Nitric Oxide-Mediated Regulation of Chemosensitivity in Cancer Cells

Nicola E. Matthews, Michael A. Adams, Lori R. Maxwell, Teneille E. Gofton, Charles H. Graham

Background: Hypoxia in tumors is associated with malignant progression, metastatic spread, and increased resistance to radiotherapy and chemotherapy. Molecular O2 is required for the cellular production of nitric oxide (NO) by the enzyme NO synthase (NOS), and NO may block components of the adaptive response to hypoxia. Hence, we hypothesized that hypoxia increases drug resistance in tumor cells by inhibiting endogenous NO production. Methods: Human breast carcinoma (MDA-MB-231) and mouse melanoma (B16F10) cells were pre-exposed to 20% O2, 5% O2, or 1% O2, incubated with a pharmacologic inhibitor of endogenous NO production, and then treated with chemotherapeutic agents. Resistance was assessed by colony-formation assays, and western blot analysis was used to measure NOS protein levels. All P values were twosided. Results: Incubation of MDA-MB-231 tumor cells in 1% O2 maximally increased their resistance to doxorubicin and 5-fluorouracil by 8.5-fold (P = .002) and 2.3-fold (P = .002), respectively, compared with incubation in 20% O2. B16F10 mouse melanoma cells preincubated in 1% O2 (versus 20% O2) for 12 hours exhibited a twofold increase in resistance to doxorubicin (P < .001). The rapid acquisition of drug resistance after exposure to 1% O2 could be mimicked by incubating the MDA-MB-231 cells for 12 hours with the NOS inhibitor Nω-monomethyl-L-arginine (fivetfold increase; P < .001). Conversely, replacement of NO activity by use of the NO-mimetic glyceryl trinitrate (GTN) and diethylenetriamine NO adduct produced statistically significant attenuations in the development of resistance of 59% (P < .001) and 40% (P < .001), respectively, in MDA-MB-231 cells. Treatment of B16F10 cells with GTN produced a 58% reduction in resistance (P < .001). MDA-MB-231 cells expressed all three isoforms of the NOS enzyme at levels that were not altered by exposure to hypoxia. Conclusions: NO mediates chemosensitivity in tumor cells, and hypoxia-induced drug resistance appears to result, in part, from downstream suppression of endogenous NO production. These results raise the possibility that administration of small doses of NO mimetics could be used as an adjuvant in chemotherapy. [J Natl Cancer Inst 2001;93:1879–85]
NO synthase (NOS). Studies (22,23) have shown that, during exposure to hypoxia, administration of NO-mimetic agents can suppress HIF-1 activity as well as the elevated expression of some of the above genes. Although drug resistance induced by hypoxia in cancer cells may involve changes in gene expression, previous studies have not examined the potential role of NO in the regulation of hypoxia-induced chemoresistance.

The fact that molecular O$_2$ is a cofactor required for the generation of NO and that exposure of cells to low levels of O$_2$ inhibits NO production (24,25) led us to postulate that phenotypic changes induced by hypoxia are causally linked to a lack of cellular NO production. Using clonogenic assays, we examined whether inhibition of NO production, as a result of exposure to hypoxia or an NO antagonist, leads to increased chemoresistance of tumor cells. Thus, this study investigated whether a critical component of the mechanism by which hypoxia increases drug resistance in tumor cells may be the inhibition of endogenous NO production.

As a corollary to this hypothesis, we postulated that NO-mimetic agents could be used to prevent acquisition of the resistance phenotype that develops in cells during exposure to hypoxia.

**Materials and Methods**

Cells. Human metastatic MDA-MB-231 breast carcinoma cells (26) and mouse B16F10 melanoma cells (from A. Chambers, London Regional Cancer Centre, London, ON, Canada) were maintained in monolayer culture in RPMI-1640 medium (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 5% fetal bovine serum (Life Technologies, Inc.).

**Culture conditions.** For incubation in various concentrations of O$_2$, 90% confluent cell cultures in 100-mm plates were placed either in a standard Sanyo carbon dioxide (CO$_2$) incubator (5% CO$_2$ in air, 37°C; Esbe Scientific, Markham, ON, Canada) or in airtight chambers (Bellco Biotechnology, Vineland, NJ) that were flushed with a 5% CO$_2$–95% nitrogen mixture. O$_2$ concentrations within these chambers were maintained at specified levels using Pro-Ox 2 regulators (Reming Bioinstruments Co., Redfield, NY). To determine whether administration of NO-mimetic drugs during exposure to hypoxia affects the survival of cells after doxorubicin or 5-fluorouracil (5-FU) treatment, we incubated randomly selected culture plates with low concentrations of glycerol trinitrate (GTN; 1 μM and 0.1 mM) or diethylenetriamine NO adduct (DETA/NO; 1 μM; Sigma Chemical Co., St. Louis, MO) administered at the beginning of the hypoxic exposure. To examine whether inhibition of endogenous NO generation influences the survival of cells treated with chemotherapeutic agents, we exposed cells to a single 0.5-mM dose of L-arginine (L-NMMA) (Calbiochem-Novobiochem, La Jolla, CA) in either 1% or 20% O$_2$ for 12 hours (after a 12-hour preincubation in hypoxia) before doxorubicin exposure. To confirm that the effects of L-NMMA were due to its actions on NOS activity and not to nonspecific pharmacologic effects of the molecule, we also performed experiments using a 0.5-mM dose of N$^\alpha$-monomethyl-L-arginine (N$^\alpha$-NMMA) (Calbiochem-Novobiochem), an inactive enantiomer of L-NMMA.

Clonogenic (colony-formation) assays to determine cell survival. After exposure of cells to various O$_2$ levels, cultures were treated with doxorubicin (1–100 μM; Sigma Chemical Co.) or 5-FU (1.5 mM; Sigma Chemical Co.) for 1 hour at 37°C in 20% O$_2$. Cells were then harvested with 0.025% trypsin–EDTA in phosphate-buffered saline (PBS), counted, and seeded at various densities in triplicate or sextuplicate. After 7–14 days, colonies were fixed with acetic acid: methanol (1:25), stained with dilute crystal violet (1:30), and counted.

**Western blot analysis of NOS expression.** To examine levels of NOS proteins, we lysed MDA-MB-231 cells, preincubated in 20%, 5%, or 1% O$_2$ for 24 hours, with buffer containing 2% sodium dodecyl sulfate (SDS), 10 mM Tris–HCl, and 0.15 M NaCl (pH 7.5) and centrifuged the lysates briefly at 4°C for 5 minutes at 14,000g. The supernatants were collected and stored at −80°C until use. SDS–polyacrylamide gel electrophoresis was performed, and resolved proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) by use of a wet-transfer apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked by incubation overnight at 4°C in a PBS solution containing 0.01% Tween 20 and 5% evaporated milk. The blots were then incubated with polyclonal rabbit antisera raised against human NOS I (1:500), II (1:800), or III (1:800) (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) and then with a horseradish peroxidase-labeled goat anti-mouse immunoglobulin G secondary antibody (Bio-Rad Laboratories). Antigen was detected by enhanced chemiluminescence (Perkin-Elmer Life Sciences Inc., Boston, MA) followed by exposure of the membrane onto DuPont Reflection NEF film (DuPont Canada, Inc., Mississauga, ON, Canada). Western blot analysis of NOS expression was performed to determine the level of NOS expression. To determine the level of NOS expression, we incubated MDA-MB-231 cells in 20%, 5%, and 1% O$_2$ for 24 hours before a 1-hour exposure to various concentrations of doxorubicin.

**Flow cytometric analysis of cell cycle.** After a 6-hour exposure to various O$_2$ concentrations in the absence or presence of 10$^{-10}$ M GTN, cells were fixed in ethanol and stained with propidium iodide (100 μg/mL). DNA content in cells was analyzed by use of a Coulter Elite Flow Cytometer (Beckman-Coulter Corp., Miami, FL).

**Calculations and statistical analysis.** Plating efficiency was calculated from the number of surviving colonies expressed as a proportion of the total number of cells seeded. In pilot studies, it was determined that seeding 5 × 10$^4$ cells of the chemotherapeutic agent-treated populations and 100–500 cells of the nontreated control groups resulted in adequate numbers of colonies for consistency and ease of counting. Surviving fractions were determined by dividing the plating efficiency of drug-treated cells by the plating efficiency of nontreated cells. The survival values of hypoxia-treated cells relative to the corresponding survival values of cells incubated in 20% O$_2$ were computed to account for the contribution of cell density to resistance. All data are presented as medians, with 95% confidence intervals. Statistical calculations were performed by use of SigmaStat® (SPSS Science, Chicago, IL). A one-way analysis of variance on ranks was performed by use of the Kruskal–Wallis method, and the P values were adjusted for multiple comparisons with the use of Dunn’s or Dunnet’s methods as indicated in the figure legends. All of the statistical tests were two-sided, and the differences were considered to be statistically significant at P < 0.05.

**Results**

**Exposure to Hypoxia and Drug Resistance.** To determine the level of hypoxia required for induction of drug resistance, we incubated MDA-MB-231 cells in 20%, 5%, and 1% O$_2$ for 24 hours before a 1-hour exposure to various concentrations of doxorubicin. In tissue culture, 20% O$_2$ is equivalent to partial pressure of O$_2$ (pO$_2$) values of approximately 150 mmHg, and 5% O$_2$ (30–40 mmHg) is similar to levels present in the venous circulation or most tissue extracellular matrices. Levels of less than 1% O$_2$ (10–15 mmHg) characterize tissues with compromised blood flow such as some cancerous tumors (3). Plating efficiencies were similar for cells cultured in either 20% or 1% O$_2$, and exposure to hypoxia alone did not affect population doubling time or the pH of the culture medium (data not shown). There was no difference in the survival of cells incubated in 5% versus 20% O$_2$ after doxorubicin treatment, whereas the survival of cells in 1% O$_2$ showed a statistically significant increase (Fig. 1, A and B; P < 0.001). Compared with cells incubated under standard conditions, cells incubated in 1% O$_2$ for 24 hours exhibited an 8.5-fold increase in survival (P = .002) after treatment with 50 μM doxorubicin (Fig. 1, A). Results revealed that as little as 6 hours of hypoxic exposure was sufficient to generate increased survival (an approximately 10-fold relative increase; P < 0.001; data not shown). Although hypoxia consistently induced drug resistance, the relative magnitude varied with duration of exposure to reduced O$_2$ levels and the concentration of the chemotherapeutic agent. Furthermore, the hypoxia-induced drug resistance was transient, because cells exposed to hypoxia for 12 hours and then returned to 20% O$_2$ completely regained their sensitivity to doxorubicin (Fig. 2, A).

The effect of hypoxia on drug resistance was not limited to MDA-MB-231 cells, because preincubation of B16F10
mouse melanoma cells in 1% O2 (versus 20% O2) for 12 hours resulted in a twofold increase in their survival after exposure to 10 μM doxorubicin for 1 hour (P<.001, Kruskal–Wallis followed by Dunnett’s Multiple Pairwise Comparison).

To determine whether hypoxia also increases the resistance of tumor cells to other chemotherapeutic agents, we exposed MDA-MB-231 cells preincubated for 6 hours in 20% or 1% O2 to 5-FU (1.5 mM) for 1 hour. Compared with cells incubated in 20% O2, clonogenic assays revealed a 2.3-fold increase in the resistance to this drug of cells preincubated in 1% O2 (P = .002, Kruskal–Wallis followed by Dunnett’s Multiple Pairwise Comparison).

Expression of the NOS Enzyme by MDA-MB-231 Cells

Western blot analysis showed that all three NOS isoforms are expressed in the human breast carcinoma cell line MDA-MB-231 (Fig. 2, B), with NOS I present at the lowest levels. Incubation in different concentrations of O2 did not affect the levels of any of the NOS isoforms.

Induction of Drug Resistance After NOS Inhibition

To establish the link between hypoxia-induced resistance to chemotherapeutic drugs and reduced NO levels (hypnotroxia), we blocked cellular NO production by administration of the NOS inhibitor L-NMMA. Compared with control cells incubated in 20% O2 without L-NMMA, cells incubated in 20% O2 for 12 hours in the presence of a single dose of L-NMMA (0.5 mM) exhibited a fivefold increase in survival (P<.001; Fig. 2, A). Moreover, cells incubated with L-NMMA displayed survival levels similar to those of cells incubated for a further 12 hours in 1% O2 but without L-NMMA treatment. Cells incubated in 1% O2 for 12 hours in the presence of L-NMMA exhibited a further twofold increase in survival (P = .004) when compared with cells incubated in 1% O2 in the absence of NOS inhibition. To confirm that these effects were due to NOS inhibition and not a result of nonspecific effects of L-NMMA treatment, we also conducted experiments in which cells were treated with 0.5 mM d-NMMA in 20% O2. Results show that cells exposed to d-NMMA did not exhibit increased survival compared with cells incubated in 20% O2 alone (P = .72; Fig. 2, A).

Effect of Low Concentrations of an NO Mimetic on the Induction of Hypoxia-Associated Drug Resistance

Addition of the NO-mimetic prodrug GTN at the onset of 12- and 24-hour exposures to hypoxia effectively attenuated the development of the hypoxia-induced drug resistance phenotype (Fig. 3, A and B). Moreover, DETA/NO, a different NO mimetic that spontaneously releases NO when in solution, was also effective at attenuating the resistance observed in cells over a 12-hour hypoxic incubation (Fig. 3, A). Specifically, the addition of a single low concentration of GTN (0.1 mM) or DETA/NO (1 μM) at the onset of the hypoxic exposure inhibited the acquisition of drug resistance found after 12 hours by 59% and 40%, respectively (P<.001; Fig. 3, A). Moreover, a single dose of GTN (0.1 mM) reduced the resistance by nearly 20% after 24 hours (P = .002; Fig. 3, B), and addition of a higher concentration of GTN (1 μM) to ensure that sufficient amounts of NO mimetic were present throughout the 24-hour hypoxic incubation, resulted in a 77% inhibition of the hypoxia-induced resistance to doxorubicin (P = .006; Fig. 3, B). In contrast, the survival of cells incubated in 20% O2 was not decreased by either GTN or DETA/NO treatment (Table 1). GTN-mediated prevention of drug resistance was further demonstrated by the use of 0.1 mM concentrations of GTN in both B16F10 cells treated with 10 μM doxorubicin (12-hour hypoxic pre-exposure, 58% inhibition of resistance; P<.001, Kruskal–Wallis followed by Dunnett’s Multiple Pairwise Comparison) and MDA-MB-231 cells treated with 1.5 mM 5-FU (6-hour hypoxic pre-exposure, 72% inhibition of resistance; P = .002, Kruskal–Wallis followed by Dunnett’s Multiple Pairwise Comparison).
Effect of a 6-Hour Hypoxic Incubation on Cell Cycle

Flow cytometric analysis of the DNA content of cells exposed to either 20% or 1% O2 with or without NO-mimetic treatment revealed no statistically significant differences in the proportions of cells at each stage of the cycle in any of the treatment populations when compared with each other. The analysis was performed in duplicate for all of the groups, and the proportion of cells ranged from 33.2% to 38.4% in G0/G1 (P = .13), from 39.2% to 43.8% in S (P = .20), and from 22.3% to 23.1% in G2/M (P = .33). There was no evidence of G0/G1 arrest in any of the groups exposed to hypoxia for 6 hours with or without NO-mimetic treatment.

DISCUSSION

The major finding of this study is that suppression of endogenous NO production (“hyponitroxia”) appears to be a key component of the underlying mechanism of hypoxia-induced drug resistance in cancer cells. Changes in chemoresistance were demonstrated in two different cancer cell lines with the use of two different anticancer agents, doxorubicin and 5-FU. The fact that the resistance phenotype could also be induced by pharmacologic inhibition of cellular NOS activity, even in high levels of O2, suggests that hypoxia and lack of NO act via a common pathway. Moreover, replacement of cellular NO by use of low doses of NO mimetics (0.1 nM to 1 μM GTN or 1 μM DETA/NO) prevented the development of the hypoxic drug resistance by up to 77%. The selectivity of this effect to hypoxic cells—those cells incubated at 20% O2 with GTN or DETA/NO were not rendered more sensitive to drug treatment—confirms that hypoxia-induced changes are indeed linked to hyponitroxia and not to the lowering of cellular O2 levels per se.

The mechanisms and kinetics of the NO release are very different in the two NO mimetics, which may account for the differences in concentrations required of the compounds used to attenuate the hypoxic resistance. These differences further strengthen the concept that NO is the effector molecule in the chemosensitivity pathway. GTN is a prodrug that is bio-transformed by a variety of enzymes in the cell, including cytochrome P450 enzymes and glutathione S-transferase (27). Thus, the effective concentrations of NO available to the cell depend on the number of cells present and on the rate of cellular biotransformation. Conversely, DETA/NO releases NO spontaneously in solution at a constant rate, with a half-life of approximately 20 hours (28). In contrast to the local release of NO from GTN, DETA/NO releases NO throughout the media (29); the released NO must then travel to the cells to have an effect.

Three distinct NOS enzymes have been identified: NOS I (neuronal NOS); NOS II (inducible NOS), which is associated with the cytotoxic NO burst in macrophages; and NOS III (endothelial...
NOS), which is constitutively expressed by endothelial cells (30). The expression of NOS II has been demonstrated in a variety of tumor cells, including the mouse B16F10 melanoma cells used in our present study (31). Western blot analysis revealed MDA-MB-231 cells express all three NOS isoforms. Although there is evidence that, in some cell lines, reduced O2 levels increase the expression of NOS II messenger RNA and protein (32, 33), our data showed that incubation in various O2 concentrations did not affect the expression of any of the isoforms in MDA-MB-231 cells. However, while NOS protein can be expressed even under hypoxic conditions, NO production may not occur during severe hypoxia because of the O2 requirement for the reaction that converts L-arginine to L-citrulline and releases NO. Indeed, there is evidence that exposure of cells to a low, but physiologically relevant, level of O2 (1%–3%) inhibits NO production by up to 90% (24, 25). Thus, we propose that a critical mechanism by which reduced O2 levels mediate resistance to chemotherapeutic agents is by limiting endogenous NO production. In our study, pharmacologic inhibition of NO production was able to increase cellular drug resistance to levels higher than those induced by hypoxia alone, suggesting that a 24-hour incubation in 1% O2 is not sufficient to fully inhibit NO production. It is not uncommon, however, for some solid tumors to exhibit regions that are almost anoxic (3).

Exposure to low levels of O2 has been shown to increase resistance to various chemotherapeutic agents in both animal and human cell lines (5, 9, 11, 34). In previous studies, the experimental conditions most commonly used were 24-hour incubations in either 1% or 20% O2. Concentrations of 20% O2 (approximately 150 mmHg) are nonphysiologic. Within most solid cancers, pO2 values range from less than 1 mmHg to about 30 mmHg (35, 36). Our data demonstrate that cells incubated in 5% O2, a level similar to that found in many nonhypoxic regions of the body and hence physiologic, are as sensitive to drug treatment as cells incubated in 20% O2.

Other mechanisms for the hypoxia-mediated increase in chemoresistance have been proposed, including the absence of O2 radicals, cell cycle disruption or arrest, DNA overreplication, and induction of stress proteins (10, 37, 38). In the experiments described here, the cells...
Table 1. Survival of cells incubated in 20% or 1% oxygen (O2) with or without nitric oxide mimic after 50 μM doxorubicin treatment*

<table>
<thead>
<tr>
<th></th>
<th>20% O2</th>
<th>1% O2</th>
<th>Fold increase in survival at 1% O2</th>
<th>% induction of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.0088</td>
<td>0.041</td>
<td>4.7</td>
<td>(3.95 to 5.45)</td>
</tr>
<tr>
<td>0.1 nM GTN</td>
<td>0.011</td>
<td>0.022</td>
<td>2.0</td>
<td>(1.91 to 2.09)</td>
</tr>
<tr>
<td>1 μM DET/NO</td>
<td>0.018</td>
<td>0.028</td>
<td>1.6</td>
<td>(1.56 to 1.64)</td>
</tr>
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*Numbers represent median surviving fraction of an experiment repeated three or more times. Ninety-five percent confidence intervals are indicated (in parentheses) for the increase in survival and the percent induction of resistance. Fold increase was calculated relative to the appropriate 20% O2 control. GTN = glyceryl trinitrate; DET/NO = diethylenetriamine nitric oxide adduct.

Inhibition of the proteasome by use of lactacystin restored chemosensitivity and topoisomerase II levels in a dose-dependent manner (42). However, since cell cycle arrest was required for these effects, it seems unlikely to be the primary mechanism of hypoxia-mediated drug resistance at earlier time points.

In summary, we describe here a novel mechanism by which the lack of production of endogenous NO, resulting from a reduction in the O2 supply to the cell or from pharmacologic inhibition of NOS, can lead to increased resistance to chemotherapy. Furthermore, we have shown that very low doses of an NO mimic can produce a statistically significant reduction in this resistance phenotype and, therefore, may have a potential therapeutic role.

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Notes

Supported by a grant from the Canadian Institutes of Health Research and by Vaxis Therapeutics Corporation. C. H. Graham was a Research Scholar from the Heart and Stroke Foundation of Ontario, Canada.

Manuscript received April 12, 2001; revised September 17, 2001; accepted October 19, 2001.