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Nitric Oxide 10 (2004) 119–129
NITRIC OXIDE
Biology and Chemistry
www.elsevier.com/locate/yniox

Endogenous production and exogenous exposure to nitric oxide augment doxorubicin cytotoxicity for breast cancer cells but not cardiac myoblasts

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Received 22 October 2003; received in revised form 23 March 2004
Available online 20 April 2004

Abstract

We studied the effect of nitric oxide (·NO) on the anticancer activity of doxorubicin. When MCF-7 human breast cancer cells were exposed to an aqueous solution of ·NO delivered as a bolus 30 min prior to doxorubicin, the cytotoxic effect as measured in a clonogenic assay was increased (doxorubicin alone, 40% survival, doxorubicin plus ·NO, 5% survival). The ·NO donor diethylamine nitric oxide, but not inactivated donor, also yielded an increase in doxorubicin cytotoxicity. The sequence was important since the simultaneous application of ·NO with doxorubicin yielded only a small augmentation of effect, and the exposure of the cells to doxorubicin prior to the ·NO obliterated the augmentation. Prior depletion of glutathione by incubation of the cells for 24 h with D,L-buthionine-*S*,*R*-sulfoximine (BSO) further increased the cytotoxicity so that BSO plus ·NO plus doxorubicin killed all of the clones. MCF-7 cells transduced with inducible nitric oxide synthase gene (iNOS) through an adenoviral vector overexpressed iNOS and produced increased amounts of nitrite, an indicator of increased ·NO production. These iNOS transduced cells were more susceptible to doxorubicin than vector control or wild-type cells. Cell cycle progression of iNOS transduced cells was not different from controls. Likewise, iNOS transduction resulted in no change in cellular glutathione levels. For comparison, we examined the effect of iNOS transduction on the sensitivity of MCF-7 to edelfosine, a membrane-localizing anticancer drug without direct DNA interaction. Insertion of the iNOS had no effect on killing of the MCF-7 cells by this ether lipid class drug. We also tested the effect of iNOS transduction on doxorubicin sensitivity of H9c2 rat heart-derived myoblasts. We found no augmentation of cytotoxicity by ·NO, and this observation offers potential therapeutic tumor selectivity by using ·NO with doxorubicin. Therefore, we conclude that ·NO produced intracellularly by iNOS overexpression or delivered as a bolus sensitizes human breast cancer cells in culture to doxorubicin, but not to a cardiac cell line or to edelfosine. This augmentation is not due to a modulation of cell cycle distribution or measurable cellular glutathione resulting from the transduction.
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Keywords: Nitric oxide; iNOS transduction; Doxorubicin; Breast cancer; MCF-7; Edelfosine; H9c2 rat heart-derived myoblasts; Tumor selectivity

Nitric oxide (·NO) has potential as an adjuvant to cancer chemotherapy. It is known that peroxynitrite ($ONOO^-$), a reaction product of ·NO with $O_2^{\cdot-}$, can damage cells [1], and induce apoptosis in a concentration-dependent manner [2,3] probably with an oxidative basis [4]. However, ·NO alone will not likely give sufficient cell killing at the concentrations of ·NO that can be delivered clinically to bring about a cure for human cancer. Therefore, we have concentrated on its potential as an adjuvant to an existing modality of therapy. In this regard, it is known that ·NO can sensitize cells to ionizing radiation [5], and photodynamic therapy [6]. It is

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doi:10.1016/j.niox.2004.03.006

known that iNOS[2] overexpression enhances the radiation sensitivity of human colorectal cancer cell lines [7].

There is also evidence that the antitumor effect of certain cancer chemotherapy agents might be augmented by the use of ·NO. For example, Wink et al. [8] reported that ·NO can enhance the effect of cisplatin in V79 cells. They speculated that reactive nitrogen oxide species inhibit DNA repair to explain the augmentation. Azizzadeh et al. [9] also found improved cisplatin cytotoxicity in Chinese hamster lung fibroblasts using long-acting, but not short-acting ·NO donors. In a study with another drug, Cook et al. [10] reported an enhancement of the anticancer effect of melphalan. Adams et al. [11] have shown that an ·NO-donating drug enhanced the cytotoxicity of the antimetabolite fludarabine for freshly obtained human chronic lymphocytic leukemia cells. They found no effect of sequence of administration and no effect on several other drugs including doxorubicin, gemcitabine, and a topoisomerase inhibitor. In a complementary manner, Matthews et al. [12] decreased intracellular ·NO production using hypoxia or drugs and showed increased resistance to doxorubicin for MDA-MB-231 human breast cancer cells and for B16F10 mouse melanoma cells, and furthermore this resistance could be attenuated with an ·NO donor drug. This therapeutically useful interaction with chemotherapy could be related to an effect on blood vessels since ·NO may also have a role in tumor angiogenesis [13]. However, iNOS overexpression inhibited the toxicity of the nitrosourea anticancer drug BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), but not that of cisplatin or temozolomide for the rat C6 glioma cell line [14]. Information on the interaction of ·NO with chemotherapy would have implications if it turns out to be a cell-derived mediator of doxorubicin effect as suggested by studies of the breast cancer line EMT-6 [15] and since doxorubicin decreases intracellular ·NO production [16].

The ether lipids are anticancer drugs of particular interest because they represent a strategy for killing neoplastic cells that is fundamentally different from doxorubicin since there is no evidence of direct interaction with DNA [17]. Their lysophospholipid structure is similar to platelet activating factor, and they are membrane localizing [18,19], where they alter the physical properties of membranes [20–22]. The drugs translocate and cluster the cell death receptor Fas in membrane rafts [23]. Their critical intracellular target remains unidentified [24]. One member of the class, miltefosine (*n*-hexadecylphosphorylcholine), is used clinically for the treatment of breast cancer, and leishmaniasis. The prototype drug is edelfosine (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine, often abbreviated ET-18-OCH_3), and it has been shown to eliminate leukemic cells from autologous bone marrow grafts without eliminating normal precursor "stem" cells [25,26].

Therefore, we carried out a study of the interaction of ·NO with chemotherapy. The human breast adenocarcinoma cell line MCF-7 was used for the ·NO studies because it is a well-characterized cell line that is sensitive to doxorubicin. Its antioxidant content is known [27]. For comparison doxorubicin was chosen as the major focus because of its importance in clinical medicine and because there is ample evidence that a portion of its toxicity is due to reactive oxygen species [28–32]. Studies with edelfosine were done for comparison to a drug with a different site of action. We examined our hypothesis that ·NO augments doxorubicin antitumor effect using ·NO delivered externally as both a bolus and ·NO donor. In addition, we examined the effect of internally generated ·NO by using cells transduced with iNOS. Modulation of cellular production of ·NO provides the strongest and most direct evidence for a potential therapeutic interaction with chemotherapy. This is the case since exogeneous ·NO may not reach critical intercellular sites at an effective concentration. To determine tumor selectivity of the ·NO, we also studied H9c2 rat heart-derived myoblasts transduced with iNOS.

Experimental procedures

Cell culture

The human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA) and was maintained in Eagle's MEM containing nonessential amino acids + 10% FBS (Gibco) in a humidified atmosphere containing 5% CO_2 at 37 °C. For experimental manipulations cells were utilized at approximately 75% confluence. They were then washed and media replaced. Experimental conditions included exposure to ·NO in a bolus (as a saturated aqueous solution, 1.8 mM), or DEANO (diethylamine nitric oxide, 1 mM), which were added to the cells 30 min before the addition of doxorubicin. After exposure to ·NO and/or chemotherapy drug, cells were trypsinized, and plated for clonogenic survival. In the glutathione-depletion experiments, cells were incubated with the indicated amount of BSO (buthionine sulfoxamine, Sigma, St. Louis, MO) for 24 h prior to exposure to ·NO or doxorubicin.

[2] *Abbreviations used:* BSA, bovine serum albumin; BSO, D,L-buthionine-*S*,*R*-sulfoximine; DEANO, diethylamine nitric oxide; DOX, doxorubicin; eNOS, endothelial nitric oxide synthase; GSH, glutathione; GSSG, glutathione disulfide; iNOS, inducible nitric oxide synthase; MEM, minimum essential medium; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PFU, plaque-forming units; SOD, superoxide dismutase; TTBS, Tween Tris-buffered saline.

The H9c2 embryonic rat heart-derived myoblasts were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, L-glutamine (1.6 mM), penicillin (80 U/ml), and streptomycin (80 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37 °C. To maintain the cardiac phenotype of the H9c2 cell line, 10 nM retinoic acid was added to the culture medium [33]. The H9c2 cells were not used after 10 passages.

·NO preparation and measurement

Nitric oxide was prepared by mixing 2 parts 1 M $NaNO_2$ with 1 part 1 M HCl in an O_2-free, gas-tight bottle. To produce ·NO-saturated tissue culture medium, the ·NO gas liberated from this reaction was bubbled for 2 h through full tissue culture medium in a glass vessel sealed with a rubber stopper (two 18-gauge needles were inserted into the stopper, one for gas inflow and one for gas outflow). Prior to the introduction of ·NO gas, oxygen purging of the tissue culture medium was accomplished by bubbling N_2 for 30 min. For ·NO treatment, ·NO-saturated medium (approximately 1.8 mM) from the flask was quickly removed and added to plates containing the cells in a manner that produced a minimum of headspace. Nitric oxide donor compound DEANO (diethylamine nitric oxide, Molecular Probes, Eugene, OR) was made as a stock solution in NaOH. Specified concentrations of DEANO were added to cells in the same manner as bolus addition at the designated times. Nitric oxide concentrations were verified using a Sievers Model 280 Nitric Oxide Analyzer (Boulder, CO).

Transduction of cells with recombinant adenoviruses

The adenoviruses, AdiNOS, and AdLacZ, were constructed at the Gene Transfer Vector Core of The University of Iowa by inserting *Escherichia coli lac-Z* cDNA or mouse *iNOS* cDNA into the E1 region of an Ad5 with deletion of entire E1 and partial E3 regions, which renders the recombinant adenovirus replication-deficient [34,35]. The cDNAs are under the control of the human CMV promoter/enhancer. Approximately 1×10^6 MCF-7 human breast cancer or 5.5×10^5 cells myoblasts were seeded into 100 mm^2 tissue culture dishes and allowed to adhere for 12 h. The cells were then washed twice with serum-free media. The adenovirus constructs were added to the cells in 5 ml serum-free medium at the desired multiplicity of infection (MOI, in units of PFU/cell). After 2 h of virus exposure, 250 μl of FBS was added to the medium. The cells were incubated with the virus constructs for 24 h. Following the incubation, the virus-containing medium was removed, and the cells were washed once with complete medium. Complete medium was added to the cells for an additional incubation of 24 h before the cells were harvested for a Western blot or chemotherapy drug exposure.

Chemotherapy drug exposure of transduced MCF-7 cells

Following the adenovirus transduction, wild-type MCF-7 cells, LacZ-infected MCF-7 cells, and iNOS-infected MCF-7 cells were exposed to chemotherapy drug at increasing concentrations for 45 min (doxorubicin) or 24 h (edelfosine). Times were chosen to give an optimal level of cytotoxicity to demonstrate augmentation for given drugs and conditions. The cells were detached with 5 mM EDTA and plated into 6-well tissue culture plates. To allow for colony formation, the plates were incubated for 14 days. The colonies were fixed with 3:1 methanol/acetic acid and stained with 0.1% crystal violet and 2.1% citric acid. Colonies containing >50 cells were counted for cell survival.

Flow cytometric assay for cell cycle phase determination

Cell cycle analysis was performed using the propidium iodide staining method of Menon et al. [36]. Briefly, cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS: KCl 2.7 mM, KH_2PO_4 1.5 mM, $NaHPO_4$ 8 mM, and NaCl 136.9 mM, pH 7.0), fixed in 70% ethanol, and stored at 4 °C. The cells were centrifuged and incubated with 100 μl RNase A (1 mg/ml) per 1×10^6 cells for 30 min followed by staining with propidium iodide (35 μg/ml) for 60 min at room temperature. Flow cytometric analysis was performed and cell cycle distribution was determined using CellQuest software (Becton–Dickinson Immunocytometry Systems, San Jose, CA).

Cell homogenization and Western blot analysis

Cells were washed in PBS, harvested, and pelleted at 1000*g* for 10 s in 1.5 ml microfuge tubes. The supernatant was removed and cells were resuspended in 50 mM phosphate buffer (pH 7.8) and sonicated on ice for 3×30 s using a Vibra Cell cup horn sonicator (Sonics and Materials, Newtown, CT) at 30% power. Total cellular proteins, 30 μg protein per well, were electrophoresed in a 10% PAGE gel according to the method of Laemmli [37]. Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell Bioscience, Keene, NH) and protein loading was determined to be equal using 0.1% ponseau S staining. The blots were blocked in 5% dried milk in TTBS (0.01 M Tris/0.15 M NaCl buffer, pH 8.0, and 0.1% Tween 20, Transduction Laboratories, Lexington, KY) at room temperature for 1 h. Blots were rinsed three times with TTBS and incubated with an iNOS primary antibody (Transduction Laboratories, Lexington, KY) diluted

1:1000 in 2% bovine serum albumin (BSA) and 0.1% TTBS, for 1 h at room temperature. After washing three times with TTBS, the blot was incubated in goat antirabbit IgG conjugated with horseradish peroxidase (Chemicon International, Temecula, CA) at a 1:10,000 dilution in 2% BSA and 0.1% TTBS for 1 h at room temperature. Blots were washed again and bands were visualized using chemiluminescence (ECL + plus Western Blotting Detection System, Amersham Biosciences, Piscataway, NJ) and exposed to film. Photographic analysis was performed using the Digital Imaging and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

Measurement of intracellular glutathione levels

Intracellular glutathione (GSH) and glutathione disulfide (GSSG) were assayed using a previously published spectrophotometric recycling assay described by Anderson [38]. The cells were scrape-harvested in PBS and centrifuged. To the cell pellet, sulfosalicylic acid (5% w/v SSA) was added immediately and the soluble fraction was used for the thiol analysis. GSH and GSSG were distinguished by adding 20 μl of a 1:1 mixture of 2-vinylpyridine and ethanol per 100 μl sample and incubating for 2 h as described by Griffith [39]. The determination of total glutathione (GSH + GSSG) was normalized for the amount of precipitated cellular protein of the acid-treated samples. The protein concentrations were determined by resuspending the cells in 5% (w/v) SDS in 0.1 M NaOH and using the bicinchoninic acid protein assay with the Micro BCA Protein Assay Kit (Pierce, Rockford, IL) with BSA as the standard.

Statistics

Student's t test or one-way analysis of variance was used for comparisons. Two-way analysis of variance that included cell type and drug concentration was used to analyze the normalized data of the transduction experiments. Within the analysis, the weighted least squares method was used to adjust for the unequal variance among the groupings. Bonferroni's adjustment was used to control the experiment wise error rate among the pairwise comparisons.

Results

·NO augments doxorubicin anticancer effect

We studied the effect of ·NO on the toxicity of a commonly used antineoplastic drug, doxorubicin. MCF-7 cells were exposed to 1 μM doxorubicin for 2 h with or without ·NO delivered as a bolus in ·NO-saturated tissue culture medium (1.8 mM ·NO), which was added 30 min prior to doxorubicin. The results are shown in Fig. 1. There was an 8-fold augmentation of doxorubicin cytotoxicity when the cells were exposed to ·NO ($p < 0.001$).

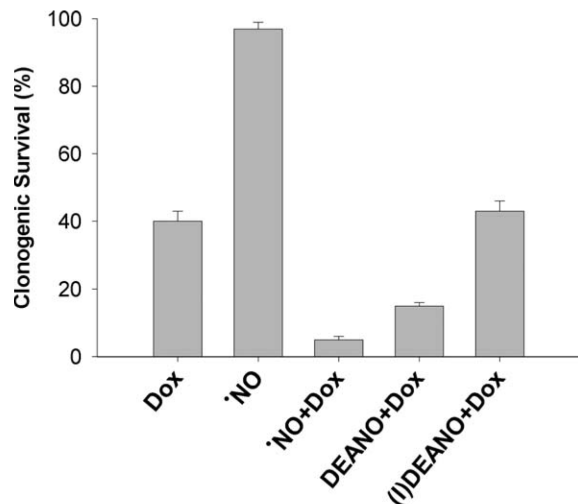

Fig. 1. Synergistic effect of ·NO on doxorubicin (DOX) clonogenic cytotoxicity. MCF-7 cells were exposed to ·NO either as a bolus of ·NO delivered as a 1.8 mM saturated solution of ·NO in tissue culture media or using the ·NO donor DEANO (1.0 mM). Cells were exposed to ·NO for 30 min, and then doxorubicin (1 μM) was added for an additional 2 h. Clonogenic survival was then determined. Abbreviations are DOX, doxorubicin; ·NO, ·NO delivered by bolus; DEANO, ·NO delivered by the ·NO-donor, DEANO; and (I) DEANO, inactivated DEANO. Shown are the means and SE of 6–7 replicates in three separate experiments. Values represent the percent survival as compared to the plating efficiency of controls.

When the ·NO was delivered by a ·NO donor, 1 mM DEANO, there was also an augmentation (2- to 3-fold) ($p < 0.005$). DEANO inactivated by setting at room temperature overnight showed no augmentation. Controls of MCF-7 cells in presence of DEANO alone and inactivated DEANO had survivals of 92 and 96%, respectively (not shown).

Additional control studies were done to rule out an effect of nitrite. The addition of 5 mM sodium nitrite to the culture medium of MCF-7 cells alone, or to cells plus either doxorubicin, bolus ·NO, or doxorubicin/DEANO had no significant effect (data not shown).

Vitamin E does not effect ·NO-induced sensitization

The addition of 100 μM vitamin E as D,L-α-tocopherol acetate to the culture medium of the MCF-7 cells had no effect on the clonogenic survival of MCF-7 cells exposed for 2 h to doxorubicin at 1 or 4 μM, or doxorubicin at those concentrations in the presence of bolus ·NO (data not shown). This lack of effect was seen whether the vitamin E was added simultaneous with the ·NO or as a 24 h pre-incubation.

Glutathione depletion increases the augmentation of doxorubicin killing by ·NO

D,L-Buthionine-*S*,*R*-sulfoximine (BSO) alters the redox environment of cells by inhibiting the synthesis of glutathione via inhibition of γ-glutamylcysteine synthetase. Glutathione is important in the reduction of cellular hydroperoxides, including lipid hydroperoxides. We utilized BSO in a recent study with ·NO [40]. In those experiments, depletion of cellular glutathione levels by BSO prior to Fe^{2+} addition resulted in a more rapid initial rate of oxygen depletion, and a shorter time for the ·NO-induced inhibition of oxygen consumption. In the current study, we incubated the MCF-7 cells with BSO for 24 h prior to adding the ·NO, then subsequently 4 μM doxorubicin. Cytotoxicity was assessed by clonogenic survival. As seen in Fig. 2A, BSO depletion had a dramatic effect. There were no surviving cells when GSH-depleted cells were exposed to ·NO then doxorubicin. This effect was dependent on [BSO] with the effect starting at 50 μM and being complete at 200 μM as shown in Fig. 2B. In separate experiments, BSO had no augmenting effect on doxorubicin toxicity in the absence of ·NO (doxorubicin 33 ± 9% normalized survival, doxorubicin plus BSO 44 ± 14% normalized survival).

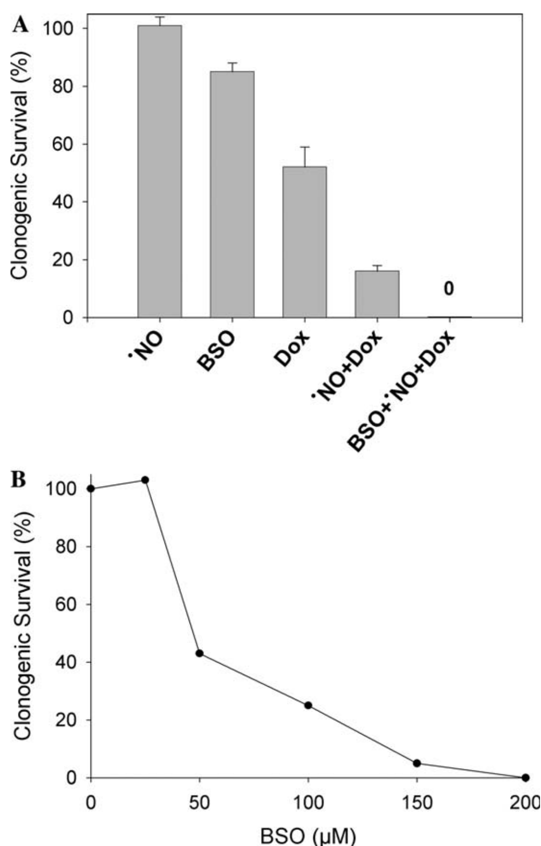

Fig. 2. (A) Glutathione depletion further augments the effect of ·NO on doxorubicin clonogenic cytotoxicity. (A) MCF-7 cells were incubated with 100 μM BSO for 24 h prior to adding the ·NO for 30 min. Then doxorubicin was added for an additional 2 h. Survival was then assessed by a clonogenic assay. BSO itself had no inhibitory effect on doxorubicin clonogenic survival (doxorubicin, 33.3%; doxorubicin + BSO, 43.9%). (B) Concentration dependence of BSO effect. MCF-7 cells were incubated with increasing concentrations of BSO for 24 h prior to adding the ·NO for 30 min. Then doxorubicin was added for an additional 2 h. Survival was then assessed by a clonogenic assay and is normalized to 100%.

Lack of augmentation when cells are exposed to doxorubicin prior to ·NO

The majority of experiments were done using a sequence of ·NO delivered to the cells 30 min prior to drug exposure. When this sequence was reversed, that is doxorubicin delivered before ·NO, there was no augmentation (Fig. 3). When the ·NO and drug were delivered simultaneously, there was a slight augmentation, but much less than when the ·NO was delivered first (data not shown).

Increased iNOS protein and ·NO production in transduced cells

Adenovirus-mediated gene transfer of iNOS may provide a way of delivering ·NO to tumor cells without directly affecting the sensitivity of normal cells. In this way an increased rate of production of ·NO is more easily maintained in the tumor over time. Also, we wanted to explore a method of avoiding the hypotension of pharmacologically delivered ·NO. We first examined the effect of iNOS transduction on ·NO production. As seen in Fig. 4, nitrite was increased in the transduced

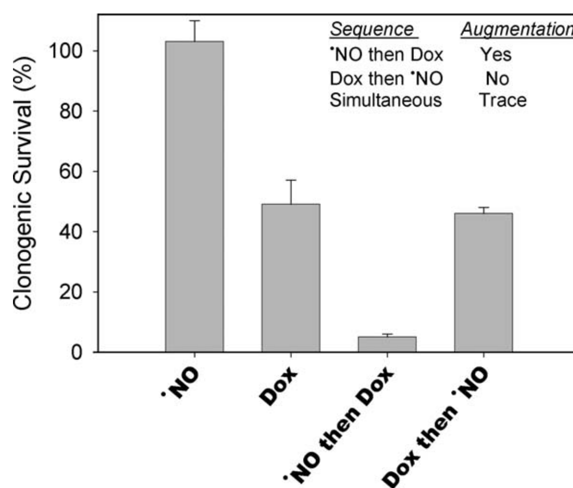

Fig. 3. The sequence of delivery is critical: ·NO delivered before doxorubicin (Dox) is effective but ·NO delivered after doxorubicin is not. Cells were exposed to doxorubicin (1 μM) for 2 h, then ·NO was delivered as a 1.8 mM bolus ("Dox then ·NO" bar), or in the sequence of ·NO then doxorubicin as described in Fig. 1 ("·NO then Dox" bar). Clonogenic survival was determined. Shown are the means and SE of three separate experiments. Abbreviations are as in Fig. 1.

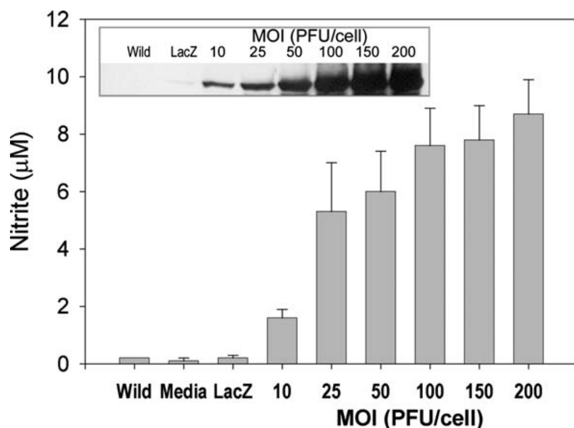

Fig. 4. Transduction of MCF-7 cells with iNOS results in ·NO production. Cells transduced with iNOS were washed and placed in full media (in one experiment, supplementation with 10 mM L-arginine did not change the result). Samples were taken at the designated time points and assayed for nitrite levels using the Sievers Nitric Oxide Analyzer. Shown are the means of 10 replicates (Wild and LacZ), 20 replicates at an MOI of 100 PFU/cell or duplicates (others). Media = MEM + 10% FBS. LacZ = Vector control cells transduced with LacZ. Inset shows that transduction of MCF-7 cells produces increased iNOS protein by Western blot.

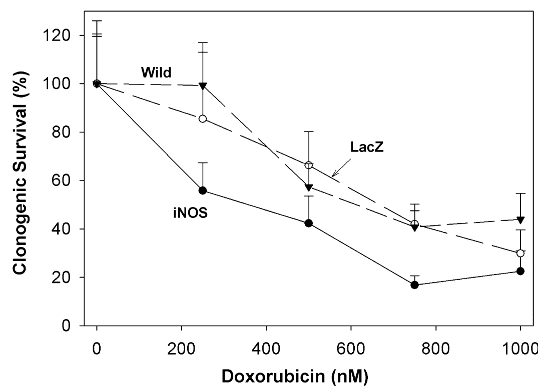

Fig. 5. iNOS transduction increases doxorubicin toxicity. MCF-7 cells were transduced with iNOS or LacZ control, both at an MOI of 100 PFU/cell, and then exposed to doxorubicin for 45 min. Clonogenic survival was determined at 14 days. The data represent means and the SE of five independent determinations. Statistical comparison was done using two-way analysis of variance.

cells at all levels of MOI. Based on this, we choose a MOI of 100 PFU/cell for further studies. iNOS protein (Western blot) increased as a function of MOI (Fig. 4, inset).

iNOS transduced cells were sensitized to doxorubicin

We compared the sensitivity of MCF-7 cells transduced with iNOS to wild-type MCF-7 cells and vector controls. As seen in Fig. 5, there was a difference in survival of the cell lines. Particularly noteworthy was that the cells transduced with iNOS had a lower survival at all concentrations of drug. In a two-way analysis of variance, the p value testing whether there is an overall difference in survival among the cell lines was $p < 0.0001$. For the pairwise comparison among the cell lines, the Bonferroni adjusted p values are as follows: wild vs iNOS $p < 0.001$ (mean difference 23, 95% confidence intervals 9.3, 36.7), LacZ vs iNOS $p < 0.005$ (mean difference 20.3, 95% confidence intervals 4.9, 35.7) and wild vs LacZ $p = 0.99$ (mean difference 2.7, 95% confidence intervals −14.3, 19.7). There was a significant difference in cell survival among the doses ($p < 0.0001$). There was not a significant interaction between cell line and dose, which indicates that the differences in cell line sensitivity to the drug did not depend upon specific drug concentrations. The non-selective inhibitor of nitric oxide synthase (N-nitro-L-arginine methyl ester) at 6.4 mM eliminated 73, 41, and 80% of the ·NO-mediated augmentation of 250, 500, and 750 nM doxorubicin concentrations, respectively.

iNOS transduction did not affect cell cycle

We examined whether iNOS transduction has an effect on cell cycle progression to explain the augmentation of doxorubicin toxicity. Table 1 shows the percentage of cells in each phase of the cell cycle prior to transduction (0 h) and at 24 and 48 h after insertion of iNOS or LacZ. There was no difference in cell cycle distribution of the iNOS transduced cells compared to wild-type cells and vector controls.

Table 1
Lack of effect of iNOS transduction on cell cycle progression[a]

	0 h	24 h			48 h		
		Wild	LacZ	iNOS	Wild	LacZ	iNOS
%G_1	43	57	55	54	54	51	51
%S	43	26	29	33	31	33	32
%G_2 + M	14	17	16	13	15	16	17

[a] Shown are values for the percentage of cells in phases of the cell cycle as determined by propidium iodide and flow cytometry and are the means of three replicates from two closely agreeing experiments. There are no meaningful differences in the cell cycle distribution of the three cell lines at 24 and 48 h.

Table 2
Lack of difference in cellular content of glutathione

Cell	GSH (nmol/mg)	GSSG (nmol/mg)
Wild	66.3 ± 14.2	0.5 ± 0.2
LacZ	47.8 ± 11.7	0.4 ± 0.2
iNOS	47.3 ± 9.0	0.4 ± 0.4

Shown are the means and SE of three separate experiments. There was no significant difference in the three cell lines by one-way analysis of variance (for GSH, $p = 0.48$; for GSSG, $p = 0.85$).

Cellular glutathione levels were not affected by transduction

Since the mechanism of augmentation of doxorubicin toxicity in the iNOS transduced cells could be due to an alteration of cellular glutathione prior to exposure to drug, we determined the levels of GSH, and GSSG (Table 2). Both transduced cells had a slightly lower level of GSH than the wild-type cells, but there was no difference in the cell lines and vector controls by one-way analysis of variance. GSSG was barely detectable, and there was no significant difference in the cell lines. In one of the three experiments, we included triplicate determinations on a cell transduced with *Bgl*II as an additional vector control, which does not contain a transgene. GSH in the *Bgl*II-transduced cell was 43.6 ± 9.4 nmol/mg while GSSG was 0.9 ± 0.1 nmol/mg. There was no significant difference in the glutathione levels of the cell lines when *Bgl*II was added to the analysis of variance statistic.

iNOS transduced H9c2 cardiac myoblasts were not sensitized to doxorubicin

We compared the doxorubicin sensitivity of iNOS transduced H9c2 cardiac cells to wild-type H9c2 cells and vector controls. As shown in Fig. 6, the three cell lines have similar cell survival and do not differ in sensitivity to doxorubicin. In a two-way analysis of variance, the p value of 0.83 showed there was no significant difference between the survival of the three cell lines. Individual comparisons for the pairwise cell lines were wild vs iNOS, $p = 0.54$ (mean difference 8.3, 95% confidence intervals −6.7, 23.4), wild vs lacZ, $p = 0.46$ (mean difference −11.7, 95% confidence intervals −31.5, 8.1), and iNOS vs LacZ, $p = 0.081$ (mean difference 20.0, 95% confidence intervals −1.8, 41.8). There was a significant effect of the dose on cell survival ($p < 0.0001$).

To confirm iNOS transduction in these myoblasts, we measured nitrite production as an estimate of ·NO production. There was a 30-fold increase in the transduced cells at the MOI level utilized (wild type, 0.2 μM; LacZ vector control, 0.2 μM; iNOS 25 MOI, 1.1 μM; iNOS 50 MOI, 1.9 μM; iNOS 100 MOI, 3.8 μM; iNOS 150 MOI, 6.1 μM; and iNOS 200 MOI, 5.8 μM).

iNOS transduced cells were not sensitized to edelfosine

We compared the edelfosine sensitivity of MCF-7 cells transduced with iNOS to wild-type MCF-7 cells and vector controls. The survival of the cell lines is shown in Fig. 7. In a two-way analysis of variance, the p value testing showed that there is not a significant difference in survival among the three cell lines ($p = 0.051$). Individual Bonferroni adjusted p values for the pairwise comparisons of the cell lines were wild vs iNOS $p = 0.99$ (mean difference −0.3, 95% confidence intervals −4.1, 3.7); wild vs LacZ, $p = 0.31$ (mean difference 1.9, 95% confidence intervals −0.9, 4.7); and iNOS vs LacZ, $p = 0.19$ (mean difference −2.2, 95% confidence intervals

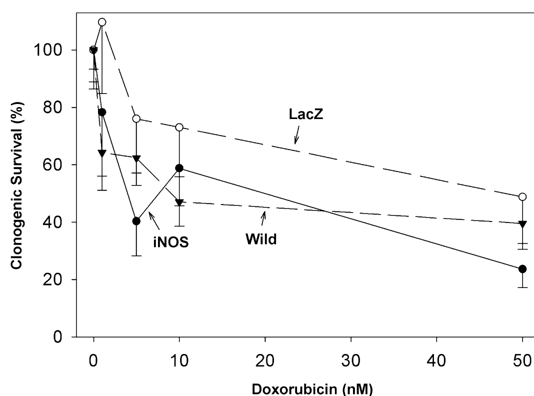

Fig. 6. iNOS transduction does not affect cardiac myoblast toxicity. H9c2 cardiac myoblasts were transduced with iNOS or LacZ control, both at an MOI of 150 PFU/cell, and then exposed to doxorubicin for 45 min. Clonogenic survival was determined at 8 days. The data represent means and the SE of four independent determinations. Statistical comparison was done using two-way analysis of variance.

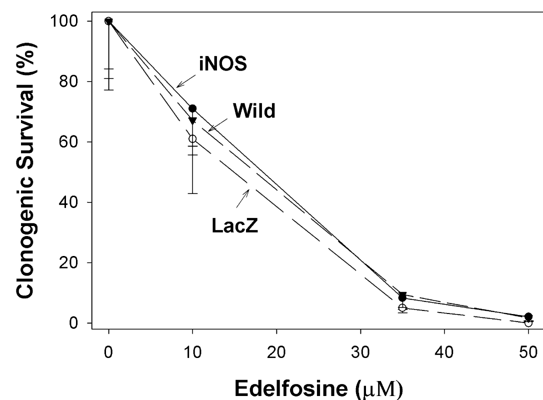

Fig. 7. iNOS transduction does not affect edelfosine toxicity. MCF-7 cells were transduced with iNOS or LacZ control, both at an MOI of 100 PFU/cell, and then exposed to edelfosine for 24 h. Clonogenic survival was determined at 14 days. The data represent means and the SE of four independent determinations (three for LacZ). Statistical comparison was done using two-way analysis of variance.

−5.0, 0.7). There was a significant effect of dose on cell survival ($p < 0.0001$).

Discussion

There are several possible mechanisms to explain the synergy of $^{\cdot}NO$ with this important chemotherapeutic agent. It may be that the doxorubicin radical produced by metabolism of the drug [41] results in an increase in $O_2^{\cdot-}$ production (Fig. 8). It is known that $^{\cdot}NO$ reacts at diffusion-limited rates with $O_2^{\cdot-}$ to produce $ONOO^-$ [1,42,43]. Any increase in $O_2^{\cdot-}$ in the presence of $^{\cdot}NO$ would lead to a subsequent increase in $ONOO^-$. This species is highly reactive with many biomolecules and may cause damage at key intracellular sites. Furthermore, such sites destabilized by the effects of $ONOO^-$ may be more susceptible to doxorubicin. We favor this mechanism. However, there are other possible nitrosative mediators of an effect on drug toxicity by $^{\cdot}NO$ including $^{\cdot}NO_2$ and N_2O_3. Doxorubicin can inhibit the cellular production of $^{\cdot}NO$ [16], therefore, the provision of higher levels of $^{\cdot}NO$ therapeutically by intervention may "counter" this effect, and in this way increase doxorubicin toxicity. The mechanism might be related to the observations of Kalivendi et al. [44] who showed that doxorubicin increases eNOS transcription in aortic endothelial cells, and that the cytotoxicity of the drug to these cells is linked to an oxidant-dependent activation by eNOS. Other possible mechanisms include an effect of $^{\cdot}NO$ on DNA repair [45,46] or lipid peroxidation.

The sensitivity of the neoplastic cell to edelfosine was not affected by $^{\cdot}NO$. This indicates that $^{\cdot}NO$ augmentation is not a general phenomenon that applies to all cytotoxic agents or to all toxic stresses. Furthermore, this specificity for doxorubicin, but not edelfosine, may allow better understanding of the mechanism of augmentation. It is possible that the augmentation is related to the DNA intercalating effect of doxorubicin since edelfosine has no direct DNA interaction.

We investigated whether the reaction of $^{\cdot}NO$ with glutathione to produce *S*-nitrosoglutathione, which lowers the cellular levels of GSH and increases susceptibility to oxidation by doxorubicin, might be the cause of the augmentation. Our observation that there is not a decrease in glutathione in iNOS transduced cells demonstrates that this is not the explanation for increased sensitivity to this oxidative drug. Similarly, there is no significant change in cellular antioxidant enzymes SOD or catalase to explain the augmentation of this oxidative drug, and there is no detectible glutathione peroxidase in MCF-7 cells [6].

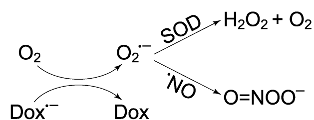

Fig. 8. Production of peroxynitrite as a possible mechanism of toxicity and role of SOD. $Dox^{\cdot-}$ in the figure is the doxorubicin semiquinone radical. It is formed by the one-electron metabolic reduction of Dox. It should be noted that the possible formation of peroxynitrite is speculative and based on the work of others since it was not measured in the present study.

Both doxorubicin and edelfosine induce oxidative events. One of the proposed mechanisms of action of doxorubicin involves oxidation since it is reduced in the cell to a semiquininone radical which can donate free electrons to O_2 [28–32]. We have reported that edelfosine enhances iron-induced lipid peroxidation [47] and generates a lipid-derived free radical [48]. Others have also reported the generation of reactive oxygen substances by edelfosine [49,50]. Since doxorubicin, but not edelfosine, is influenced by $^{\cdot}NO$, the sensitization does not apply generally to a modulation of cellular redox environment, at least not qualitatively.

Traditional cytotoxic chemotherapy is limited because of its toxicity to normal tissues especially those with a high turnover rate such as the gut and bone marrow. There is reason to postulate that the therapeutic use of $^{\cdot}NO$ might confer a greater toxicity to tumor vs normal tissues. SOD competes with $^{\cdot}NO$ for $O_2^{\cdot-}$ as shown in Fig. 8 [51]. Since SOD is low in most neoplastic tissue, these aberrant cells will have a chemical competitive advantage in the formation of the oxidatively toxic $ONOO^-$, thereby conferring a potential selective toxicity.

There are several other possible reasons for a selectivity when $^{\cdot}NO$ is given with chemotherapy. Tumors are relatively hypoxic and have a lower pH compared to normal tissues [52]. It has been reported that lipid peroxidation induced by $ONOO^-$ is greater at acid pH [53]. Therefore, to the extent that $ONOO^-$ mediates toxicity, there is rationale for relative tumor selectivity. In this regard, lower pH also increases Fe^{2+}-induced lipid peroxidation, which provides a way of magnifying oxidatively mediated reactions within tumors [54]. Lastly, tumors are known to be relatively low in the antioxidant enzymes SOD [55] and catalase. If there is a specific threshold below which the cellular antioxidant capacity becomes fatally limiting then nitrosative stress may have an effect on neoplastic tissues with lower antioxidant capacity, but not tissues with normal levels.

To achieve the full augmentation of $^{\cdot}NO$ on doxorubicin, it is crucial that the $^{\cdot}NO$ be present prior to the drug. One possible explanation for this is based on the pharmacology of doxorubicin. Doxorubicin has a double or sometimes triple exponential decay with a first half-life of about 5 min. Therefore, it is possible that the concentration of doxorubicin rapidly decreases below a critical level and is not sufficient for interaction when $^{\cdot}NO$ is added later. Another, perhaps more likely,

possibility to explain the necessity of delivering the ·NO first is simply that it requires a minimum amount of time to modulate the cellular biologic systems prior to the initiation of the apoptotic program by the anticancer drug.

Our observations are important because they provide a potential strategy to increase the efficacy of traditional chemotherapy in killing tumor cells. Concentrations of doxorubicin used in our study are well within that obtained after therapeutic administration to humans [56]. Clinically, ·NO can be given by sublingual, transdermal, p.o., i.v., aerosol, or intranasal spray delivery [57]. It can be delivered using ·NO donor compounds [58]. There has also been a good deal of interest in ·NO inhalational therapy in the last decade [59]. Alternatively, iNOS gene can also be delivered efficiently by single or multiple intratumoral injection of an adenovirus iNOS construct. This is a technique currently used in gene therapy trials because of its ability to precisely target tumors.

Most existing cancer chemotherapy is toxic to normal cells as well as neoplastic ones. It has been hoped that differences in the biochemistry, signaling events or vascularity will yield effective strategies to achieve selectivity for cancer. We have shown that the administration of ·NO augments the cytotoxicity of doxorubicin for a breast cancer cell line, but not for a cardiac cell line. In this regard, ·NO donors have been reported to protect myocytes from *t*-butyl hydroperoxide-induced oxidative damage [60]. Another group found that NOS inhibitors protected myocytes from doxorubicin, but concluded from studies of non-inhibitory enantiomers that the effect was likely due to the production of low levels of ·NO due to their ability to react with intracellular ascorbic acid [61]. Although the mechanism of our observations remains unknown, the nitrosative, and oxidative chemistry involved may provide selectivity of some antineoplastic drugs for the abnormal cell with relative sparing of susceptible normal tissue and afford a future clinical strategy to achieve therapeutic tumor selectivity.

In addition, our studies provide further information about the biology of this complex molecule. These results could be seen as unexpected based on earlier work showing that ·NO is an antioxidant [40]. The two faces of ·NO are well recognized and the paradox of prooxidant under some conditions and antioxidant under others has not been fully explained. Our observations demonstrate a synergy with an important apoptosis-inducing drug that has a prooxidant mechanism to provide insights to help explain its biologic mechanisms.

Acknowledgments

Statistical advice and calculation of the two-way analysis of variance was provided by Dr. Justine Ritchie of The University of Iowa College of Public Health. Ling Li and Dr. Douglas Spitz of the Antioxidant Enzyme Core Laboratory provided technical assistance with the glutathione experiments, and Sean Martin of the Electron Spin Resonance Core Laboratory assisted with the nitrite experiments. Dennis Charkowski of the Holden Comprehensive Cancer Center Flow Cytometry Core facility assisted with the cell cycle studies and Dr. Prabhat Goswami gave overall advice regarding these experiments. We thank Dr. Donald Heistad and the Gene Transfer Vector Core for providing the AdiNOS for use in these studies. This investigation was supported by Grants P01 CA66081 and R01 CA84462 awarded by the National Cancer Institute, Department of Health and Human Services, The Iowa Leukemia and Cancer Research Fund, The Dr. Richard O. Emmons Memorial Fund, and The Mamie C. Hopkins Fund. The Electron Spin Resonance Core is supported by P01 CA66081. The Antioxidant Enzyme Core Laboratory (glutathione determinations) is supported by P01 CA66081 and by Department of Energy Grant DE-FG02-02ER63447 (the latter to E.I. Azzam, Principal Investigator, and D.R. Spitz, Co-Investigator).

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