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Protection by Glutathione Against the Antiproliferative Effects of Nitric Oxide

DEPENDENCE ON KINETICS OF NO RELEASE

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ABSTRACT. Pretreatment by L-buthionine sulfoximine (BSO), which inactivates γ-glutamylcysteine synthetase and, therefore, inhibits glutathione (GSH) synthesis, greatly increased the sensitivity of tumor cells to the antiproliferative effects of several NO-donating compounds. The sensitization that resulted from depletion of cellular GSH pools was observed in tumor cells exhibiting different degrees of resistance to NO. In contrast, GSH depletion of tumor target cells did not affect their sensitivity to the cytostatic activity of activated macrophages and other NO-producing cells (EMT6 cells treated by interferon γ and LPS). The kinetics for NO generation is a parameter that may differentiate NO-producing cells and short-lived NO donors. To study the relationship between the magnitude of NO fluxes and the increased toxicity on BSO-pretreated cells, two NO-releasing zwitterions derived from polyamines (NONOates) with different half-lives were selected. NO fluxes as a function of time were simulated, according to the donor half-life and initial concentration, and antiproliferative effects on control and BSO-treated cells were compared. GSH depletion increased the sensitivity of tumor cells in the case of the less stable NO donor only. We, thus, propose that intracellular GSH is specifically protective against high fluxes of NO. BIOCHEM PHARMACOL 52;2:205–212, 1996.

KEY WORDS. nitric oxide; glutathione; buthionine sulfoximine; macrophage; cytotoxicity

Murine-activated macrophages exert nonspecific cytotoxicity toward tumor cells. The mechanism of growth inhibition involves the generation of nitric oxide (NO), which reacts with a range of structures in proteins, particularly haem and Fe-S centers, tyrosinyl radicals, and thiols [1–3]. It has been demonstrated that NO inhibits ribonucleotide reductase activity essential for DNA synthesis, inactivates several Fe-S center-containing enzymes in the mitochondrial electron transport chain (complex I and II) and the tricarboxylic acid cycle (aconitase), and decreases glyceraldehyde-3-phosphate dehydrogenase activity in the glycolytic pathway [4–6]. The attack upon such enzymes may account for NO-dependent cytostasis.

However, NO activates or inhibits many other targets, including bioregulatory systems (guanylate cyclase, cyclooxygenase, protein kinase C, $p21^{ras}$, etc.) and enzymes implicated in oxidative stress reactions (NADPH oxidase, catalase, xanthine oxidase, indoleamine 2-3 dioxygenase, and NO synthases, etc.) [7–9].

Because the functional importance of the potential molecular targets of NO action might differ from one cell type to another, we attempted to establish whether or not all tumor cells were equally sensitive to the antiproliferative effects of NO and what kind of manipulations might increase their sensitivity.

GSH§, the most abundant low-molecular-weight intracellular thiol, is essential for maintenance of protein thiols and protects against oxidative stress, free-radical damage, and cell injury by some chemotherapeutic drugs [10]. It has been shown that fibroblasts depleted of glutathione display reduced viability on exposure to NO [11, 12]. Because these studies utilized NO gas or NO-donating compounds, it was important to compare the role of glutathione in tumor cells exposed either to activated macrophages or to NO donors.

NO is synthesized from L-arginine by a group of isoenzymes collectively termed NO synthases [13]. During inflammatory and immunological reactions, NO is generated in large quantities, and for long periods of time, by an inducible NOS isoform (iNOS). Maximal induction of iNOS depends upon synergistic combinations of stimuli; the most effective stimuli for murine peritoneal macrophages are IFN-γ and LPS [14, 15]. Different NO-donating compounds are often used to mimic the effects of activated

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§ *Abbreviations:* BSO, L-buthionine-(S,R)-sulfoximine; DETA/NO, N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2,-ethylenediamine; E/T, effector to target ratio; FCS, fetal calf serum; GSH, glutathione; GSNO, S-Nitroso-L-glutathione; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NMMA, N-monomethyl-L-arginine; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; SOD, superoxide dismutase; Spermine-NO, 2,2′-(hydroxynitrosohydrazono)bis-ethanamine; TDM, trehalose dimycolate.

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macrophages. However, NO fluxes generated by these two types of NO sources may be substantially different on a kinetic basis because most NO donors have short half-lives, whereas macrophages release NO at a constant rate for hours. Furthermore, the nitrogen oxide reactive species generated by activated macrophages is still an object of debate.

In the present study, we have observed that among a panel of 6 different tumor cell lines, P815 mastocytoma was particularly sensitive to the cytostatic effect of NO, whereas K562 erythroleukemia was clearly more resistant than the average. We have shown that, in both cell types, depletion of cellular GSH enhanced the toxicity of all the NO donors tested, with the exception of the most stable one. However, it did not modify the macrophage antiproliferative effect.

MATERIALS AND METHODS

Macrophage and Tumor Cell Cultures

Specific pathogen-free (C57B1/6 × DBA/2)F1 mice obtained from Iffa-Credo (l'Arbresle, France) were maintained under barrier conditions and received sterile food and water. Peritoneal cells, collected as previously described [16], were resuspended in RPMI 1640 with HEPES and glutamine and supplemented with 5% FCS of low LPS content (< 0.1 ng/mL) (Gibco, Grand Island, NY, U.S.A.), and antibiotics at concentrations varying from 0.037 to 1.2 × 10^6 macrophages/mL. The macrophages were allowed to adhere in culture dishes at 37°C in a 5% CO_2 humidified atmosphere for at least 3 hr; then, nonadherent cells were washed out.

Various tumor cell lines were used: P815, a murine mastocytoma from DBA2; L929 fibroblast-like cells of murine origin; EMT6, a murine mammary adenocarcinoma from BALB/c; K562 cells derived from a human leukemia; U937, a human promonocytic cell line; and HL60, a human promyelocytic cell line. All these cell lines were negative for mycoplasma contamination and were maintained in RPMI 1640 supplemented with glutamine, HEPES, antibiotics, and 10% FCS.

NO-Producing Cells

Three types of cells were used:

1. Macrophages primed *in vivo* by TDM. TDM from *Mycobacterium tuberculosis*, strain Peurois, was suspended in water [16] and injected i.p. (50 μg TDM/mouse) 7 days before macrophage harvesting. NOS activity was induced in TDM-primed macrophages by a short exposure (4 hr) to low doses of LPS (1 to 10 ng/mL) [17]. LPS was from Sigma (L 6011) (St. Louis, MO, U.S.A.).
2. Inflammatory macrophages were elicited by thioglycollate: 1.5 mL of thioglycollate broth (Sanofi-Pasteur Diagnostics, France) were injected i.p. 4 days before macrophage harvesting. NOS activity was induced in thioglycollate-elicited macrophages by 24-hr treatment with IFN-γ (2 to 10 U/mL) and LPS (1 to 10 ng/mL) [18]. Murine recombinant IFN-γ was kindly provided by Dr. G. R. Adolf (Ernst-Boehringer Institut für Arzneimittel Forschung, Vienna, Austria).
3. EMT6 cells were treated with IFN-γ (50 U/mL) and LPS (100 ng/mL) for 18 hr [19].

NO-Generating Compounds

Various NO-generating compounds were used: GSNO and SNAP were obtained from Alexis (Switzerland); SIN-1 was a kind gift from Dr. Winicki, Hoechst; Spermine/NO and DETA/NO were obtained from RBI (Natick, MA, U.S.A.).

NO production by iNOS-expressing cells, thionitrites, and SIN-1 was measured by nitrite accumulation in the culture medium. Nitrite concentration was measured by using Griess reagent, as previously described [19]. Decomposition of NONOates was followed spectrophotometrically, at 37°C, in 0.1 M phosphate buffer, pH 7.4. For stimulation of NO production, it was considered that NONOates release 2 equivalents of NO and that NO autoxidizes in aqueous solutions according to the following equations [20]:

$$4\ NO^\circ + O_2 + 2H_2O \rightarrow 4\ NO_2^- + 4\ H^+$$

$$\text{with } -d[NO^\circ]/dt = 4\ k\ [NO^\circ]^2\ [O_2]$$

$[O_2]$ being constant and equal to 220 μM.

The second integration method of Runge-Kutta was used [21]. A small integration step was used to simulate the reactions at the initial times and the integration step was increased as the reaction proceeded with time; as a rule, the integration step size was of the order of one hundredth of the reaction time of the fastest reaction. The calculations and graphics were performed under the spreadsheet QuattroPro (Borland-Novell).

GSH Depletion and Assay

For GSH depletion, tumor cells in exponential growth were treated for 18 hr with 100 μM BSO (from Sigma), a transition state inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step of GSH synthesis. Cells were centrifuged and resuspended at 0.3 × 10^6 cells/mL in fresh culture medium. Total glutathione (GSH plus glutathione disulfide (GSSG)) was determined in cell lysates prepared in 0.1% Triton X100 or 0.6% sulfosalicylic acid by the GSSG reductase-5,5′-dithiobis-2-nitrobenzoate recycling method [22, 23]. All data were normalized per mg protein; protein content of cell lysates was measured by the method of Bradford.

Measurement of Tumor Cell Proliferation

Tumor cells (0.3 × 10^6 cells/mL), pretreated or not with BSO, were distributed in microtest plates (200 μL/well). In

some experiments, the microtest wells contained an adherent monolayer of activated macrophages and, in others, NO-generating compounds were added. Tumor cell growth was monitored by [^{3}H]-thymidine incorporation. Thymidine ([5-^{3}H]-thymidine from Amersham, Les Ulis, France, supplemented with cold thymidine to obtain a specific activity of 1 GBq/mmol), was added to cultures in microtest plates (37 kBq/well). Four or 18 hr later, the DNA of each well was collected on glass fiber filters with a cell harvester (Skatron, Norway) and the radioactivity incorporated into the DNA was measured in a β-scintillation spectrometer. All assays were performed in triplicate. Results are expressed as residual tumor growth after exposure to NO, in percent of tumor growth in untreated cultures.

Statistical Analysis

Data are presented as mean ± SD of at least 3 independent experiments. Statistical significance of differences was assessed by unpaired Student's *t*-test.

RESULTS

Sensitivity of Various Tumor Cell Lines to the Antiproliferative Effects of NO

The antiproliferative effects of exogenously administered NO were determined by exposing tumor cells of various origins to increasing concentrations of NO donors. The cytostatic action of NO was quantified by calculation of residual growth, measured by [^{3}H]-thymidine incorporation. For most cell lines, incubation in the presence of 100 μM SNAP reduced proliferation by 30% (Fig. 1). However, 2 cell lines presented an original profile: K562 cells were more resistant (10% growth inhibition in the presence of 200 μM SNAP) and P815 cells were more sensitive (60% growth inhibition in the presence of 100 μM SNAP) (Fig. 1). Similar results were obtained with 3 other NO donors (GSNO, Spermine-NO and DETA-NO) and in the presence of SIN-1 plus SOD (Table 1): the concentration of NO donor required to reduce tumor cell growth by 50% was 3 to 8 times higher for K562 cells than for P815 cells. Conversely, K562 cells were sensitive to SIN-1, which releases stoichiometric amounts of NO and superoxide anion (Table 1).

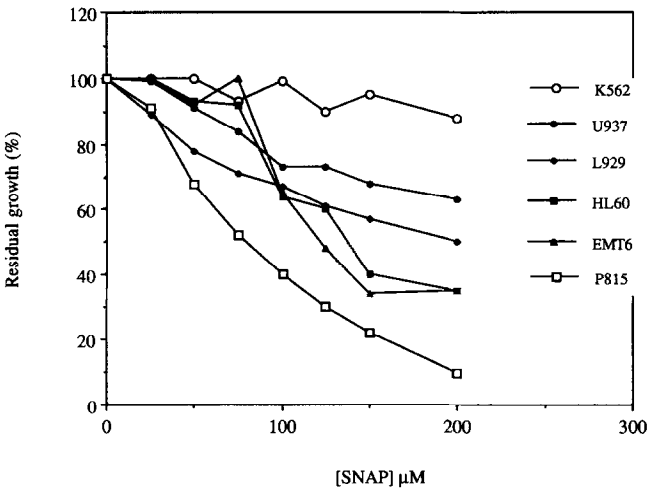

FIG. 1. Sensitivity of various tumor cell lines to the antiproliferative effect of NO generated from SNAP. Tumor cells (0.3 × 10^6 cells/mL) were plated in microtest wells (200 μL/well) in RPMI 1640 supplemented with FCS and antibiotics. Increasing doses of SNAP were added; after 2 hr of incubation at 37°C, tritiated thymidine was added. Thymidine incorporation was measured after 18 hr in triplicate wells. Results from an experiment representative of 3.

The sensitivity of P815 and K562 cells to the cytostatic effects of activated murine peritoneal macrophages was also different, as shown in Fig. 2. The discrimination was possible when the experimental conditions were selected to give a production of NO corresponding to an accumulation of nitrite inferior to 20 μM after 18 hr. A moderate production of NO was obtained either by decreasing macrophage density to 0.3 × 10^6 macrophages/mL and below (Fig. 2) or by reducing the stimulant doses applied on macrophages plated at high density (for example, IFN γ 0.5 U/mL and LPS 1 ng/mL added to 1.2 × 10^6 macrophages/mL; Table 3, line 1). With fully activated macrophages at a conventional E/T ratio of 4, a complete growth arrest (residual growth < 2% of controls) was observed with both cell lines.

Effects of GSH Depletion of the Sensitivity of Tumor Cells to NO

In a first attempt to determine the mechanisms of resistance to NO antiproliferative action, we investigated the effect of depletion of intracellular glutathione. The concentration of total glutathione was similar in P815 and K562 cells (12.1 ± 1.0 nmoles/mg cellular proteins and 10.5 ± 0.9 nmoles/mg, respectively). Pretreatment of tumor cells for 18 hr with 100 μM BSO reduced intracellular glutathione by 95% (total glutathione was 0.07 ± 0.005 nmoles/mg and 0.06 ± 0.008 nmoles/mg in BSO-treated P815 and K562 cells, respectively). Glutathione depletion had no effect on the proliferative capacity of the two cell lines: thymidine incorporation by BSO-pretreated cells was within ± 10% of the incorporation measured for control cells (usually 100,000 cpm, see Table 2 legend for actual figures).

TABLE 1. Concentrations of NO donors required to inhibit tumor growth by 50%

NO donor	IC_{50} for P815 (μM)	IC_{50} for K562 (μM)
SNAP	70 ± 10	600 ± 50
GSNO	84 ± 12	⩾600
SIN-1	103 ± 20	82 ± 8
SIN-1 + SOD	90 ± 20	⩾600
Spermine/NO	100 ± 20	300 ± 50
DETA/NO	220 ± 40	600 ± 50

SOD, 500 U/mL. Experimental procedure as in Fig. 1. Results are the mean ± SD of at least 3 independent experiments.

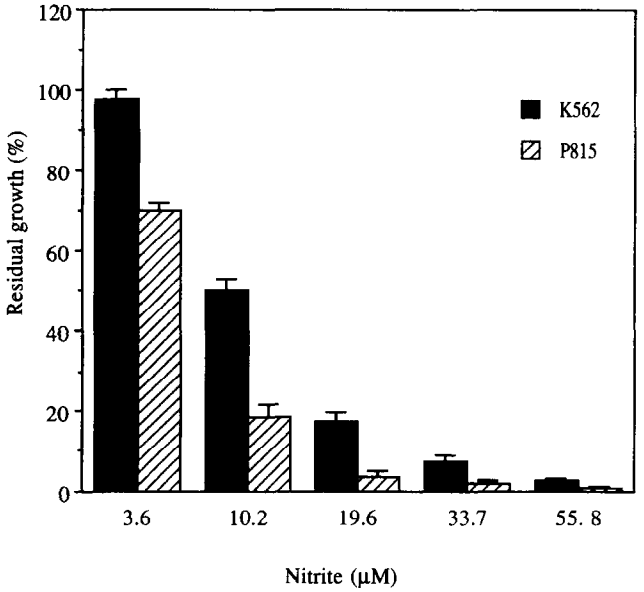

FIG. 2. The sensitivity of P815 and K562 cells to the cytostatic effect of activated macrophages is different. Thioglycollate-elicited macrophages were suspended in RPMI 1640 supplemented with FCS and antibiotics at different concentrations: 0.037, 0.075, 0.15, 0.3, and 0.6 x 10^6 macrophages/mL. Cell suspensions were plated in microtest wells (200 μL/well). After 4 hr, macrophage monolayers were washed and interferon γ (20 U/mL) was added. Twenty hours later, LPS (ng/mL) was added. Four hours after LPS addition, the culture medium was replaced by tumor cell suspensions (0.3 x 10^6 cells/mL in RPMI 1640 medium supplemented with FCS and antibiotics), and LPS and tritiated thymidine were added. For each macrophage concentration, 6 wells did not receive thymidine and were used, instead, for nitrite determination. Nitrite accumulation and thymidine incorporation were measured 18 hr after addition of tumor cells. Results are presented as mean ± SD of 5 independent experiments. The E/T ratios increased from 0.1 (first pair of columns) to 2 (last pair of columns). As a control, measurements were also conducted at an E/T ratio of 4 (1.2 x 10^6 macrophages/mL): nitrite accumulation was 77 ± 5 μM and the residual growth of tumor cells was reduced to 0.5 ± 0.1% and to 1.8 ± 0.2% of controls for P815 and K562 cells, respectively.

Depletion of glutathione resulted in a significant increase in the sensitivity of both cell lines to NO released from SNAP (Table 2): the concentrations of SNAP that inhibited control cell growth by 50% reduced the growth of BSO-pretreated cells to small percentages.

Experiments were repeated using peritoneal macrophages as a NO source. Macrophages were elicited by thioglycollate and activated *in vitro* for iNOS expression by IFN-γ and LPS. We verified that the cytostatic effect of macrophages against target cells treated or not by BSO was blocked by NMMA, an analog of arginine inhibiting iNOS (in the presence of 0.2 mM NMMA, the residual growth of BSO-treated tumor cells cultured for 4 hr in the presence of 0.15 x 10^6 activated macrophages was 77% and 91% of controls for P815 and K562 cells, respectively; compare to data of Table 3, line 3). We verified, also, that BSO, added to macrophages after iNOS induction, did not reduce NOS activity (100 μM BSO reduced nitrite accumulation over 18 hr by 10% ± 3%). As shown in Table 3, GSH depletion by BSO did not increase the sensitivity of either P815 or K562 cells to the cytostatic effects of macrophages.

This result was repeatedly observed under various experimental conditions: macrophage density and stimulation mode were varied to modify the possible contribution of macrophage-secreted products other than NO. In some experiments, the duration of the cytostasis assay was reduced to 4 hr to compare macrophages with short-lived NO donors more accurately (Table 3, line 3). In other experiments, 100 μM BSO was added to the cocultures of activated macrophages and BSO-pretreated tumor cells: no significant difference was observed in the level of proliferation measured in cultures that had received BSO or not during the cytostasis assay (data not shown). In addition to thioglycollate-elicited macrophages, 2 other types of NO-producing cells were used: macrophages preactivated *in vivo* by TDM and stimulated *in vitro* by LPS, and EMT6 adenocarcinoma cells cultured *in vitro* in the presence of IFN-γ and LPS. In both cases, cytostatic activity was not modified by pretreatment of tumor cells with BSO (Table 3).

Glutathione Protects Against High Fluxes of NO

An adherent monolayer of macrophages in standard culture dishes produces a NO flux that generates a steady-state concentration of NO of 5 μM in the vicinity of the effector cells at the maximum (1.2 x 10^6 fully activated macrophages/mL) [24]. Reducing macrophage density, as in the experiments reported in Table 3, will reduce the steady-state concentration of NO between 0.5 and 1 μM [24]. Fluxes generated by NO donors will vary according to the donor half-life and initial concentration; for some NO-donating compounds, supplementary factors (temperature, pH, thiol concentration, etc.) have to be taken into account. We selected two donors that belong to the same family of zwitterions derived from polyamines, but which have different half-lives: spermine/NO ($t_{1/2}$ = 39 min, at 37°C) and DETA/NO ($t_{1/2}$ = 20 hr) [25]. The kinetics of NO production were simulated as described in Materials and Methods. As shown in Fig. 3, NO is released from spermine/NO as a burst: 5 μM NO is produced in the first few min from 300 μM spermine/NO but, after 5 hr, the concentration of NO is reduced to 0.1 μM. In contrast, the release of NO from 300 μM DETA/NO gives a plateau in the μM range for 20 hr. The protecting effect of glutathione was compared for tumor cells exposed to both NONOates (Table 4). In the presence of the less stable NO-donor (spermine/NO), glutathione-depleted tumor cells were clearly more susceptible to NO injury than their normal counterparts. In contrast, when the NO donor had a longer half-life (DETA/NO), similar levels of cytostasis were observed for glutathione-depleted cells and controls cells, a situation reminiscent of that observed with macrophages.

TABLE 2. Glutathione-depleted tumor cells exhibited an increased sensitivity to NO generated from SNAP

SNAP added (μM)	Nitrite produced (μM)	P815 residual growth (%)		K562 residual growth (%)	
		Control	BSO-treated	Control	BSO-treated
150	53.7 ± 10.6	38.5 ± 13	5.3 ± 1.5	88.5 ± 10	19.1 ± 2.7
300	104.0 ± 16.0	9.4 ± 3.6	2.3 ± 1.0	65.5 ± 13	5.8 ± 2
600	172.0 ± 13.6	3.8 ± 2.8	1.4 ± 0.9	58.7 ± 14	2.2 ± 0.6

Tumor cell residual growth and nitrite accumulation were measured 18 hr after the addition of SNAP. Data are the mean ± SD of 4 independent experiments. P815 control cells vs K562 control cells: $P = 0.001$. BSO-treated K562 cells vs K562 control cells: $P < 0.004$. BSO-treated P815 cells vs P815 control cells, with 150 μM SNAP: $P = 0.002$. BSO-treated P815 cells vs P815 control cells, with 300 μM SNAP: $P = 0.01$. BSO-treated P815 vs P815 control cells, with 600 μM SNAP: $P = 0.12$. Thymidine incorporation was 93,900 ± 6300 cpm and 92,900 ± 1100 cpm for control and BSO-treated P815 cells, respectively, $P = 0.77$ (paired Student's t-test); it was 114 700 ± 7300 and 108,500 ± 12,900 for control and BSO-treated K562 cells, respectively, $P = 0.55$.

DISCUSSION

Glutathione is the major intracellular reserve of nonprotein free thiols, and is capable of interacting with oxidizing agents. Two groups have recently proposed that NO mediates the clonogenic inactivation of cells through a pathway that can be inhibited significantly by cellular glutathione. The authors observed that glutathione depletion by BSO resulted in enhanced cytotoxicity on exposure to a bolus of NO: in fact tumor cells were exposed either to 1.7 mM NO [12] or to 2 mM DEA/NO [11], a NO donor of extremely short half-life ($t_{1/2}$ = 2.1 min, at 37°C) [25]. In this report, we confirm these observations: with two tumor cell lines exhibiting a large difference in sensitivity to NO antiproliferative effects, we demonstrated that depletion of intracellular glutathione by pretreatment with BSO increased the cytostatic activity of SNAP.

In contrast, we demonstrated that glutathione did not interfere with the cytostic activity of NO-producing cells (activated macrophages or stimulated EMT6 cells). It has been established that BSO inhibits NOS induction but does not interfere with production of NO by activated cells [26, 27, and this report].

Several hypotheses may be proposed to explain that SNAP acts through a glutathione-sensitive pathway and NO-producing cells through a glutathione-insensitive pathway: (1) fluxes of NO (concentration and duration) may be different in both cases; (2) the reactive intermediates produced may be different; and (3) the cytostatic activity observed with NO-producing cells may be the result of the synergistic action of NO and another molecule. The last hypothesis seems improbable because it is unlikely that the 3 cell types used as a NO source (thioglycollate-elicited and TDM-preactivated macrophages and EMT6 adenocarcinoma cells) produce the same cytotoxines. For example, we noticed that TDM-preactivated macrophages do not release TNFα, even after LPS addition and that EMT6 cells stimulated by IFNγ and LPS do not release superoxide or hydrogen peroxide upon TPA triggering [our unpublished results].

In an attempt to validate the first hypothesis, we established by simulations that NO fluxes generated by short- or long-lived NO donors are, actually, different. Furthermore, the nature of the reactive species generated may depend upon the NO concentration. Recently, Wink *et al.* [11] studied the formation of S-NO complexes with stopped-flow techniques: the kinetics clearly showed that the reaction of two molecules of NO with one of O_2 must occur before the formation of an S-NO adduct, the nitrosating species being a reactive nitrogen oxide, NO_x. Moreover,

TABLE 3. Pretreatment of tumor cells by BSO did not increase their sensitivity to the antiproliferative action of NO-producing cells

NO-producing cells	Density ($\times 10^{-6}$) cells/mL	Stimulation		Assay (h)	Nitrite (μM)	P815 residual growth (%)		K562 residual growth (%)	
		IFNγ (U/mL)	LPS (ng/mL)			Control	BSO-treated	Control	BSO-treated
Thio-MΦ	1.2	0.5	1	18	17 ± 4	20.2 ± 4.5	23.3 ± 6.4	56.7 ± 2.8	52.7 ± 8.2
Thio-MΦ	0.075	20	10	18	12 ± 2.5	14.4 ± 2.0	15.6 ± 1.0	51.1 ± 3.9	49.3 ± 4.0
Thio-MΦ	0.15	20	10	4	4.7 ± 0.9	21.2 ± 2.3	18.6 ± 3.5	42.1 ± 3.4	55.6 ± 10
TDM-MΦ	0.6	–	10	18	26 ± 5	1.8 ± 0.4	1.8 ± 0.5	34.0 ± 4	43.6 ± 3
TDM-MΦ	0.3	–	10	18	12 ± 3	37.1 ± 5	26.3 ± 6	100	100
EMT-6	0.1	50	100	18	17 ± 1.5	13.1 ± 0.5	16.3 ± 3	54.9 ± 7	58.6 ± 6

Macrophages were either elicited by thioglycollate broth (Thio-MΦ) or primed *in vivo* by TDM (TDM-MΦ). Nitrite accumulation was identical in cultures that received untreated tumor cells and BSO-treated cells. Results are presented as mean ± SD of 3 independent experiments.

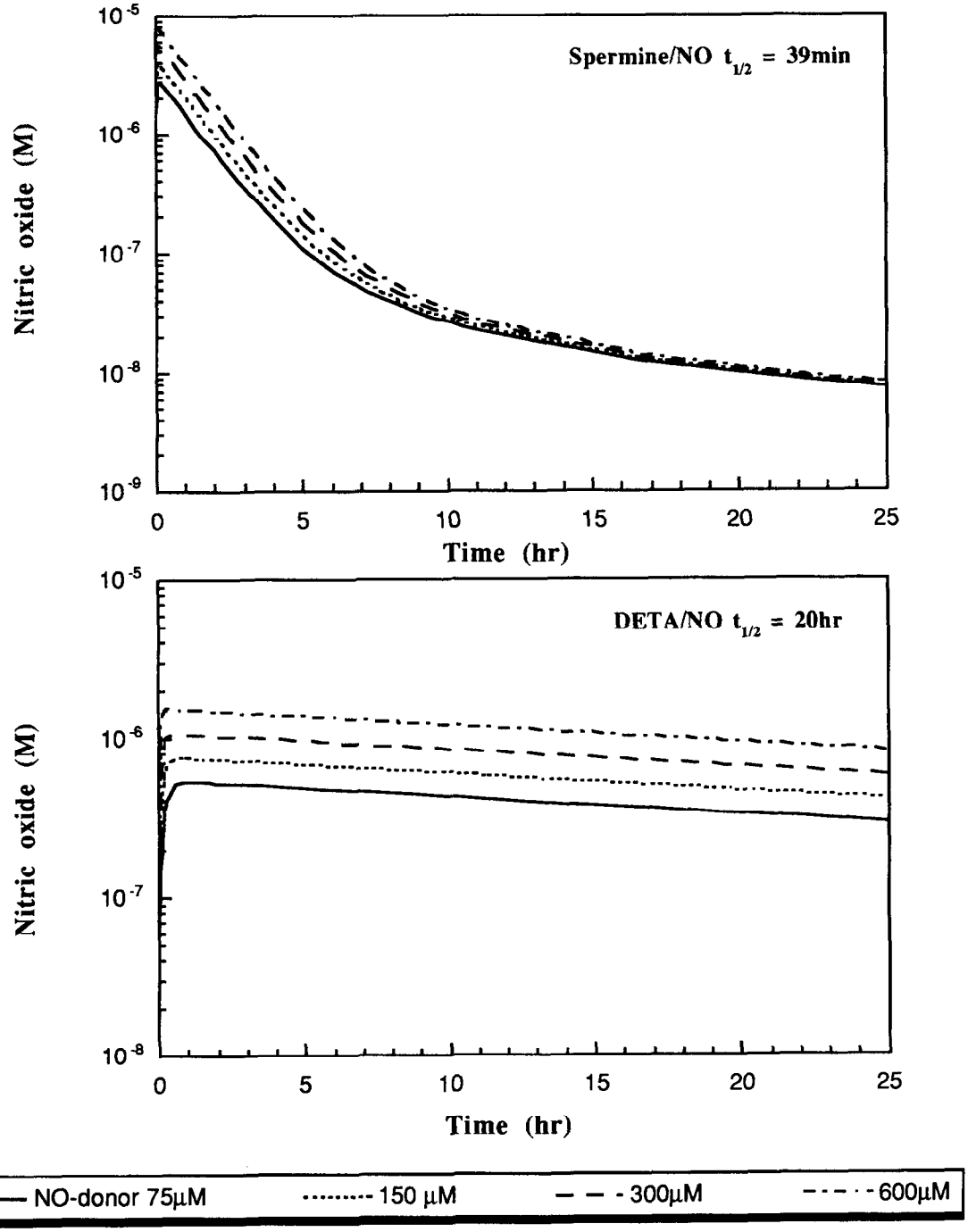

FIG. 3. Stimulation of NO fluxes generated by two NO donors with different half-lives. The molar concentration of NO generated by the spontaneous decomposition of spermine-NO ($t_{1/2}$ = 39 min at 37°C), upper panel, or DETA-NO ($t_{1/2}$ = 20 hr at 37°C), lower panel, was simulated as a function of time for increasing initial concentrations of the NO-donating compounds (75, 150, 300, and 600 μM). The concentration of oxygen was assumed to be constant and equal to 220 μM.

anaerobic solutions of NO are not capable of nitrosating glutathione. The second-order dependency on NO for the formation of NO_x implies that, at low NO concentrations, cytotoxicity will be mostly mediated by NO itself through its direct reaction with metalloproteins or tyrosinyl radicals. However, as the NO concentration increases, the amount of NO_x species generated will increase rapidly and contribute to the observed cytotoxicity. Because NO_x (unlike NO) preferentially reacts with thiols, glutathione may serve to scavenge NO_x or regenerate oxidized protein thiols. Thus, the protective action of intracellular GSH may be restricted to situations where high fluxes of NO (and NO_x) are produced.

From the experiments reported here, it can be concluded that the cytostatic effects of long-lived NO donors and activated macrophages plated at low density are due to NO.

TABLE 4. Glutathione determines cell sensitivity to high fluxes of NO

NO donor	Concentration (μM)	P815 residual growth (%)		K562 residual growth (%)	
		Control	BSO-treated	Control	BSO-treated
Spermine NO	75	61.7 ± 1.7	34.5 ± 2	104.0 ± 6	48.1 ± 4
	150	33.7 ± 5	3.6 ± 0.9	69.3 ± 2	4.7 ± 1
	300	4.3 ± 0.9	0.3 ± 0.1	42.2 ± 3	0.2 ± 0.05
DETA/NO	150	70.5 ± 3	78.0 ± 4	100.5 ± 4	93.3 ± 5
	300	37.0 ± 4	32.1 ± 5	90.0 ± 5	79.5 ± 7
	600	8.3 ± 1	8.6 ± 2	52.0 ± 6	25.3 ± 5

Experimental procedure as in Fig. 1. Results are presented as mean ± SD of 3 independent experiments. Differences between control cells and BSO-pretreated cells were always significant with spermine/NO as a NO donor ($P < 0.04$); they were never significant in the case of DETA/NO ($P > 0.3$), except for 600 μM DETA/NO added on K562 cells ($P = 0.001$).

In contrast, the cytostatic effects of short-lived NO donors (and perhaps of macrophages cultured at high density) may be due in part to NO_x, which selectively reacts with thiols. Primary targets of reactive nitrogen oxide species may be different in cells submitted to low (≤1 μM NO) or high steady-state concentrations of NO. Taking into account the diffusion and oxidation of NO, it can be calculated that a stationary NO concentration of 1 μM in the vicinity of the producing cells corresponds to a rate of nitrite production of about 1.8 μM/hr, which is obtained *in vitro* when activated macrophages are plated at a density of 10^5 macrophages/mL [24]. Such a concentration is in the same order of magnitude as the concentration of endogenous free NO directly measured with a porphyrinic microsensor, reported to be 320 nM at the rat alveolar macrophage surface 300 min after addition of IFNγ [28] and 1.30 μM on the endothelial cell membrane after injection of bradykinin [29].

The main difference between cells expressing constitutive or inducible NO synthase is the period of time (min vs hr) during which they are able to produce NO. Thus, different NO-donating compounds have to be used to mimic the release of NO by cells expressing the inducible or constitutive forms. It may also be possible to use pharmacological NO-donors of differing stability to attack different molecular targets (thiol groups vs metal centers).

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