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Hypoxia Potentiates Nitric Oxide-mediated Apoptosis in Endothelial Cells via Peroxynitrite-induced Activation of Mitochondria-dependent and -independent Pathways*

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Nitric oxide ($NO^{\cdot}$) at low concentrations is cytoprotective for endothelial cells; however, elevated concentrations of $NO^{\cdot}$ (≥1 μmol/liter), as may be achieved during inflammatory states, can induce apoptosis and cell death. Hypoxia is associated with tissue inflammation and ischemia and, therefore, may modulate the effects of $NO^{\cdot}$ on endothelial function. To examine the influence of hypoxia on $NO^{\cdot}$-mediated apoptosis, we exposed bovine aortic endothelial cells (BAEC) to (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate (diethylenetriamine NONOate, DETA-NO) (1 mmol/liter) under normoxic or hypoxic conditions (pO_2 = 35 mm of Hg) and measured the indices of apoptotic cell death. BAEC treated with DETA-NO under normoxic conditions demonstrated increased levels of histone-associated DNA fragments, which was confirmed by terminal dUTP nick-end labeling assay, and hypoxic conditions augmented this response. To determine whether mitochondrial dysfunction was one mechanism by which $NO^{\cdot}$ initiated apoptosis under hypoxic conditions, we evaluated mitochondrial membrane potential in (ψ_m). Exposure to DETA-NO resulted in a decrease in ψ_m and concomitant release of cytochrome *c* and caspase-9 activation, which were enhanced by hypoxia. By utilizing Rho_0 BAEC (Rho_0-EC), which lack functional mitochondria, we demonstrated that dissipation of ψ_m was associated with increased reactive oxygen species generation and peroxynitrite formation. Moreover, in Rho_0-EC we identified activation of caspase-8 as part of the mitochondrial-independent pathway of apoptosis. To establish that peroxynitrite mediated mitochondrial damage and apoptosis, we treated BAEC and Rho_0-EC with the peroxynitrite scavenger uric acid and found that the indices of apoptosis were decreased significantly. These findings confirm that high flux of $NO^{\cdot}$ under hypoxic conditions promotes cell death via mitochondrial damage and mitochondrial-independent mechanisms by peroxynitrite.

Nitric oxide ($NO^{\cdot}$), an endogenous signaling molecule that modulates vessel wall function, has both cytoprotective and cytotoxic effects on the vascular endothelium (1). At nanomolar concentrations, as are present under basal conditions, $NO^{\cdot}$ is protective against apoptosis-inducing stimuli such as tumor necrosis factor-α, serum starvation, hydrogen peroxide, and hypoxia (1), and endothelial cells isolated from mice lacking the endothelial isoform of nitric-oxide synthase demonstrate increased sensitivity to apoptotic stimuli (2). In contrast, $NO^{\cdot}$ when present at micromolar concentrations, which may be achieved during inflammation, initiates apoptosis in both endothelial and non-endothelial cells (1, 3, 4). The importance of $NO^{\cdot}$-mediated apoptosis has been recognized clinically as contributing to endothelial dysfunction, atherosclerosis, and transplant vascular disease (5–8).

The mechanism(s) by which $NO^{\cdot}$ modulates apoptosis highlights the central role of the mitochondrion in $NO^{\cdot}$-mediated cell death. Prolonged exposure to elevated levels of $NO^{\cdot}$ suppresses mitochondrial respiration by inhibition of cytochrome oxidase (complex IV), resulting in a decrease in inner mitochondrial membrane potential (ψ_m) and induction of mitochondrial permeability transition to effect release of cytochrome *c* into the cell cytosol. These events activate caspase-9, which in turn activates downstream executioner caspases to commence intracellular proteolysis, internucleosomal DNA fragmentation, and eventual cell death (9–11).

Nitric oxide-induced uncoupling of mitochondrial respiration additionally facilitates apoptosis by increasing mitochondrial reactive oxygen species (ROS)[1] production (12). ROS promote mitochondrial DNA damage, and inhibition of mitochondrial protein synthesis in vascular endothelial cells increases susceptibility to $NO^{\cdot}$-mediated apoptosis. ROS may also react with $NO^{\cdot}$ in a diffusion-limited manner to form peroxynitrite and other reactive nitrogen species. In fact, long term incubation with $NO^{\cdot}$ via peroxynitrite formation has been shown to inhibit NADH:ubiquinone reductase (complex I) activity (13). Peroxynitrite itself is a highly reactive oxidizing species and exerts its cytotoxic effects further by inducing mitochondrial membrane depolarization (14), activating both caspase-9 and caspase-8 (15), and inactivating ATP synthetase, aconitase, and creatine kinase (12).

Hypoxia, which is associated with tissue inflammation and ischemia, has been shown to increase mitochondrial ROS generation in endothelial cells (16) and to potentiate $NO^{\cdot}$-mediated

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[1] The abbreviations used are: ROS, reactive oxygen species; BAEC, bovine aortic endothelial cells; Rho_0-EC, Rho_0 BAEC; DETA-NO, (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate diethylenetriamine NONOate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DPI, diphenyleneiodonium.

apoptosis in both fibroblasts and neurons (17). To date the combined influence of hypoxia and NO• on mitochondria-dependent apoptosis in endothelial cells has not been determined. In the present study we examined the effect of hypoxia on NO•-mediated apoptosis in vascular endothelial cells and demonstrated the relevance of peroxynitrite formation in mitochondrial dysfunction and cell death.

MATERIALS AND METHODS

Cell Culture—Bovine aortic endothelial cells (BAEC) (Cell Systems Co.) were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged twice weekly by harvesting with 0.5% trypsin/EDTA. Experiments were conducted on cells from passages 4–12. To generate endothelial cells devoid of mitochondria, so-called Rho_0 BAEC (Rho_0-EC), BAEC were incubated in Dulbecco's modified Eagle's medium containing ethidium bromide (100 ng/ml) and uridine (50 μg/ml) for 96 h. Normoxic conditions were defined as pO_2 = 150 mm of Hg. Hypoxic conditions (pO_2 = 35 mm of Hg) were achieved by purging a modular incubation chamber (Billups-Rothenberg) with 95% N_2, 5% CO_2 for 15 min. After purging, the chamber was sealed and maintained inside a humidified 37 °C incubator. Experiments on hypoxic cells were performed inside an inflatable glove bag purged with 95% N_2, 5% CO_2 to maintain hypoxic conditions.

Measurement of Cell Death—Cell necrosis was determined by measuring lactate dehydrogenase levels in the media (Sigma). Apoptotic cell death was determined using the cell death detection ELISA^{Plus} (Roche Applied Science) to detect cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Data were normalized for comparison by total protein concentration (Bio-Rad). Apoptosis was confirmed using the DeadEnd™ Fluorometric terminal dUTP nick-end labeling System (Promega). Apoptotic cells were visualized using a Nikon Eclipse TE300 inverted fluorescent microscope (Nikon).

Bcl-2 Family Member Immunoblotting—Bcl-2 family member expression was determined by loading 50 μg of total cell protein per lane and resolved by SDS-PAGE on a 15% gel, transferred to polyvinylidene fluoride membranes, and blocked with 5% milk. The membranes were then incubated with a 1:500 dilution of a polyclonal anti-Bax (Stressgen) or anti-Bcl-X (Lab Vision) overnight at 4 °C and visualized using the ECL detection system (Amersham Biosciences).

Measurement of Mitochondrial Membrane Potential (ψ_m)—Inner mitochondrial membrane potential (ψ_m) was assessed using the MitoTag JC-1 dye (Chemicon International). BAEC and Rho_0-EC were grown to 90–95% confluence and treated in 96-well plates. After treatment, cells were incubated in the dark for 15 min at 37 °C in Dulbecco's modified Eagle's medium containing the JC-1 dye. Cells were washed three times with phosphate-buffered saline (PBS) (1%), and ψ_m was determined in a microplate fluorimeter (SpectraMax Gemini, Molecular Devices). The dye localizes within the mitochondria in proportion to ψ_m and, when in the mitochondria, forms aggregates that fluoresce red (excitation 550 nm; emission 600 nm). When ψ_m dissipates, the JC-1 dye leaks into the cytoplasm and fluoresces green (excitation 485 nm; emission 535 nm). Inner mitochondrial membrane potential normalized to the number of cells was calculated by dividing mitochondrial (red) fluorescence by cytosolic (green) fluorescence.

Cytochrome c Immunohistochemistry—BAEC were grown to confluence on glass chamber slides and exposed to experimental conditions. Cells were washed with PBS, fixed to the slide with methanol:acetone (1:1) for 15 min at 25 °C, and blocked in 1% bovine serum albumin for 1 h. The cells were incubated for 2 h with 1 μg/ml anti-cytochrome *c* monoclonal antibody (Lab Vision) at 37 °C. The cells were then washed with 0.1% bovine serum albumin, air-dried, incubated with a rhodamine-conjugated secondary antibody (Chemicon International), and visualized using a Nikon Eclipse TE300 inverted fluorescent microscope.

Measurement of Caspase Activity and Expression—Caspase-8 and caspase-9 enzymatic activities were measured with fluorometric assay kits using substrates specific to each caspase (R&D Systems). Data were normalized using total protein concentration. Caspase expression was determined by immunoblotting as described for Bcl-2 family members using a 1:500 dilution of a polyclonal anti-capase-9 (Stressgen) overnight at 4 °C.

Dichorodihydrofluorescein Fluorescence—BAEC and Rho_0-EC were grown to confluence and exposed to experimental conditions. One hour before treatment was completed, cells were washed with phenol red-free Dulbecco's modified Eagle's medium and then incubated with 20 μmol/liter 6-carboxy-2′-7′ dichlorodihydrofluorescein diacetate di(acetoxymethyl) ester (Molecular Probes) for 1 h at 37 °C in the dark. Reactive oxygen species accumulation was determined in a microplate fluorimeter (SpectraMax Gemini, Molecular Devices). Data were normalized for comparison by total protein concentration (Bio-Rad).

Peroxynitrite ELISA—Peroxynitrite formation was determined using the Bioxytech® Nitrotyrosine™ EIA assay (Oxis Int.). Data were normalized for comparison by total protein concentration (Bio-Rad).

Peroxynitrite Immunohistochemistry—Cells were grown to confluence on glass chamber slides, subjected to experimental conditions, washed once in PBS, fixed in methanol:acetone (1:1) at 25 °C for 15 min, and incubated with 3% H_2O_2 for 5 min. After washing with PBS, the slides were incubated in 10% bovine serum albumin for 1 h at 25 °C, washed in PBS, and incubated with a rabbit polyclonal anti-3-nitrotyrosine antibody (Santa Cruz Biotechnology, Inc.) at a 1:50 dilution overnight at 4 °C. After the slides were washed in PBS, a biotinylated secondary antibody (Vector Laboratories) was applied and incubated for 1 h at 25 °C. Nitrotyrosine was visualized using the 3,3′-diaminobenzidine substrate method (Vector Laboratories). The slides were visualized using an Olympus BX41 microscope.

Statistical Analysis—All experiments were performed in duplicate a minimum of three times. Continuous data were expressed as the mean ± S.E. Comparison between groups was performed by Student's paired two-tailed *t* test. Two-way analysis of variance was used to examine differences in response to treatments between groups, with *post hoc* analysis performed by the method of Student-Newman-Keuls. A p value of < 0.05 was considered significant.

RESULTS

Hypoxia Enhances NO•-mediated Cell Death—To examine the effect of hypoxia on cell death in endothelial cells exposed to high concentrations of NO•, BAEC were treated with the NO• donor diethylenetriamine NONOate (DETA-NO), which yields 3 μmol/liter NO• flux, comparable with amounts produced *in vivo* by the inducible isoform of nitric oxide synthase (18). Cells were treated with DETA-NO (0 or 1 mmol/liter) for 24 h under normoxic (pO_2 = 150 mm of Hg) or hypoxic (pO_2 = 35 mm of Hg) conditions, and cell death was determined by lactate dehydrogenase release. At 24 h BAEC exposed to 1 mmol/liter DETA-NO under normoxic conditions did not display a marked increase in cell death as compared with BAEC in the absence of DETA-NO (345 ± 2 *versus* 356 ± 3 units/ml, $p < 0.05$). Under our experimental conditions, hypoxia alone did not increase cell death; however, BAEC treated with 1 mmol/liter DETA-NO under hypoxic conditions demonstrated a significant increase in cell death as compared with cells under normoxic conditions (356 ± 3 *versus* 516 ± 12 units/ml, $p \leq 0.001$). This increase in cell death represented 26% of total cell mass as determined by lactate dehydrogenase levels measured after total cell lysis. Because there was evidence of ongoing cell necrosis at 24 h, we elected to examine cells for markers of apoptosis at 18 h.

To determine whether cell death resulted from apoptosis, cytosolic histone-associated DNA fragmentation, a marker of apoptotic cell death, was measured in BAEC (Fig. 1*A*). Compared with untreated BAEC under normoxic conditions, BAEC exposed to 1 mmol/liter DETA-NO for 18 h demonstrated a significant increase in internucleosomal DNA fragments (323 ± 65 *versus* 1567 ± 236 units/mg of protein, $p \leq 0.001$). Hypoxia alone caused a small increase in histone-associated DNA fragments (323 ± 65 *versus* 466 ± 27 units/mg of protein, $p \leq 0.05$), and this effect was augmented by DETA-NO (466 ± 27 *versus* 8362 ± 200 units/mg of protein, $p < 0.001$). To confirm apoptosis in our system, we performed an *in situ* fluorometric terminal dUTP nick-end labeling assay and calculated an apoptosis index based on the number of apoptotic nuclei/number of cells per high power field (Fig. 1*B*). Under normoxic conditions, compared with untreated cells BAEC exposed to 1 mmol/liter DETA-NO demonstrated a significant increase in apoptosis (2 ± 1 *versus* 48 ± 13 cells/high power field, $p < 0.001$). Under hypoxic conditions there was an increase in apoptosis (2 ± 1 *versus* 21 ± 5 cells/high power field, $p < 0.01$), and this effect was enhanced significantly in cells

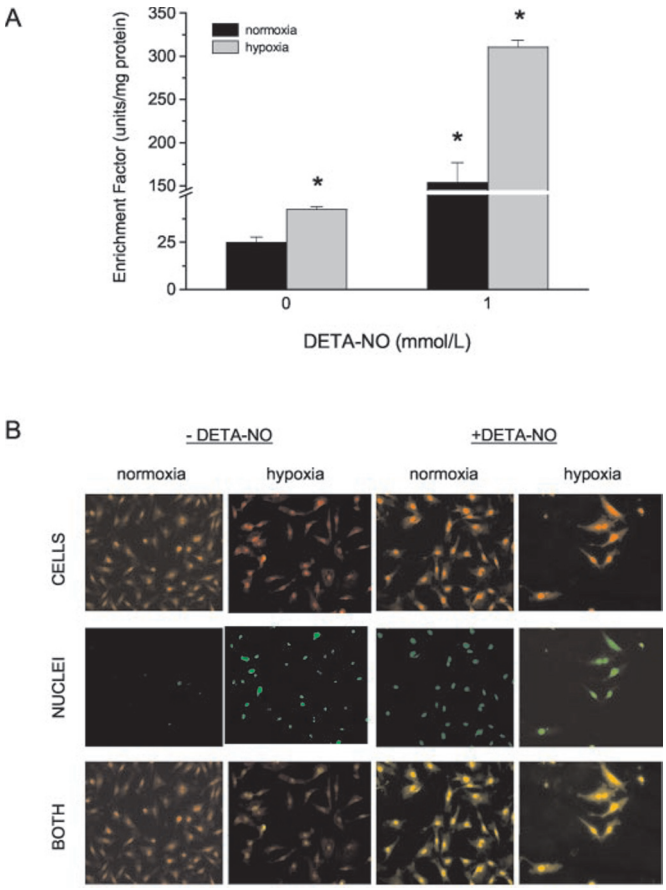

FIG. 1. **Hypoxia augments NO·-mediated apoptosis in endothelial cells.** *A*, BAEC were exposed to DETA-NO (0 or 1 mmol/liter) under normoxic or hypoxic conditions for 18 h, and cytosolic histone-associated DNA fragments were measured as an index of apoptosis. Data are expressed as the mean ± S.E. *, $p < 0.05$ *versus* DETA-NO (0 mmol/liter), normoxia. *B*, apoptosis in BAEC exposed to 0 or 1 mmol/liter DETA-NO under normoxic or hypoxic conditions for 18 h was demonstrated using a fluorometric terminal dUTP nick-end labeling assay. In the *upper panels* (*CELLS*), all cells examined for apoptosis are labeled. In the *middle panel* (*NUCLEI*), apoptotic nuclei are labeled with fluorescein-12-dUTP. In the *lower panel* (*BOTH*) a composite analysis demonstrates the number of apoptotic cells per field.

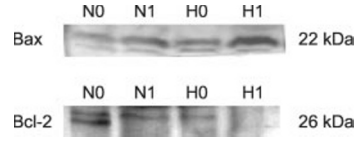

FIG. 2. **Hypoxia augments NO·-mediated changes in Bcl-2 family protein expression.** BAEC were exposed to DETA-NO (0 or 1 mmol/liter) under normoxic or hypoxic conditions for 18 h, and Bax expression (*upper panel*) and Bcl-2 expression (*lower panel*) were determined by immunoblotting. Representative blots are shown. *NO*, normoxia, 0 mmol/liter DETA-NO; *N1*, normoxia 1 mmol/liter DETA-NO; *H0*, hypoxia 0 mmol/liter DETA-NO; *H1*, hypoxia 1 mmol/liter DETA-NO.

treated with DETA-NO (21 ± 5 *versus* 93 ± 6 cells/high power field, $p < 0.001$). Taken together, these data demonstrate that NO· induces apoptotic cell death in BAEC and hypoxia potentiates this response.

Bcl-2 Family Member Expression Is Modulated by Nitric Oxide and Hypoxia—Members of the Bcl-2 family of apoptosis regulators have been shown to mediate mitochondrial function and thereby influence apoptosis (5). To determine the effect of exposure to DETA-NO and hypoxia on Bcl-2 family member expression, we analyzed protein expression of Bax, a pro-apoptotic homologue, and Bcl-2, an anti-apoptotic homologue (Fig. 2). In BAEC exposed to 1 mmol/liter DETA-NO under normoxic conditions for 18 h there was an increase in Bax expression that was enhanced under hypoxic conditions. In contrast, in BAEC treated with DETA-NO, there was a decrease in Bcl-2 expression, which was suppressed further under hypoxic conditions. These findings suggest that exposure to NO· under hypoxic conditions promotes expression of pro-apoptotic effectors and decreases expression of anti-apoptotic effectors.

Nitric Oxide-mediated Cell Death Involves a Mitochondria-dependent Pathway—To determine whether NO·-mediated apoptosis occurred via a mitochondria-dependent pathway, we measured inner mitochondrial membrane potential (ψ_m) (Fig. 3*A*). After 18 h ψ_m was significantly decreased in BAEC exposed to DETA-NO under normoxic conditions (100 ± 1 *versus* 89 ± 4% control, $p < 0.05$), an effect that was enhanced under hypoxic conditions (100 ± 1 *versus* 72 ± 4% control, $p < 0.001$). To demonstrate whether a decrease in ψ_m resulted in release of cytochrome *c*, we performed immunohistochemistry with a rhodamine-conjugated secondary antibody (Fig. 3*B*). Under normoxic conditions, in the absence of DETA-NO, cytochrome *c* was localized to mitochondria and appeared as punctate spots. In contrast, in BAEC exposed to DETA-NO and hypoxic conditions for 18 h, cytochrome *c* was released to the cytoplasm and appeared as diffuse staining. Because release of cytochrome *c*, a pro-apoptotic effector, activates pro-caspase-9, we next measured caspase-9 activity (Fig. 3*C*). Caspase-9 activity was increased significantly in BAEC after 18 h of exposure to DETA-NO under normoxic conditions (472 ± 5 *versus* 599 ± 36 units/mg of protein, $p < 0.05$) and increased further under hypoxic conditions (472 ± 5 *versus* 810 ± 68, units/ml/mg of protein, $p < 0.01$). These findings were confirmed by immunoblotting (Fig. 3*D*), which demonstrated an increase in both pro-caspase-9 and caspase-9 breakdown products (molecular mass = 37, 34, 18, and 10 kDa), consistent with apoptosis in BAEC treated with DETA-NO under hypoxic conditions as compared with untreated BAEC under normoxic conditions. These data demonstrate that high concentrations of NO· induce mitochondria-dependent apoptosis and that hypoxia enhances these effects.

Mitochondria and NO·-mediated Apoptosis under Hypoxic Conditions—To evaluate the importance of functional mitochondria in our system, Rho_0 cells, which lack mitochondrial DNA and functional mitochondria, were created from BAEC (Rho_0-EC). To confirm Rho_0 status, ψ_m was measured (Fig. 4*A*) and found to be decreased by 75% compared with BAEC (4.4 ± 1.0 *versus* 1.1 ± 0.03 × 10^{-4} units, $p < 0.05$). Rho_0 status was also confirmed by labeling cells with MitoTag JC-1 dye, which localizes within the mitochondria in proportion to ψ_m and, when in the mitochondria, forms aggregates that fluoresce red; when ψ_m dissipates, as occurs in Rho_0 cells, the JC-1 dye leaks into the cytoplasm and fluoresces green (Fig. 4*B*).

Rho_0-EC were treated with DETA-NO (0 or 1 mmol/liter) under normoxic or hypoxic conditions for 24 h and evaluated for markers of cell death. In the absence of DETA-NO under normoxic conditions there was no significant difference between BAEC and Rho_0-EC with respect to cell death, as determined by media lactate dehydrogenase (349 ± 13 *versus* 359 ± 12 units/ml, p = NS). Interestingly, in Rho_0-EC exposed to DETA-NO under normoxic conditions, there was an increase in cell death, and this effect was more pronounced than that observed in BAEC (556 ± 23 *versus* 356 ± 3 units/ml, $p < 0.001$). Similarly, Rho_0-EC treated with DETA-NO under hypoxic conditions demonstrated increased cell death compared with BAEC (750 ± 20 *versus* 516 ± 12 units/ml, $p < 0.001$). This finding suggested that endothelial cells lacking functional mitochondria were more sensitive to NO·-mediated cell death under both normoxic and hypoxic conditions.

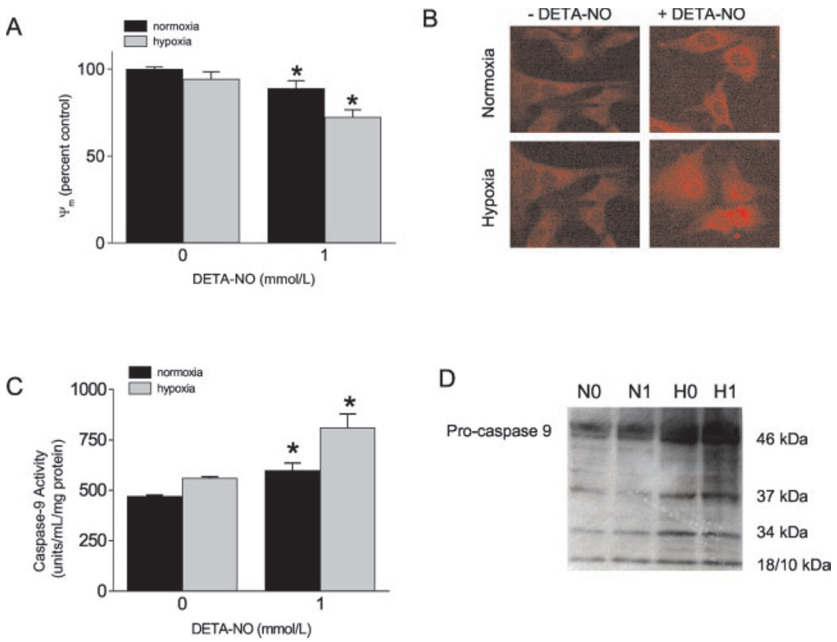

FIG. 3. **Hypoxia initiates NO˙-mediated apoptosis via a mitochondria-dependent pathway.** BAEC were exposed to 0 or 1 mmol/liter DETA-NO under normoxic or hypoxic conditions for 18 h. *A*) inner mitochondrial membrane potential (ψ_m) was measured. *B*, cytochrome *c* release was demonstrated by immunohistochemistry using a fluorescent rhodamine-conjugated secondary antibody. Caspase-9 activity was measured (*C*), and breakdown of pro-caspase-9 and production of caspase-9 breakdown products was determined by immunoblotting (*D*). Data are expressed as the mean ± S.E. *, $p < 0.05$ *versus* DETA-NO (0 mmol/liter), normoxia.

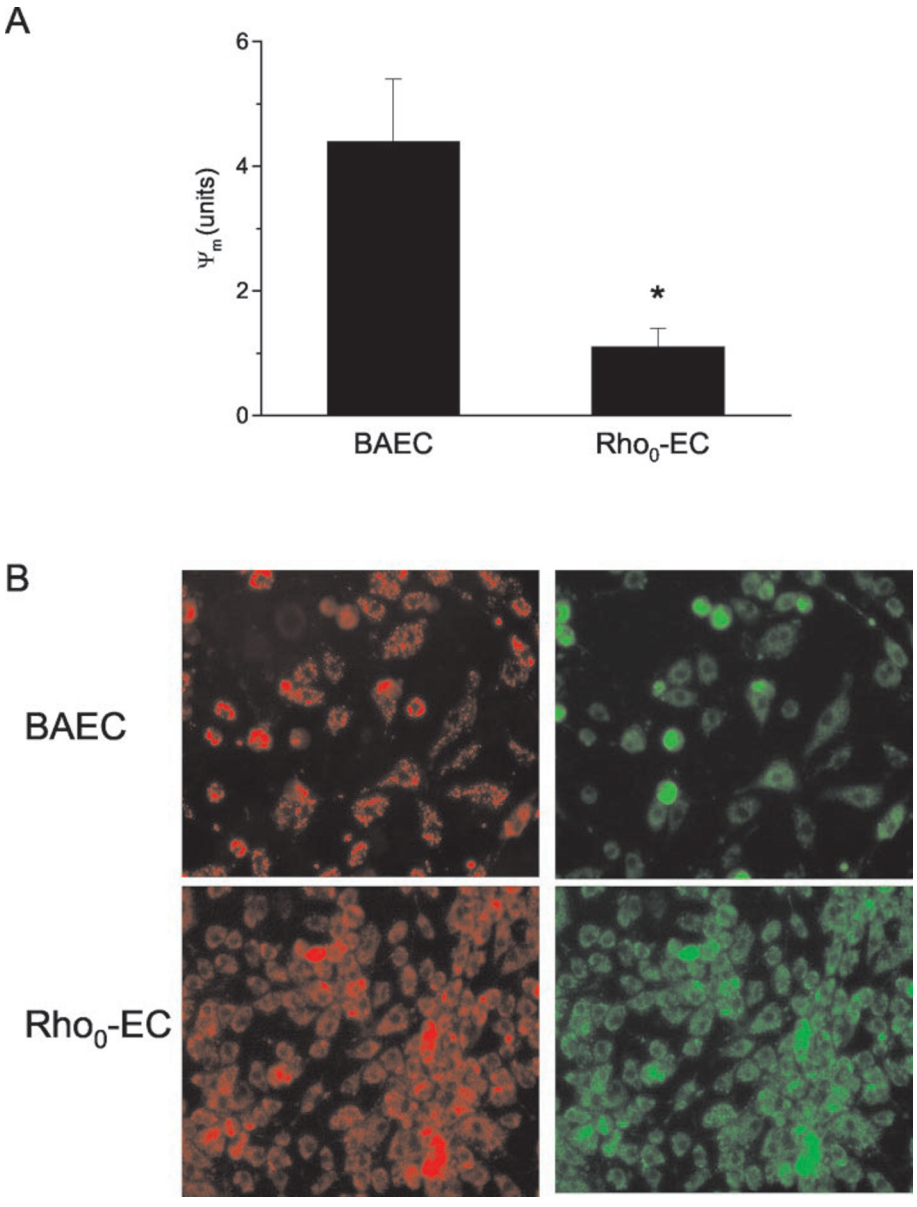

FIG. 4. **Rho_0 endothelial cells.** BAEC were treated with ethidium bromide (100 ng/ml) and uridine (100 μg/ml) for 96 h to eliminate a functional mitochondrial electron transport chain. Rho_0 status was confirmed by measuring the inner mitochondrial membrane potential (ψ_m) (*A*) and visualized using Mitotag JC-1 dye (*B*). The dye localizes within the mitochondria in proportion to membrane potential and when in the mitochondria forms aggregates that fluoresce red (excitation 550 nm; emission 600 nm). When mitochondrial membrane potential dissipates, the JC-1 dye leaks into the cytoplasm and fluoresces green (excitation 485 nm; emission 535 nm). *, $p < 0.05$ *versus* BAEC.

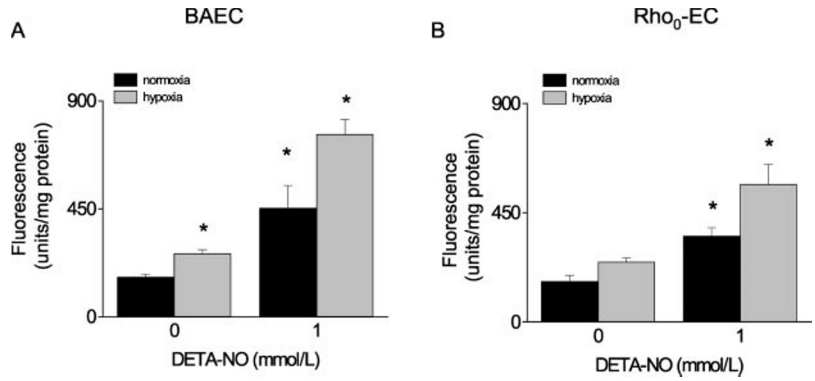

FIG. 5. **Reactive oxygen species production.** BAEC or Rho_0-BAEC were exposed to 0 or 1 mmol/liter DETA-NO under normoxic or hypoxic conditions for 18 h, and ROS production was measured by 6-carboxy-2′-7′ dichlorodihydrofluorescein diacetate di(acetoxymethyl) ester (*DCF*) fluorescence. Data are expressed as mean ± S.E. *, $p < 0.05$ compared with cells exposed to DETA-NO (0 mmol/liter) under normoxic conditions.

We next examined Rho_0-EC for markers of apoptosis at 18 h. There was no significant difference between Rho_0-EC and BAEC in levels of histone-associated DNA fragments in the absence of DETA-NO under normoxic conditions (323 ± 25 *versus* 340 ± 13 units/mg of protein, p = NS); however, compared with BAEC, Rho_0-EC cells exposed to the DETA-NO under normoxic conditions demonstrated a significant increase in internucleosomal DNA fragments (1567 ± 236 *versus* 4704 ± 260 units/mg of protein, $p < 0.001$) as did Rho_0-EC treated with DETA-NO under hypoxic conditions (8362 ± 200 *versus* 12,663 ± 128 units/mg of protein). These observations confirm that endothelial cells lacking functional mitochondria demonstrate increased sensitivity to NO·-mediated apoptosis under both normoxic and hypoxic conditions.

Exposure to High Levels of NO· Increases Oxidant Stress—Previous studies have suggested that increased ROS production is one mechanism by which NO· mediates mitochondrial damage to initiate apoptosis. To examine this hypothesis, we measured ROS accumulation under our experimental conditions (Fig. 5*A*). ROS accumulation was elevated significantly in BAEC exposed to the NO· donor under normoxic conditions compared with untreated cells (166 ± 13 *versus* 454 ± 94 arbitrary units/mg of protein, $p < 0.001$) and to a greater extent in BAEC treated with the NO· donor under hypoxic conditions (166 ± 13 *versus* 760 ± 63 arbitrary units/mg of protein, $p < 0.001$). To determine the source of ROS production, we treated BAEC with L-N^G-nitroarginine methyl ester. (1 mmol/liter), oxypurinol (100 μmol/liter), indomethacin (10 μmol/liter), diphenyleneiodonium (DPI) (10 μmol/liter), or rotenone (10 μmol/liter) to inhibit endothelial nitric-oxide synthase, xanthine oxidase, cyclooxygenase, NAD(P)H oxidase(s), and mitochondria, respectively (Table I). Only the addition of indomethacin, DPI, or rotenone decreased ROS production, suggesting that mitochondria are in part a source of ROS production; however, indomethacin inhibits both cyclooxygenase and mitochondrial respiration, and DPI inhibits ROS production by all flavin-containing enzymes, including NAD(P)H oxidases as well as mitochondrial complex I (19, 20). Therefore, to confirm the role of mitochondria in ROS production, we next measured ROS accumulation in Rho_0-EC that lack mitochondrial DNA (Fig. 5*B*). Interestingly, ROS accumulation was increased in Rho_0-EC cells exposed to DETA-NO under normoxic conditions (168 ± 24 *versus* 354 ± 34 arbitrary units/mg of protein, $p < 0.001$) and to a greater extent in cells exposed to the NO· donor under hypoxic conditions (168 ± 24 *versus* 567 ± 82 arbitrary units/mg of protein, $p < 0.001$), albeit ROS levels were significantly less than those observed in BAEC under the same conditions (567 ± 82 *versus* 1562 ± 112 arbitrary units/mg of protein, $p < 0.001$). To determine the source of ROS production in Rho_0-EC, we exposed cells to the same inhibitors as BAEC (Table I). ROS production was attenuated in Rho_0-EC treated with indomethacin or DPI, suggesting that cyclooxygenase and NAD(P)H oxidase may contribute to elevated ROS levels. Taken together these findings confirm that exposure to elevated levels of NO· increases both mitochondrial and non-mitochondrial ROS production in endothelial cells, and hypoxia augments this response.

Peroxynitrite Is Formed in Cells Treated with DETA-NO—To determine whether increased mitochondrial ROS production was associated with peroxynitrite formation in our experimental system, we first measured 3-nitrotyrosine levels by ELISA (Fig. 6*A*). In BAEC exposed to DETA-NO under normoxic conditions for 18 h, 3-nitrotyrosine levels were increased compared with untreated cells (4.1 ± 1.1 *versus* 6.7 ± 1.3 nmol/liter/mg of protein, $p < 0.05$), and this effect was more pronounced under hypoxic conditions (4.6 ± 1.2 *versus* 9.0 ± 1.2 nmol/liter/mg of protein, $p < 0.05$). To confirm these findings, we performed immunohistochemistry using a polyclonal antibody to 3-nitrotyrosine (Fig. 6*B*). In BAEC treated with the NO· donor under normoxic conditions, there was increased 3-nitrotyrosine immunostaining that was enhanced under hypoxic conditions. Similarly, in Rho_0-EC, peroxynitrite formation was increased and to a greater extent than that observed in BAEC under similar experimental conditions (Fig. 6*C*). In Rho_0-EC, 3-nitrotyrosine levels were increased in cells exposed to DETA-NO under normoxic conditions compared with untreated cells (4.5 ± 0.9 *versus* 7.8 ± 0.4 nmol/liter/mg of protein, $p < 0.05$) and increased further under hypoxic conditions (6.1 ± 0.3 *versus* 16.2 ± 2.4 nmol/liter/mg of protein, $p < 0.001$). In fact, when compared with BAEC exposed to 1 mmol/liter DETA-NO under hypoxic conditions, peroxynitrite formation was increased significantly in Rho_0-EC under similar experimental conditions (9.0 ± 1.2 *versus* 16.2 ± 2.4 nmol/liter/mg of protein, $p < 0.05$). These findings were supported by cell immunostaining for 3-nitrotyrosine (Fig. 6*D*).

Apoptosis Results from Increased Peroxynitrite Formation—To demonstrate that peroxynitrite plays a significant role in NO·-mediated apoptosis in endothelial cells under normoxic or hypoxic conditions, we treated BAEC with uric acid (0.1 mmol/liter) to scavenge peroxynitrite and measured the indices of apoptosis. In the presence of uric acid, apoptosis, as determined by cytosolic histone-associated DNA fragments, was decreased significantly in BAEC exposed to DETA-NO under both normoxic (1567 ± 236 *versus* 448 ± 77 units/mg of protein, $p < 0.01$) and hypoxic (8362 ± 204 *versus* 665 ± 289 units/mg of protein, $p < 0.001$) conditions (Fig. 7*A*). Interestingly, when peroxynitrite was scavenged by uric acid in BAEC, mitochondrial membrane potential was maintained after exposure to DETA-NO under either normoxic (88 ± 3 *versus* 98 ± 2% control, $p < 0.05$) or hypoxic conditions (73 ± 2 *versus* 99 ± 4% control, $p < 0.01$), suggesting that peroxynitrite initiated apoptosis by inducing mitochondrial damage.

To determine whether scavenging peroxynitrite would influence apoptosis in Rho_0-EC, in which the source of peroxynitrite

TABLE I

Source of ROS production in BAEC and Rho_0-EC exposed to DETA-NO and hypoxic conditions

ROS production is measured as arbitrary fluorescent units/mg of protein and expressed as mean ± S.E. L-NAME, L-N^G-nitroarginine methyl ester.

	No addition[a]	L-NAME[a] (1 mmol/liter)	Oxypurinol[a] (100 μmol/liter)	Indomethacin[a] (10 μmol/liter)	DPI[a] (10 μmol/liter)	Rotenone[a] (10 μmol/liter)
BAEC						
Normoxia						
−DETA-NO	193 ± 14	196 ± 12	214 ± 19	216 ± 11	182 ± 18	200 ± 16
+DETA-NO	878 ± 30	805 ± 54	813 ± 42	744 ± 27	363 ± 15[b]	338 ± 18[b]
Hypoxia						
−DETA-NO	762 ± 70	624 ± 43	678 ± 26	580 ± 29[c]	355 ± 19[b]	318 ± 10[b]
+DETA-NO	1562 ± 112	1361 ± 97	1263 ± 73	1094 ± 51[c]	454 ± 26[b]	401 ± 20[b]
Rho_0-EC						
Normoxia						
−DETA-NO	146 ± 3	147 ± 4	137 ± 3	137 ± 3	100 ± 3[b]	140 ± 6
+DETA-NO	341 ± 8	343 ± 15	331 ± 8	230 ± 5[b]	208 ± 4[b]	327 ± 8
Hypoxia						
−DETA-NO	217 ± 7	224 ± 3	223 ± 5	178 ± 4[c]	156 ± 6[b]	223 ± 3
+DETA-NO	577 ± 19	591 ± 13	595 ± 9	437 ± 16[b]	364 ± 15[b]	563 ± 15

[a] $p < 0.05$ for means between treatment groups by analysis of variance.
[b] $p < 0.001$ *vs.* No addition.
[c] $p < 0.01$ *vs.* No addition.

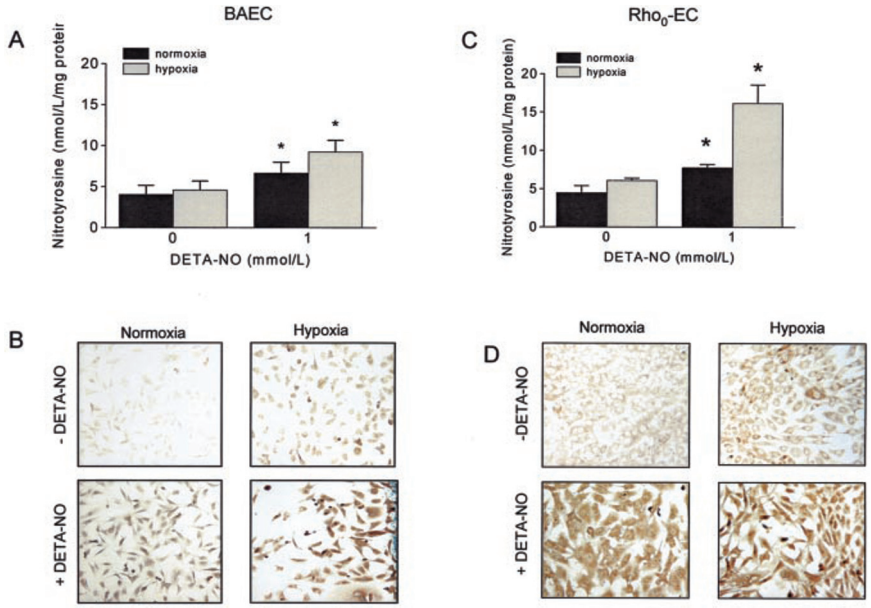

FIG. 6. **Peroxynitrite formation.** *A*, peroxynitrite formation was measured by ELISA in BAEC exposed to 0 or 1 mmol/liter DETA-NO under normoxic or hypoxic conditions for 18 h. Data are expressed as mean ± S.E. *, $p < 0.05$ compared with cells exposed to DETA-NO (0 mmol/liter) under normoxic conditions. *B*, peroxynitrite formation was demonstrated by immunohistochemistry using an antibody to 3-nitrotyrosine.

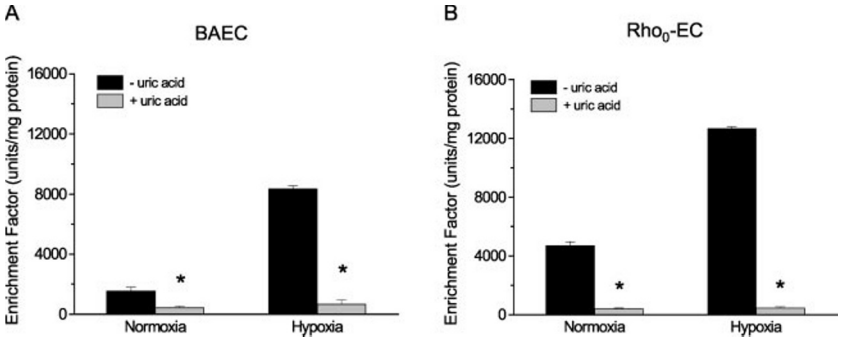

FIG. 7. **Scavenging peroxynitrite prevents apoptosis.** BAEC (*A*) or Rho_0-BAEC (*B*) were exposed to 0 or 1 mmol/liter DETA-NO under normoxic or hypoxic conditions for 18 h in the presence or absence of uric acid (0.1 mmol/liter) and apoptosis was determined by measuring cytosolic histone associated DNA fragments . Data are expressed as the mean ± S.E. *, $p < 0.05$ compared with cells exposed to DETA-NO (1 mmol/liter), −uric acid.

was extra-mitochondrial, we treated Rho_0-EC with uric acid and measured histone-associated DNA fragments. In the presence of uric acid, apoptosis was decreased significantly in Rho_0-EC exposed to DETA-NO under normoxic conditions (4,704 ± 260 *versus* 421 ± 32 units/mg of protein, $p < 0.001$) and under hypoxic conditions (12,663 ± 128 *versus* 463 ± 84 units/mg of protein, $p < 0.001$) (Fig. 7*B*).

Because Rho_0-EC lack functional mitochondria yet demonstrate increased indices of apoptosis when exposed to $NO^{\cdot}$ under hypoxic conditions, we speculated that peroxynitrite must also modulate apoptosis via a mitochondria-independent mechanism. To investigate this hypothesis we measured the activity of caspase-8, which is activated independently of mitochondria and stimulates similar downstream effector caspases as caspase-9. In Rho_0-EC exposed to DETA-NO under normoxic conditions, caspase-8 activity was increased significantly compared with untreated cells (61 ± 10 *versus* 184 ± 5 units/ml/mg of protein, $p < 0.001$) and increased further under hypoxic conditions (75 ± 11 *versus* 250 ± 9 units/ml/mg of protein, $p < 0.001$) (Fig. 8*B*). To determine whether this mechanism was

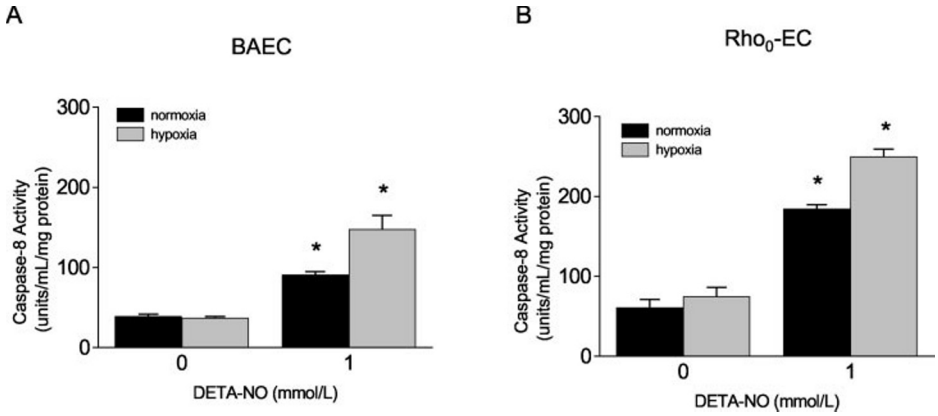

FIG. 8. **Hypoxia initiates NO·-mediated apoptosis via a mitochondrial-independent pathway.** BAEC (*A*) or Rho_0-BAEC (*B*) were exposed to 0 or 1 mmol/liter DETA-NO under normoxic or hypoxic conditions for 18 h, and caspase-8 activity was measured. Data are expressed as mean ± S.E. *, $p < 0.05$ *versus* DETA-NO (0 mmol/liter).

also operative in endothelial cells with intact mitochondria, we measured caspase-8 activity in BAEC. Under normoxic conditions, BAEC treated with DETA-NO demonstrated increased caspase-8 activity (39 ± 3 *versus* 91 ± 4 units/ml/mg of protein, $p < 0.001$), and this response was also augmented by hypoxia (37 ± 2 *versus* 148 ± 17 units/ml/mg of protein, $p < 0.001$) (Fig. 8*A*), thereby confirming that peroxynitrite initiates apoptosis by mitochondria-dependent as well as -independent pathways in endothelial cells.

DISCUSSION

In the present study we examined the influence of hypoxia on NO·-mediated apoptosis in vascular endothelial cells. Our findings demonstrate that high concentrations of NO· induce apoptosis in endothelial cells by increasing ROS production and peroxynitrite formation, which in turn initiate mitochondrial damage, cytochrome *c* release, and activation of caspase-9, and that hypoxia potentiates these responses. Furthermore, by utilizing Rho_0-EC, which lack functional mitochondria, we demonstrate that peroxynitrite formed by exposure to high concentrations of NO· under hypoxic conditions additionally activates caspase-8, a mitochondria-independent mechanism of apoptosis.

Hypoxia has been shown to modulate NO·-induced apoptosis in rat fibroblasts. Interestingly, hypoxia alone did not enhance cell death in fibroblasts, and cells grown under hypoxic conditions (1.5% O_2) exhibited similar levels of apoptosis as fibroblasts under normoxic conditions. Only when exposed to NO· under hypoxic conditions, at levels shown to be non-toxic under normoxic conditions, was apoptotic cell death markedly increased (4). In our studies performed in vascular endothelial cells, exposure to hypoxic conditions for 24 h did not induce cell death; however, markers of apoptosis were increased, suggesting impending cell death. Other investigators have reported that the effect of hypoxia alone on endothelial cell death appears to be dependent upon the origin of the cells and the duration of hypoxia. For example, in human umbilical vein endothelial cells, exposure to hypoxia for 24 h resulted in only 2% cell death and 4% of cells positive for apoptotic markers. After 48 h of hypoxia, cell death was increased and only 55% of cells were viable with 35% positive for apoptotic markers. In this cell line p53 levels were altered, yet there was no change in Bcl-X_L or Bax expression (21). In contrast, in human aortic endothelial cells exposed to hypoxia for 24 h, there was a significant decrease in cell number due to necrosis and an increased number of apoptotic cells exhibiting DNA fragmentation compared with cells grown under normoxic conditions. These investigators implicated NF-κB-induced Bcl-2 suppression as the mechanism for hypoxia-mediated apoptosis (22). These observations suggest that hypoxic conditions may serve to sensitize cells to apoptosis-inducing stimuli, such as elevated levels of NO·.

Although NO· at low concentrations confers a cytoprotective benefit on the endothelium, high levels of NO·, as may be achieved by the inducible isoform of nitric oxide synthase during inflammatory states, have been shown to injure endothelial cells and may contribute to apoptosis (23). This finding is not surprising as elevated concentrations of NO· stimulate apoptosis in macrophages, thymocytes, pancreatic islets, neurons, and some tumor cell lines (24). Furthermore, overexpression of *Nos3* to increase local NO· levels similarly induces apoptosis in neuronal cells (25), and exposure to NO· donors has been shown to sensitize A549 and Jurkat T cells to apoptosis-inducing stimuli by inhibiting NF-κB (26). In our experiments prolonged exposure to DETA-NO under normoxic conditions increased apoptosis in BAEC, and this effect was enhanced under hypoxic conditions. Previous studies performed in endothelia that have examined the effect of high concentrations of NO· on cell viability have failed to demonstrate apoptosis after a 24-h exposure period in the absence of a second insult, such as withdrawal of glucose from the media; however, these studies utilized DPTA-NONOate as the NO· donor, which has a considerably shorter half-life than DETA-NO (3 *versus* 20 h), and examined a late marker of apoptosis, annexin V (27).

Several mechanisms have been proposed to explain NO·-mediated apoptosis including increased p53 levels, ROS production, and/or mitochondrial permeability transition (26, 28). Recent studies have focused on the central role of the mitochondrion in NO·-mediated apoptosis. Nitric oxide directly influences mitochondrial function by inhibiting mitochondrial respiration via inactivation of NADH:ubiquinone reductase (complex I) and cytochrome *c* oxidase (complex IV) and by stimulating mitochondrial production of ROS and peroxynitrite, which in turn can influence the mitochondrial permeability transition and dissipation of membrane potential (28). Loss of mitochondrial membrane potential results in the release of cytochrome *c*, which itself has been recognized as a pro-apoptotic signal (9).

In our studies we found that NO·-mediated apoptosis in endothelial cells under hypoxic conditions was associated with an increase in ROS production, peroxynitrite formation, and mitochondrial membrane damage. Moreover, by demonstrating increased ROS production in Rho_0-EC, which lack functional mitochondria, it appears that mitochondrial as well as extramitochondrial sources contribute to ROS accumulation. In BAEC, ROS production was attenuated by treatment with indomethacin, DPI, or rotenone. In addition to inhibiting cyclooxygenase and NAD(P)H oxidases, respectively, indomethacin and DPI reduce mitochondrial ROS production, and indomethacin has been shown to decrease NAD(P)H oxidase activity in neutrophils (19, 20). Previous studies in endothelial cells have implicated ROS production in mitochondria-dependent apoptosis. It has been demonstrated that treatment with

rotenone, an inhibitor of NADH:ubiquinone reductase (complex I), which generates ROS, limits tumor necrosis factor-α-mediated ROS production, activation of caspase-3, and apoptosis (9). In addition, high density lipoprotein has been shown to inhibit mitochondrial-dependent apoptosis by decreasing ROS formation and mitochondrial membrane potential dissipation and by inhibiting cytochrome *c* release. Interestingly, increased ROS accumulation in endothelial cells may also promote mitochondrial DNA damage by decreasing mitochondrial protein synthesis, resulting in increased susceptibility to NO$^{\bullet}$-mediated apoptosis (29). In vascular endothelial cells treated with chloramphenicol to inhibit mitochondrial protein synthesis, exposure to the NO$^{\bullet}$ donor DPTA-NONOate in glucose-free media enhanced apoptosis in association with a decreased ratio of cytochrome *c* oxidase (complex IV) to cytochrome *c* and increased reactive oxygen and nitrogen species generation (27). Of note, these investigators did not measure peroxynitrite levels, and therefore were unable to assess the contribution of peroxynitrite to the process.

Peroxynitrite formation is favored in mitochondria exposed to high levels (>1 μmol/liter) of NO$^{\bullet}$. At these levels NO$^{\bullet}$ oxidizes ubiquinol, thereby promoting ubisemiquinone auto-oxidation and concomitant generation of peroxynitrite. Once formed, peroxynitrite inhibits NADH:ubiquinone reductase (complex I) activity and ATP synthetase (complex V) to promote the membrane permeability transition and apoptosis (30). In our experiments, we demonstrated increased peroxynitrite levels by ELISA and confirmed peroxynitrite formation by immunohistochemistry; yet the sources of peroxynitrite generation remains unclear. Although mitochondria are a recognized source of peroxynitrite formation, it is also possible that peroxynitrite formed in extra-mitochondrial sites may diffuse into and influence mitochondrial function. Indeed, our studies with Rho_0-EC, which lack functional mitochondria, suggest that the latter possibility may be as important in our experimental system as mitochondrial sources. In fact, we measured increased peroxynitrite formation in Rho_0-EC and demonstrated enhanced immunohistochemical staining for peroxynitrite compared with BAEC. There are several plausible explanations for these findings. The first is that Rho_0-EC lack manganese-superoxide dismutase, which serves as an important mitochondrial anti-oxidant enzyme to limit peroxynitrite formation. Second, Rho_0-EC may be deficient in reducing equivalents, such as ascorbate, which has been shown to reside in the mitochondrial matrix, influence NO$^{\bullet}$ generation from peroxynitrite, and thereby decrease peroxynitrite toxicity (31). Finally, extra-mitochondrial peroxynitrite formation may initiate apoptosis via a mitochondrial-independent mechanism. Interestingly, we demonstrated activation of caspase-8 in Rho_0-EC, and to a lesser extent, in BAEC, a finding that has been confirmed in HL-60 cells exposed to peroxynitrite (15). Regardless of the site of peroxynitrite formation, the observation that urate inhibited mitochondrial membrane potential dissipation and apoptosis in both endothelial cells and Rho_0-EC exposed to DETA-NO under hypoxic conditions implicates peroxynitrite as the primary mediator of apoptosis in our studies. Further understanding of how decreased extracellular oxygen tension exacerbates the cytotoxic effects of high concentrations of NO$^{\bullet}$ on endothelial cells may serve to elucidate the basic mechanisms that underlie endothelial dysfunction associated with vascular disease.

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