large batch of the recombinant adenovirus was done by two consecutive cesium chloride centrifugations, and it was stored at −80 °C in 10% glycerol, 10 mM Tris-HCl, pH 7.4, and 1 mM $MgCl_2$ with a stock concentration of 1.6×10^{11} pfu/ml.

Infection of HUVECs—Subconfluent HUVECs were washed once with warmed complete medium and incubated at a multiplicity of infection (m.o.i.) indicated with control adenovirus or IκBαM adenovirus in complete medium. After 72 h at 37 °C, TNF-α, IL-1β, LPS, or IL-4 was added to the HUVECs.

Immunoblots—After experimental treatment of HUVECs, cell monolayers were detached from plastic culture dishes with a cell scraper, washed in cold phosphate-buffered saline, and incubated in 50 μl of Buffer A (10 mM Hepes, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 200 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5% Nonidet P-40) for 10 min at 4 °C. The cytoplasmic protein was collected in the supernatant following microcentrifugation. The pellet was incubated in 20 μl of Buffer B (20 mM Hepes, pH 8.0, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 20% glycerol) for 10 min at 4 °C. The nuclear protein was collected in the supernatant following microcentrifugation and subjected to electrophoretic mobility shift assay. Cytosolic extracts from treated HUVECs were electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 5% methanol at 100 V for 1 h at 4 °C. Filters were blocked overnight with TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk. Immunoblots were analyzed for IκBα protein using antiserum at a 1:1000 dilution. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham Pharmacia Biotech) using 1:10,000 horseradish peroxidase-linked anti-rabbit secondary antiserum. Blots were exposed to film for 1–10 min.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotide containing the E-selectin NF-κB site (5′-AGCTTAGAGGGGATTTCCGAGAGGA-3′) (43) was end-labeled with [^{32}P]ATP (50 μCi at 3000 Ci/mmol, NEN Life Science Products) and the Klenow fragment of *Escherichia coli* DNA polymerase I. Binding reactions in 20 μl contained 20 μg of nuclear extract protein, binding buffer (10 mM Tris, pH 7.5, 20 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μg of single-stranded salmon testis DNA, 1 μg of poly(dI-dC)), and 100,000 cpm ^{32}P-labeled DNA. Reactions were incubated at room temperature for 60 min and analyzed by electrophoresis on a 4% nondenaturing polyacrylamide gel at 200 V for 2 h. After electrophoresis, gels were dried, and DNA-protein complex was localized by autoradiography for 18 h.

Enzyme-linked Immunosorbent Assay—TNF-α, IL-1β, LPS, or IL-4 was added to uninfected and adenovirus-infected HUVECs. After 6 h at 37 °C, cells were washed twice with phosphate-buffered saline and incubated with 5 μg/ml of the anti-VCAM-1 monoclonal antibody (clone 4B9) conjugated with horseradish peroxidase in RPMI medium containing 2% fetal bovine serum at 37 °C for 1 h. The antibody solution was aspirated, and the cells were subsequently washed three times with phosphate-buffered saline. Cells were developed using 1 mg/ml of *o*-phenylenediamine in 0.1 M sodium citrate, pH 4.5, with 0.01% H_2O_2 (v/v). Absorbance at 490 nm was measured for each well using a plate reader (Bio-Tec Instruments, Winooski, VT).

Viability Assay—TNF-α, IL-1β, LPS, or IL-4 was added to uninfected and adenovirus-infected HUVECs with or without CHX. After 24 h at 37 °C, medium was removed and replaced with medium containing 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and incubated at 37 °C for 5 h. The medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbance at 570 nm was determined for each well.

RESULTS

IL-1β and LPS, as Well as TNF-α, Induce Endothelial Cell Apoptosis—Consistent with previous data (24, 25), TNF-α, IL-1β, and LPS treatment of HUVECs induced cell death only in the presence of CHX (Fig. 1*A*). This result suggests that HUVEC survival following exposure to IL-1β or LPS, as well as to TNF-α, is dependent upon synthesis of a cytoprotective protein(s). The caspase inhibitor, zVAD-fmk (44), inhibited cell death induced by TNF-α, IL-1β, or LPS with CHX (Fig. 1*B*), indicating that in the presence of CHX, TNF-α, IL-1β, or LPS activates a caspase-mediated cell death pathway in HUVECs.

IL-1β- or LPS-induced Apoptosis Does Not Involve Fas-Fas Ligand—The mechanism by which TNF-α activates caspases and induces apoptosis has been well defined, but the signaling pathway leading to apoptosis following IL-1β or LPS stimulation has not been characterized. We recently showed that overexpression of a FADD-dominant-negative construct in a human microvascular endothelial cell line protected the cells from apoptosis following co-incubation with LPS and a protein synthesis inhibitor but that a neutralizing anti-Fas antibody did not (39). Nevertheless, in other model systems, oxidized LDL-induced apoptosis of endothelial cells was blocked by neutralizing mAb to Fas ligand (FasL) (45) and IL-1-induced apoptosis of Langerhans cells (46) and thyrocytes (47) was shown to involve Fas-FasL. However, blockade of the Fas-FasL apoptotic

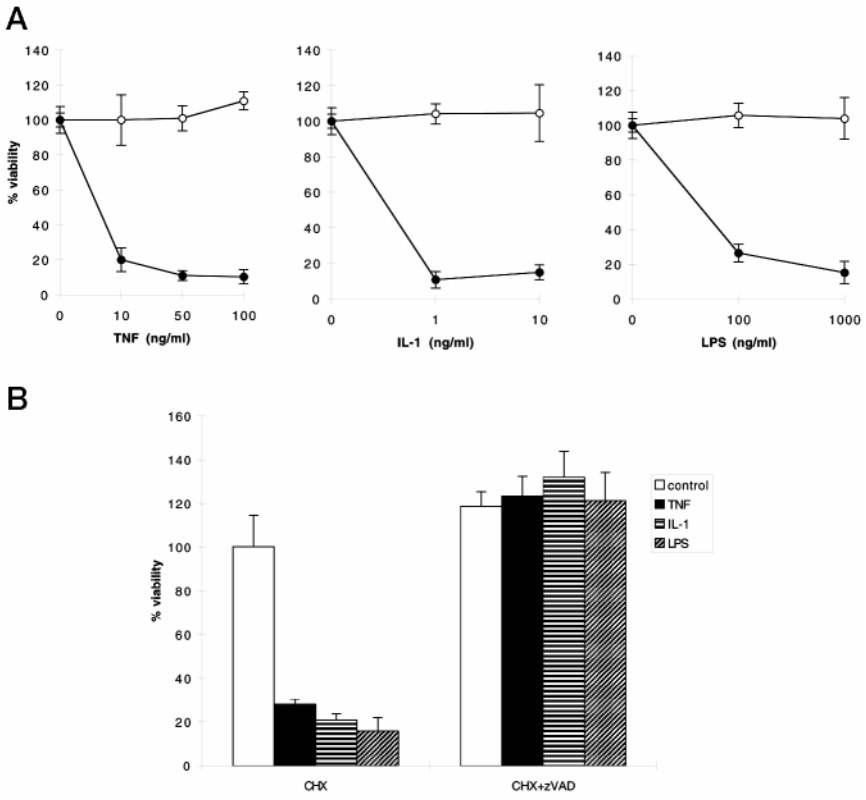

FIG. 1. **TNF-α, IL-1β, or LPS induces cell death in the presence of CHX.** *A,* HUVECs were incubated with or without CHX (10 μg/ml) for 0.5 h, and then untreated (*open circle*) or CHX-treated (*closed circle*) HUVECs were incubated with the indicated amounts of TNF-α, IL-1β, or LPS. An MTT assay was performed after 24 h incubation. Percentage of viability was expressed as the proportion of untreated cells. Results shown are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments. *B,* HUVECs were pretreated with zVAD-fmk (100 μM) for 2 h and then incubated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), or LPS (1 μg/ml) with CHX (10 μg/ml). After 24 h, an MTT assay was performed, and percentage of viability was expressed as the proportion of untreated cells. Results shown are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments.

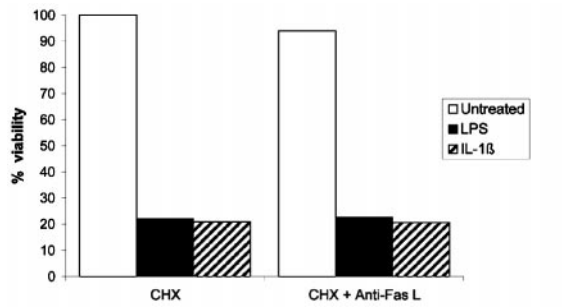

FIG. 2. **IL-1- or LPS-induced apoptosis does not involve Fas-FasL.** HUVECs were incubated in medium containing CHX (5 μg/ml) with or without IL-1β (10 ng/ml) or LPS (100 ng/ml) and with or without the neutralizing anti-FasL mAb 4H9 (10 μg/ml). An MTT assay was performed after 24 h incubation. Percentage of viability was expressed as the proportion of cells treated with CHX alone. Results shown are the means of duplicate wells in a single experiment and are representative of two separate experiments.

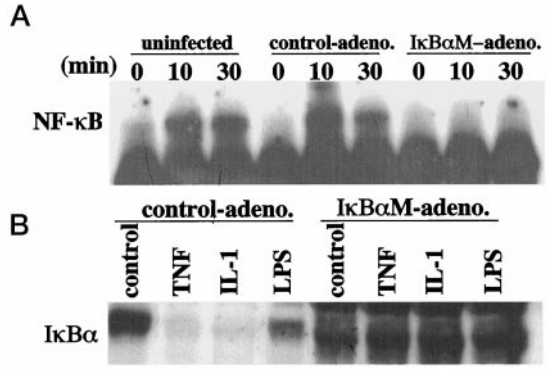

FIG. 3. **Activation of NF-κB is blocked in IκBαM-expressing HUVECs.** *A,* HUVECs were infected with 1000 m.o.i. of control adenovirus or IκBαM adenovirus for 72 h and then exposed to TNF-α (10 ng/ml) for 0, 15, and 30 min. After treatment, nuclear proteins were extracted and subjected to gel mobility shift assay for NF-κB-DNA binding activity as described under "Experimental Procedures" (20 μg protein/lane). *B,* HUVECs were infected with 1000 m.o.i. of control adenovirus or IκBαM adenovirus for 72 h, and then exposed to TNF-α (10 ng/ml), IL-1β (10 ng/ml) for 15 min, or LPS (1 μg/ml) for 60 min. After treatment, cytoplasmic protein (50 μg/lane) was subjected to immunoblot analysis for IκBα.

pathway by the neutralizing anti-FasL mAb, 4H9, (45, 47) did not prevent IL-1β- or LPS-induced apoptosis in HUVECs (Fig. 2).

Adenovirus-transduced Dominant-negative IκB Mutant Blocks NF-κB Activation—To examine the role of NF-κB in activation of the cytoprotective pathway(s), we generated recombinant adenovirus containing an IκBαM gene with serine to alanine mutations at residues 32 and 36 (Ad.PGK-IκBαM). This mutant IκBα protein inhibits signal-induced phosphorylation and subsequent proteasome-mediated degradation of IκBα, thereby preventing translocation of NF-κB to the nucleus (40). Gene transduction using recombinant adenovirus is one of the most effective strategies to express genes in HUVECs (4, 48). First, transfection efficiency of adenovirus was determined in HUVECs. An X-gal assay was performed 48 h after adding Ad.RSV-βgal to HUVECs, and efficiency of transfection was observed to be 100% at 1000 m.o.i. of recombinant adenovirus (data not shown). Next, we determined whether overexpression of the IκBαM inhibited NF-κB activation and IκBα degradation. Gel mobility shift experiments with the E-selectin-κB site as the probe showed that Ad.RSV-βgal-infected cells, as well as uninfected cells, exhibited strong induction of NF-κB after TNF-α treatment, whereas κB binding activity was not observed in the IκBαM-expressing cells (Fig. 3*A*). Similar inhibition of NF-κB binding activity was observed in Ad.PGK-IκBM-transduced HUVECs treated with IL-1β or LPS (data not shown). Immunoblot analysis confirmed that TNF-α, IL-1β, or LPS induced IκBα degradation in control adenovirus-infected HUVECs, but overexpressed IκBαM was not degraded following stimulation by TNF-α, IL-1β, or LPS (Fig. 3*B*), presumably because it was not phosphorylated. Finally, we tested whether

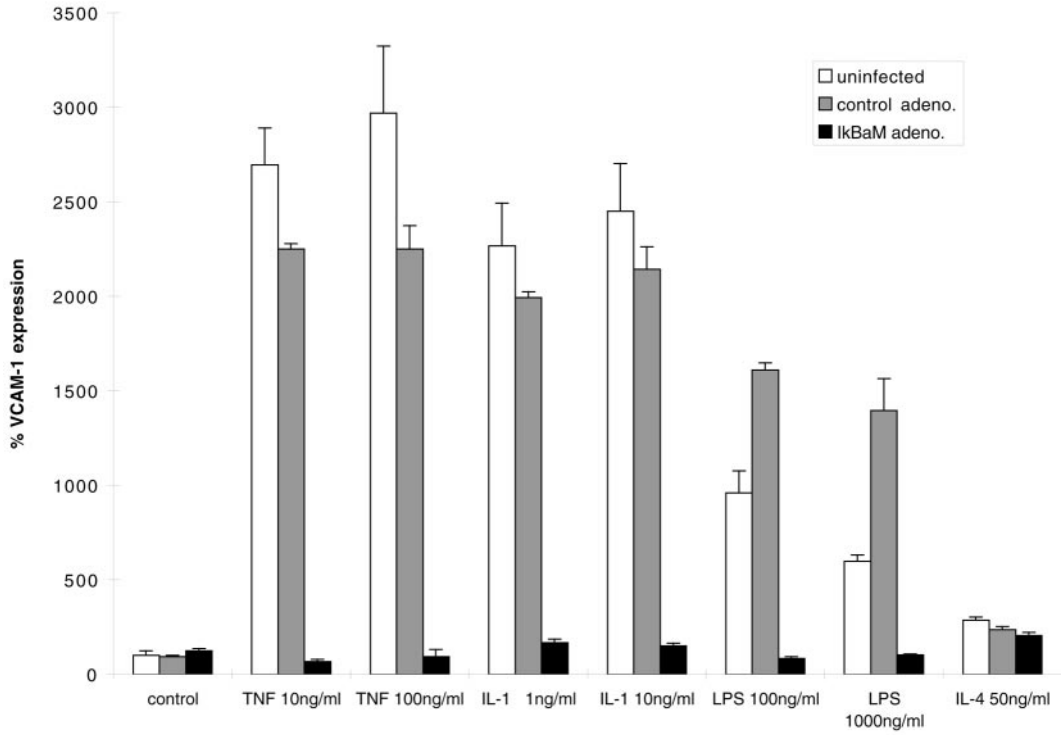

FIG. 4. **VCAM-1 protein expression is suppressed by overexpression of IκBαM.** HUVECs were infected with 1000 m.o.i. of Ad.RSV-βgal or Ad.PGK-IκBαM for 72 h. VCAM-1 protein expression was examined by enzyme-linked immunosorbent assay after 6 h incubation with the indicated concentrations of TNF-α, IL-1β, LPS, or IL-4. Percentage of VCAM-1 expression was expressed as the proportion of untreated cells. Results shown are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments.

expression of IκBαM regulated VCAM-1 protein expression, as TNF-α-induced VCAM-1 expression is known to be dependent upon NF-κB activation (3, 4). As shown in Fig. 4, the induction of VCAM-1 protein expression by TNF-α, IL-1β, or LPS was suppressed in IκBαM-expressing cells. Infection with Ad.PGK-IκBαM did not inhibit IL-4-induced VCAM-1 expression, consistent with the previous report that IL-4 induction of VCAM-1 expression is NF-κB-independent (49).

Inhibition of NF-κB Activation Sensitizes HUVECs to TNF-α- but Not IL-1β- or LPS-induced Apoptosis—Wang *et al.* (19) showed that TNF-α induced cell death in IκBαM-expressing HT1080 cells but not in control HT1080 cells. Therefore, we tested this cell line in our assay. X-gal stained almost all of the HT1080 cells at 500 m.o.i. of Ad.RSV-βgal at 48 h after infection of cells (data not shown). As reported (19), TNF-α induced cell death in IκBαM-expressing HT1080 cells infected by the recombinant adenovirus (Fig. 5*A*). As shown in Fig. 5*B,* TNF-α also induced apoptosis in IκBαM-expressing HUVECs, consistent with results of Bach *et al.* (20) in porcine aortic endothelial cells.

Notably, neither IL-1β nor LPS induced cell death in the IκBαM-expressing HUVECs. Although inhibition of VCAM-1 protein level was dependent upon the m.o.i. of recombinant adenovirus, cell viability with IL-1β and LPS treatment was unaffected at all m.o.i. levels tested (Fig. 6).

It has been reported that IL-1 activation may involve the PI 3-kinase and p38 MAP kinase signaling pathways (50–52). The PI-3/Akt kinase signaling cascade provides a survival signal that protects a variety of cells from apoptosis (53, 54). Recent studies also suggest that p38 kinase regulates cell survival (55). In the present study, neither the PI 3-kinase inhibitor LY294002 (20 μM) nor the p38 kinase inhibitor SB203580 (20 μM) provoked cell death in HUVECs exposed to IL-1β or LPS (data not shown).

DISCUSSION

Consistent with our previous studies (24), we found that IL-1β, LPS, or TNF-α induced HUVEC death in the presence of CHX. The caspase inhibitor, zVAD-fmk, inhibited cell death in this setting. We previously reported that overexpression of the cytoprotective Bcl-2 homologue A1 prevented death of human microvessel endothelial cells exposed to TNF-α + actinomycin D (56) or LPS + CHX (57). These results indicate that endothelial cell death induced by IL-1β, LPS, or TNF-α in the presence of CHX is due to apoptosis.

Although it is well established that signaling through the TNF receptor and Fas can induce apoptosis in normal or transformed cells under a number of circumstances, there are limited studies of apoptosis induced by LPS (39) or IL-1β (46, 47, 58). We recently showed that overexpression of a FADD-dominant-negative construct in a human microvascular endothelial cell line protected the cells from apoptosis following co-incubation with LPS and a protein synthesis inhibitor but that a neutralizing anti-Fas antibody did not (39). In other model systems, oxidized-LDL induced apoptosis was blocked by neutralizing mAb to FasL (45), and IL-1-induced apoptosis of Langerhans cells (46) and thyrocytes (47) was shown to involve Fas-FasL. However, blockade of the Fas-FasL apoptotic pathway by a neutralizing anti-FasL mAb did not prevent IL-1β- or LPS-induced apoptosis in HUVECs.

It is not clear how the FADD-dominant-negative inhibited the LPS-stimulated death pathway (39). As discussed previously, TLR4 is essential for LPS signaling *in vivo* (33–35). Human endothelial cells were shown to express TLR4 and TLR2, and LPS activated NF-κB in these cells through IL-1/Toll signaling mediators (37). FADD has been shown to associate with CD95 (Fas, Apo-1), TNF receptor-1 and DR3 (death receptor 3) and to transmit a death signal by activating caspase-8 (59). The death domain on FADD binds directly to

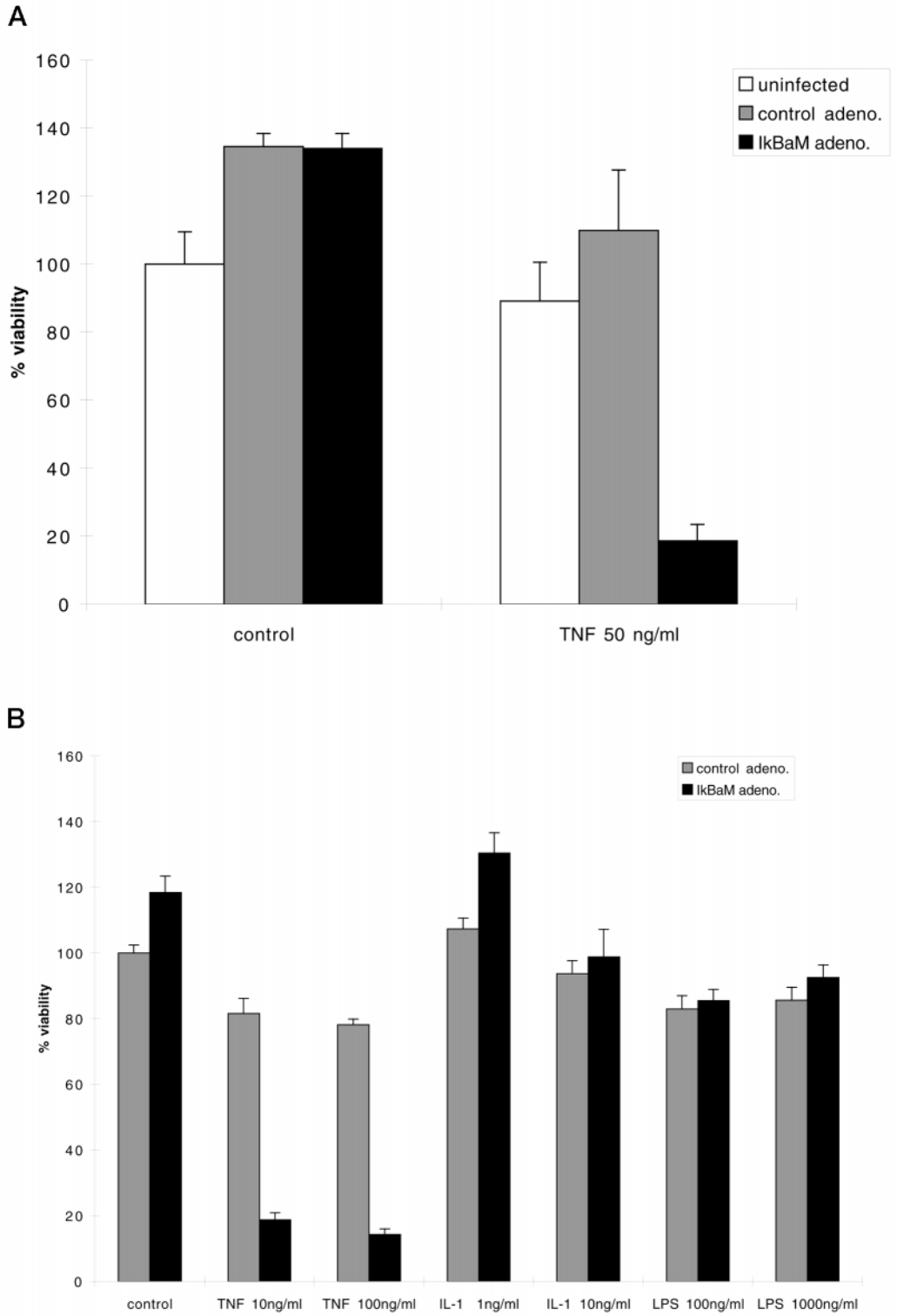

FIG. 5. **TNF-α, but not IL-1β or LPS, induces cell death in IκBαM-expressing HUVECs.** *A,* HT1080 cells were infected with 500 m.o.i. of Ad.RSV-βgal or Ad.PGK-IκBαM for 72 h and then exposed to TNF-α (50 ng/ml) for 24 h. Percentage of viability in MTT assay was expressed as a proportion of untreated cells. *B,* HUVECs were treated by 1000 m.o.i. of control adenovirus or IκBαM adenovirus for 72 h and then exposed to the indicated concentrations of TNF-α, IL-1β, or LPS for 24 h. Percentage of viability in the MTT assay was expressed as a proportion of control adenovirus infected cells. Results shown are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments for HT1080 cells and five separate experiments for HUVECs.

the death domain on Fas and uses the adapter molecule designated TNF receptor-associated death domain to associate with TNF receptor-1 and DR3. FADD has not previously been shown to associate with any of the Toll-like receptors. The intracellular portion of the TLR family of receptors does not have a death domain; thus, if FADD associates with TLR4 (or TLR2), it presumably will require an adapter molecule with a death domain. The TLR family of receptors possesses an intracellular Toll/IL-1R homology domain, and this is known to associate with the Toll/IL-1R homology domain on the C-terminal region of MyD88 (60). MyD88 also has an N-terminal death domain sequence (60), and it is a potential candidate for an adapter

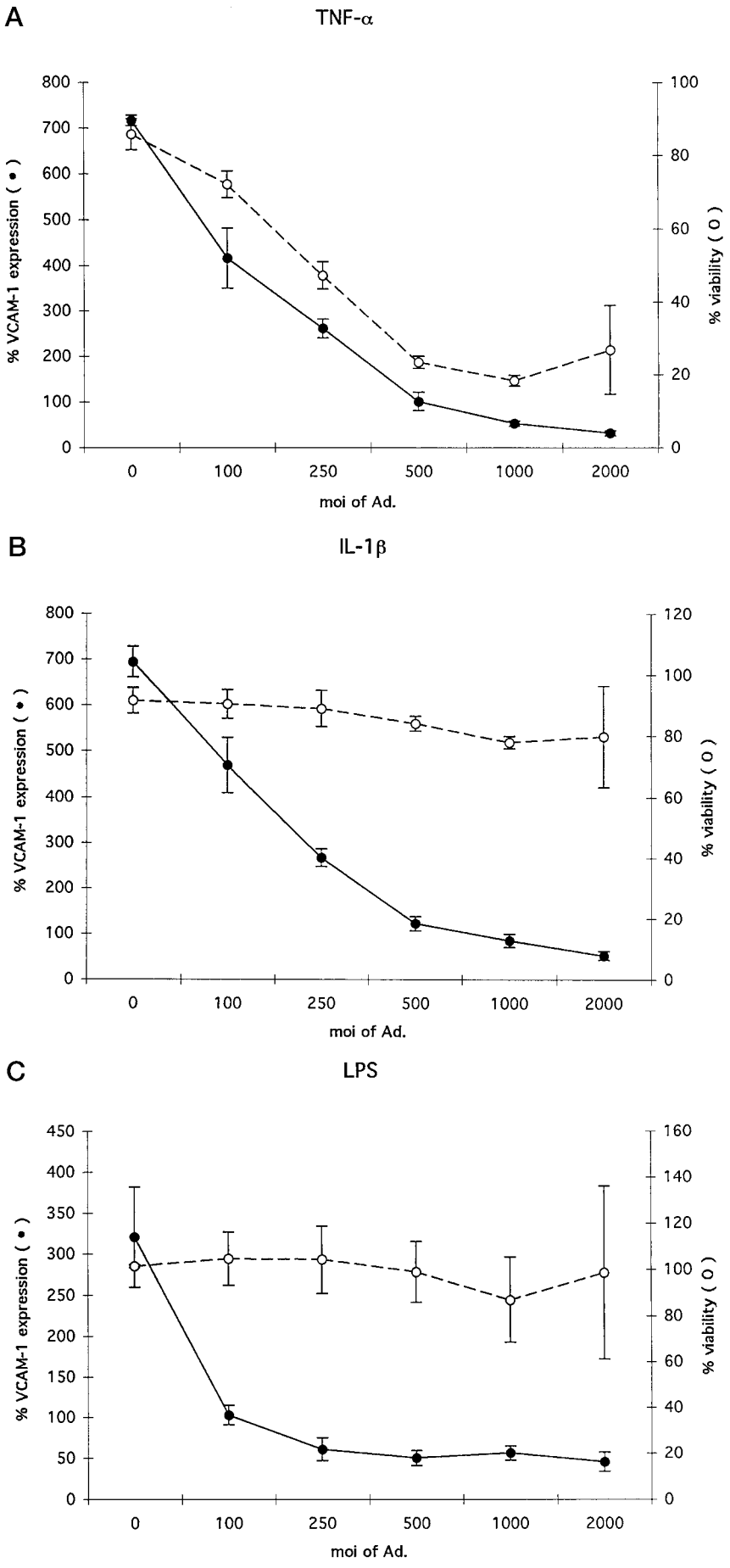

FIG. 6. **Effect of IκBαM adenovirus on VCAM-1 protein expression and cell viability following stimulation with TNF-α, IL-1β, or LPS.** HUVECs were treated with the indicated m.o.i. of recombinant adenovirus for 72 h and then exposed to 10 ng/ml of TNF-α (*A*), 10 ng/ml of IL-1β (*B*), or 1 μg/ml of LPS (*C*) for 6 h in the VCAM-1 enzyme-linked immunosorbent assay (*closed circles*) or for 24 h in the MTT viability assay (*open circles*). VCAM-1 expression and viability are expressed as percentage of untreated cells. Results shown are the means ± S.D. of triplicate wells in a single experiment and are representative of three separate experiments.

molecule linking FADD with the LPS receptor. Clearly, additional studies are required to define the death pathway stimulated by these Toll-family receptors.

Recently, cell survival following exposure to a variety of stimuli was shown to be dependent upon signaling pathways that regulate expression or function of cytoprotective genes. Inhibition of protein synthesis has been shown to sensitize a wide variety of cell types to TNF-α-mediated cytotoxicity. In a previous study, we showed that CHX sensitized HUVECs to IL-1β or LPS as well as to TNF-α and suggested that death induced by TNF-α, IL-1β, or LPS in the presence of CHX resulted from inhibition of synthesis of a cytoprotective protein(s) by CHX (24). Multiple reports have now demonstrated that NF-κB-dependent synthesis of cytoprotective proteins inhibits apoptosis induced by TNF-α or other agents (16–23). Therefore, we examined whether TNF-α, IL-1β, and LPS induced cell death in HUVECs when activation of NF-κB was blocked by overexpression of IκBαM transduced by adenoviral vector. The ability of overexpressed IκBαM to block TNF-α-, IL-1β-, or LPS-induced NF-κB activation in the HUVECs was shown by gel shift assay. Endogenous IκBα should be degraded following TNF-α-induced phosphorylation of IκBα with subsequent release of NF-κB. The lack of NF-κB-DNA binding in the gel shift assay in the IκBαM-overexpressing HUVECs may reflect rapid binding of free NF-κB released by degradation of endogenous IκBα to exogenous nonphosphorylated IκBαM protein, which is not subject to proteolytic degradation. Alternatively, Wrighton *et al.* (4) proposed that LPS failed to induce degradation of IκBα in IκBα-wild type-overexpressing porcine endothelial cells because phosphorylation induced by LPS was limiting. LPS-stimulated phosphorylation was sufficient for the phosphorylation of endogenous levels of IκBα but not for the much higher amounts of protein generated by the recombinant adenovirus (4).

In the MTT viability assay (Fig. 4), TNF-α induced cell death in both IκBαM-expressing HT1080 cells and HUVECs, consistent with the previous reports (19, 20). The NF-κB-dependent cytoprotective gene(s) involved in HUVECs have not been identified. Beg and Baltimore (17) demonstrated that transfection of A20 into Rel $A^{-/-}$ 3T3 cells was unable to prevent cell death, suggesting that other genes (or multiple genes) might be required to protect cells from TNF-α cytotoxicity. Chu *et al.* (61) showed that the human inhibitor of apoptosis protein 2 (IAP2) protected TNF-treated HeLa cells. Recently, we demonstrated that the Bcl-2 homologue A1 was induced by TNF-α and IL-1β (56) and that overexpressed human A1 protected a human microvessel endothelial cell line from TNF-α + actinomycin D-mediated cytotoxicity (62). In separate studies, we found that induction of A1 mRNA by LPS (57) was inhibited in IκBα-overexpressing HUVECs, demonstrating that it is NF-κB-dependent and, thus, A1 is also a candidate cytoprotective protein in this setting.

Of note, in contrast to TNF-α, neither LPS nor IL-1β induced cell death in IκBαM-expressing HUVECs, although both IL-1β and LPS induced caspase-mediated cell death in CHX-treated HUVECs. Even incubation with IκBαM adenovirus for up to 96 h failed to sensitize HUVECs to killing by IL-1β or LPS (data not shown). TNF-α and IL-1 signaling pathways, and perhaps also LPS signaling, are believed to converge at NIK, the kinase acting proximal to IκB kinase α and β (8). Nonetheless, although this pathway appears to be the major mechanism by which TNF-α activates cytoprotective mechanisms in HUVECs, IL-1β and LPS appear to utilize alternative or additional pathways, not requiring NF-κB activation. Blockade of PI 3-kinase or p38 kinase did not sensitize HUVECs to killing by IL-1 or LPS, suggesting that these potential antiapoptotic pathways are not involved in the survival signal. Further studies are necessary to characterize the signaling pathway(s) involved in cell survival with IL-1β and LPS treatment and to identify the constitutive or induced cytoprotective protein(s).

In summary, we studied the relationship between cell death and NF-κB activation in HUVECs. NF-κB activation was required for protection of HUVECs from TNF-α-induced apoptosis, whereas protection from IL-1β- and LPS-induced cell death was not dependent on NF-κB activation in HUVECs. We conclude that the cytoprotective mechanisms involved in endothelial cell survival are dependent upon the specific stimulus.

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