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) at low concentrations is cytoprotective for endothelial cells; however, elevated concentrations of NO (≥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 on endothelial function. To examine the influence of hypoxia on NO-mediated apoptosis, we exposed bovine aortic endothelial cells (BAEC) to (Z)-1-(2-aminoethyl)-N-(2-ammonioethyl) amino diazene-1-i um-1,2-diolate (diethylenetriamine NONOate, DETA-NO) (1 mmol/liter) under normoxic or hypoxic conditions (pO₂ = 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-initiated apoptosis under hypoxic conditions, we evaluated mitochondrial membrane potential (Ψₘ). Exposure to DETA-NO resulted in a decrease in Ψₘ and concomitant release of cytochrome c and caspase-9 activation, which were enhanced by hypoxia. By utilizing Rh₀ BAEC (Rh₀ EC), which lack functional mitochondria, we demonstrated that dissipation of Ψₘ was associated with increased reactive oxygen species generation and peroxynitrite formation. Moreover, in Rh₀ 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 Rh₀ 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 under hypoxic conditions promote cell death via mitochondrial damage and mitochondrial-independent mechanisms by peroxynitrite.

Nitric oxide (NO), 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 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 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-mediated apoptosis has been recognized clinically as contributing to endothelial dysfunction, atherosclerosis, and transplant vascular disease (5–8).

The mechanism(s) by which NO modulates apoptosis highlights the central role of the mitochondrion in NO-mediated cell death. Prolonged exposure to elevated levels of NO suppresses mitochondrial respiration by inhibition of cytochrome oxidase (complex IV), resulting in a decrease in inner mitochondrial membrane potential (Ψₘ) 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-mediated apoptosis. ROS may also react with NO in a diffusion-limited manner to form peroxynitrite and other reactive nitrogen species. In fact, long term incubation with NO 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-mediated

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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 trypsinization with 0.5% trypsin/EDTA. Experiments were conducted on cells from passages 4–12. To generate endothelial cells devoid of mitochondria, so-called Rh0- 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 pO₂ = 150 mm Hg. Hypoxic conditions (pO₂ ≈ 35 mm Hg) were achieved by purging a modular incubation chamber (Billups-Rothenberg) with 95% N₂, 5% CO₂ 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% N₂, 5% CO₂ 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 ELISAPlus (Roche Applied Science) to detect cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Data were normalized for comparison by total protein concentration (Bio-Rad).

**Measurement of Mitochondrial Membrane Potential (ΔΨm)—** Inner mitochondrial membrane potential (ΔΨm) was assessed using the MitoTag JC-1 dye (Chemicon International). BAEC and Rh0-EC 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) at 25 °C, and incubated with 3% H₂O₂ 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. The next day 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.

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**Measurement of Caspase Activity and Expression—** Caspase-8 and -9 enzymatic activities were measured with fluorometric assay kits using substrates specific to each caspase (R&D Systems). Data were normalized for comparison by total protein concentration (Bio-Rad).

**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 (iNOS). Cells were treated with DETA-NO (0 or 1 mmol/liter) for 24 h under normoxic (pO₂ = 150 mm Hg) or hypoxic (pO₂ = 35 mm Hg) conditions. Cell death was determined using 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 < 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. 1A). A decrease in histone-associated DNA fragments (323 ± 65 versus 1567 ± 236 units/mg of protein, p ≤ 0.001). Hypoxia alone caused a small increase in histone-associated DNA fragments (323 ± 65 versus 466 ± 27 units/mg of protein, p ≤ 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. 1B). 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 exposed to 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.

**Bel-2 Family Member Expression Is Modulated by Nitric Oxide and Hypoxia**—Members of the Bel-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 Bel-2 family member expression, we analyzed protein expression of Bax, a pro-apoptotic homologue, and Bel-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 Bel-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 (ψm) (Fig. 3A). After 18 h, ψm 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 ψm resulted in release of cytochrome c, we performed immunohistochemistry with a rhodamine-conjugated secondary antibody (Fig. 3B). 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 caspase-9, we next measured caspase-9 activity (Fig. 3C). 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/mg of protein, p < 0.01). These findings were confirmed by immunoblotting (Fig. 3D), 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, Rho0 cells, which lack mitochondrial DNA and functional mitochondria, were created from BAEC (Rho0-EC). To confirm Rho0 status, ψm was measured (Fig. 4A) 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). Rho0 status was also confirmed by labeling cells with MitoTag JC-1 dye, which localizes within the mitochondria in proportion to ψm and, when in the mitochondria, forms aggregates that fluoresce red; when ψm dissipates, as occurs in Rho0 cells, the JC-1 dye leaks into the cytoplasm and fluoresces green (Fig. 4B).

Rho0-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 Rho0-EC with respect to cell death, as determined by media lactate dehydrogenase (349 ± 13 versus 359 ± 12 units/ml, p = NS). Interestingly, in Rho0-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, Rho0-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 (Δψₘ) 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. **Rho₀ 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. Rho₀ status was confirmed by measuring the inner mitochondrial membrane potential (Δψₘ) (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.
We next examined Rho0-EC for markers of apoptosis at 18 h. There was no significant difference between Rho0-EC and BAEC in levels of histone-associated DNA fragments in the absence of DETA-NO under normoxic conditions (323 ± 5 versus 340 ± 13 units/mg of protein, p = NS); however, compared with BAEC, Rho0-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 Rho0-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. 5A). 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 1-NAD'-nitroarginine methyl ester (1 mmol/liter), oxyypurinol (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, cytochrome oxidase, 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 cytochrome oxidase 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 Rho0-EC that lack mitochondrial DNA (Fig. 5B). Interestingly, ROS accumulation was increased in Rho0-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 Rho0-EC, we exposed cells to the same inhibitors as BAEC (Table I). ROS production was attenuated in Rho0-EC treated with indomethacin or DPI, suggesting that cytochrome oxidase 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 nonmitochondrial 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. 6A). 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. 6B). In BAEC treated with the NO donor under normoxic conditions, there was increased 3-nitrotyrosine immunostaining that was enhanced under hypoxic conditions. Similarly, in Rho0-EC, peroxynitrite formation was increased and to a greater extent than that observed in BAEC under similar experimental conditions (Fig. 6C). In Rho0-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 Rho0-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. 6D).

**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. 7A). 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 Rho0-EC, in which the source of peroxynitrite...
was extra-mitochondrial, we treated Rho0-EC with uric acid and measured histone-associated DNA fragments. In the presence of uric acid, apoptosis was decreased significantly in Rho0-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. 7B).

Because Rho0-EC lack functional mitochondria yet demonstrate increased indices of apoptosis when exposed to NO 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 Rho0-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. 8B). To determine whether this mechanism was
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. 8A), 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\(_2\) 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 Rh0\(_2\)-EC, which lack functional mitochondria, we demonstrate that peroxynitrite formed by exposure to high concentrations of NO\(_2\) under hypoxic conditions additionally activates caspase-8, a mitochondria-independent mechanism of apoptosis.

Hypoxia has been shown to modulate NO\(_2\)-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\(_2\) 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-\( \kappa \)B-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:\(_2\).

Although NO\(_2\) at low concentrations confers a cytoprotective benefit on the endothelium, high levels of NO\(_2\), as may be achieved by the inducible isofrm 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\(_2\) stimulate apoptosis in macrophages, thymocytes, pancreatic islets, neurons, and some tumor cell lines (24). Furthermore, overexpression of Nos3 to increase local NO\(_2\) levels similarly induces apoptosis in neuronal cells (25), and exposure to NO\(_2\) donors has been shown to sensitize A549 and Jurkat T cells to apoptosis-inducing stimuli by inhibiting NF-\( \kappa \)B (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\(_2\) 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\(_2\) 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 mitochondrial NO\(_2\)-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\(_2\)-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 Rh0\(_2\)-EC, which lack functional mitochondria, it appears that mitochondrial as well as extra-mitochondrial 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-depend- ent apoptosis. It has been demonstrated that treatment with...
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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-mediated apoptosis (29). In vascular endothelial cells treated with chlor-aminphenicol to inhibit mitochondrial protein synthesis, exposure to the NO 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. At these levels NO oxidizes ubiquinol, thereby promoting ubiquinone 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 Rh0-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 Rh0-EC and demonstrated enhanced immunohistochemical staining for peroxynitrite compared to BAEC. There are several plausible explanations for these findings. The first is that Rh0-EC lack manganese-superoxide diamutase, which serves as an important mitochondrial anti-oxidant enzyme to limit peroxynitrite formation. Second, Rh0-EC may be deficient in reducing equivalents, such as ascorbate, which has been shown to reside in the mitochondrial matrix, influence NO 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 Rh0-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 Rh0-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 on endothelial cells may serve to elucidate the basic mechanisms that underlie endothelial dysfunction associated with vascular disease.

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