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 two-sided. 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 two-fold 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 (fivefold 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 O2 is a cofactor required for the generation of NO and that exposure of cells to low levels of O2 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 NOS 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., Grand Island, NY) supplemented with 5% fetal bovine serum (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) and antibiotics. MDA-MB-231 cells were maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum (Life Technologies, Inc.) and 10% fetal bovine serum (Remel Biostatistics Co., Redfield, NY). To determine whether administration of NO-mimetic drugs during exposure to hypoxia affects the survival of cells after doxorubicin treatment, we incubated cells in either 20%, 5%, or 1% O2 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 anti-rat NOS raised against unfractionated rat NOS 1 (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). Anti-NOS 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).

Flow cytometric analysis of cell cycle. After a 6-hour exposure to various O2 concentrations in the absence or presence of 10−8 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 × 104 cells of the chemo- therapeutic 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% O2 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® (SSPS 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 Dunnett’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 <.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% O2 for 24 hours before a 1-hour exposure to various concentrations of doxorubicin. In tissue culture, 20% O2 is equivalent to partial pressure of O2 (pO2) values of approximately 150 mmHg, and 5% O2 (30–40 mmHg) is similar to levels present in the venous circulation or most tissue extracellular matrices. Levels of less than 1% O2 (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% O2, 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% O2 after doxorubicin treatment, whereas the survival of cells in 1% O2 showed a statistically significant increase (Fig. 1, A and B; P <.001). Compared with cells incubated under standard conditions, cells incubated in 1% O2 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 <.001; data not shown). Although hypoxia consistently induced drug resistance, the relative magnitude varied with duration of exposure to reduced O2 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% O2 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 two-fold increase in their survival after exposure to 10 μM doxorubicin for 1 hour (P<.001, Kruskal–Wallis followed by Dunn’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 Dunn’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 (hypnotroxaemia), 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 five-fold 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 two-fold 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 (1 μM) 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 47% 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 mimic 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 Dunn’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 Dunn’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 GTN for 6 hours 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 prevented the development of the hypoxia-induced 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 biotransformed 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).

Fig. 3. Effect of a single dose of glyceryl trinitrate (GTN) or diethylaminetriamine nitric oxide adduct (DETA/NO) on the hypoxia-induced resistance to doxorubicin. A) Cells were treated with 10⁻¹⁰ M GTN or 10⁻⁶ M DETA/NO at the onset of a 12-hour incubation in 1% O2 before exposure to 50 μM doxorubicin. * indicates a statistically significant difference in survival compared with cells incubated in 20% O2 (P<.001, Kruskal–Wallis followed by Dunn’s Multiple Pairwise Comparison). ** indicates a statistically significant difference in survival compared with cells incubated in 1% O2 alone (P<.001, Kruskal–Wallis followed by Dunn’s Multiple Pairwise Comparison). B) Cells were treated with either 0.1 nM or 1 μM GTN at the beginning of a 24-hour incubation in 1% or 20% O2 before exposure to 25 μM doxorubicin. Bars represent survival (median ± 95% confidence intervals) from three independent experiments, each conducted in triplicate. * indicates a statistically significant difference in survival compared with cells incubated 20% O2 (P = .003, Kruskal–Wallis followed by Dunn’s Multiple Pairwise Comparison). ** indicates a statistically significant difference in survival compared with cells incubated in 1% O2 alone (P = .002 and P = .006, Kruskal–Wallis followed by Dunn’s Multiple Pairwise Comparison). All P values in A and B were two-sided.

Journal of the National Cancer Institute, Vol. 93, No. 24, December 19, 2001 REPORTS 1883
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.

REFERENCES


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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>
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<td>0.0088</td>
<td>0.041</td>
<td>4.7</td>
<td>100</td>
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<tr>
<td>0.1 mM GTN</td>
<td>0.011</td>
<td>0.022</td>
<td>(1.91 to 2.90)</td>
<td>(42.98 to 43.02)</td>
</tr>
<tr>
<td>1 μM DETA/NO</td>
<td>0.018</td>
<td>0.028</td>
<td>1.6</td>
<td>34</td>
</tr>
</tbody>
</table>

*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; DETA/NO = diethylenetriamine nitric oxide adduct.


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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.