SUPPRESSION OF NUCLEAR FACTOR-KAPPA B ACTIVITY BY NITRIC OXIDE AND HYPOXIA IN OXYGEN RESISTANT CELLS

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SUMMARY:

Inhaled nitric oxide (iNO) is used clinically to treat pulmonary hypertension in newborns, often in conjunction with hyperoxia (NO/O₂). Prolonged exposure to NO/O₂ causes synergistic lung injury and death of lung epithelial cells. To explore the mechanisms involved, oxygen-resistant HeLa-80 cells were exposed to NO±O₂. Exposure to NO and O₂ induced a synergistic cytotoxicity, accompanied with apoptotic characteristics, including elevated caspase-3-like activity, Annexin V incorporation, and nuclear condensation. This apoptosis was associated with a synergistic suppression of NF-κB activity. Cells lacking functional NF-κB p65 subunit were more sensitive to NO/O₂ than their wildtype counterparts. This injury was partially rescued by transfection with a p65 expression construct, suggesting an inverse relationship between NF-κB and susceptibility to the cytotoxicity of NO/O₂. Despite the reduced NF-κB activity in cells exposed to NO±O₂, IκBα was degraded, suggesting that pathways regulating the steady state levels of IκB were not involved. However, exposure to NO/O₂ caused a marked reduction in nuclear localization and an increase in protein carbonyl formation of NF-κB p65 subunit. These results suggest that NO/O₂-induced apoptosis occurs by suppressing NF-κB activity.

Key Words: nitric oxide, hyperoxia, NF-κB, apoptosis, nuclear localization, protein carbonyl
INTRODUCTION:

Inhaled nitric oxide (iNO) is used clinically as a therapeutic modality to selectively manipulate the pulmonary vasculature for the treatment of persistent pulmonary hypertension of the newborn. In most cases, these patients receive simultaneous oxygen therapy with supraphysiological concentrations of oxygen. However, studies have shown that prolonged exposure to the combination of NO and hyperoxia (NO/O₂) causes significantly more lung injury than hyperoxia alone in several animal models, including piglets (1) and rats (2). This increased lung injury is associated with increased apoptosis of lung cells (3). We have previously shown that exposure of cultured pulmonary cells to NO and hyperoxia causes a synergistic cytotoxicity (4), similar to that observed in lungs of exposed animals. In addition, significantly more apoptosis has been found when isolated human neutrophils and primary cultures of normal human lung fibroblasts were exposed to NO/O₂ than those exposed to O₂ alone (5,6). These studies suggest that prolonged exposure to the combination of NO and hyperoxia has direct toxic effects on lung cells and the resulting injury and death of pulmonary cells may lead to impaired pulmonary function and lung injury.

NF-κB is a key redox-sensitive transcription factor in inflammatory diseases, where it regulates the inflammatory response by modulating the gene expression of cytokines, chemokines, and adhesion molecules (7,8). In addition, NF-κB has been shown to play a pivotal role in mediating cell proliferation and survival against a variety of cell death stimuli, including oxidative and nitrosative stress (9-12, 29-31). NO has also been shown to be closely associated with NF-κB. NF-κB is an essential transcription factor for the production of endogenous NO and acts by mediating the gene expression of iNOS (13,14). Exogenous NO can also affect NF-κB transcriptional activity by directly interacting with or modulating upstream pathways of its
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activation (15). The role of exogenous NO in modulating NF-κB activity in vitro is cell line-, NO donor-, and dose-dependent (15,16). In human peripheral blood mononuclear cells, NF-κB is activated by NO (17), while in human and bovine vascular endothelial cells, exogenous NO inhibits TNF-mediated NF-κB activation (18,19).

All normal cells are sensitive to hyperoxia, and suffer oxygen toxicity. Reactive oxygen species (ROS), especially superoxide, are thought to have a pivotal role in oxygen toxicity. In the presence of ROS, NO can form highly reactive species, including peroxynitrite, resulting in enhanced cytotoxicity (15). To delineate the role of the pathways induced by hyperoxia in the absence of oxygen toxicity, we utilized HeLa-80 cells, a mutant oxygen-resistant cell line. HeLa-80 cells, derived from HeLa cells, a cervical epithelial cell line, are capable of stable proliferation in 80% O₂, a lethal dose to many other cell types (20,21). In this study, we examined the cytotoxicity resulting from the exposure to NO±O₂ in HeLa-80 cells, determined the mode of cell death, and examined the role of NF-κB regulation in the cytotoxicity of NO/O₂.
EXPERIMENTAL PROCEDURES:

**Cell culture** - HeLa-80 cells and human lung adenocarcinoma A549 cells were grown and maintained at 37 °C as described previously (22). NIH 3T3 wildtype and RelA−/− cells (23) were cultured in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin in 95% room air and 5% CO₂. Cells were exposed to 0.5-7.5 mM DETA NONOate, a NO donor from Cayman Chemical, Ann Arbor, MI, in the presence or absence of 80% O₂ for up to 6 d as previously described (4,22). Media and gases were refreshed daily. Cell death/viability was determined by trypan blue exclusion or MTT assays (41). All experiments were performed independently and at least twice. The data were expressed as Mean ± standard error (SE), and analyzed for statistical significance using the unpaired Student’s T-test and analysis of variance (ANOVA) with p<0.05 considered significant.

**Assays for apoptosis** - The Annexin V incorporation assay, which detects the flipping of phosphatidylserine (PS) from the cytosolic surface to the extracellular surface, was performed as previously described (22). Briefly, cells were trypsinized and combined with cells detached during the exposure. After centrifugation, cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the vendor’s instructions (R&D systems, Minneapolis, MN). Ten thousand cells from each sample were analyzed on a Becton Dickson Flow Cytometer. Both Annexin V binding and cell shrinkage were used to characterize apoptosis (Cell Quest software, Becton Dickson, Franklin Lakes, NJ). Relative fluorescence units (FITC) higher than 210 or cell sizes smaller than 502 relative units (the Forward Scatter) were considered positive for apoptosis, while cells with relative fluorescence units (PI) higher than 250 were deemed necrotic. To assess caspase activation, cells were trypsinized and centrifuged at 200 x g for 5 min. The cell pellet was resuspended in cold cell lysis buffer at 25
μl per 1 x 10^6 cells according to the vendor’s instructions (R&D systems, Minneapolis, MN). At least 5 x 10^6 cells were collected for the caspase-3-like activity assay for each sample. Equal volumes of cell lysate and 2 x reaction buffer were mixed with DEVD-pNA for 2 h at 37°C. Colorimetric reactions were analyzed at 405 nm. Results were normalized to total protein content of each sample.

*Western Blotting* - After cell lysates were collected, protein concentrations were determined, and western blot analyses were performed as previously described (22). Briefly, 5 µg of protein of each sample were loaded onto 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) for electrophoresis. Polyclonal antibodies directed against IκBα, NF-κB p65 subunit, actin (Santa Cruz Biotechnology, Santa Cruz, CA) and nitrotyrosine (Cayman Chemical) were used for western blot analysis.

*Protein Carbonyl Analysis of p65* - Modification of NF-κB p65 subunit was assessed by determining the presence of carbonyl groups using a standard kit (Oxy-blot, Intergen Co., Purchase, NY, USA) following derivatization with 2,4-dinitrophenylhydrazine (DNPH) in the presence of trifluoroacetic acid according to the vendor's protocol. Five µg of each derivatized protein sample were loaded on 10% SDS-polyacrylamide gels and blots were probed with the primary and secondary antibodies supplied in the kit and developed as western blots. To determine whether some protein carbonyl bands contain NF-κB p65 subunit, blots were stripped and reprobed with a 1:1000 dilution of anti-p65 polyclonal antibody. To further confirm protein carbonyl modification of NF-κB p65 subunit, total p65 protein from cell lysates was immunoprecipitated by Agarose-conjugated with anti-p65 polyclonal antibody (Santa Cruz
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Biotechnology, Santa Cruz, CA) according to the vendor’s protocol. Protein carbonyl formation was then determined as described above in the resulting immunopurified p65.

**Immunofluorescent Analysis** - Cells grown on chamber slides were washed twice with PBS and fixed for 10 minutes in 10% buffered formalin as previously described (22). Briefly, slides were rinsed with PBS and incubated in a 1% BSA in PBS solution (Panvera Corp., Madison, WI) for 30 minutes. Cells were then incubated with anti-p65 NF-κB polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for one hour. Subsequently, the slides were washed and incubated for one hour with anti-rabbit IgG-rhodamine antibody (Boehringer Mannheim, Indianapolis, IN). The cells were then stained with 5 µg/ml DAPI (4’-6-Diamidine-2-phenylindole dihydrochloride; Boehringer-Mannheim, Indianapolis, IN) for 10 minutes.

**Cell Fractionation** - Nuclear extracts from HeLa-80 and A549 cells were prepared as described (24) from at least 4 x 10⁷ cells per sample. To prepare mitochondrial extracts, cells were washed with PBS, trypsinized, and pelleted at 1,500 x g. Cells were then washed and homogenized in cold fractionation buffer (0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 2 mM sodium citrate, 1 mM sodium succinate) using a Dounce homogenizer. Disruption of the plasma membrane was monitored by trypa blue staining. Lysates were centrifuged twice for 5 minutes at 2,000 x g to pellet nuclei and other debris. The supernatant was then centrifuged at 10,000 x g for 10 minutes. The remaining pellet was resuspended in a buffer containing 50 mM HEPES, pH 7.0, 500 mM NaCl, and 1% NP-40, supplemented with a cocktail of protease inhibitors, and collected as the mitochondrial extracts.

**NF-κB reporter assay** - To determine the levels of NF-κB transactivation activity, a reporter gene expression assay was performed as described (22). Briefly, HeLa-80 cells were
transiently cotransfected with pCMV-SPORT-β-galactosidase, pBlue, and a reporter plasmid containing 5 NF-κB binding sites inserted upstream of the luciferase reporter (Stratagene, La Jolla, CA). Cells were exposed to NO and 80% O₂ 24 h post-transfection. NF-κB activity was determined by luciferase activity (luminescence) with a Lumat LB9501 luminometer and normalized to β-galactosidase expression.

**Transient transfection of p65 mutant cells** - 3T3 RelA⁻/⁻ cells were transiently transfected with a plasmid containing the gene encoding NF-κB p65 subunit. Transient transfections were performed using the LipofectAMINE Reagent kit (Invitrogen, Carlsbad, CA) according to the vendor’s protocol.

**Electrophoretic Gel Shift (EMSA) assay** - Double-stranded NF-κB oligonucleotide (Promega, Madison, WI) was end-labeled with [³²P]γATP (PerkinElmer, Boston, MA) using T4 polynucleotide kinase in a Gibco labeling buffer at 37°C for 60 minutes. Binding reactions were performed by mixing 100,000 cpm of the above [³²P]-labeled probe with 5 µg of nuclear proteins, 1 µg poly dI-dC, 1 µg poly L-lysine, 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM NaCl, 1 mM DTT, 0.2% Tween20 (w/v), 30 mM KCl in a volume of 20 µl. Reactions were incubated for 30 minutes at room temperature before electrophoretic analysis on 6% non-denaturing polyacrylamide gels in 0.5x TBE buffer (0.045 M Tris-borate, 1 mM EDTA). Supershift analyses were performed by incubating nuclear proteins with polyclonal antibodies against NF-κB p65 (Santa Cruz Biotechnology) prior to adding [³²P]-labeled probe. The DNA-protein complexes were visualized by autoradiography at -80 °C for 12-24 h.
RESULTS:

Synergistic cytotoxicity of NO and hyperoxia in oxygen-resistant epithelial cells - To determine if the synergistic cytotoxicity induced by the exposure to NO/O_2 is primarily due to oxygen toxicity, HeLa-80 cells were exposed to 80% O_2 and 0.5 mM DETA NONOate either separately or in combination. These cells were viable and proliferated for 6 d when cultured either in room air or 80% O_2 (Fig. 1A). However, a decrease of cell viability was observed in cells exposed to NO/O_2 after 5 d, while cells exposed to NO alone were alive after 6 d (Figure 1A). To determine whether there is an increase in cell death in cells exposed to NO/O_2 than to NO, we determined the cytotoxicity of DETA NONOate) in HeLa-80 cells. Exposure to up to 2.5 mM DETA NONOate for 24 h caused little or no significant cell death, while 5-7.5 mM DETA NONOate induced marked cell death (data not shown). We therefore used 5 mM DETA NONOate in the subsequent experiments. After 24 h exposure to 5 mM NO, the amount of cell death was increased to 24±3.6%, while 81±8.1% (p<0.05) cells exposed to both 80% O_2 and 5 mM DETA NONOate were dead (Figure 1B). Results derived from MTT analysis further validated these results (data not shown). Because HeLa-80 cells tolerate 80% O_2, these data demonstrate that synergistic cytotoxicity of NO and hyperoxia is not due to pleiotropic oxygen toxicity.

Exposure to NO, either alone or in combination with hyperoxia, induces apoptosis - Stress from both ROS and reactive nitrogen species (RNS) have been shown to induce either apoptosis or necrosis in a dose- and cell type-dependent manner (25,26). To determine the mode of cell death in HeLa-80 cells exposed to 5 mM DETA NONOate, either alone or in combination with 80% O_2, Annexin V incorporation, cell shrinkage, nuclear condensation, and caspase activation were measured as indicators of apoptosis. Externalization of PS from the cytosolic
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surface to the extracellular surface is an early event in apoptosis (27). Using FITC-conjugated Annexin V to detect PS translocation and PI exclusion to detect membrane permeability, we analyzed the mode of cell death. More than 90% of the cells cultured in room air or hyperoxia were alive based on PI exclusion and Annexin V incorporation analyses (Table 1). Exposure to NO, either alone or in combination with hyperoxia, led to increased apoptosis (Table 1). At 24 h, NO induced apoptosis in 18±3.1% of the exposed cells, while NO/O₂ increased apoptosis to 79±3.8%. After 2 d, 68±0.5% and 95±1.4% of cells exposed to NO alone, or with O₂, respectively, were apoptotic. To demonstrate that apoptosis can be distinguished from necrosis, cells were exposed to 0.01% NP-40 for 0.5 min, and 73% were found to die by necrosis (Table 1).

To further characterize the mode of cell death, we examined cell size and nuclear morphology. Fig. 2A shows the nuclear morphology of exposed cells visualized with DAPI staining. Condensed chromatin was evident in the nuclei of cells exposed to NO±O₂ (Figure 2A). In addition, apoptotic bodies were apparent in cells exposed to NO/O₂ for 24 h (Figure 2A). As expected, cells cultured in room air or in hyperoxia alone showed no signs of nuclear condensation (Figure 2A). Cell shrinkage was quantified by flow cytometry analysis as illustrated in Figure 2B. Cell shrinkage was apparent in cells exposed to NO/O₂ (79% at 24 h, 97% at 48h) compared to those exposed to NO alone (25% at 24 h, 71% at 48 h). Figure 2C demonstrates the activation of caspase-3-like caspases using synthetic substrates. Caspase activity was induced in cells exposed to NO alone for 24 h, whereas such an increase in caspase activity was detected much earlier in NO/O₂ exposed cells (6 h). To test whether caspase activation plays a causal role in cell death induced by these exposures, cells were pretreated for 60 min with 50 µM Z-DEVD-FMK (Calbiochem), a cell-permeable caspase-3-like inhibitor,
prior to the exposure to NO±O₂. Ninety nine (± 1.0 %) or 90±1.6% of cell death, induced by either exposure to NO alone or to NO/O₂, respectively, was prevented or delayed with this pre-treatment, suggesting that caspase activation is necessary for NO±O₂-induced cell death. These data demonstrate that 5 mM DETA NONOate induces significant amounts of apoptosis in HeLa-80 cells, which is significantly enhanced with hyperoxia, even though hyperoxia alone is not toxic to these cells.

*Suppressed NF-κB activity in cells exposed to NO and hyperoxia* - NF-κB has been shown to play a crucial role in mediating cell survival under nitrosative stress (11,12). To determine whether NF-κB activity is regulated upon exposure to NO±O₂, we assayed for the transcriptional activity of NF-κB using a luciferase reporter assay as described previously (22). Constitutive NF-κB activity was detected in HeLa-80 cells (Figure 3). There was a moderate decrease in NF-κB activity when cells were grown in 80% O₂ compared to basal levels in room air (Figure 3A). However, NF-κB activity was significantly suppressed in cells exposed to NO. This NO-induced suppression of NF-κB activity was further pronounced by the addition of hyperoxia. Figure 3A shows that the synergistic suppression of NF-κB activity was apparent by 6h (p<0.05). By 24 h, most of the NF-κB activity was inhibited in cells exposed to NO either alone or in combination with hyperoxia. In addition, NF-κB binding activity in nuclei of HeLa-80 cells was determined by EMSA. As a positive control, A549 cells were treated with TNFα for 30 min to induce NF-κB activation (22). A similar NF-κB DNA binding complex was detected in nuclear extracts prepared from cells cultured in room air. Exogenous addition of anti-p65 antibodies supershifted this band, suggesting that p65 is one of the components of this complex. Exposure to NO/O₂ for 16 h significantly reduced this binding activity (Figure 3B).
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Repression of NF-κB activity increases susceptibility to the cytotoxicity of NO/O₂ - We have previously demonstrated that NF-κB provides a protective role in hydrogen peroxide-induced apoptosis in epithelial cells (22). To investigate any causal effects of NF-κB suppression in NO/O₂-induced cell death, we exposed fibroblasts lacking a functional NF-κB p65 subunit, RelA(p65)−/−, to 95% O₂ and 0.5 mM DETA NONOate. Similar to the observations in HeLa-80 cells and lung epithelial A549 cells, exposure of fibroblasts to the combination of 0.5 mM DETA NONOate and 95% O₂ induces a synergistic cytotoxicity (data not shown). Figure 4 shows that mutant cells lacking functional NF-κB were hyper-sensitive to NO/O₂ exposure relative to their wild type counterparts. After 2 d of exposure to NO/O₂, 46±2.9% of wildtype cells died compared to 96±1.2% of the mutant cells (Fig. 4). This difference in cytotoxicity in response to the exposure to NO/O₂ is not due to a difference in their growth rate because mutant cells proliferate at a similar rate as the wildtype fibroblasts (data not shown). To test whether the addition of functional p65 can rescue such injury, RelA−/− cells were transfected with a p65 expression construct. This resulted in the rescue of (36±5% after 1 d and 37±4% after 2 d, p<0.05) from injury caused by exposure to NO/O₂. These data indicate that repression of NF-κB increases susceptibility to the cytotoxicity induced by NO/O₂.

Degraded IκBα and decreased nuclear localization of NF-κB in exposed cells - Inactive NF-κB is sequestered in the cytoplasm by IκB proteins, the inhibitors of NF-κB. Upon activation, IκB is phosphorylated and degraded (28). NF-κB is then released and translocates to the nucleus, regulating gene expression. It has been shown that NO regulates NF-κB activity by increasing the steady state levels of IκB by enhancing its mRNA stability or reducing its phosphorylation (18,19). To determine the status of IκB in cells exposed to NO±O₂, we
examined the steady-state levels of IκBα by western blots. As illustrated in Figure 5, IκBα levels were decreased in cells exposed to NO for 24 h. This reduction was more pronounced in cells exposed to NO/O2 for 24 h. Therefore, a lack of degradation of IκB is clearly not responsible for the reduction of NF-κB activity. We then examined if the reduced activity is due to decreased NF-κB expression. While the protein level of NF-κB p65 subunit was maintained upon exposure to NO/O2 for up to 16 h, a decrease was detected in cells exposed to NO/O2 for 24 h (Figure 5). However, the decrease was only moderate, compared to the 85±2% reduction of NF-κB activity observed after only 6 h exposure (Figure 3). As a loading control, the steady-state levels of actin were examined. Figure 5 shows that there was no substantial changes in the levels of actin upon exposure to NO±O2, suggesting that the effects of NO±O2 on IκBα and p65 is specific. These data suggest that the reduction of NF-κB activity upon exposure to NO±O2 cannot be attributed primarily to either decreased p65 expression or increased expression of its inhibitor.

We further assessed the level of nuclear p65 to determine whether the reduced NF-κB activity in cells exposed to NO±O2 was due to its decreased nuclear translocation. Corresponding to the constitutive NF-κB activity in HeLa-80 cells, NF-κB p65 subunit was evident in the nuclear fraction of these cells. Exposure to NO±O2 decreased the level of nuclear p65 compared to the controls (Figure 6). As a positive control for nuclear translocation, A549 cells were exposed to 10 ng/ml TNFα for 30 min. This treatment induced significant p65 nuclear translocation (Figure 6), as shown previously (22). We further performed immunofluorescent studies in HeLa-80 cells to confirm the results derived from cell fractionation studies of p65 nuclear translocation. As shown in Figure 7, room air control cells had marked nuclear
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localization of the p65 subunit, while less was observed in cells exposed to either NO or NO/O₂, with nuclear evacuation in some cells exposed to NO/O₂. These studies demonstrate that the suppression of NF-κB activity correlates with a reduction in nuclear p65 in cells exposed to NO±O₂.

To characterize the mechanism responsible for the reduced nuclear p65 in cells exposed to NO/O₂, we assayed for protein carbonyl modifications. Figure 8 shows that exposure to NO/O₂ increased protein carbonyl formation. We observed that a protein carbonyl band around 65 kD (Lane 3 in Figure 8) co-migrated with NF-κB p65 subunit (Lane 7). To further confirm protein carbonyl formation on NF-κB p65 subunit, p65 was immunoprecipitated and protein carbonyl formation was determined on the resulting immunopurified p65. Lanes 9 and 10 in Figure 8 show that protein carbonyl formation on NF-κB p65 subunit was indeed increased in cells exposed to NO/O₂, compared to the controls.
DISCUSSION:

In this report, we demonstrate that exposure to NO and hyperoxia are synergistic in inducing caspase-mediated apoptosis in oxygen resistant HeLa-80 cells. The extent of apoptosis correlated with the degree of NF-κB suppression. Instead of increasing the steady-state levels of IκBα or decreasing protein levels of the p65 subunit, exposure to NO±O₂ suppressed NF-κB activity by markedly reducing the nuclear localization of NF-κB p65 subunit. Using fibroblasts lacking functional NF-κB, we further demonstrated an inverse relationship between NF-κB and cell susceptibility to NO/O₂.

Studies presented in this report indicate that oxygen resistant HeLa-80 cells are not only resistant to hyperoxia, but also less sensitive to RNS-induced cytotoxicity. The mode of cell death under oxidative and nitrosative stresses is dose-dependent (25,26). In this report, high doses of NO (up to 5 mM DETA NONOate) were used to induce cell death. We anticipated that such high concentrations of NO would cause necrotic cell death, especially when these cells are simultaneously exposed to hyperoxia. However, our data clearly demonstrated that cells exposed to NO had typical apoptotic morphological characteristics (cell shrinkage, nuclear and chromatin condensation) and hyperoxia potentiated this response. In addition, increased Annexin V incorporation, augmented caspase activity, and attenuated apoptosis in the presence of synthetic inhibitors against caspase activation indicate that apoptotic pathways are intact under high doses of nitrosative stress. It is unclear how HeLa-80 cells metabolize the potentially high levels of ROS and RNS generated during the exposure to high concentrations of hyperoxia and NO. Joenje and colleagues have shown that activities of the classic antioxidant enzymes, such as SOD and catalase, in HeLa-80 cells are not induced as compared to their parental cells (20).
addition, similar levels of constitutive NF-κB activities were observed between HeLa-80 and the parental, non hyperoxia resistant HeLa cells (WFR and LLM, unpublished results). Regardless, studies presented in this report suggest that the mechanisms underlying oxygen resistance in these cells may also protect against nitrosative stresses.

Results presented in this report indicate that NF-κB plays a pivotal role in regulating NO/O2-induced cytotoxicity. This is supported by the correlation between the suppressed NF-κB activity and the cytotoxicity observed in HeLa-80 cells, as well as by studies using p65 mutant cells. First, transactivation of NF-κB reporter genes in cells exposed to NO±O2 revealed a reduction of NF-κB activity (Figure 3A). In addition, EMSA analysis in HeLa-80 nuclear extracts indicated that exposure to NO/O2 suppresses NF-κB DNA binding activity (Figure 3B). This suppression of NF-κB activity is correlated with the cell death observed in HeLa-80 cells. Furthermore, mutant fibroblasts with no NF-κB p65 have increased susceptibility to the toxic effects of NO/O2 (Figure 4) and this increased susceptibility can be rescued by an expression of NF-κB p65.

NF-κB activation can be modulated by NO at three or more different steps (15). The first step involves the activation of pathways upstream of IκB degradation by enhancing IκBα at the mRNA and protein levels, thereby reducing NF-κB activity (12,18,19,32,33). To our surprise, exposure to NO±O2 enhanced IκBα degradation in HeLa-80 cells, demonstrating that modulation of IκBα degradation is not contributing to the suppression of NF-κB activity in these cells. Binding to its consensus DNA sequence is another site for regulating NF-κB activity. The DNA binding activity of NF-κB has shown to be inhibited by NO through S-nitrosylation of the DNA binding subunit, (p50) of NF-κB in lung epithelial cells and macrophages (18,34).
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Although our study did not focus on the p50 subunit, it is likely that the S-nitrosylation of p50 subunit takes place in the exposed cells due to the high concentrations of NO used in these studies. In addition, we have detected nitrotyrosine formation on p65 (WRF and LLM, unpublished results) and p65 is one of the components in the DNA binding complex (Figure 3B). However, it is unclear whether this modification plays any role in the reduced DNA binding activity because exposure to NO/O₂ significantly reduced nuclear translocation of p65 (Figures 6-7). The third site for modulation involves nuclear translocation of NF-κB. We have demonstrated that exposure to NO±O₂ induced a marked reduction in nuclear localization of NF-κB p65 subunit (Figures 6-7). Although the mechanism for reduced nuclear localization of p65 is unclear, our studies indicate that exposure to NO/O₂ induced protein carbonyl modification of the p65 subunit (Figure 8). While there is no direct evidence that protein carbonyl modification reduces nuclear translocation, carbonyl modification of the nuclear localization sequence (NLS) of HIV-1 proteins by reverse transcriptase inhibitors prevents the binding of NLS to the nuclear transportation machinery (35). In addition to altering post-translational modification, NF-κB activity could also be down-regulated via decreased gene expression. However, exposure to the combination of NO and hyperoxia did not significantly reduce NF-κB at the protein level until 24 h (Figure 5, Figure 8, lanes 5 and 7). This reduction was much later than that of the suppressed NF-κB transactivation activity (6 h) (Figure 3A). These data further support the notion that post-translational modification of NF-κB plays a major role in reducing its transcription activity.

Although iNO in combination with hyperoxia is used in the management of pulmonary hypertension in newborn infants, there are no clearly established dose guidelines for the clinical application of iNO/O₂ or the method of weaning this therapy. Concentrations as high as 80 ppm,
which corresponds to approximately 3.2 uM NO (39), have been used in clinical trials for critically ill patients (40, 42). This is comparable to the amount of NO generated by 1.6 mM DETA NONOate in cell culture (4). The overall effect of iNO on hyperoxic lung injury is somewhat controversial, perhaps reflecting the differences in the doses of iNO and O\textsubscript{2} applied, the systemic effects and the response of different animal species. Nevertheless, studies in cell cultures indicate that direct exposure to NO has a deleterious effect on hyperoxic cell injury, although the extent is cell type- and donor-dependent (4-6). Studies presented in this report further demonstrate that the combination of NO and hyperoxia act synergistically in inducing cytotoxicity in oxygen-resistant cells, even when concentrations of each agent were individually non-cytotoxic (Figure 1A). Such cytotoxicity is at least partially due to suppressed NF-κB activity via reduced nuclear localization of NF-κB p65 subunit in cells exposed to NO±O\textsubscript{2}. Therefore, in light of the approved clinical use of iNO in newborn infants, we recommend considering the potential harmful effects of such therapy to the lung, especially to lung epithelial cells that are directly exposed during long term-application. Perhaps, relatively lower doses of NO and shorter exposure times during the therapy would limit or minimize such toxicity.
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REFERENCES:


FIGURE LEGENDS:

**Figure 1.** Synergistic cytotoxicity induced by the exposure to NO and hyperoxia in oxygen-resistant HeLa-80 cells. Subconfluent cell cultures were exposed to NO donor (DETA NONOate) in the presence or absence of O_2 (80%) for up to 6 d as indicated. Trypan Blue Inclusion was used to determine cell death. A. 0.5 mM DETA NONOate. B. 5 mM DETA NONOate. Values are means ± SE (n=9).

**Figure 2.** Exposure to NO and hyperoxia induces apoptosis. HeLa-80 cells, seeded either on chamber slides (A), or on six-well plates (B), or on 100 mm plates (C), were exposed to 5 mM DETA NONOate and 80%O_2, either alone or in combination, for up to 48. Cells cultured in room air were used as controls. A. **Nuclear morphology.** Cells cultured on chamber slides were stained with DAPI to visualize all nuclei. B. **Cell size.** Cell sizes were determined using the forward scatter with a Becton-Dickson Flow Cytometer. Cells smaller than 502 RFU were scored as apoptotic. C. **Caspase-3-like activity.** Caspase-3-like activity was assayed by colorometric reactions using caspase-3 substrates in total cell lysates as described in the Experimental Procedures. The caspase-3 activity in cells exposed to NO and hyperoxia, either alone or in combination, were normalized to total proteins. A significant increase in the caspase-3-like activity was observed with increased exposure to NO and hyperoxia over the cells cultured in room air. Values are means ± SE (n=9).

**Figure 3.** Suppression of NF-κB activity in cells exposed to NO in combination with hyperoxia. A. **Transactivation assay.** Activity of NF-κB was assayed in the exposed cells that were transiently transfected with a luciferase reporter and β-galactosidase constructs. NF-κB activity was normalized to β-galactosidase expression and synergistically decreased over time.
when exposed to NO in the presence of hyperoxia. A significant reduction of NF-κB activity was observed after exposure to NO, either alone or in combination with hyperoxia. Values are means ± SE (n=9). **B. DNA binding assay.** 

$^{32}$P-Labeled probe was incubated with nuclear extracts prepared from HeLa-80 cells either cultured in room air (RA) or exposed to the combination of NO and hyperoxia for 16 h (NO/O$_2$). The DNA-protein complex indicated by the Arrow I was detectable only in the room air sample. Such a complex was not detectable upon exposure to NO/O$_2$. This complex was supershifted (indicated by the Arrow II) in the presence of anti-NF-κB p65 (lane 2). As a positive control for the NF-κB DNA complex and its supershift product with the p65 subunit, EMSA assay was performed in nuclear extracts prepared in A549 cells treated with TNFα (Lanes 5-6). This supershifted band was very close to the top of gels, similar to the observation by others (43). “+” and “-“ indicate whether EMSA was performed in the presence or absence of anti-NF-κB p65.

**Figure 4. Abolishing NF-κB function increases the susceptibility to NO and hyperoxia-induced cytotoxicity.** Both wild type (WT) NIH 3T3 cells and cells lacking the functional NF-κB p65 subunit (Rel A$^{-/-}$) were exposed to NO/O$_2$ (0.5 mM DETA NONOate/95% O$_2$) for up to 3 d. Cell viability was determined by Trypan Blue Exclusion. Significant difference in cell viability after the exposure was seen between WT and mutant cells. Values are means ± SE (n=9).

**Figure 5. Molecular mechanisms of suppression of NF-κB activity by NO and hyperoxia.** Five µg of total cell lysate from HeLa-80 cells exposed to 5 mM DETA NONOate and 80% O$_2$, either alone or in combination, for the times indicated. The steady state levels of IκBα, NF-κB p65 subunit, and actin were determined by western blots.
Figure 6. Decreased nuclear NF-κB in NO±O$_2$ exposed cells. HeLa-80 cells, grown on 100 mm plates, were exposed to 5 mM DETA NONOate and 80% O$_2$, either alone or in combination, for 16 h. Levels of p65 subunit in nuclear extracts were determined using SDS-PAGE/western blots with antibodies against NF-κB p65 subunit. As a positive control for the presence of p65 in nuclear extracts, levels of the p65 subunit were also determined in nuclear extracts prepared in A549 cells with or without the treatment of TNFα for 30 min (Lanes 4-5).

Figure 7. Suppression of NF-κB nuclear localization by NO and hyperoxia. HeLa-80 cells, grown on chamber slides, were exposed to 5 mM DETA NONOate in the presence or absence of 80% O$_2$, for 16 h. Levels of p65 subunit were determined in intact cells via immunofluorescence with antibodies against NF-κB p65 subunit.

Figure 8. Protein carbonyl modification of NF-κB p65 subunit. Lanes 1-4 show the analysis of protein carbonyl formation carried out as described in the Experimental Procedures. Lanes 5-8, the same blot showed in lanes 1-4 was stripped and re-probed with antibodies against NF-κB p65 subunit. “+” and “-“ indicate whether the sample was treated with DNPH for detecting the protein carbonyl formation. Lanes 9-10, protein carbonyl analysis performed with the immunoprecipitated p65 subunit. Lane 1, 2, 5, 6 and 9 are room air samples; lanes 3, 4, 7, 8 and 10 are samples from exposed cells. RA, room air control; NO/O$_2$, cells were exposed to NO/O$_2$ for 16 h.
Table 1. Flow cytometry analysis of HeLa-80 cells exposed to NO±O₂. Annexin V incorporation and PI incorporation was measured by flow cytometry to assess the mode of cell death. Relative Fluorescence Units represents the intensity of Annexin V incorporation or PI incorporation. The percent cells undergoing apoptosis or necrosis was determined as described in the Experimental Procedures. Cells treated with NP-40 were used as controls for necrosis. Values are Means±SE (n=6).

<table>
<thead>
<tr>
<th></th>
<th>RA(%)</th>
<th>O₂(%)</th>
<th>NO(%)</th>
<th>NO/O₂(%)</th>
<th>NP40(%)</th>
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<tr>
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<td>24 h</td>
<td>16 h</td>
<td>24 h</td>
<td>48 h</td>
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<td>Normal</td>
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<td>Necrotic</td>
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<td>4±0.2</td>
<td>4±0.7</td>
<td>8±1.9</td>
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</tbody>
</table>
Figure 3

A

Relative Activity

RA  O₂  6  16  24

Exposure (h)

B

RA  NO/O₂  TNFₐ

-  +  -  +  -  +

1  2  3  4  5  6
Figure 4

The graph shows the cell viability (%) over different exposure times (d) to NO/O2. The x-axis represents the exposure time, and the y-axis represents the cell viability. The data is plotted for two groups: WT and Rel A -/-.
Figure 5

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- **IkBα**
- **NF-κB**
- **Actin**
Suppression of nuclear factor-KAPPA B activity by nitric oxide and hyperoxia in oxygen resistant cells

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