Nitric Oxide–Induced Cytotoxicity: Involvement of Cellular Resistance to Oxidative Stress and the Role of Glutathione in Protection

M. WHIT WALKER, MICHAEL T. KINTER, ROBERT J. ROBERTS, AND DOUGLAS R. SPITZ

University of Virginia Health Sciences Center, Division of Neonatology, Departments of Pediatrics and Pathology, University of Virginia Hospital, Charlottesville, Virginia 22908 [M.W.W., M.T.K., R.J.R.], and Mallinckrodt Institute of Radiology, Radiation Oncology Center, Section of Cancer Biology, Washington University Medical Center, St. Louis, Missouri 63108 [D.R.S.]

ABSTRACT

A series of experiments were designed to examine the potential cytotoxicity of nitric oxide (NO), or reactive species derived from NO, in HA1 fibroblasts and H₂O₂-resistant variants of this cell line, designated OC14 cells. A 1-h exposure at 37°C to a 1.7 mM bolus dose of NO, prepared in N₂-gassed medium, significantly reduced clonogenic survival in the HA1 fibroblasts line to 60% of control cells treated with N₂-gassed medium alone. The OC14 cells were found to be completely resistant (100% survival) to NO-mediated injury in comparable experiments. A second set of experiments was designed to determine the role of the intracellular antioxidant, glutathione, in protection against NO-mediated injury. Depletion of total glutathione resulted in a significant reduction in HA1 and OC14 clonogenic survival to 8% and 50% when compared with respective control cells. The effect of total glutathione depletion on NO-initiated toxicity in HA1 cells was dose- and cell-density dependent and was observed to occur within 5 min of exposure to NO. Further evidence of cytotoxicity was demonstrated by loss of trypan blue dye exclusion properties in glutathione-depleted HA1 cells after

NO exposure. Other experiments demonstrated that nitrate and nitrite exposure produced no cytotoxicity in glutathione-depleted HA1 cells and that coincubation of NO-saturated medium with oxyhemoglobin inhibited NO-induced cytotoxicity in glutathione-depleted HA1 cells. These results demonstrate that 1) nitric oxide, or an NO-derived reactive nitrogen species other than nitrites or nitrates, is responsible for reduction in clonogenic survival and trypan blue dye exclusion capabilities *in vitro*; 2) biochemical pathways associated with cellular resistance to oxidative stress also confer resistance to NO-mediated injury in this cell model; and 3) total glutathione content determines a significant portion of cell sensitivity to NO-mediated cytotoxicity. (*Pediatr Res* 37: 41–49, 1995)

Abbreviations

BSO, L-buthionine-S,R-sulfoximine
NO, nitric oxide
NO₂, nitrogen dioxide
MEM, minimal essential medium

NO is a free radical molecule endogenously produced by a variety of cell types. NO has been demonstrated to participate in physiologic reactions involving regulation of vasomotor tone, neurotransmission, immune system function, and platelet aggregation (1–4). Recent clinical trials have demonstrated some efficacy for inhaled NO in treatment of neonatal pulmonary hypertension and adult respiratory distress syndrome (5–7). Paradoxically, elevated concentrations of atmospheric NO are known to be harmful in many animal models, and lethal exposures at high concentrations have been reported in both animals and humans (8–10). This same paradoxical beneficial and/or toxic effect of NO has been observed *in vitro*. Several

investigators have reported that endogenously derived NO, or NO released from pharmacologic agents, can function in cytoprotective responses to injury initiated by ischemia-reperfusion or other pathologic conditions (11–14). Conversely, other investigators report that endogenously generated or exogenously administered NO may initiate or contribute to cytotoxic reactions seen in neural, cardiac, and endothelial tissue after similar experiments designed to evaluate the interactions of NO and ischemia-reperfusion (15–18). Clearly, NO can have beneficial, as well as cytotoxic, properties depending on the system and initial conditions that are being studied.

Recently, Wink *et al.* (19) demonstrated that aqueous phase NO in the presence of oxygen reacted to form a reactive nitrogen oxide that is both a potent oxidant and a nitrosating agent. This reactive nitrogen oxide, NO_x, was determined not to be one of the commonly proposed reactive nitrogen species, such as peroxyxynitrite (OONO[•]), NO₂[•], N₂O₃, or NO⁺ (19).

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Correspondence: Dr. M. Whit Walker, Department of Neonatology, Greenville Hospital Systems, 701 Grove Road, Greenville, SC 29605.

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Because this NO_x species derived from NO is a potent oxidant, we hypothesized that biochemical pathways associated with resistance to oxidative stress might also confer resistance to NO-mediated toxicity associated with exposure to NO in the aqueous phase.

The goal of our study was to characterize the toxic potential of bolus exposure to aqueous NO in a cell culture system and to investigate mechanisms of resistance to oxidative stress as potential modifiers of NO-mediated cytotoxicity. Specifically, we chose to examine the clonogenic survival response and trypan blue dye exclusion capabilities of HA1 Chinese hamster fibroblasts after exposure to NO in comparison with similar experiments with OC14 fibroblasts, which have been selected for resistance to hydrogen peroxide-mediated oxidative stress (20). We also depleted the intracellular total glutathione content of both cell types with BSO to test the hypothesis that intracellular stores of free, nonprotein, reduced thiols, known to actively bind NO, would contribute to resistance from NO-mediated cytotoxicity.

METHODS

Cells and culture conditions. Chinese hamster fibroblasts (HA1) and H₂O₂-resistant variants derived from HA1 cells (designated OC14 cells) were cultured as described (20). Briefly, cells were passaged in Eagle's MEM with 10% FCS and grown to confluence (8×10^6) in T-75 cm² flasks over 48 h. Cells were trypsinized (0.1% trypsin, 0.53 mM EDTA, in Puck's saline) and plated in 60-mm dishes such that at the beginning of each experiment (36–48 h postplating), each dish contained $1\text{--}2 \times 10^6$ cells. Cultures were maintained in a room air, 5% CO₂, humidified, 37°C incubator (NUAIRE, Plymouth, MN) for the duration of the experiment. Treatment medium was Eagle's MEM without FCS or phenol red (to prevent inadvertent binding of NO). BSO (Sigma Chemical Co., St. Louis, MO), was added to selected dishes in a 250 μM concentration and allowed to incubate for 16 h before exposure to treatment medium to deplete total intracellular glutathione. After BSO pretreatment, total cellular glutathione was below the limits of assay detection (0.2 μg/mg protein). Concentrations of NO, oxyhemoglobin, and nitrites or nitrates used are specified in the figures. All treatments were for a duration of 1 h at 37°C unless otherwise indicated.

Clonogenic survival and trypan blue exclusion. Clonogenic survival was determined in trypsinized cultures by a method used routinely in our laboratory (20, 21). Normal sham-treated plating efficiencies for HA1 and OC14 cells range from 60 to 90% and were determined in each experiment. Sham-treatment consisted of exposure to medium prepared by nitrogen gassing. Survival data from reactive nitrogen species-treated cells were compared with sham-treated or BSO-treated control plating efficiencies and plotted as a function of time or dose. Trypan blue dye exclusion was done *in situ* by washing the treated cells with saline and adding 0.04% trypan blue dye in isotonic saline. After a 5-min incubation, the dye was washed off the dish with saline and the number of cells excluding dye was expressed as a percentage of 100 cells counted from two randomly chosen areas of the dish. All experiments were

accomplished in duplicate, unless otherwise stated. Within each experiment, treatment groups were tested in triplicate and each treatment culture dish was subsequently plated for clonogenic survival in triplicate.

Preparation of NO solutions. N₂-gassed medium was prepared at 25°C by bubbling FCS-free, phenol red-free Eagle's MEM (GIBCO, Grand Island, NY) for 15 min with N₂ in gas-tight containers fitted with gas-tight stoppers and a bleeder valve. For experiments, NO-containing medium was prepared by bubbling approximately 100 mL of 99.99% NO gas (Matheson Gas Co., Cucamonga, CA) into 100 mL of N₂-gassed medium for 3 min. Because NO bubbling in excess of 4–7 mL gas/100 mL medium resulted in "supersaturated" solutions of NO, pH of the medium was monitored and adjusted with NaOH or HCl to maintain a range of 7.2 to 7.8. Dilutions of saturated NO medium were made by mixing appropriate concentrations of the N₂-gassed control medium with NO-containing medium in gas-tight syringes. NO, dissolved in medium, was assayed using a chemiluminescence detection method based on the reaction of NO with ozone. Quantitative measurements of NO in medium were made with a Sievers Research Redox Chemiluminescence Detector, model NOA 270 (Sievers Research, Boulder, CO). The lower limit of detection by this method is 22 pmol (22, 23).

Nitrite determination and oxyhemoglobin preparation. Nitrite (NO₂[−]) content was determined spectrophotometrically using a procedure based on the Griess reaction (24). Sample aliquots were mixed with the Griess reagent that contained 1 part 0.1% naphthylethylenediamine dihydrochloride in distilled H₂O plus 1 part 1% sulfanilamide in 5% concentrated H₃PO₄, and absorbance was determined at 546 nm. For quantification of nitrite in the medium, culture medium was deproteinized with 35% sulfosalicylic acid and centrifuged at $10\,000 \times g$ for 15 min, and the supernatant was combined with 5% aqueous NH₄Cl buffer and 5% NaOH before analysis. Oxyhemoglobin was prepared by reducing bovine crystalline Hb (Sigma Chemical Co.) with 10 M equivalents of sodium dithionite. Excess reducing agent was removed from the Hb solution by passage over a Sephadex G25 column and spectrophotometric analysis confirmed the presence of bovine deoxyhemoglobin by the absorbance at 431 nm (25). Oxyhemoglobin was formed upon exposure of deoxyhemoglobin to 21% oxygen and confirmed spectrophotometrically by the absorbance peak at 414 nm. Oxyhemoglobin was added, at 100 μM final concentration, to selected treatment cultures immediately before NO addition. Spectrophotometric analysis for the presence of methemoglobin, the final metabolic product of the NO-oxyhemoglobin reaction, was performed after NO treatment by absorbance peak determination at 406 nm.

Glutathione assay. Total glutathione (reduced glutathione + oxidized glutathione) was determined by an oxidized glutathione reductase recycling assay routinely used in our laboratory (26). All data were normalized per mg protein.

Statistical analysis. Analysis of clonogenic survival was performed using analysis of variance for multiple points. Statistical significance at $p < 0.05$ was confirmed using Bonferroni's comparison. Analysis of trypan blue dye exclusion and total

glutathione were performed using the *t* test with statistical significance at $p < 0.05$.

RESULTS

The cytotoxicity of exogenously administered NO was determined by exposing immortalized Chinese hamster fibroblasts, HA1 cells, to known concentrations of NO for specific periods of time. Injury was quantitated by clonogenic cell survival and trypan blue dye exclusion. As shown in Figure 1, exposure of HA1 fibroblasts to saturated, 1.7 mM NO-containing medium for 1 h caused a 40% reduction in clonogenic cell survival relative to control cells exposed to N₂-gassed medium alone. These conditions produced no differences in the percentage of cells capable of excluding trypan blue dye between control and NO-treated HA1 cells (>95% exclusion). Figure 1 also demonstrates that depletion of total intracellular glutathione, performed before NO exposure, results in a significant reduction in clonogenic cell survival in HA1 cells to <20%. Glutathione depletion alone had no effect on HA1 clonogenic survival. An additional manifestation of NO-mediated cytotoxicity seen in total glutathione-depleted HA1 cells was the loss of the ability to exclude trypan blue dye (>95% loss) (Fig. 1). Thus, the total glutathione-depleted HA1 cells were significantly more sensitive to NO-mediated toxicity than non-glutathione-depleted HA1 cells as determined by both clonogenic survival and trypan blue dye exclusion.

The significant reduction in clonogenic cell survival in NO-treated, total glutathione-depleted HA1 cells was both dose dependent and cell density dependent. Total glutathione-depleted HA1 cells had an almost linear reduction in clono-

genic cell survival as NO concentration increased from 250 to 1700 μ M (Fig. 2A). Increasing the cell density per culture dish of total glutathione-depleted HA1 cells resulted in increased clonogenic cell survival after exposure to saturated, 1.7 mM, NO-containing medium (Fig. 2B).

Clonogenic survival of total glutathione-depleted HA1 cells was markedly decreased after NO-treatment as brief as 5 min (Fig. 3). This rapid reduction in clonogenic cell survival was not significantly enhanced by longer exposures to saturated concentrations (1.7 mM) of NO. However, exposure of total glutathione-depleted HA1 cells to a relatively low dose of NO (0.17 mM) for up to 6 h did not significantly affect clonogenic survival (data not shown).

Experiments to determine the cytotoxic effects of NO, with and without intracellular total glutathione depletion, were repeated in OC14 cells to further delineate the protective contribution of adaptation to oxidative stress and total glutathione to NO-induced cytotoxicity. OC14 cells are known to contain 2- to 3-fold greater concentrations of total glutathione compared with HA1 cells (26). In contrast to HA1 cells, OC14 cells did not exhibit significant reductions in clonogenic cell survival after 1-h exposures to saturated NO solutions (Fig. 4). NO exposure resulted in a significant reduction in clonogenic cell survival in total glutathione-depleted OC14 cells to 50% of controls. However, OC14 cells were relatively more resistant to NO-induced injury after total glutathione depletion than were the total glutathione-depleted HA1 cells (50 *versus* 8% surviving fraction in experiments with equal cell-plating densities) despite total glutathione levels below the limits of assay detection (0.2 μ g/mg protein) in both cell lines. Both HA1 and OC14 cell types exhibited significantly decreased total gluta-

Figure 1. Effects of total glutathione depletion on clonogenic survival and trypan blue exclusion demonstrated by NO-treated HA1 cells. Effects of a 60-min exposure to saturated (1.7 mM) NO solutions on clonogenic survival and trypan blue dye exclusion of HA1 fibroblasts with and without BSO pretreatment. Total glutathione content in BSO-treated HA1 cells was below the assay limit of detection. Data expressed as mean $\pm$ SD. An asterisk represents $p < 0.05$ vs respective control.

Figure 2. A, Effects of increasing concentrations of NO on clonogenic survival of total glutathione-depleted HA1 cells. B, Effects of increasing cell density on clonogenic survival of 1.7 mM NO-treated, total glutathione-depleted HA1 cells. Clonogenic survival of BSO-pretreated HA1 fibroblasts after a 60-min exposure to increasing concentrations of NO. Data expressed as mean $\pm$ SD. An asterisk represents $p < 0.05$ vs control.

thione levels after exposure to NO alone (Fig. 5). The disappearance of total glutathione in both cell lines after NO treatment suggests glutathione was being consumed during the treatment interval. This result, in combination with results using total glutathione-depleted HA1 and OC14 cells (Figs. 1 and 4), indicate that reactions involving glutathione play an important cytoprotective role during exposure to NO.

Aqueous NO is rapidly converted to other species after exposure to oxygen (19). Chemiluminescent quantification of saturated NO solutions demonstrated a NO concentration of 1.7 mM at 25°C before exposure to oxygen. No nitrites could be detected in stock N_2 -gassed phenol red- and serum-free MEM using the Griess reaction. No brown gas (indicative of gas phase NO_2 formation after reaction of NO + oxygen) was

Figure 3. Effects of exposure duration of total glutathione-depleted HA1 cells exposed to saturated NO-containing solutions. Clonogenic survival of BSO pretreated HA1 fibroblasts after increasing durations of exposure (5–60 min) to saturated NO-containing medium. Data expressed as mean $\pm$ SD. An *asterisk* represents $p < 0.05$ vs control.

Figure 4. Effects of total glutathione depletion on clonogenic survival of NO-treated OC14 fibroblasts. Clonogenic survival of OC14 fibroblasts with and without BSO pretreatment after a 60-min exposure to saturated concentrations (1.7 mM) of NO. Total glutathione content in BSO-treated OC14 cells was below the assay limit of detection. Data expressed as mean $\pm$ SD. An *asterisk* represents $p < 0.05$ vs respective control.

present in any medium bottle at any time during the experiment. To evaluate whether nitrites or nitrates could represent the cytotoxic agent responsible for clonogenic inactivation in the previous NO experiments, total glutathione-depleted HA1 cells were incubated with 5 mM nitrite or nitrate. No signifi-

cant change in clonogenic survival was seen with either nitrate or nitrite after 1 h of treatment (Fig. 6).

Finally, experiments were designed to evaluate the protective effects of coincubating NO with oxyhemoglobin, a molecule known to have potent, irreversible binding affinity for NO

Figure 5. Total cellular glutathione content of HA1 and OC14 fibroblasts after exposure to saturated NO-containing solutions. Total glutathione content of HA1 and OC14 fibroblasts before and after a 60-min exposure to 1.7 mM NO-containing medium solutions. An asterisk represents $p < 0.05$ vs control.

(2). Addition of oxyhemoglobin to NO-saturated medium immediately before cell exposure resulted in a significant increase in clonogenic cell survival when total glutathione-depleted HA1 cells were exposed to saturated solutions of NO (Fig. 7).

DISCUSSION

Our observations demonstrated that exposure of HA1 fibroblasts to aqueous NO prepared in N_2 -gassed medium resulted in a significant reduction in clonogenic cell survival. In addition, the concentration of endogenous intracellular total glutathione in HA1 and OC14 cells is reduced after exposure to exogenous NO. Previous depletion of HA1 intracellular glutathione (a major intracellular reserve of nonprotein free thiols capable of interacting with oxidizing agents) (46, 47) significantly enhances the NO-mediated cytotoxicity in a dose- and cell density-dependent fashion. Total glutathione depletion, before exposure to NO, resulted in a significant reduction in the ability of HA1 cells to exclude trypan blue dye, another acute indicator of cytotoxicity. NO-mediated cytotoxic effects on clonogenic cell survival in total glutathione-depleted HA1 cells are rapid (5 min) and dose dependent. These results are consistent with the hypothesis that NO or other reactive nitrogen species derived from the aqueous NO-oxygen reaction mediate the clonogenic inactivation of cells through a pathway that can be inhibited to a significant extent by cellular glutathione.

In another series of experiments, we have shown that the OC14 cell line, which demonstrates a stable H_2O_2 -resistant phenotype (20), is cross-resistant to the toxicity associated with exposure to aqueous NO. OC14 cells are known to have elevated concentrations of intracellular total glutathione that are two to three times the amount of glutathione found in HA1 cells (26). When OC14 cells were depleted of total glutathione to absolute levels less than HA1 cells and exposed to NO, they demonstrated a significant reduction in clonogenic cell survival. Comparison of clonogenic survival between glutathione-depleted HA1 cells and OC14 cells plated at equal densities and exposed to equal doses of NO demonstrates that OC14 cells survive at a significantly greater extent (50%) than do HA1 cells (8%). Thus, OC14 cells remain significantly more resistant to reactive nitrogen species injury, relative to HA1 cells, despite glutathione depletion to levels below the limits of assay detection ($0.2 \mu\text{g}/\text{mg}$ protein). Experiments designed to determine the effects of exposing glutathione-depleted HA1

Figure 6. Effects of nitrites (NO_2^-) and nitrates (NO_3^-) on clonogenic survival of HA1 cells with total glutathione depletion. Effects of 60 min of exposure to 5 mM nitrite (NO_2^-), 5 mM nitrate (NO_3^-), and 1.7 mM NO on the clonogenic survival of HA1 fibroblasts with BSO pretreatment. Data expressed as mean $\pm$ SD. An asterisk represents $p < 0.05$ vs respective control.

Figure 7. Oxyhemoglobin protection of total glutathione-depleted HA1 cells from NO-mediated injury. Effects of 100 μ M oxyhemoglobin (OxyHb) on the clonogenic survival of HA1 fibroblasts with BSO pretreatment after 60 min of exposure to 1.7 mM NO-containing medium solution. Data expressed as mean $\pm$ SD. Asterisk represents $p < 0.05$ vs respective control.

cells to nitrite or nitrate, potential toxic metabolites from the reaction of NO and oxygen, demonstrated no effect on cell clonogenic survival. However, the ability of oxyhemoglobin to protect HA1 cells from saturated NO-containing medium demonstrates that the reactive nitrogen species involved in producing the cytotoxic response in this cell model originate from NO.

NO has been reported to contribute to cytotoxic and cytoprotective reactions in a number of *in vitro* cell experiments (11–18). Controversy about the role of endogenous or exogenous NO exists in a number of scenarios including ischemia-reperfusion injury (18), neurotoxicity (15–17), and leukocyte-mediated and autoimmune diseases (3, 27, 28). Two critical characteristics of NO that may account for the apparent conflicting reports in the literature include the observation that NO is known to exist in a number of oxidation states (29) and that NO is an extremely reactive free radical with a $t_{1/2}$ of 3–5 s in physiologic conditions (4). NO has been hypothesized to exist in the reduced state, NO $\cdot$, a state observed when NO is in the gaseous state, when NO is physiologically secreted, or when NO is released by pharmacologic agents such as 3-morpholin-oxysynonimine (SIN-1) (29). As a reduced free radical, NO has been shown to avidly react with oxygen and superoxide anion to yield potentially cytotoxic agents such as NO $_2\cdot$ and peroxynitrite (OONO $^-$) (29–31). The reaction of NO and oxygen is complete upon formation of nitrite and nitrate. Although nitrite and nitrate are not toxic in our *in vitro* model, other reactive nitrogen species may be involved. Reduced NO is also

capable of avidly binding with sulfhydryl groups, such as those found in thiols, and with ferrous heme molecules, such as those found in guanylate cyclase and Hb (30, 48, 49). Other investigators have postulated that some of the physiologic effects of NO are mediated via formation of stable nitrosothiol compounds that results in a decreased pool of highly reactive reduced NO (32).

Formation of stable nitrosothiol-intermediate compounds is hypothesized as an explanation for our experimental results in which cytotoxic responses followed exposure to bolus concentrations of reduced aqueous NO. We speculate that intracellular glutathione pools act to scavenge reactive species derived from NO directly or act as cofactors in reactions that detoxify oxidized cell components, such as lipids, after damaging reactions initiated by NO-derived species. These types of glutathione-mediated reactions could effectively limit the cytotoxicity seen in HA1 and OC14 cells after NO exposure. The formation of nitrosothiols or other glutathione conjugates could also explain the loss of total glutathione content seen in HA1 and OC14 cells after NO exposure, because these compounds cannot be measured by our assay. Experimental support for the hypothesis that nitrosothiol formation results from reactions between NO and glutathione in aqueous medium and that subsequent glutathione depletion results in enhanced toxicity to NO or NO-donating pharmacologic agents has been reported by others (49). Thus, glutathione appears to protect cells from toxicity associated with exposure to the products of the NO/oxygen reaction. Exposure of increasing numbers of total

glutathione-depleted HA1 cells to saturated NO-containing medium resulted in increased clonogenic survival. This result (Fig. 2B) can be explained by assuming that the total cell population has reduced toxicity due to the collective protection provided by some non-glutathione-dependent mechanism(s). Specifically, metabolism of NO to other nontoxic products or a decrease in the dose of NO per cell may explain the protection afforded by increasing cell density.

The participation of other, non-glutathione-dependent, protective pathways is also demonstrated by the observation that glutathione-depleted OC14 cells are less sensitive to the toxicity of NO-containing medium than are similarly glutathione-depleted HA1 cells despite both cell lines having nondetectable levels of total glutathione. OC14 cells are known to have elevated activities of several antioxidant systems when compared with HA1 cells including total glutathione (2–3 times greater), catalase (20 times greater), CuZn superoxide dismutase (1.8 times greater), glutathione peroxidase (2 times greater), and glutathione transferase (2 times greater) (20, 26). It is possible that the elevations of these other antioxidant enzymes or the activity of other intracellular protective mechanisms associated with resistance to oxidative stress (*i.e.* repair of oxidative damage) may contribute to the decreased sensitivity of OC14 cells to NO exposure in the absence of glutathione.

NO-mediated toxicity occurs very rapidly in our cell model. Other investigators have reported rapid NO inhibitory effects on the mitogenic capabilities of other cell lines *in vitro* (33, 34). Additional reports have suggested the NO, or NO-derived, species are capable of causing oxidative injury (35–45, 49). Aqueous NO in the presence of oxygen has been shown to generate a species that is a potent oxidant capable of reacting with glutathione (19, 49). Our experimental results, which demonstrated a role for glutathione in protection from NO-mediated injury and that cellular resistance to oxidative stress also confers resistance to aqueous NO, indirectly support a causal role for NO-mediated oxidative injury in this model of NO toxicity. However, caution is warranted in extrapolating the results of these experiments to the clinical situation due to the inherent toxicity differences existing between NO bolus dosing in *in vitro* studies and the continuous steady state exposure to NO that is likely to occur *in vivo*. Additional verification studies are needed.

In conclusion, this cell model demonstrates that 1) NO, or some other reactive nitrogen species derived from NO (other than nitrites or nitrates), is responsible for decreases in clonogenic cell survival and trypan blue dye exclusion capabilities; 2) biochemical pathways associated with cellular resistance to oxidative stress also confer resistance to NO-mediated cytotoxicity; and 3) the cellular content of total glutathione determines a significant portion of cell sensitivity to NO-mediated cytotoxicity. Future investigation will focus on specifically identifying the injurious reactive nitrogen species, the critical biomolecules damaged during production of this injury, and the involvement of other antioxidant systems in protection against NO-mediated cytotoxicity. Finally, although the bolus doses of NO used in our *in vitro* experiments are higher than could be achieved using <100 ppm inhaled NO *in vivo*, we

urge continued evaluation of inhaled NO therapy, particularly in neonates who have demonstrated an enhanced susceptibility to oxidative injury. Special emphasis should be placed on establishing NO dose-response curves and toxicity-exposure outcomes.

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