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The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells

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Abstract

Nitric oxide (NO) plays an important role in the cytotoxic activity of macrophages towards tumour cells and microbial pathogens. We investigated whether alteration of intracellular thiol levels modulates the cytotoxic effects of different NO donors and lipopolysaccharide-induced NO in the murine macrophage cell line J774A.1. The NO-releasing compound *S*-nitroso-*N*-acetylpenicillamine caused a significant concentration-dependent loss of viability of the macrophages only under glucose-limiting conditions. The cytotoxic effect of *S*-nitroso-*N*-acetylpenicillamine was prevented by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO). Depletion of total glutathione before exposure to *S*-nitroso-*N*-acetylpenicillamine further decreased cell viability while pretreatment with *N*-acetylcysteine was protective. Comparing equimolar concentrations of various NO donors including *S*-nitrosoglutathione, *S*-nitrosocysteine and 3-morpholino-sydnonimine hydrochloride, cytotoxicity appeared to be related to the relative stability of the test compound. Both the order of stability and the order of potency for cell killing was *S*-nitrosoglutathione > *S*-nitroso-*N*-acetylpenicillamine > *S*-nitrosocysteine = 3-morpholino-sydnonimine hydrochloride. Stimulation of the macrophages with lipopolysaccharide and interferon-γ resulted in dose-dependent cell injury and NO production. Glutathione depletion prior to stimulation considerably decreased macrophage viability as well as the NO production. In contrast to the protective effect on *S*-nitroso-*N*-acetylpenicillamine-mediated injury, pretreatment with *N*-acetylcysteine did not influence the lipopolysaccharide-mediated cytotoxicity. These results demonstrate that (a) reduction in the availability of glucose and intracellular glutathione renders the cells more vulnerable to the cytotoxic effects of NO donors, (b) in this model of cytotoxicity, long-lived NO donors were more cytotoxic than short-lived NO donors, (c) the differential effects of *N*-acetylcysteine on *S*-nitroso-*N*-acetylpenicillamine-induced and bacterial lipopolysaccharide-mediated cytotoxicity support the existence of other toxic species different from NO or NO-related compounds with a potent cytotoxic activity in immunostimulated macrophages, and (d) other non-protein thiols like *N*-acetylcysteine may substitute for glutathione as a major component of the cellular antioxidant defense system.

Keywords: Nitric oxide (NO); Nitrosothiol; Glutathione; *N*-Acetylcysteine; Glucose; Lipopolysaccharide; Cytotoxicity; Macrophage

1. Introduction

The demonstration of the synthesis of nitrite and nitrate (NO_2^-/NO_3^-) by activated murine macrophages (Stuehr and Marletta, 1985) and the identification of the radical nitric oxide (NO) as the intermediate in the enzymatic conversion of L-arginine to nitrogen oxides by macrophages (Marletta et al., 1988) suggested that NO may play a more extensive role as an effector molecule in immunological and inflammatory reactions. Indeed, the phagocytosis-independent cytotoxic effects of murine macrophage-derived NO have been shown to be a primary mechanism of defense against tumour cells (Stuehr and Nathan, 1989) and a number of intracellular and extracellular microorganisms (Hibbs et al., 1990; Nathan and Hibbs, 1991). NO is a highly reactive free radical with a broad aqueous chemistry involving other interrelated redox forms that may also contribute to the cytotoxic effects of activated macrophages (Stamler et al., 1992). Thus, the cytotoxicity of NO must also be viewed within the context of the intracellular redox milieu.

The mechanisms by which NO (or NO-related species) exerts cytotoxicity have not been completely characterized, but they include inhibition of different enzymes containing catalytically active non-haem iron coordinated to sulphur atoms, release of intracellular iron from target cells and damage of DNA (Hibbs et al., 1990). Interestingly, NO is

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cytostatic in some cells and cytolytic in others, suggesting that the different sensitivity to NO may be dependent on the relative importance of Fe-S-centred enzymes in the target cells (Moncada et al., 1991). In this respect, previous studies showed that tumour target cells co-cultivated with cytotoxic activated macrophages stopped dividing and rapidly died unless substrate for glycolysis, a metabolic pathway which does not involve catalytically active iron, was present in their environment. Because macrophage-induced cytotoxicity almost completely inhibited cellular respiration, and hence mitochondrial ATP production, injured neoplastic cells became dependent on glucose supply for chemical energy (Granger et al., 1980). Furthermore, the macrophages themselves are subject to the cytotoxic effects of NO production by the L-arginine/NO pathway (Tucker et al., 1991) and the expression of NO synthase activity is associated with rapid rates of glucose uptake and glycolysis (Albina and Mastrofrancesco, 1993).

Mammalian cells have evolved protective mechanisms to minimize injury by oxygen-based and other radicals. In this respect, glutathione has a major role as scavenger of free radicals and is probably the most important cellular antioxidant (Meister, 1994). This cysteine-containing tripeptide accounts for the majority of the intracellular non-protein thiols in mammalian cells. Its depletion results in a higher sensitivity of the cells to the toxic effects of nitric oxide, different oxidants and radiation (Meister, 1994; Walker et al., 1995; Deneke and Fanburg, 1989).

Although it is well established that free sulfhydryl group-containing compounds are potential targets for reactive nitrogen oxides (Stamler et al., 1992), there are only few data regarding the effect of thiol depletion on NO-induced cytotoxicity (Walker et al., 1995) and to our knowledge, none regarding the protective role against NO-mediated cell death of other non-protein thiols, e.g., *N*-acetylcysteine. Compounds which generate NO or related redox species have been used in several studies to explore the toxicity of NO towards mammalian cells (e.g., Tepperman et al., 1994; Szabó and Salzman, 1995). The present study was thus performed in order to investigate whether alteration of intracellular thiol levels and changes in the glucose content of the culture media modulate the cytotoxic effects of NO donors and endogenously produced NO in the J774A.1 murine monocytic leukaemia cell line.

2. Materials and methods

2.1. Drugs

S-Nitroso-*N*-acetyl-D,L-penicillamine was kindly provided by Dr. Martin Feelisch (Schwarz Pharma, Monheim, Germany). *S*-Nitrosoglutathione and *S*-nitrosocysteine were synthesized as previously described (Zamora and Feelisch, 1994). L-Cysteine hydrochloride, reduced glutathione, *N*-acetyl-D,L-penicillamine, hydroxylamine, glutathione disulphide reductase, NADPH, 5,5′-dithiobis-2-nitrobenzoic acid, *N*-acetyl-L-cysteine, L-buthionine-[*S*,*R*]-sulfoximine, 3-amino-7-dimethyl-2-methylphenazine hydrochloride (neutral red) and lipopolysaccharide (phenol-extracted *Salmonella typhosa*) were purchased from Sigma (St. Louis, MO, USA). Sodium nitrite was from Merck (Darmstadt, Germany). N^G-Monomethyl-L-arginine was a gift from Dr. Salvador Moncada (Wellcome Research Laboratories, Beckenham, UK). Recombinant rat interferon-γ was from Holland Biotechnology (Leiden, Netherlands). 3-Morpholino-sydnonimine hydrochloride was from Cassella (Frankfurt, Germany). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) was from Alexis (Läufelfingen, Switzerland). All cell culture media and supplements were from Gibco (Paisley, UK).

2.2. Cells and culture medium

The murine monocyte/macrophage cell line J774A.1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium containing 1000 mg/l glucose and supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Experiments were performed in 1000 mg/l or 250 mg/l glucose-containing medium.

2.3. Experimental protocol

Cells were seeded in 24-well plates (1 ml) or in 96-well microtiter plates (250 μl) at a concentration of 10^6 cells/ml and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h. Thereafter, cells were washed with warm medium to remove non-adherent cells and medium was replaced with either 1000 mg/l glucose-containing medium (control) or 1000 mg/l glucose-containing medium plus buthionine sulfoximine or *N*-acetylcysteine at concentrations ranging from 1 to 1000 μM. After 24 h, the medium was replaced by fresh medium in the presence or absence of various concentrations of the NO donors or lipopolysaccharide plus interferon-γ as described in Section 3. Following another 24 h incubation period the medium was collected, centrifuged and stored at −20°C for later NO_2^-/NO_3^- determination.

2.4. Determination of total glutathione levels

The total cellular glutathione content (GSH + GSSG) was determined using a modified procedure of the GSSG reductase recycling assay as previously described (Anderson, 1985). Briefly, the monolayer ((7–8) × 10^6 cells/culture flask) was washed three times with ice-cold phosphate-buffered saline and carefully scraped off after addition of 1 ml 100 mM Sorensen phosphate buffer (20 mM KH_2PO_4, 89 mM Na_2HPO_4, pH 7.4). The protein content of the cell suspension was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL,

USA) as previously described (Smith et al., 1985). The cell suspension was then mixed vigorously with 5% 5-sulfosalicylic acid and centrifuged at $3000 \times g$ for 15 min to remove protein residues. The supernatant was assayed for glutathione by measuring the colour change of 5,5′-dithiobis-2-nitrobenzoic acid at 415 nm in the presence of GSSG reductase and NADPH. All data were normalized to mg protein.

2.5. NO_2^-/NO_3^- measurement

NO production was assayed by measuring the accumulation of NO_2^-/NO_3^- in the cell supernatant. Sample proteins were precipitated with 30% (w/v) $ZnSO_4$ solution and centrifuged. After reducing NO_3^- in the supernatant to NO_2^- using acid-washed cadmium granules (Thomsen et al., 1991), NO_2^- concentrations were measured using the Griess reaction (Schmidt et al., 1988). Briefly, 200 μl of cell-free supernatant was transferred to a 96-well microtiter plate and mixed with 40 μl of 6.5 M HCl and 37.5 mM sulfanilic acid (1 : 1). After 10 min, 20 μl 12.5 mM *N*-(1-naphthyl)-ethylene diamine was added. After 30 min at 37°C the absorbance was read at 540 nm using a Titertek Multiskan MCC/340 plate reader (Lab Systems). NO_2^- concentration was calculated from a standard curve of $NaNO_2$ in the medium. The detection limit of this assay is 1 μM.

2.6. Cell viability

The cytotoxic effect of the test compounds was measured using a previously described assay (Löwik et al., 1993) based on the incorporation of the supravital dye neutral red by viable cells. Briefly, following exposure of the cells to the test agent (sample) or medium alone (control), fresh medium containing neutral red (0.01%) was added and the plates were returned to the incubator for 1–1.5 h to allow for uptake of the dye. After removal of the dye solution by inverting the plates, the cells were rapidly washed 2 times with phosphate-buffered saline by vigorously emptying the plates and blotting them dry on a piece of filter paper. The dye was then extracted from the cells by addition of 0.05 M NaH_2PO_4 in 50% ethanol. An aliquot of 100 μl was then transferred to a 96-well microtiter plate and the optical density (OD) was read at 540 nm. Background absorbance (blank) was determined from control wells containing extracting solution only. The viability was calculated according to the formula:

$$\%\ \text{viability} = \frac{OD_{540}\ \text{sample} - OD_{540}\ \text{blank}}{OD_{540}\ \text{control} - OD_{540}\ \text{blank}} \times 100$$

2.7. Statistics

All values are expressed as mean ± S.E.M. of n experiments performed in duplicate or triplicate. Comparisons were made by the unpaired Student's test. A P-value less than 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of the NO-donating nitrosothiol S-nitroso-N-acetylpenicillamine

J774 cells were treated with various concentrations of the spontaneous NO donor *S*-nitroso-*N*-acetylpenicillamine and assayed for cytotoxicity after a 24 h incubation (Fig. 1). In 1000 mg/l glucose-containing medium, incubation with *S*-nitroso-*N*-acetylpenicillamine did not result in significant cytolysis and even at 1000 μM *S*-nitroso-*N*-acetylpenicillamine cells maintained about 80% viability. Assuming that NO-exposed cells may survive because of the sufficient supply of glucose for glycolysis and ATP production, we repeated the incubation with *S*-nitroso-*N*-acetylpenicillamine in 250 mg/l glucose-containing medium. Indeed, limiting glucose affected the viability in a dose-dependent manner with only ±20% cell viability at 1000 μM *S*-nitroso-*N*-acetylpenicillamine. In control cells, addition of *N*-acetyl-D,L-penicillamine or the *S*-nitroso-*N*-acetylpenicillamine metabolites *N*-acetyl-D,L-penicillamine disulfide and NO_2^- alone, in the same concentration range as *S*-nitroso-*N*-acetylpenicillamine, did not affect the viability. Since glucose concentrations below 250 mg/l significantly decreased the viability of *S*-nitroso-*N*-acetylpenicillamine-treated cells as well as of control cells, further experiments were performed in 250 mg/l glucose-containing medium. The cytotoxic effect of *S*-nitroso-*N*-acetylpenicillamine was inhibited by the NO scavenger

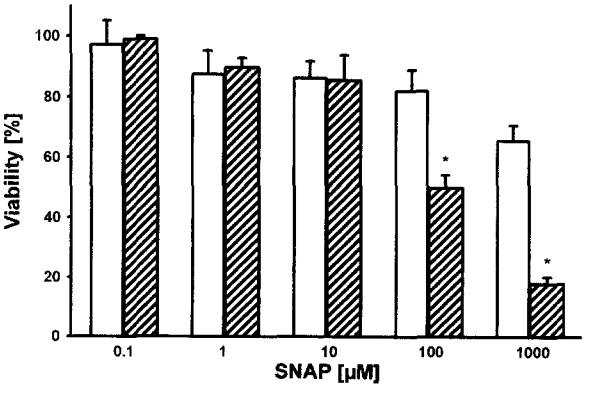

Fig. 1. Cytotoxicity of *S*-nitroso-*N*-acetylpenicillamine (SNAP) in J774A.1 macrophages and influence of the medium glucose concentration. After 24 h culture in 1000 mg/l glucose-containing medium (open bars) or 250 mg/l glucose-containing medium (hatched bars) plus SNAP (0.1–1000 μM), the medium was replaced with medium containing 0.01% neutral red. After 1.5 h, the dye was extracted with 0.05 M NaH_2PO_4 in 50% ethanol for measuring the optical density at 540 nm. Values are expressed as percentage of viability compared to control cells and are the mean ± S.E.M. of 3 experiments performed in triplicate. * $P < 0.05$.

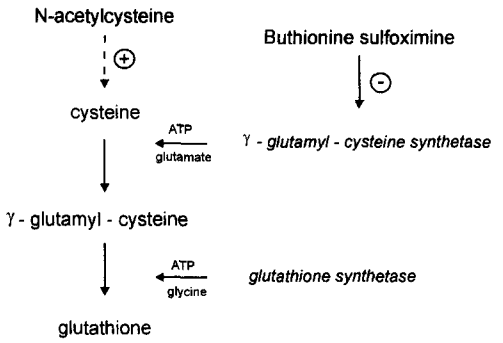

Fig. 2. Simplified diagram of the glutathione biosynthesis.

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO). Addition of the latter (100 μM) together with *S*-nitroso-*N*-acetylpenicillamine (100 or 1000 μM) increased the cell viability from 50.0 ± 4.9% to 93.1 ± 4.7% and from 17.9 ± 3.6% to 41.2 ± 2.4%, respectively ($n = 3$).

3.2. Effect of cellular glutathione depletion on S-nitroso-N-acetylpenicillamine-mediated cytotoxicity and NO_2^-/NO_3^- production

Glutathione depletion was achieved by pretreating the cells for 24 h with buthionine sulfoximine, a potent inhibitor of γ-glutamylcysteine synthetase (Deneke and Fanburg, 1989) and hence glutathione biosynthesis (Fig. 2). Buthionine sulfoximine in a concentration of 1000 μM, as suggested by Berger et al. (1994), reduced the intracellular glutathione from about 7 μg/mg protein to nondetectable levels. As shown in Fig. 3, glutathione depletion increased the *S*-nitroso-*N*-acetylpenicillamine-mediated injury. Whereas 100 μM *S*-nitroso-*N*-acetylpenicillamine caused only 50% cell death in control cells, viability was further reduced to 20% in buthionine sulfoximine-pretreated cells. Pretreatment with 1000 μM buthionine sulfoximine alone did not exert cytotoxicity nor did it affect the production of NO_2^- from *S*-nitroso-*N*-acetylpenicillamine (Table 1).

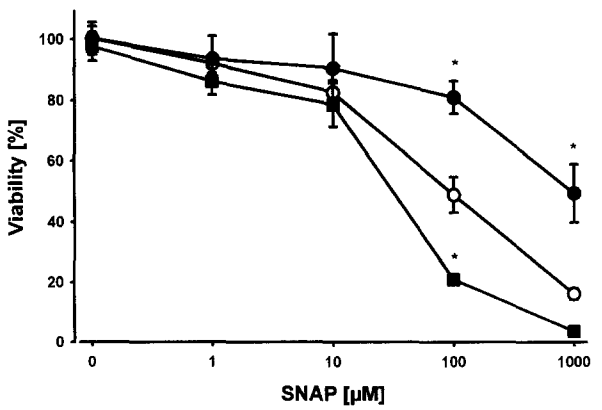

Fig. 3. Effect of buthionine sulfoximine (BSO) and *N*-acetylcysteine (NAC) pretreatment on *S*-nitroso-*N*-acetylpenicillamine (SNAP)-induced injury of J774A.1 macrophages. After 24 h pretreatment with BSO (■) or NAC (●) (both 1000 μM) in 1000 mg/l glucose-containing medium, cells were exposed to different concentrations of SNAP in 250 mg/l glucose-containing medium for 24 h. Control cells (○) were preincubated in culture medium alone. Data are expressed as the mean ± S.E.M. of 3 experiments performed in triplicate. * $P < 0.05$.

3.3. Effect of N-acetylcysteine pretreatment on S-nitroso-N-acetylpenicillamine-mediated cytotoxicity and NO_2^-/NO_3^- production

Assuming a direct protective role for glutathione against NO-mediated injury, we next determined whether incubation with the cysteine precursor *N*-acetylcysteine might protect against the cytotoxic effects of *S*-nitroso-*N*-acetylpenicillamine. *N*-Acetylcysteine is taken up and deacetylated (Sjödin et al., 1989; Yim et al., 1994) by a wide variety of cells, thus providing them with cysteine, a necessary substrate for the synthesis of glutathione. Surprisingly, *N*-acetylcysteine dose-dependently decreased cellular glutathione, thus mimicking at higher concentrations the effect of buthionine sulfoximine on glutathione levels (Fig. 4). The effect on *S*-nitroso-*N*-acetylpenicillamine-induced cell loss, however, was quite different: *N*-acetylcysteine pretreatment clearly protected the cells and almost completely restored cell viability at a concentration of 100 μM *S*-nitroso-*N*-acetylpenicillamine, which proved to be very toxic to control and buthionine sulfoximine-pretreated cells (Fig. 3). Pretreatment with *N*-acetylcysteine alone (1–1000 μM) did not exert cytotoxicity nor did it affect the production of NO_2^- from *S*-nitroso-*N*-acetylpenicillamine (Table 1).

3.4. Cytotoxicity related to endogenous NO production and the effect of buthionine sulfoximine

Stimulation of the macrophages with different concentrations of lipopolysaccharide plus interferon-γ (100 U/ml)

Table 1
Concentrations of NO_2^- in supernatant of J774A.1 cells cultured with *S*-nitroso-*N*-acetylpenicillamine (SNAP)

SNAP (μM)	NO_2^- (μM) pretreatment		
	Control	BSO	NAC
0.1	< d.l.	< d.l.	< d.l.
1	< d.l.	< d.l.	< d.l.
10	6.6 ± 1.3	3.4 ± 0.1	3.2 ± 0.3
100	53.8 ± 3.9	55.8 ± 4.0	51.0 ± 3.6
1000	275.4 ± 15.9	292.3 ± 35.6	258.3 ± 16.6

J774A.1 cells were incubated in 1000 mg/l glucose-containing medium alone (control) or 1000 mg/l glucose-containing medium plus buthionine sulfoximine (BSO) or *N*-acetylcysteine (NAC) (both 1000 μM) for 24 h. The medium was then removed and the cells were incubated in fresh medium (250 mg/l glucose) containing SNAP at the concentrations indicated. After 24 h, supernatants were collected for NO_2^- determination (see Section 2). Values are the mean ± S.E.M. of 4–11 determinations from 3 independent experiments. (d.l. = detection limit).

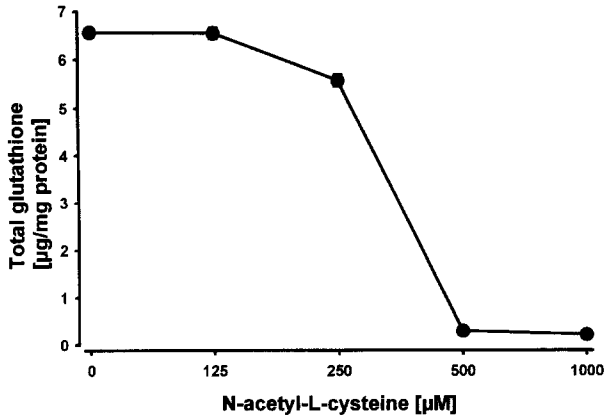

Fig. 4. Effect of *N*-acetylcysteine (NAC) on intracellular glutathione levels in J774A.1 macrophages. After 24 h incubation in 1000 mg/l glucose-containing medium plus NAC (●) at the indicated concentrations, the cells were washed three times with cold phosphate-buffered saline and tested for glutathione content (see Section 2). Control cells were incubated in culture medium alone. Data are the mean ± S.E.M. of 3 determinations. Error bars are obscured by the symbols.

resulted in a dose-dependent loss of cell viability and increased NO_2^- production (Fig. 5). These effects were prevented by addition of 0.5 mM N^G-monomethyl-L-arginine, a known inhibitor of NO synthases (Moncada et al., 1991). Glutathione depletion of the cells with 1000 µM buthionine sulfoximine prior to stimulation with lipopolysaccharide enhanced macrophage cell death considerably. Whereas in control cells 0.1 µg/ml lipopolysaccharide was not cytotoxic, viability decreased to less than 50% in buthionine sulfoximine-pretreated cells (Fig. 5A). Buthionine sulfoximine pretreatment also decreased the NO_2^- production in a concentration-dependent manner (Fig. 5B), without affecting the NO_3^- concentration. The total NO production, however, decreased as compared to control cells (Fig. 6). Results are shown for 1 µg/ml lipopolysaccharide plus 100 U/ml interferon-γ.

3.5. Effect of N-acetylcysteine pretreatment on lipopolysaccharide plus interferon-γ-induced injury

In contrast to the protective effect on *S*-nitroso-*N*-acetylpenicillamine-mediated cell injury, pretreatment with *N*-acetylcysteine (1000 µM) did not prevent the lipopolysaccharide-induced cytotoxicity (Fig. 5A). The endogenous NO production (NO_2^-/NO_3^-) was not affected as compared to control cells (Fig. 5B and Fig. 6).

3.6. The effect of other NO donors with different decomposition rates on macrophage viability

In order to investigate whether the J774 macrophages display a similar susceptibility towards other NO-generating compounds, *S*-nitrosoglutathione, *S*-nitrosocysteine, and the sydnonimine derivative 3-morpholino-sydnonimine hydrochloride were used as sources of NO. Incubation of the cells for 24 h with equimolar concentrations (100 µM or 1000 µM) of these compounds differentially affected cell viability: while 3-morpholino-sydnonimine hydrochloride and *S*-nitrosocysteine were not toxic, *S*-nitroso-*N*-acetylpenicillamine and *S*-nitrosoglutathione induced cell death even at the lower concentration (Fig. 7). Assuming that these differences may be related to the kinetics of NO release, we compared the stability of the NO donors with the potency for cell killing. Detection of NO_2^- was used as an indicator of NO production from NO donors. When authentic NO is introduced into water in the presence of oxygen, it partially oxidizes to NO_2. The latter combines with the remaining NO to form N_2O_3, which hydrolyses to NO_2^-. However, in physiological systems the yield of NO_3^- has also to be considered. The stability was thus estimated by measuring the NO_2^- and NO_3^- concentrations at several time points in solutions of the test compounds (100 µM) in complete medium containing 250 mg/l glucose at 37°C

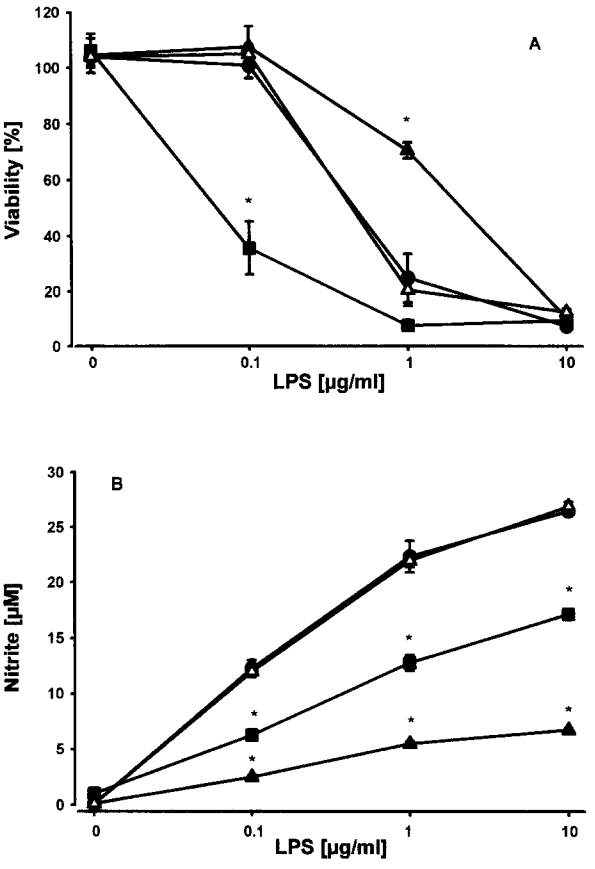

Fig. 5. Effect of buthionine sulfoximine (BSO) and *N*-acetylcysteine (NAC) pretreatment on viability (A) and NO_2^- production (B) of immunostimulated J774A.1 macrophages. After 24 h pretreatment with BSO (■) or NAC (●) (both 1000 µM) in 1000 mg/l glucose-containing medium, cells were stimulated with lipopolysaccharide plus interferon-γ (100 U/ml) in 250 mg/l glucose-containing medium for an additional 24 h. The supernatants were then collected for NO_2^- determination and medium containing 0.01% neutral red was added for 1.5 h. Control cells were incubated either in culture medium alone (△) or in medium containing 0.5 mM N^G-monomethyl-L-arginine (▲). Data are expressed as the mean ± S.E.M. of 3 experiments performed in triplicate. * $P < 0.05$.

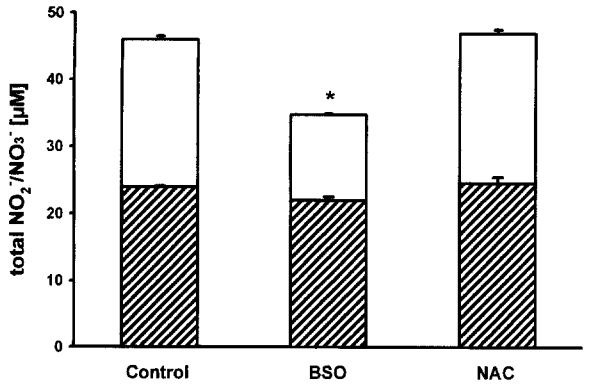

Fig. 6. Effect of buthionine sulfoximine (BSO) and *N*-acetylcysteine (NAC) pretreatment on total NO_2^-/NO_3^- production of immunostimulated J774A.1 macrophages. After 24 h pretreatment with BSO or NAC (both 1000 μM) in 1000 mg/l glucose-containing medium, cells were stimulated with 1 μg/ml lipopolysaccharide plus interferon-γ (100 U/ml) in 250 mg/l glucose-containing medium for an additional 24 h. The supernatants were then collected for NO_2^-/NO_3^- determination. Control cells were incubated in culture medium alone. Hatched bars represent NO_3^- values. Data are expressed as the mean ± S.E.M. of 3 experiments performed in triplicate. * $P < 0.05$.

(not shown). In the case of the nitrosothiols, we observed a ratio of about 3 : 2 for NO_2^-/NO_3^-. The half-lives were found to be 10 min (3-morpholino-sydnonimine hydrochloride), 15 min (*S*-nitrosocysteine), 300 min (*S*-nitroso-*N*-acetylpenicillamine) and > 360 min (*S*-nitrosoglutathione). Both the order of stability of the test compounds and the order of potency for cell killing was *S*-nitrosoglutathione > *S*-nitroso-*N*-acetylpenicillamine > *S*-nitrosocysteine = 3-morpholino-sydnonimine hydrochloride.

3.7. Effect of hydroxylamine (NH_2OH) on macrophage viability

In addition to the former compounds which release NO spontaneously, we also examined the effects of NH_2OH, a mutagenic agent known to generate NO via an enzymatic mechanism (Craven et al., 1979). Like *S*-nitrosocysteine and 3-morpholino-sydnonimine hydrochloride, NH_2OH was not toxic to the cells at 100 μM (Fig. 7) and was less toxic than *S*-nitroso-*N*-acetylpenicillamine and *S*-nitrosoglutathione at 1000 μM.

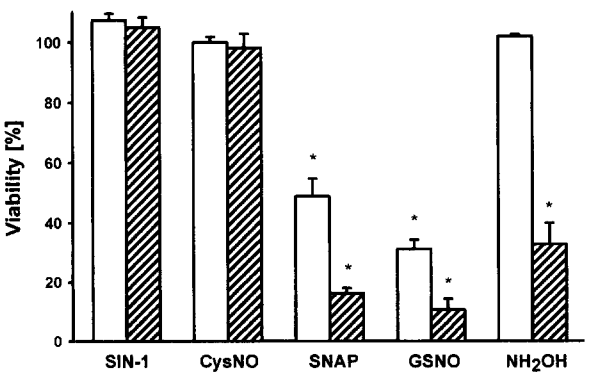

Fig. 7. Effect of 3-morpholino-sydnonimine hydrochloride, *S*-nitrosocysteine, *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosoglutathione and NH_2OH on viability of J774A.1 macrophages. Cells were incubated in 250 mg/l glucose-containing medium with the different NO donors at 100 μM (open bars) or 1000 μM (hatched bars) for 24 h. Data are expressed as percentage of viability compared to untreated cells and are the mean ± S.E.M. of 3 experiments performed in triplicate. * $P < 0.05$.

4. Discussion

It is well demonstrated that NO is involved in the cytotoxic effects of immunostimulated macrophages towards tumour cells (Stuehr and Nathan, 1989) and that macrophages themselves are susceptible to endogenously produced NO (Tucker et al., 1991). However, little information is available about the conditions under which this NO-related toxicity leads to cell death. Our observations demonstrate that exposure of J774.A1 murine macrophages to the NO donor *S*-nitroso-*N*-acetylpenicillamine resulted in a significant loss of viability depending on both the glucose and the *S*-nitroso-*N*-acetylpenicillamine concentration. Control solutions of *N*-acetyl-D,L-penicillamine, its disulfide or NO_2^- alone in the same concentration range as *S*-nitroso-*N*-acetylpenicillamine did not affect viability. Carboxy-PTIO, a known inhibitor of NO-mediated biological responses (Akaike et al., 1993), prevented cell injury by *S*-nitroso-*N*-acetylpenicillamine. This points to NO or an NO-derived species as mediator of the *S*-nitroso-*N*-acetylpenicillamine-induced cell death. The cytotoxic effect of *S*-nitroso-*N*-acetylpenicillamine was significantly enhanced under glucose-limiting conditions. This confirms the importance of the presence of glucose in the culture medium for cell survival, as demonstrated for macrophage-injured tumour cells which rapidly die if glucose or other glycolytic substrate is exhausted from their medium (Granger et al., 1980; Hibbs et al., 1990). Glucose might not only provide a source of ATP but other cytosolic reducing equivalents, i.e., NADPH, generated via activation of the hexose monophosphate shunt. NADPH is necessary for the conversion of oxidized glutathione to the reduced form, a reaction catalyzed by glutathione reductase. In that way, glucose may play a role in maintaining the efficiency of glutathione as antioxidant. This is supported by the observation that glutathione depletion enhances the cytotoxic effects of *S*-nitroso-*N*-acetylpenicillamine in this study, and of authentic NO as previously described (Walker et al., 1995). Since part of the toxicity of NO is believed to be due to the reaction with superoxide ($O_2^{\cdot -}$) giving peroxynitrite ($ONOO^-$), we can also speculate about a reaction between glucose and $ONOO^-$. Indeed, the latter was recently found to react with glucose (and other compounds containing an alcohol functional group) in a process suggested to be an additional detoxification pathway for $ONOO^-$ (Moro et al., 1995). There is no evidence for direct chemical trapping of NO by glucose, although this cannot be ruled out. However, the exact

mechanisms underlying this protective role of glucose remain to be investigated. Thus, apart from the cytostatic or antiproliferative effect demonstrated for cytotoxic activated macrophage-derived NO (Hibbs et al., 1990), our results show a cytolytic effect for exogenous NO when the ATP-producing glycolytic pathway is limited. Since in most studies the NO donors are used at mM concentrations, lowering the content of glucose in culture media may be a useful means to potentiate the cytotoxic activity of NO donors in other in vitro models as well.

Recent evidence shows the involvement of cellular resistance to oxidative stress in general and the role of glutathione in particular in the protection against NO-induced cytotoxicity (Meister, 1994; Walker et al., 1995). However, the role of other non-protein thiols has not been investigated. In the present study, intracellular glutathione depletion of J774 macrophages with buthionine sulfoximine further increased the *S*-nitroso-*N*-acetylpenicillamine-mediated cytotoxicity, as previously shown for Chinese hamster fibroblasts (HA1) exposed to authentic NO (Walker et al., 1995), thus confirming the involvement of NO or NO-related species in the *S*-nitroso-*N*-acetylpenicillamine-induced injury. Moreover, we also found that buthionine sulfoximine-mediated glutathione depletion decreased the viability of macrophages stimulated with bacterial lipopolysaccharide plus interferon-γ, an injury partially prevented by addition of N^G-monomethyl-L-arginine. In buthionine sulfoximine-pretreated cells the total NO production was also decreased, but NO_3^- concentrations were higher than NO_2^- values. This is not surprising, since the decrease of glutathione levels favours the formation of oxygen radicals (Meister, 1994) and consequently of $ONOO^-$, which mainly decomposes to NO_3^- (Pryor and Squadrito, 1995). Although the protective activity of NO synthase inhibitors indicates the participation of the L-arginine/NO pathway in the lipopolysaccharide-mediated cytotoxicity, the finding that glutathione-depleted cells produced less NO is in agreement with a recent study suggesting the existence of additional pathways that contribute to immunostimulation-mediated cell injury in this cell line (Szabó et al., 1996). Whether or not the synthesis of NO or NO-related compound(s) induces the formation of other cytotoxic agents remains to be elucidated.

The observation that glutathione depletion increased both exogenous and endogenous NO-mediated cell death prompted us to investigate whether pretreatment with the cysteine precursor *N*-acetylcysteine prevents NO-mediated cytotoxicity. It has been previously demonstrated that *N*-acetylcysteine is effectively hydrolysed to yield cysteine by rat, mouse and human tissue, and this metabolic step is probably a prerequisite for most of its protective effects (Sjödin et al., 1989). Our results clearly demonstrate that *N*-acetylcysteine pretreatment diminished the *S*-nitroso-*N*-acetylpenicillamine-mediated cytotoxicity in the J774 macrophages but not the cell damage related to the lipopolysaccharide-induced NO synthase activity. This selective protection is still unclear. As pointed out earlier, other factors besides NO may contribute to the immunostimulation-mediated cell injury.

Previous reports suggest that generation of NO at the cell surface, either enzymatically or nonenzymatically, is the most likely mechanism of nitrosothiol activity (Mathews and Kerr, 1993). In order to verify whether the different pretreatments influenced the cell membrane in a way that the decomposition of *S*-nitroso-*N*-acetylpenicillamine was also affected, cell-free supernatants were harvested at the end of the culture period and assayed for the presence of NO_2^-. There were no significant differences between NO_2^- levels in untreated controls and *N*-acetylcysteine-pretreated cells, thus the protective effect of *N*-acetylcysteine was not due to suppression of NO production.

Contrary to what was expected, glutathione levels were decreased by *N*-acetylcysteine pretreatment. Therefore, the protective effect of *N*-acetylcysteine in the J774 macrophages was clearly not mediated by glutathione. Thus, alternative pathways must exist by which *N*-acetylcysteine itself or its metabolite cysteine may have limited the NO-mediated injury. First, reduced thiols (RSH) can react with $O_2^{\cdot -}$ or $HO_2^{\cdot}$ (Wefers and Sies, 1983; Aruoma et al., 1989):

$$O_2^{\cdot -} \xrightarrow{H^+} HO_2^{\cdot} \xrightarrow{RSH} RS^{\cdot} + H_2O_2 \quad (1)$$

$$RS^{\cdot} + RS^{\cdot} \rightarrow RSSR \quad (2)$$

$O_2^{\cdot -}$ is formed in all aerobically metabolizing cells (Pryor, 1986) and its reaction with NO (Eq. (3)) gives $ONOO^-$, recently shown to cause a more pronounced inhibition of cell respiration in J774.2 macrophages as compared to the NO donors *S*-nitroso-*N*-acetylpenicillamine and diethylamine NONOate (Szabó and Salzman, 1995):

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^- \quad (3)$$

Moreover, the decomposition of $ONOO^-$ (Eq. (4)) results in the formation of a powerful oxidant, probably a high-energy form of peroxynitrous acid ($HOONO^*$), with hydroxyl radical-like activity (Pryor and Squadrito, 1995). Since thiols such as glutathione and cysteine can be oxidized by $ONOO^-$ (or its protonated form) in a bimolecular reaction (Koppenol et al., 1992), it is very likely that the cysteine precursor *N*-acetylcysteine may act as peroxynitrite scavenger as well.

$$ONOO^- + H^+ \rightleftharpoons HOONO \rightleftharpoons HOONO^* \quad (4)$$

Thus, *N*-acetylcysteine may protect against NO-mediated damage by reacting with $O_2^{\cdot -}$, thereby preventing the formation of the more toxic $ONOO^-$, and by scavenging the latter or its protonated form.

The influence of thiols on the decomposition of nitrosothiols (RSNOs) is complex. Thiols (e.g., *N*-acetylcysteine or cysteine), which mostly react as thiolate (RS^-),

can react with RSNOs (e.g., *S*-nitroso-*N*-acetylpenicillamine) to give hydroxylamine (NH_2OH) via nitroxyl (NO^-) formation (Arnelle and Stamler, 1996; Doyle et al., 1988):

$$RSH \rightarrow RS^- + H^+ \quad (5)$$

$$RSNO + RS^- \rightarrow RSSR + NO^- \quad (6)$$

$$NO^- + 2RSH \rightarrow RSNHOH + RS^- \quad (7)$$

$$RSNHOH + RSH \rightarrow RSSR + NH_2OH \quad (8)$$

If NH_2OH is formed in our system according to the above reactions, it should be less toxic to the macrophages than *S*-nitroso-*N*-acetylpenicillamine. Indeed, we found that NH_2OH was less cytotoxic than *S*-nitroso-*N*-acetylpenicillamine at the same concentrations, thus partial conversion of *S*-nitroso-*N*-acetylpenicillamine by reacting with *N*-acetylcysteine or cysteine to give NH_2OH may contribute to the observed *N*-acetylcysteine-mediated protection.

Furthermore, the demonstration of the metabolic hydrolysis of *N*-acetylcysteine to yield cysteine by different tissues (Sjödin et al., 1989) led us to hypothesize that after uptake and deacetylation of *N*-acetylcysteine by the macrophages, transfer of the nitroso group from *S*-nitroso-*N*-acetylpenicillamine to the newly generated cysteine may take place. Transnitrosation of nitrosothiols with other thiols has been shown to occur very readily in aqueous solution (Barnett et al., 1994). It was suggested that the NO release in vivo may be possible by transnitrosation from a relatively stable nitrosothiol (e.g., *S*-nitrosoglutathione) to yield a much more labile nitrosothiol. Moreover, the transnitrosation of cysteine by *S*-nitrosoglutathione has been reported at physiological pH (Park, 1988). Thus, it is conceivable that the reaction between *S*-nitroso-*N*-acetylpenicillamine and cysteine yields the more unstable *S*-nitrosocysteine. Of course, the assumption of *S*-nitrosocysteine formation would necessarily imply that this NO donor is less toxic to the macrophages. Indeed, equimolar concentrations of *S*-nitrosocysteine were less cytotoxic than *S*-nitroso-*N*-acetylpenicillamine and *S*-nitrosoglutathione under the same experimental conditions. This also suggests a possible correlation between the cytotoxic potency of the spontaneously NO-generating compounds and the rate at which NO is liberated. These results are thus consistent with a previous assumption that a sustained exposure to moderate amounts of NO has a more pronounced effect than a brief exposure to larger NO concentrations (Stuehr and Nathan, 1989). Detection of NO_2^- is commonly used as an index of NO production from NO donors. The oxidation of NO in the presence of oxygen in water yields nearly exclusively NO_2^-. However, in physiological systems the formation of NO_3^- needs also to be considered. For the half-life studies with the NO donors, we measured the NO_3^- concentrations as well. The order of stability was *S*-nitrosoglutathione > *S*-nitroso-*N*-acetylpenicillamine > *S*-nitrosocysteine, thus confirming the relative values obtained by measuring NO_2^- formation. Therefore, NO_3^- values do not provide additional information on NO donor stability. It should be noted, however, that the half-life of the nitrosothiols is markedly influenced by the assay conditions (pH, buffer composition and transition metal contamination) and the initial concentration. Thus, the absolute values we have reported may differ from other values found in the literature but the order of relative stability is consistent with previous studies (Mathews and Kerr, 1993; Gaston et al., 1994).

Recently, *N*-acetylcysteine has been shown to increase the glucose consumption in healthy volunteers (Ammon et al., 1992). Thus, another possible mechanism by which *N*-acetylcysteine protects against NO-mediated cytotoxicity could be by increasing the glucose transport into the cell, and hence the ATP production. However, the exact role of *N*-acetylcysteine in the glucose transport system in this cell line remains to be elucidated.

In summary, our results with the J774A.1 murine macrophage-like cell line demonstrate that (1) reductions in the availability of glucose and intracellular glutathione render the cells more vulnerable to the cytotoxic effect of NO donors. Therefore, in future experiments investigating the cytotoxicity of NO, it may be desirable to lower the glucose supply, (2) long-lived NO donors, which more closely resemble the steady-state production of NO in vivo, are more cytotoxic than short-lived NO donors. Thus, in cytotoxicity studies in vitro, the use of the former may be more effective than the use of the short-lived NO donors or authentic NO, (3) the differential effects of *N*-acetylcysteine on *S*-nitroso-*N*-acetylpenicillamine-induced and bacterial lipopolysaccharide-mediated cytotoxicity suggest the existence of other toxic species different from NO or NO-related compounds with a potent cytotoxic activity in immunostimulated macrophages, and (4) other non-protein thiols like *N*-acetylcysteine may substitute for glutathione as a major component of the cellular antioxidant defense system.

Finally, our results may well have clinical relevance. During ischaemia, glucose (Doukas et al., 1994) as well as glutathione levels drop (Park et al., 1991) which, at a certain stage, will render the cells more vulnerable to the cytotoxic effects of NO. The latter may originate from the activity of the inducible NO synthase present in activated macrophages at the level of an atherosclerotic plaque or after balloon angioplasty (Bosmans et al., 1994). Paradoxically, it also questions the beneficial effect of the long-term use of high concentrations of the more stable NO donors to treat ischaemic vascular diseases. Normalization of the intracellular glucose and/or thiol levels might be necessary to avoid their possible cytotoxic effect. Co-administration of *N*-acetylcysteine may fulfill some of these criteria.

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