Mechanisms of Hypoxia-induced Endothelial Cell Death

ROLE OF p53 IN APOPTOSIS*

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Endothelial cell death may contribute to tissue injury from ischemia. Little is known, however, about the characteristics of endothelial cell death in response to hypoxia. Using an *in vitro* model, we found that human umbilical vein endothelial cells were resistant to hypoxia-induced cell death with only a 2% reduction in viability at 24 h and 45% reduction in viability at 48 h. Overexpression of a mutant, IκBα, via adenoviral vector did not potentiate cell death in hypoxia, indicating that nuclear factor-κB activation was not involved in cytoprotection. Cell death in hypoxia was determined to be apoptotic by 3′ labeling of DNA using terminal deoxynucleotidyl transferase staining and reversibility of cell death with a caspase inhibitor. Exposure of endothelial cells to hypoxia did not alter levels of proapoptotic and antiapoptotic Bcl-2 family members Bax and Bcl-X$_L$ by immunoblot analysis. In contrast, changes in p53 protein levels correlated with the induction of apoptosis in hypoxic endothelial cells. Inhibition of the proteasome increased p53 protein levels and accelerated cell death in hypoxia. Overexpression of p53 by adenoviral transduction was sufficient to initiate apoptosis of normoxic endothelial cells. These data provide a framework for the study of factors regulating endothelial cell survival and death in hypoxia.

Tissue injury from ischemia and reperfusion causes significant morbidity and mortality in cardiovascular disease. Endothelial cell (EC)[1] death may contribute to the hypoxic as well as the reperfusion components of this injury. The mechanisms of cell death in hypoxia are not known but may involve calcium influx, derangements in mitochondrial function, or purine nucleotide depletion (1, 2). Limited studies suggest a role for apoptosis induced by the tumor suppressor gene p53 in hypoxia-induced cell death. Graeber *et al.* (3) found that tumor cells containing wild-type p53 were more sensitive to hypoxia-induced apoptosis when compared with tumor cells lacking functional p53. Long *et al.* (4) showed a role for p53 in cardiomyocyte apoptosis in response to hypoxia. In contrast, Amellem *et al.* (5) demonstrated that hypoxia-induced apoptosis occurred independent of p53 protein level in MCF-7 cells. There are few data, however, on the relative susceptibility of EC to hypoxia-induced cell death or the molecular mechanisms involved. Because EC are invariably exposed to hypoxia in ischemic conditions, this question has important therapeutic implications for the prevention of ischemic tissue damage.

We used an *in vitro* model to examine mechanisms of EC death during hypoxia. We found that EC underwent significant cell death with features of apoptosis only after exposure to 48 h of hypoxia. Inhibition of nuclear factor-κB (NF-κB) activation by adenoviral-mediated overexpression of a dominant negative IκBα mutant did not potentiate apoptosis in hypoxic EC. There was no correlation between Bax/Bcl-X$_L$ ratios and cell death. However, there was an increase in p53 protein levels concomitant with EC death. In addition, overexpression of wild-type p53 protein in EC by adenoviral gene transduction was sufficient to cause apoptosis. We conclude that a major component of EC death in response to hypoxia is attributable to apoptosis. EC survival in hypoxia does not appear to depend on the activation of an NF-κB-dependent pathway(s). Apoptosis in hypoxia correlated with p53 protein levels but not with alterations in Bcl-X$_L$ and Bax proteins.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords with collagenase as described previously (6). Cells were cultured in RPMI 1640 medium supplemented with 20% bovine calf serum (Sigma), maintained at 37 °C in 5% CO$_2$, and used at passage 2–3. Hypoxia was induced by placement of cells in an anaerobic chamber (Plaslabs, Lansing, MI) filled with 5% CO$_2$, 85% N$_2$, and 10% H$_2$ and heated to 37 °C. O$_2$ concentration was maintained at <14 torr as measured by a Clark electrode (Yellow Springs Instrument, Yellow Springs, OH) by the catalytic conversion of O$_2$ and H$_2$ to H$_2$O by palladium crystals. Human dermal microvascular endothelial cells (HMEC-1) (7) were a gift of Dr. E Ades (Centers for Disease Control, Atlanta, GA) and Dr. T. Lawley (Emory University, Atlanta, GA) and were cultured in RPMI 1640 medium supplemented with 10% bovine calf serum and endothelial cell growth factor (25 μg/ml) prepared from bovine hypothalamus. Construction of HMEC-bclx and HMEC-neo has been previously described (8).

Recombinant human tumor necrosis factor-α (TNF-α) was purchased from R & D systems (Minneapolis, MN). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), calpain inhibitor I (ALLN) and calpain inhibitor II (*N*-acetyl-leu-leu-methioninal) were purchased from Sigma. Anti-p53, anti-Bcl-2, anti-Bcl-X$_L$, and anti-Bax antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Kamiya Bio Co. (Seattle, WA). An Apotag kit was purchased from Oncor (Gaithersburg, MD).

[1] The abbreviations used are: EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HMEC, human microvascular endothelial cell; ALLN, *N*-acetyl-leu-leu-norleucinal; zVAD-fmk, Z-Val-Ala-Asp-fluoromethylketone; Ad, adenovirus; m.o.i., multiplicity of infection; ELISA, enzyme-linked immunosorbent assay.

Construction of Recombinant Adenovirus

Construction of the p53 adenovirus carrying the human wild-type p53 gene under the control of the cytomegalovirus promotor has been described previously (9). IκBα mutant (serine to alanine at 32 and 36 residues) cDNA was kindly provided by Drs. M. Karin and J. DiDonato (University of California, San Diego, CA) (10). This cDNA was inserted into the vector pXCJL1 under the transcriptional control of the phosphoglycerokinase promoter, upstream of the bovine growth hormone polyadenylation signal. The E1A-deficient recombinant adenovirus was generated similar to the previously described generation of recombinant control adenovirus Ad-Rous sarcoma virus-β-galactosidase (11). Briefly, E1A-deficient adenovirus was recombined with the pXCJL.1-IκBα mutant plasmid and pJM17 in 293 cells. Purification of a large batch of the recombinant adenovirus was done by two consecutive cesium chloride centrifugations with storage at −80 °C in 10% glycerol, 10 mM Tris-HCl, pH 7.4, and 1 mM MgCl$_2$.

Infection of HUVEC

Subconfluent HUVEC were washed once with warmed complete medium and incubated at a multiplicity of infection (m.o.i.) indicated with control adenovirus (AdLacZ), p53 adenovirus (Adp53), or mutant IκBα adenovirus (AdIkBm) in complete medium.

Immunoblot Analysis

After experimental treatment of HUVEC in 100-mm plates, cell monolayers were detached from plastic culture dishes with a cell scraper, washed in cold phosphate-buffered saline, and incubated in 50 μl of lysis buffer (0.5% Nonidet P-40 with 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 30 min at 4 °C. The protein was collected by microcentrifugation at 12,000 rpm for 15 min. Cytosolic extracts from treated HUVEC were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, and 5% methanol at 100 V for 1 h at 4 °C. Filters were blocked overnight with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 containing 5% skim milk. Immunoblots were analyzed for p53 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-goat secondary antiserum. Blots were exposed to film for 1–10 min. Immunoblotting for Bcl-2, Bcl-X$_{\text{L}}$, and Bax was identical except for the use of an anti-hamster second antibody for Bcl-2 and anti-rabbit second antibody for the others.

Detection of Apoptosis

DNA Ladder—Endothelial cells were removed from tissue culture plates by a 2-min incubation with 0.05% trypsin and 0.02% EDTA in Hanks' balanced salt solution and pelleted and resuspended in a lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, and 0.5% SDS followed by incubation with 100 μg/ml proteinase K for 4 h at 37 °C. DNA was extracted using phenol and chloroform followed by ethanol precipitation. The pellet was resuspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA) and treated with DNase-free RNase for 3 h at 37 °C. The DNA was ethanol precipitated and resuspended in distilled water. The concentration of DNA was measured by spectrophotometer, and 4 μg was fractionated by electrophoresis on a 1.5% agarose gel containing ethidium bromide. We evaluated the electrophoretic patterns of DNA extracted from cells subjected to hypoxia as well as normoxia. HUVEC were exposed to hypoxia for 24, 36, or 48 h, whereas hepatoma cells were exposed for 4, 12, or 24 h.

Terminal Deoxynucleotidyl Transferase-mediated Biotinylated UTP Nick End Labeling Staining—The 3′ ends of DNA were labeled according to the manufacturer's recommendation. Briefly, endothelial cells were removed from tissue culture as described above with 0.05% trypsin and 0.02% EDTA in Hanks' balanced salt solution, isolated by centrifugation, resuspended in 1% paraformaldehyde in phosphate-buffered saline, and fixed on ice for 15 min. Cells were washed twice, resuspended in 70% ice-cold ethanol, and held at −20 °C until ready for use. The 3′ ends of DNA had digoxigenin nucleotides catalytically coupled to them using terminal deoxynucleotidyl transferase. Digoxigenin was detected with fluorescein isothiocyanate-labeled anti-digoxigenin antibody, and total DNA was determined by staining with propidium iodide. Double staining allowed simultaneous display of viable and apoptotic cells. Cells that stained positive for both fluorescein isothiocyanate and propidium iodide were considered to be apoptotic and those that stained only with propidium iodide were considered normal. The percent apoptotic cells was determined as the ratio of cells
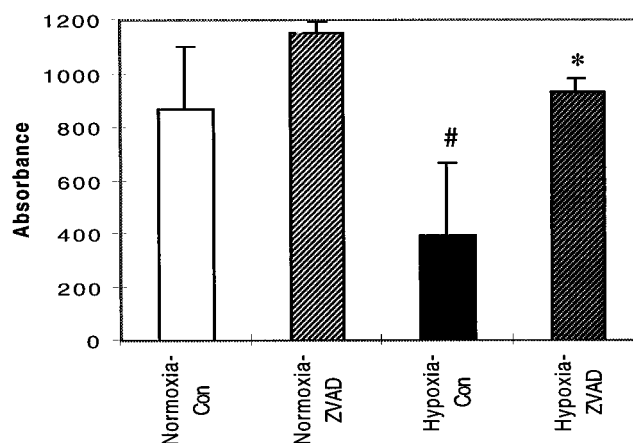

FIG. 1. **HUVEC death in hypoxia is mediated by caspases.** MTT cell viability assay was performed after 48 h of exposure of HUVEC to hypoxia with or without pretreatment with the caspase inhibitor fmk-zVAD (100 μM). Values represent the mean ± S.E. of five experiments. ∗, $p < 0.05$ *versus* hypoxic control (*Con*); #, $p < 0.05$ *versus* normoxic control (*Con*).

positive for fluorescein isothiocyanate relative to those positive for propidium iodide. Specimens were analyzed on an Epic XL cytometer (Coulter, Fullerton, CA). At least 20,000 events were evaluated.

MTT Assay—The MTT assay of cell viability was performed as described previously (8). Briefly, after treatment, medium containing 1 mg/ml MTT was added to cells for a final concentration of 0.5 mg/ml and incubated at 37 °C for 5 h. The medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbance at 630 nm (background absorbance) was subtracted from absorbance at 570 nm for each well.

Nucleosome Enzyme-linked Immunosorbent Assay (ELISA)—Nucleosome ELISA (Calbiochem, Cambridge, MA) was performed according to the manufacturer's instructions. Briefly, cells were treated with adenoviral constructs and then harvested and lysed at appropriate time points. After freezing lysates at −20 °C for at least 18 h, specimens were thawed and pipetted into wells of a pretreated ELISA plate. The plate was then incubated with detector antibody followed by streptavidin conjugate. After washing, substrate solution was added, and plates were incubated in the dark for 30 min. The reaction was stopped with stop solution, and absorbance was measured on a plate reader at dual wavelengths of 450 and 595 nm.

Northern Analysis

HUVEC were subjected to normoxia or hypoxia, and RNA was extracted at 2, 8, 18, 24, and 36 h with Trizol (Life Technologies) per the manufacturer. Total cellular RNA was separated on agarose-fromaldehyde gels, blotted onto nitrocellulose filters, and hybridized with ^{32}P-labeled probes for A1 (8) or A20 (gift of V. Dixit, Genentech, South San Francisco, CA).

Statistical Analysis

Analysis for statistical significance was performed on Excel using Student's paired *t* test.

RESULTS

HUVEC were found to be relatively resistant to hypoxia-induced cell death. The MTT assay revealed 98% viability after 24 h and 55% viability after 48 h of continuous exposure to <14 torr of oxygen (Fig. 1). HUVEC death was evaluated by several techniques to determine whether this death was apoptotic in nature. Significant DNA laddering was present after 48 h of exposure of HUVEC to hypoxia but was not seen in control HUVEC at the same time point (Fig. 2). Approximately 35% of cells were apoptotic when evaluated by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining at 48 h (Fig. 3). In contrast, RH7777 hepatoma cells, previously shown to be sensitive to hypoxia-induced apoptosis (12), underwent significant apoptosis after only 12 h of exposure to hypoxia (Fig. 3).
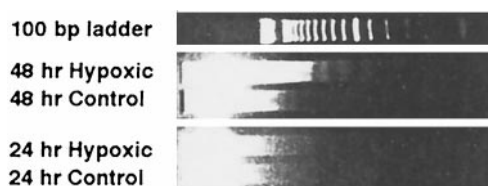
FIG. 2. **HUVEC death in hypoxia is associated with DNA laddering.** HUVEC exposed to hypoxia or normoxia for 24 and 48 h were lysed, and DNA was extracted. DNA was fractionated by electrophoresis and stained by ethidium bromide.
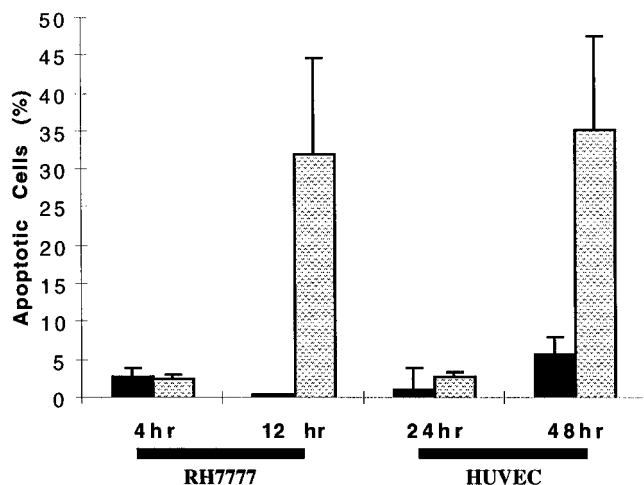

FIG. 3. **HUVEC are more resistant to hypoxia-induced apoptosis than hepatoma cells.** Annexin V binding to HUVEC and rat hepatoma cells (RH7777) was performed at various time points after exposure to hypoxia. Approximately 35% of HUVEC were apoptotic at 48 h. Values represent the means ± S.E. of three experiments.

FIG. 4. **Bax and Bcl-X$_L$ protein levels and ratios do not change during hypoxia.** Immunoblot analysis for Bax and Bcl-X$_L$ protein was performed at 6, 12, and 24 h after exposure to hypoxia.

FIG. 5. **p53 protein levels increase with hypoxia and are potentiated by a proteasome inhibitor.** Immunoblot analysis of p53 protein was performed at 2, 6, 12, and 24 h after exposure to hypoxia in the absence or presence of the proteasome inhibitor ALLN (100 μM). Three separate experiments yielded identical results.

The caspase family of proteases is thought to be the final execution pathway in apoptosis (13). To establish the role of the caspase pathway in hypoxia-induced apoptosis of EC, we treated HUVEC with the caspase inhibitor zVAD-fmk (14). Fig. 1 shows that pretreatment of HUVEC with zVAD-fmk largely prevented cell death seen after 48 h of hypoxia, further establishing that hypoxia-induced death of EC is apoptotic.

The experiments represented by Figs. 1–3 established the time course of EC death in hypoxia and confirmed that it was attributable primarily to apoptosis. Next, we examined the role of two Bcl-2 family members in the response of EC to hypoxia. Based on studies in other cell types demonstrating changes in Bcl-2/Bax ratios with apoptosis (15), we performed immunoblot analysis of Bcl-X$_L$ and Bax protein levels in response to hypoxia. These proteins were chosen because they have been shown to be the predominant cytoprotective and proapoptotic Bcl-2 family proteins in HUVEC (16), There was no significant change in their ratios after 24 h of hypoxia in three successive experiments (Fig. 4). Northern blot analysis for the inducible EC cytoprotective molecules A1 and A20 also showed no change in mRNA levels after exposure to hypoxia (data not shown). As we have previously shown (16), Bcl-2 protein was barely detectable in HUVEC and showed no increase by immunoblot analysis in response to hypoxia (data not shown).

Having excluded that hypoxia-induced changes in the protein levels of the predominant Bcl-2 family members expressed in HUVEC accounted for initiation of apoptosis, we next determined whether p53 protein levels were altered before cell death. Fig. 5 shows an immunoblot analysis of lysates of HUVEC exposed to normoxia or progressively longer periods of hypoxia. Minimal levels of p53 protein were present in normoxia or early in hypoxia. However, there was a significant increase in p53 protein level by 24 h of hypoxia.
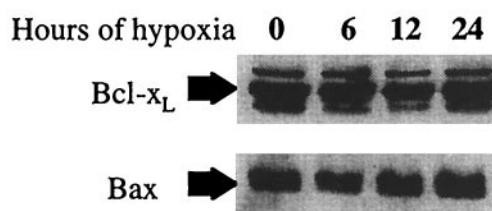
The proteasome, a multicatalytic protein involved in intracellular protein turnover (17), is known to contribute to the normally short half-life (20 min) of p53 protein (18). The addition of the proteasome inhibitor ALLN, which inhibits p53 degradation (19) caused an accelerated accumulation of p53 protein in hypoxic HUVEC (Fig. 5). Concomitant with its ability to cause an early increase in p53 protein levels, treatment of hypoxic HUVEC with ALLN resulted in a potentiation of hypoxia-induced apoptosis (Fig. 6). There was complete loss of viability of nearly all hypoxic ALLN-treated HUVEC at 24 h. In contrast, treatment of normoxic HUVEC with ALLN for 24 h did not cause significant toxicity (Fig. 6). This effect was not attributable to the inhibitory effects of ALLN on calpain, because the related calpain inhibitor II, a weak inhibitor of the proteasome (20, 21), had no effect on HUVEC viability in hypoxia.

Further experiments were performed to assess whether the effect of ALLN was attributable to potentiation of an apoptotic pathway *versus* a nonspecific toxic effect. HMEC-1 overexpressing the cytoprotective molecule Bcl-X$_L$ (HMEC-bclx) were exposed to ALLN in hypoxic conditions. HMEC-1 expressing Bcl-X$_L$ were completely protected from apoptosis after a 24-h exposure to both hypoxia and ALLN (Fig. 7).

To determine whether p53 protein alone was sufficient to initiate apoptosis in HUVEC, we used a strategy of adenovirus infection to transduce the p53 gene in HUVEC. Studies with an adenovirus encoding β-galactosidase (AdLacZ) showed near 100% infection efficiency after 48 h of exposure to 500 m.o.i. of AdLacZ (data not shown). These data are comparable to the m.o.i. used by others for adenoviral mediated transduction of EC (22). Immunoblot analysis of normoxic HUVEC infected with 500 m.o.i. of an adenovirus encoding p53 (Adp53) showed increased levels of p53 protein 24 h after infection compared with uninfected cells and cells infected with AdLacZ (Fig. 8). There was a marked reduction in HUVEC viability in normoxia as early as 48 h after infection with Adp53 and near complete loss of viability 72 h after infection (Fig. 9*A*). Viability of AdLacZ-infected normoxic cells was similar to control, uninfected HUVEC. Phase contrast microscopy showed a normal cobblestone appearance of AdLacZ-infected cells compared with detachment, membrane blebbing, and cellular fragmentation of HUVEC 72 h after infection with 500 m.o.i. of Adp53. Experiments performed with Adp53- and AdLacZ-infected cells in hypoxic conditions showed complete loss of viability of Adp53-
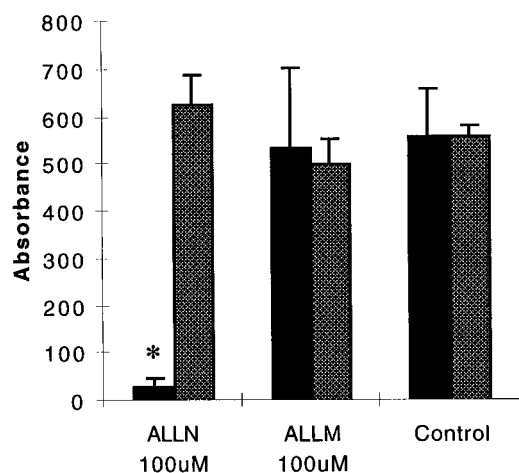
FIG. 6. **Proteasome inhibitor potentiates HUVEC death in hypoxia.** HUVEC were treated with ALLN (100 μM) or calpain inhibitor II (*ALLM*, 100 μM) and then exposed to hypoxia. Viability was determined by MTT assay after 24 h. Values represent the mean $\pm$ S.E. of three experiments. *, $p < 0.005$ *versus* control.
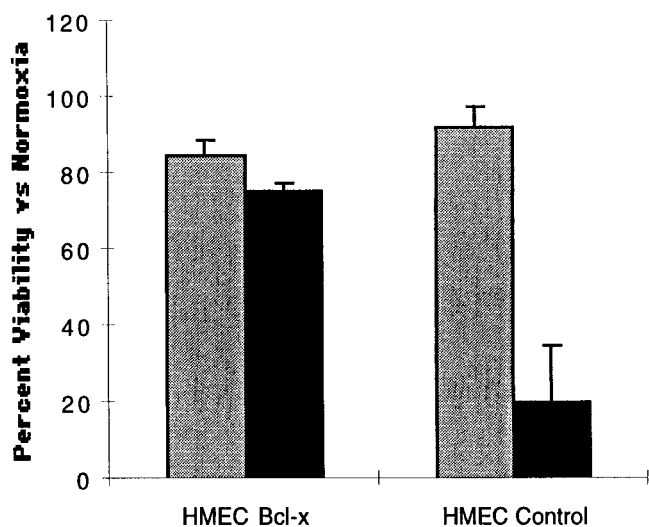

FIG. 7. **Overexpression of Bcl-X$_L$ protects EC from death induced by hypoxia.** HMEC-1 cells transduced with the cytoprotective molecule Bcl-X$_L$ or neomycin control were maintained in the presence or absence of the proteasome inhibitor ALLN (100 μM). Viability was assessed by MTT assay after 24 h of hypoxia. Values represent the means $\pm$ S.E. of three experiments. *, $p < 0.002$ *versus* HMEC Bcl-x.
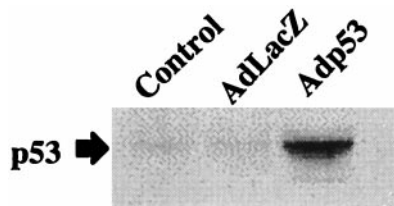

FIG. 8. **Infection with Adp53 results in high levels of p53 protein expression.** HUVEC were untreated or infected with AdLacZ or Adp53 at 500 m.o.i. for 24 h. Cell lysates were prepared and subjected to immunoblot analysis for p53 protein.

infected cells within 24 h of exposure to hypoxia (data not shown). Measurement of free nucleosomes was performed under identical conditions to evaluate whether this death was predominantly attributable to apoptosis (Fig. 9*B*). Confirmation that Adp53-induced death was dependent in part on caspase activation was established through the use of the caspase inhibitor ZVAD-fmk. Pretreatment of HUVEC with ZVAD-fmk partially reversed the loss of viability seen with

Adp53 treatment (Fig. 10).

In addition to its ability to block degradation of p53, inhibition of the proteasome by ALLN has been shown to inhibit activation of NF-κB (21). To determine whether potentiation of hypoxia-induced apoptosis of EC by ALLN was in part caused by inhibition of NF-κB, an adenoviral construct encoding a mutant form of IκBα (IkBm) was used. Serine residues 33 and 36 are converted to alanine in the mutant protein, preventing phosphorylation. The overexpressed mutant protein cannot be degraded by the proteasome but remains bound to NF-κB in the cytoplasm, inhibiting its translocation to the nucleus (10). To confirm that this strategy was effective in inhibiting NF-κB-mediated transcription, assessment of vascular cell adhesion molecule-1 (VCAM-1) expression in response to TNF-α stimulation was measured by ELISA. As previously shown in our lab,[2] control HUVEC and HUVEC infected with AdLacZ exhibited appropriate increases in VCAM expression after 18-h treatment with TNF-α (10 ng/ml), whereas HUVEC infected with AdIκBm for 24 h showed complete inhibition of VCAM-1 protein expression in response to TNF-α (Fig. 11). To ensure maximal inhibition of NF-κB activity, HUVEC were exposed to AdIκBm or AdLacZ at 1000 m.o.i. for 72 h before exposure to hypoxia. Although lower levels of AdIκBm were sufficient to inhibit TNF-induced VCAM expression, longer exposures at higher m.o.i. were necessary to completely abrogate the cytoprotective effect of NF-κB under TNF stimulation. Overexpression of the mutant IκB did not change the extent of EC apoptosis in response to hypoxia at 24 h (Fig. 12). Longer treatments with either AdLacZ or AdIκBm resulted in equivalent cytotoxicity compared with uninfected cells. As reported by others, addition of TNF-α to both control and hypoxic EC treated with an inhibitor of NF-κB translocation resulted in significant loss of viability of these HUVEC compared with AdLacZ-infected or noninfected cells at 24 h (22). Therefore, although NF-κB activation is necessary for survival in response to TNF-α, it does not appear to play a role in survival during hypoxia.

DISCUSSION

Tissue injury occurs both during hypoxia and after reoxygenation. In some instances the degree of tissue damage is greater after brief hypoxia followed by reperfusion than with more prolonged hypoxia alone. We sought to elucidate the time course and mechanisms of EC death during hypoxia in an effort to understand tissue resistance to hypoxic injury. We found that EC were resistant to hypoxia, requiring a prolonged period of exposure to induce cell death. A major component of hypoxia-induced death was attributable to apoptosis, as shown by DNA laddering, increased terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining, and inhibition of cell death by a caspase antagonist. These results are consistent with those found in several other cell lines. However, EC appear to be able to survive a more prolonged period of hypoxia without significant loss of viability when compared with other primary cell lines such as cardiomyocytes, neurons, and renal tubular cells (4, 23–25).

One explanation of EC resistance to hypoxia would be an increased expression of cytoprotective members of the Bcl-2 family in response to exposure. Alternatively, a decrease in death-promoting members of the Bcl-2 family could have a similar beneficial effect on cell survival (26). However, we found no change in the protein levels of either Bcl-X$_L$ or Bax during hypoxia. Thus, changes in expression of these two molecules are unlikely to explain early cytoprotection. Although it

A

B

FIG. 9. *A*, overexpression of p53 is sufficient to cause EC death in the absence of hypoxia. HUVEC were untreated or infected with AdLacZ or Adp53 at 500 m.o.i.. They were assessed for viability by MTT assay at 24, 48, and 72 h after infection. Compared with uninfected control cells, there was a significant decrease in viability of Adp53-infected cells at 48 and 72 h. Values represent means ± S.E. of three experiments. *, $p < 0.05$ *versus* AdLacZ. *B*, Adp53 infection results in increase in free nucleosome levels . HUVEC were infected with AdLacZ, Adp53, or control serum. Release of nucleosomes was measured by ELISA at 24, 48, and 72 h. This figure is representative of two separate experiments.
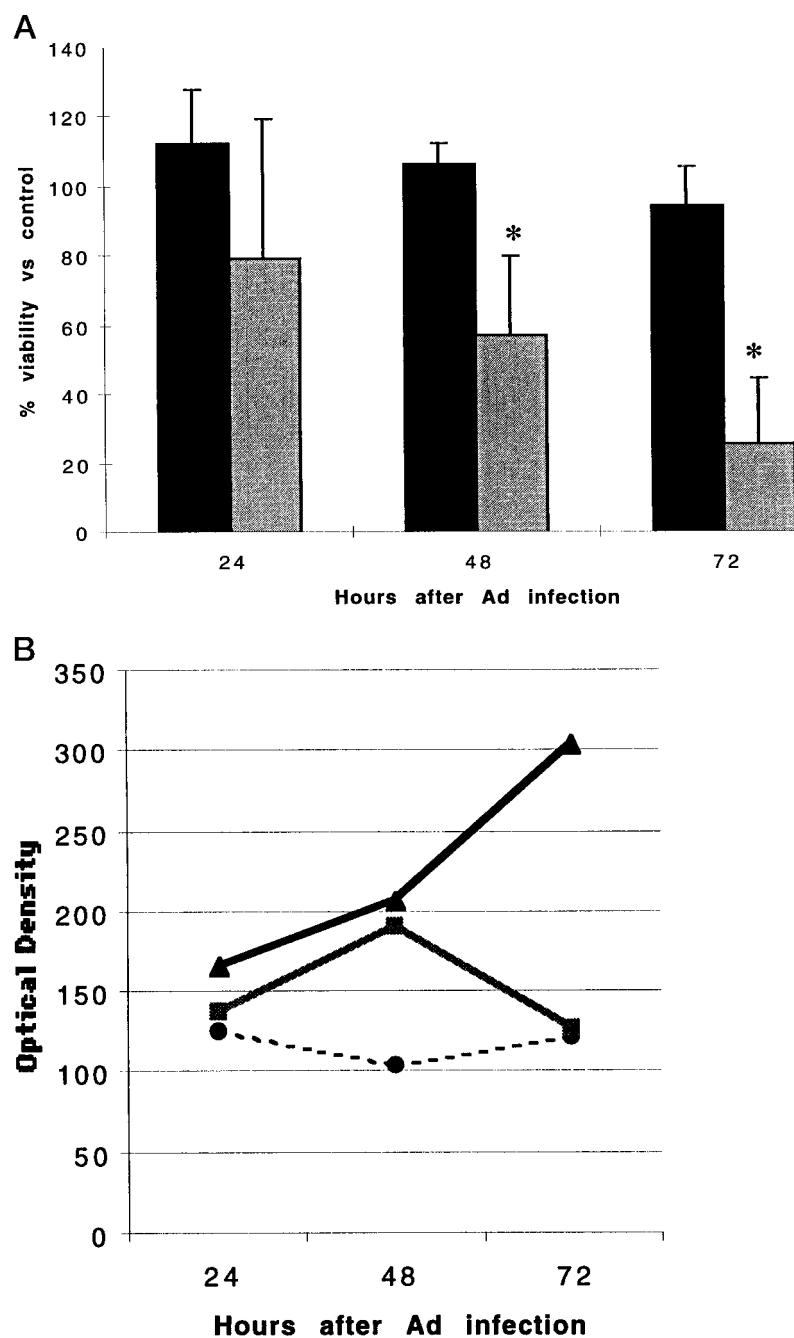
is possible that other members of this family may be modulated by hypoxia, Bcl-X_L and Bax appear to be the dominant members of the Bcl-2 family in HUVEC (16). Alternatively, phosphorylation or translocation of the Bcl-2 family member to the mitochondrial membrane may play a role in cytoprotective or proapoptotic effects independent of changes in protein level (27, 28). Further experiments to inhibit synthesis or function of these members will be necessary to define more completely their role in hypoxia-induced apoptosis of EC.

The tumor suppressor gene p53 is best known for its role in cell cycle arrest and apoptosis in response to direct genomic stress such as ionizing radiation and chemotherapeutic agents (29). Recently, it has been implicated in the death program of cells in response to other physiological stimuli, including hypoxia and oxidative stress (4, 30). Also, tumor cells lacking a functional p53 gene have been observed to have a prolonged survival in hypoxia (3), and p53 is stabilized by induction of

hypoxia-inducible factor 1α (31). However, in some cell lines p53 protein does not appear to play a role in hypoxia-induced apoptosis (32). In addition, elevated p53 protein expression may also be a secondary effect of other apoptotic pathways. Vaziri *et al.* (33) showed that p53 protein levels increased as a result of byproducts of the apoptotic process such as PARP cleavage fragments.

We found that endogenous p53 protein levels correlated with the initiation of cell death in hypoxic HUVEC and that elevation of endogenous p53 protein levels via inhibition of the proteasome potentiated apoptosis in hypoxic HUVEC. We also showed that an EC line transduced with Bcl-X_L was resistant to apoptosis at 24 h after exposure to ALLN and hypoxia. This finding is consistent with results in other cells showing that p53-induced death can be inhibited by antiapoptotic members of the Bcl-2 family (34–36). In addition, overexpression of p53 protein via an adenoviral vector was sufficient to induce apo-

FIG. 10. **The caspase inhibitor zVAD attenuates Adp53-induced apoptosis.** Cells were treated with fmk-zVAD (100 μM) at the time of adenoviral infection. Viability was assessed by MTT assay after 48 and 72 h of Adp53 infection (*Ad Rx*) at 500 m.o.i. Values represent the means $\pm$ S.E. of three experiments. *, $p < 0.05$ *versus* untreated Adp53.
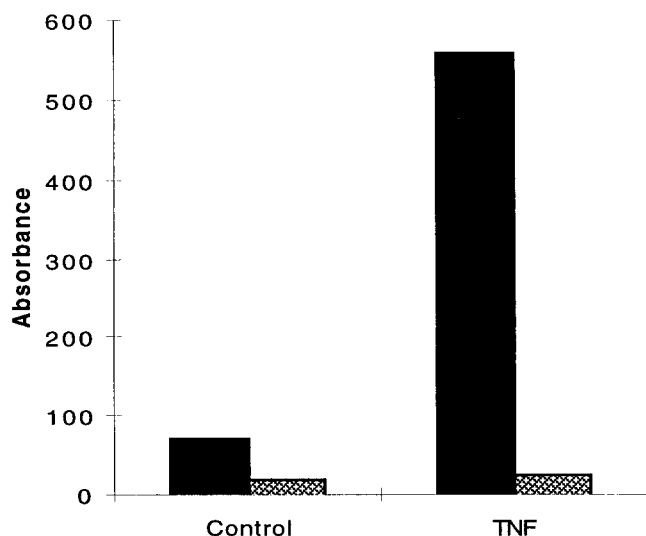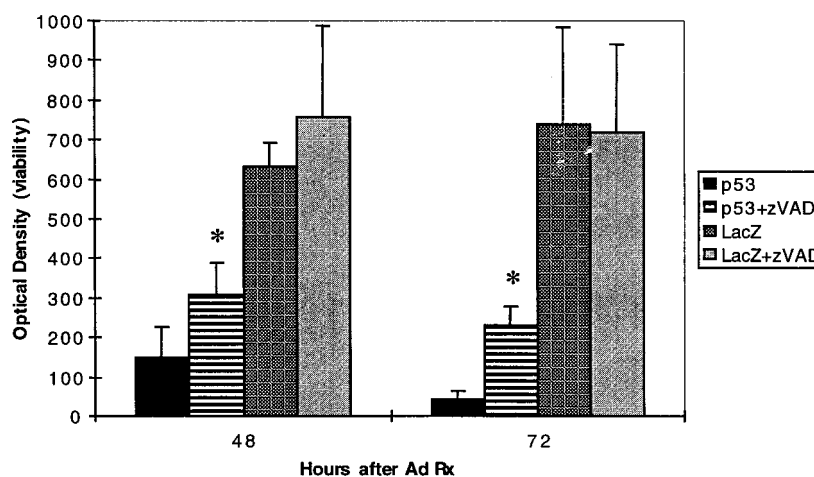

FIG. 11. **Inhibition of NF-κB abrogates VCAM-1 induction by TNF-α.** HUVEC were untreated (*Control*) or infected with 1000 m.o.i. AdLacz or AdIκBm for 24 h then treated with 10 ng/ml TNF-α for 18 h. VCAM-1 expression was then assessed by ELISA. This is representative of three separate experiments.
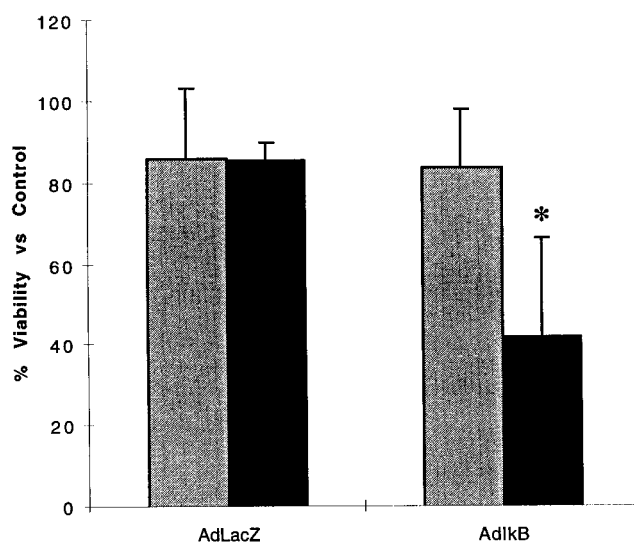

FIG. 12. **Inhibition of NF-κB potentiates cell death in response to TNF-α but not hypoxia.** HUVEC were untreated (*Control*) or infected with 1000 m.o.i. AdLacz or AdIκBm for 72 h and then subjected to hypoxia alone or hypoxia and TNF-α (5 ng/ml). After 24 h viability was assessed by MTT assay. Values represent the means $\pm$ S.E. of six experiments. *, $p = 0.002$ *versus* AdLacZ cells.

ptosis in normoxic HUVEC. These data are similar to those seen in the terminally differentiated cells, cardiomyocytes, and primary neurons (4, 9). Although the increase in p53 protein before cell death suggests a causal role, definitive experiments in which endogenous p53 function is inhibited are necessary to establish firmly that it mediates hypoxia-induced apoptosis of EC. In addition, Adp53 death was only partially reduced by the addition of the caspase inhibitor fmk-zVAD. The lack of complete protection may have been attributable to excessive activation of the death program in the absence of a stimulus (*e.g.* hypoxia) that up-regulates cytoprotective pathways. This hypothesis is supported by the finding that inhibition of transcription with actinomycin D treatment before the hypoxic insult markedly potentiates EC death (data not shown). This phenomenon is similar to the response of actinomycin D-treated EC to TNF-α, interleukin-1, or lipopolysaccharide. In these cases actinomycin D inhibits the synthesis of cytoprotective proteins, thereby provoking apoptotic cell death (8, 37, 38). Alternatively, recent data suggest that some death pathways in fact may be potentiated by caspase inhibition (39).

Studies have shown that hypoxia induces NF-κB activation in HUVEC (40). Activation of NF-κB is necessary for survival after exposure to TNF-α in multiple cell types, including HUVEC (41, 42). In addition, NF-κB activation protects

against death induced by ionizing radiation and daunorubicin in tumor cell lines and inhibits antibody-mediated apoptosis of murine B cells (43, 44). However, activation of the transcription factor NF-κB does not appear to be necessary for EC survival during hypoxic stress. Although the potentiation of hypoxia-induced apoptosis by the proteasome inhibitor may potentially have been secondary to its ability to inhibit translocation of NF-κB to the nucleus, inhibition of NF-κB translocation by an adenovirus encoding a mutant IκBα super-repressor did not potentiate HUVEC apoptosis in hypoxia. Consistent with other studies, inhibition of NF-κB did potentiate apoptosis of both normoxic and hypoxic EC treated with TNF-α (22).

These findings are of potential clinical relevance. Activation of NF-κB in hypoxia has been implicated in the induction of chemokines and adhesion molecules (45–47). These products are known to potentiate the inflammatory response after reperfusion. Activation of endothelium may be responsible for clinical events such as reperfusion injury and graft failure (48). Our *in vitro* results suggest that therapies targeted at inhibition of NF-κB in response to hypoxia and reoxygenation should not provoke or potentiate EC apoptosis. Conversely, antagonism of p53 may potentially reduce EC damage during ischemia.

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