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Effect of L-buthionine-(S,R)-sulphoximine, an inhibitor of γ-glutamylcysteine synthetase on peroxynitrite- and endotoxic shock-induced vascular failure

Salvatore Cuzzocrea, Basilia Zingarelli, Michael O'Connor, Andrew L. Salzman & [1]Csaba Szabó

Children's Hospital Medical Center, Division of Critical Care, 3333 Burnet Avenue, Cincinnati, Ohio 45229, U.S.A.

1 Peroxynitrite, a cytotoxic oxidant formed from the reaction of nitric oxide (NO) and superoxide is a mediator of cellular injury in ischaemia/reperfusion injury, shock and inflammation. Here we investigated whether L-buthionine-(**S**,**R**)-sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, alters endothelial and vascular smooth muscle injury in response to peroxynitrite *in vitro* and during endotoxic shock *in vivo*.

2 In human umbilical vein endothelial cells and in rat aortic smooth muscle cells, BSO (1 mM, for 24 h) enhanced, whereas glutathione (3 mM) or glutathione ethyl ester (3 mM) attenuated the peroxynitrite (100–1000 μM)-induced suppression of mitochondrial respiration (measured by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan), formation of nitrotyrosine (detected by Western blotting), protein oxidation (measured by detection of 2,4 dinitrophenylhydrazine-reactive carbonyls), and DNA single strand breakage and activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS) (measured by the incorporation of radiolabelled NAD^+ into nuclear proteins and by the alkaline unwinding assay, respectively). Glutathione ethyl ester treatment reduced the BSO-induced enhancement of peroxynitrite-induced cytotoxicity.

3 In rat isolated thoracic aortic rings, BSO treatment (*in vivo*, at 1 g kg^{-1} intraperitoneally (i.p.) for 24 h) enhanced, whereas pretreatment with glutathione (*in vitro*, 3 mM) attenuated the peroxynitrite-induced reduction of the contractions to noradrenaline, and the peroxynitrite-induced impairment of the endothelium-dependent relaxations to acetylcholine.

4 In BSO-pretreated rats, treatment with bacterial lipopolysaccharide (LPS, 15 mg kg^{-1}, i.p., for 6 h) caused a more pronounced vascular hyporeactivity and endothelial dysfunction *ex vivo*. BSO pretreatment also increased the degree of nitrotyrosine staining (detected by imunohistochemistry) in the aorta after LPS treatment.

5 In conclusion, our results demonstrate that L-buthionine-(**S**,**R**)-sulphoximine, an inhibitor of γ-glutamylcysteine synthetase enhances peroxynitrite- and endotoxic shock-induced vascular failure. Based on these findings, we suggest that endogenous glutathione plays an important protective role against peroxynitrite- and LPS-induced vascular injury.

Keywords: Endothelium; endothelium-derived factors; free radicals; antioxidants; shock

Introduction

Nitric oxide (NO) produced by the inducible isoform of NO synthase (NOS) is an important mediator of inflammation, and contributes to the pathogenesis of the vascular failure in circulatory shock (Rees, 1995; Szabó, 1995). The systemic inflammatory response is also associated with the production of oxygen-derived free radicals (Youn *et al*., 1991; McCord, 1993). There is now substantial evidence that much of the cytotoxicity is due to a concerted action of oxygen- and nitrogen-derived free radicals and oxidants. An important part of the oxidative injury associated with simultaneous production of NO and oxyradicals may be mediated by peroxynitrite ($ONOO^-$), a toxic oxidant formed from the reaction of NO and superoxide (Crow & Beckman, 1995; Szabó, 1996a).

Peroxynitrite is cytotoxic via a number of mechanisms. Its acute cytotoxic effects include tyrosine nitration initiation of lipid peroxidation, and inactivation of variety of enzymes, including mitochondrial respiratory enzymes and membrane pumps (Crow & Beckman, 1995; Szabó, 1996a). There are also more complex mechanisms which contribute to peroxynitrite toxicity in macrophages and smooth muscle cells (Crow & Beckman, 1995; Szabó, 1996a,b; Szabó *et al*., 1996a,b; Zingarelli *et al*., 1996). One of these pathways involve DNA single strand breakage, and consequent activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS). Massive poly-ADP-ribosylation of nuclear proteins by PARS results in cellular energy depletion and injury. (Szabó, 1996a,b; Szabó *et al*., 1996a,b; Zingarelli *et al*., 1996). Another mechanism of peroxynitrite-induced toxicity may be related to interference by peroxynitrite of membrane signal transduction pathways (Gow *et al*., 1996; Berlett *et al*., 1996; Elliott, 1996).

Peroxynitrite is a potent oxidant, and, therefore, it is conceivable that endogenous antioxidant mechanisms counteract its toxicity. In *in vitro* studies, it has been established that antioxidants such as cysteine, glutathione, ascorbic acid and α-tocopherol are scavengers of peroxynitrite and inhibitors of its oxidant capacity (Radi *et al*., 1991; Van der Vliet *et al*., 1994; Pryor & Squadrito, 1995; Vatassery, 1996). A marked depletion by exogenous or endogenous peroxynitrite of cellular antioxidants including glutathione has been demonstrated in the plasma, as well as in various cell types including endothelial cells and smooth muscle cells (Van der Vliet *et al*., 1994; Vatassery, 1996; Phelps *et al*., 1996; Szabó *et al*., 1996b).

[1] Author for correspondence.

The aim of the present study was to investigate the role of endogenous glutathione, as a protective factor in relation to peroxynitrite-induced vascular failure *in vitro* and *in vivo*. Specifically, we have investigated whether L-buthionine-(**S**,**R**)-sulphoximine (BSO), and inhibitor of γ-glutamylcysteine synthetase affects peroxynitrite-induced injury in cultured endothelial and smooth muscle cells, in vascular rings exposed *in vitro*, and during endotoxic shock, a systemic inflammatory condition which is associated with the production of peroxynitrite (Szabó, 1996a). Moreover, we also investigated whether excess extracellular glutathione effects the peroxynitrite-induced cytotoxic effects *in vitro*.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection, Rockville, MD) were cultured in F12K medium, 10% foetal bovine serum, 100 μg ml^{-1} heparin, and 30 μg ml^{-1} endothelial cell growth supplement as previously described (Hoshi *et al*., 1984). Rat aortic smooth muscle (RASM) cells were grown in RPMI medium with 10% foetal bovine serum as described by Szabó *et al*. 1996b. Cells were cultured in 96-well plates (200 μl medium/well) until 90% confluence. Cells were exposed to various concentrations of peroxynitrite for 10 min (for the measurement of PARS activity) or 1 h (for all other assays). We have previously demonstrated that these represent optimal time points for the respective determinations (Szabó *et al*., 1996a).

For the inhibition of γ-glutamylcysteine synthetase in HUVECs and RASM cells, cells were treated with 1 mM BSO (or vehicle) for 24 h (Harlan *et al*., 1984; Andreoli *et al*., 1986). Total reduced thiol concentrations were measured with 5.5′-dithiobis (2-nitrobenzoic acid as described by Szabó *et al*. (1996b). Using the above protocol of BSO treatment (1 mM, 24 h), and in agreement with previous observations (Harlan *et al*., 1984; Andreoli *et al*., 1986), we have confirmed that cellular reduced thiol concentration was depleted by more than 95%, to levels below the detection limit of the assay used ($n=8$).

In another sets of experiments, cells were treated with glutathione (GSH, 100 μM – 3 mM), or glutathione ethyl ester (100 μM – 3 mM), 10 min before peroxynitrite exposure. In another experiments, in order to investigate the role of intracellular vs extracellular effects of glutathione and its ethyl ester, cells were exposed to glutathione or glutathione ethyl ester for 4 h, followed by replacement of the medium by two consecutive washouts, and, subsequently, peroxynitrite exposure. In a final set of experiments, the ability of glutathione or glutathione ethyl ester to reverse the effects of BSO was investigated. In these latter studies, cells were exposed to BSO for 24 h as described above, followed by a 10 min exposure to glutathione or glutathione ethyl ester, and, subsequently, peroxynitrite. Mitochondrial respiration was measured as 1 h after peroxynitrite exposure in all cases.

To induce the expression of the inducible NO synthase (iNOS) in the RASM cells, cells were treated with LPS (10 μg ml^{-1}) and murine γ-interferon (IFN, 50 μ ml^{-1}) for 24 h (Szabó *et al*., 1996b). These studies were performed in control and in BSO-treated cells, both in the presence and in the absence of the NOS inhibitor N^{G}-methyl-L-arginine (L-NMA, 3 mM).

Measurement of nitrite production in cell culture supernatants

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of RASM cells at 24 h after LPS and IFN stimulation, by the spectrophotometric Griess reaction (Szabó *et al*., 1996b). Aliquots of supernatant (100 μl) were mixed with 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthyl ethylenediamine in 5% phosphoric acid). The optical density at 550 nm (OD_{550}) was measured with a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were calculated by comparison with OC_{550} of standard solutions of sodium nitrite prepared in culture medium.

Measurement of mitochondrial respiration

At 60 min after peroxynitrite exposure, or at 24 h after treatment with LPS and IFN, cell respiration was assessed by the mitochondrial-dependent reduction of MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (Szabó *et al*., 1996b). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg ml^{-1}) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in dimethylsulphoxide (DMSO; 100 μl). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550} with the Spectramax 250 microplate reader.

Determination of DNA single strand breaks

At 10 min after peroxynitrite exposure, the formation of strand breaks in double-stranded DNA was determined by the alkaline unwinding method, as previously described (Szabó *et al*., 1996a,b).

Measurement of cellular PARS activity

At 10 min after peroxynitrite exposure, the culture medium in 12-well plates was replaced with 0.5 ml of 56 mM HEPES buffer, pH 7.5 containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 125 nmol NAD^+ spiked with 0.25 μCi [^{3}H]-NAD^+. PARS activity was then measured as described by Szabó *et al*., 1996a,b.

Oxidized protein detection by SDS-PAGE and Western blotting

For the detection of oxidized proteins in whole cells, at 60 min after peroxynitrite exposure, cells were scraped in phosphate-buffered saline (PBS) into microfuge tubes and centrifuged at 14,000 *g* for 20 s. Supernatant was removed and 400 μl of RIPA lysis buffer with pepstatin A (10 μg ml^{-1}) and phenylmethylsulphonyl fluoride (0.5 mM) was used to resuspend the cells. RIPA buffer contains 1% Tergitol (Nonidet P-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sylphate (SDS). DNA was sheared by repeated passage through a 22 gauge needle and the lysates incubated on ice for 30 min. Cell lysates were then centrifuged in an Eppendorf 5402 centrifuge at 14,000 *g* for 30 min at 4°C. The OxyBlot oxidized protein detection kit (Oncor, Gaithersburgh, MD) was used to detect the oxidized carbonyl groups of protein side-chains as previously described (Szabó *et al*., 1997a).

Detection of nitrotyrosine by Western blotting

At 60 min after peroxynitrite exposure, cells were washed with 1.0 ml cold PBS than scraped in 1.0 ml cold PBS and transferred to a microfuge tube. Tubes were spun in Eppendorf microfuge for 20 s. PBS was then removed and 150 μl RIPA buffer (see above) was added to each tube of DNA sheared by repeated passage through 22 gauge needle. Cells were incubated on ice for 30 min, then centrifuged at 14,000 *g* for 20 min at 4°C. Then 30 μg of each sample was diluted in SDS-treated buffer and heated to 95°C for 5 min. Samples were then loaded into 8–16% Tris-glycine. Gels were run at 125 V for 2 h, then transferred to 0.45 μm nitro-cellulose at 25 V for 60 min with the Towbin buffer system (1.45 g Tris, 7.2. glycine, 800 ml di H_2O and 200 ml MeOH). Membrane was blocked in 1% BSA: 1% non-fat-milk in PBS-T for 1 h then probed with rabbit anti-nitrotyrosine (Upstate Biotechnology, Saranac Lake, NY) 1 μg ml^{-1} in PBS-T (0.05%) overnight at 4°C. The blot was washed three times with PBS-T, once with H_2O, then incubated for 1.5 h with secondary antibody, goat anti-rabbit-horseradish peroxidase, conjugated (1: 3000). The blot was washed three times with PBS-T, once with di H_2O, then 1.5 ml mixed ECL chemiluminescence was added for 1 min. The blot was then exposed to X-ray film for 60 s.

Measurement of isometric force in vascular rings

For the inhibition of γ-glutamylcysteine synthetase in rats, animals were treated with 1 g kg^{-1} BSO for 24 h. This regimen of BSO treatment has been shown to cause a marked reduction of intracellular glutathione levels, as measured in the lung, liver and kidney in the rat (Drew & Miners, 1984; Standeven & Wetterhahn, 1991).

From vehicle-treated or BSO-treated animals, thoracic aortae were obtained. Vessels were cleared of adhering periadventitial fat and cut into rings of 3–4 mm width. Rings were mounted in organ baths (5 ml) filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7. Experiments were performed in the presence of indomethacin (10 μM), to prevent the generation of cyclo-oxygenase metabolites. Isometric force was measured with isometric transducers as described by Szabó *et al*. (1996b). A tension of 1 g was applied and the rings were equilibrated for 60 min. Fresh Krebs solution was provided at 15 min intervals.

In experiments in which the changes in contractility were investigated, cumulative concentration-response curves to noradrenaline (10^{-9}–10^{-5} M) were obtained. To investigate changes in endothelium-dependent relaxant responsiveness, rings were precontracted with noradrenaline (10^{-6} M), and then concentration-response curves to acetylcholine (10^{-8}–10^{-5} M) were obtained. To investigate changes in endothelium-independent relaxant responsiveness, rings were precontracted with noradrenaline (10^{-6} M), and then concentration-response curves to the NO donor compound S-nitroso-N-acetyl-DL-penicillamine (SNAP, 10^{-6}–10^{-4} M) were obtained.

These vascular studies were performed in four groups of rings: in control rings (obtained from animals pretreated with saline, the vehicle for BSO), in control rings obtained from animals pretreated with BSO, in rings treated with peroxynitrite *in vitro* (750 μM for 30 min), and in rings from BSO-treated animals treated with peroxynitrite *in vitro*. In an additional series of experiments, rings were exposed to glutathione (3 mM) for 5 min, and then exposed to peroxynitrite (750 μM for 30 min). Following a 30 min incubation with peroxynitrite, rings were mounted and isometric contractions and relaxations were recorded.

Endotoxic shock protocol

Male Wistar rats (Charles River Laboratories, Wilmington, MA) were injected with *E. coli* LPS (15 mg kg^{-1}, i.v.) or vehicle at time 0. Rats were killed at 6 h after LPS injection, plasma was taken to measure nitrite and nitrate and aortae were taken for measurement of contractions, endothelium-dependent relaxations, for nitrotyrosine immunohistochemistry and for iNOS determination (Szabó *et al*., 1993; 1995).

Injection of LPS (or vehicle) was given to groups of animals pretreated with BSO, and to animals pretreated with BSO vehicle (saline). Thus, the following groups of animals were used: control; control + BSO; LPS and LPS + BSO ($n = 7$–8 rats in each group).

Immunohistochemical localization of nitrotyrosine in vascular tissue

Thoracic descending aortae were immediately excised and perfused for 15 min with fresh 3.5% cacodylate-buffered paraformaldehyde and cryostat sections were prepared from the fixed aorta tissue. Endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Non-specific adsorption was minimized by incubating the section in 3% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotine or avidin binding sites were blocked by sequential incubation for 15 min each with avidin and biotin (biotin blocking kit, Vector Laboratories, Burlingame, CA). The sections were then incubated overnight with primary anti-nitrotyrosine polyclonal rabbit (Upstate Biotech, Saranac Lake, NY) serum or control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Burlingame, CA).

Nitric oxide synthase assay

Aortae were taken from the animals and homogenized on ice, in a buffer composed of 50 mM Tris·HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4), with a Tissue Tearor 985-370 homogenizer (Biospec Products, Racine, WI, U.S.A.). Calcium-independent conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline, an indicator of iNOS activity, was measured in the homogenates as described by Szabó *et al*. (1993, 1995). Briefly, homogenates (30 μl) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and EGTA (2 mM) for 20 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting.

Plasma nitrite/nitrate measurements

In plasma samples, nitrate is the major degradation product of NO. Nitrate was converted to nitrite by incubation with 60 mu nitrate reductase and 25 μM NADPH for 180 min. Nitrite was then measured as previously described (Szabó *et al*., 1996b) by

adding Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to plasma samples diluted (1:10) in phosphate buffered saline. The optical density at 550 nm (OD_{550}) was measured by using the Spectramax 250 microplate reader.

Materials

Cell culture medium, heparin and foetal calf serum were obtained from Gibco (Grand Island, NY). S-nitroso-N-acetyl-DL-penicillamine (SNAP) was purchased from Calbiochem (San Diego, CA). Interferon was from Genzyme (Boston, MA). Bacterial lipopolysaccharide (LPS, *E. coli,* serotype No. 0127:B8), and all other chemicals were from Sigma (St. Louis, MO), Peroxynitrite was synthesized and kindly provided by Dr H. Ischiropoulos (University of Pennsylvania). Details of the preparation of peroxynitrite and on the confirmation that the cytotoxicity of this preparation towards cultured cells is due to peroxynitrite and not due to various contaminating species can be found elsewhere (Szabó *et al*., 1996a).

Data analysis

All values in the figures and text are expressed as mean ± s.e.mean of n observations, where n represents the number of wells or vascular rings studies (6–9 wells or rings from 2–3 independent experiments). Data sets were examined by one- and two-way analysis of variance. After the ANOVA had been performed, a *post-hoc* analysis was done, whereby individual group means were compared with Student's unpaired t test. A P value less than 0.05 was considered significant.

Results

Effect of BSO pretreatment on peroxynitrite-induced cytotoxicity in HUVECs and RASM cells

Exposure of HUVECs and RASM cells to peroxynitrite (100 μM – 1 mM) caused a dose-dependent suppression of mitochondrial respiration at 1 h (Figure 1a,b). BSO treatment

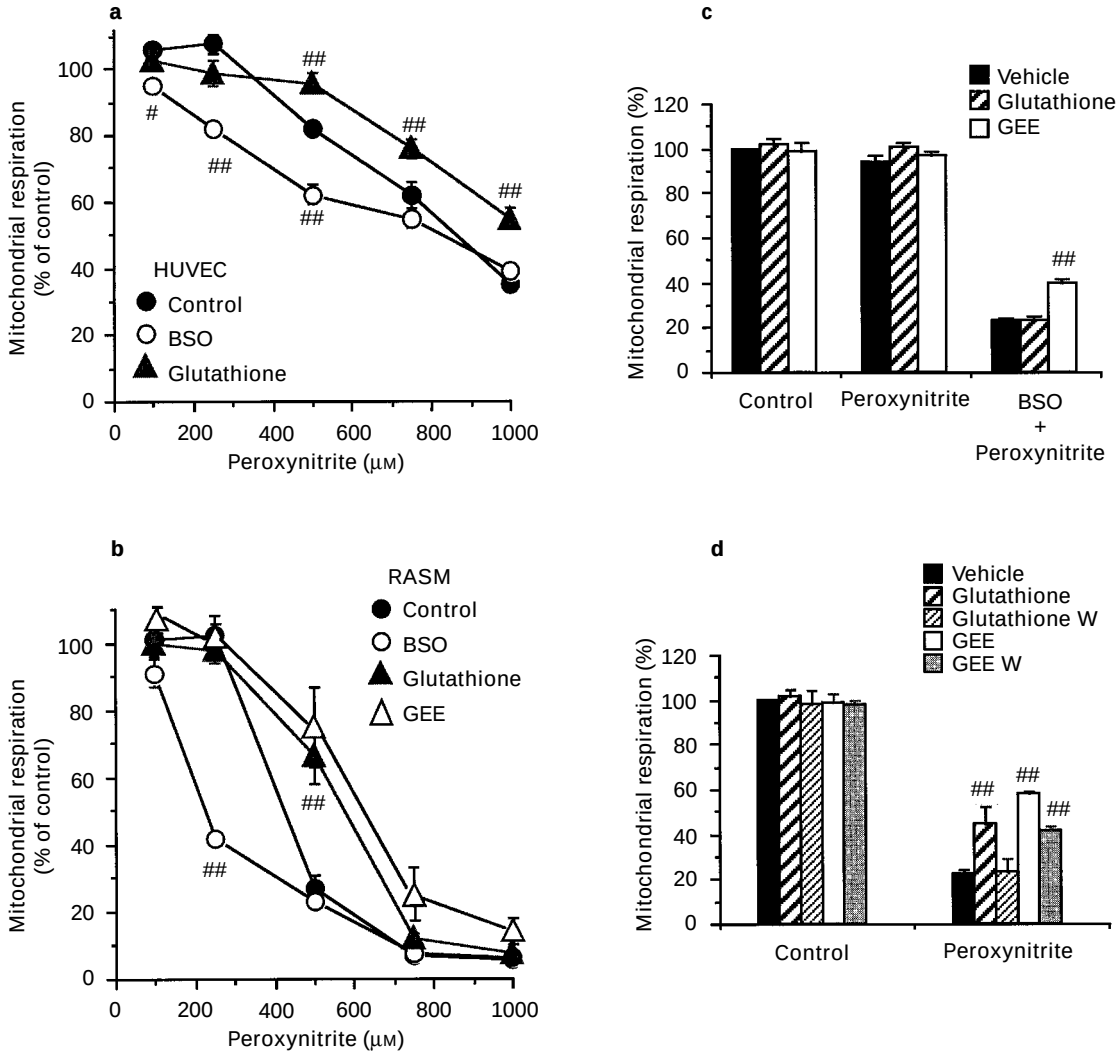

Figure 1 Effect of peroxynitrite (100 μM – 1000 μM) on the mitochondrial respiration in cultured HUVECs (a) and RASM cells (b); effect of pretreatment with BSO, glutathione (3 mM) and glutathione ethyl ester (GEE, 3 mM). In (c), the effect of glutathione or glutathione ethyl ester pretreatment (3 mM) on the response to peroxynitrite (250 μM) is shown. In (d), the effect of glutathione or glutathione ethyl ester pretreatment, in the absence or presence of washouts (W) on the peroxynitrite-induced (750 μM) suppression of mitochondrial respiration is shown. $^{\#,\#\#}$Represent differences between the effect of peroxynitrite after BSO, glutathione or glutathione ethyl ester treatment, when compared to the response to peroxynitrite in the absence of any pharmacological agents ($P<0.05$ and $P<0.01$, respectively). Formazan production in control HUVEC and RASM cells amounted to 0.11 ± 0.03 and 0.29 ± 0.02 μg/well min^{-1}, respectively. Data represent means of $n = 6-12$ observations; vertical lines show s.e.mean.

for 24 h alone had no significant effect on the cellular respiration. However, the effects of peroxynitrite were markedly enhanced in cells which had been exposed to BSO (Figure 1a,b). The enhancement was pronounced at lower peroxynitrite concentrations, while at higher concentrations of peroxynitrite, control and BSO-treated cells showed a similar degree of suppression of mitochondrial respiration in response to peroxynitrite. Pretreatment of the cells with glutathione (3 μM), or glutathione ethyl ester (3 mM) (an analogue of glutathione with better cellular uptake characteristics) alone, had no significant effect on mitochondrial respiration in HUVEC or RASM cells. However, pretreatment of the cells with glutathione (3 mM) or glutathione ethyl ester (3 mM) reduced the degree of suppression of mitochondrial respiration in response to peroxynitrite (Figure 1).

In the RASM cells, we also investigated the reversibility of the BSO-induced enhancement of the peroxynitrite-induced cytotoxicity by glutathione, and by glutathione ethyl ester. We found that glutathione ethyl ester, but not glutathione protects against the suppression of mitochondrial respiration in response to peroxynitrite in the BSO-treated cells (Figure 1c). We also investigated the effect of glutathione and glutathione ethyl ester on peroxynitrite-induced cytotoxicity after a 4 h incubation and repeated washouts, in order to determine whether extracellular, or intracellular neutralization of peroxynitrite is responsible for the protection against peroxynitrite-induced cytotoxicity. The results, shown in Figure 1d, demonstrate that washouts abolish the protection provided by glutathione. In contrast, glutathione ethyl ester maintains some of its protective effects even after washouts (Figure 1d).

The peroxynitrite-induced cellular toxicity is, in part, mediated by DNA strand breakage and activation of the nuclear enzyme PARS (Szabó, 1996b). We have therefore also investigated the effect of BSO pretreatment on DNA strand

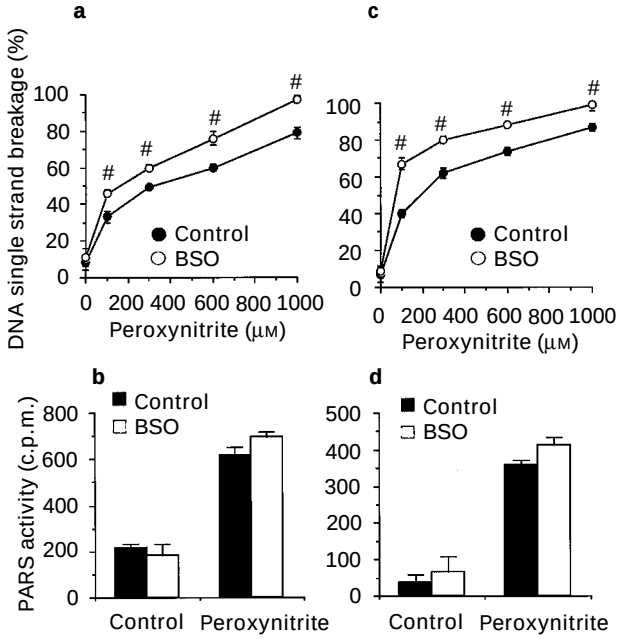

Figure 2 (a and c) Effect of peroxynitrite (100 μM – 1000 μM) on the percentage of DNA single strand breaks in (a) cultured HUVECs and (b) RASM cells; effect of BSO. (b and d) Effect of peroxynitrite (750 μM) on the activity of PARS in (b) cultured HUVECs and (d) RASM cells; effect of BSO pretreatment. #Represents significant enhancement of the effect of peroxynitrite after BSO treatment ($P<0.05$). Data represent means of $n=4-6$ observations; vertical lines show s.e.mean.

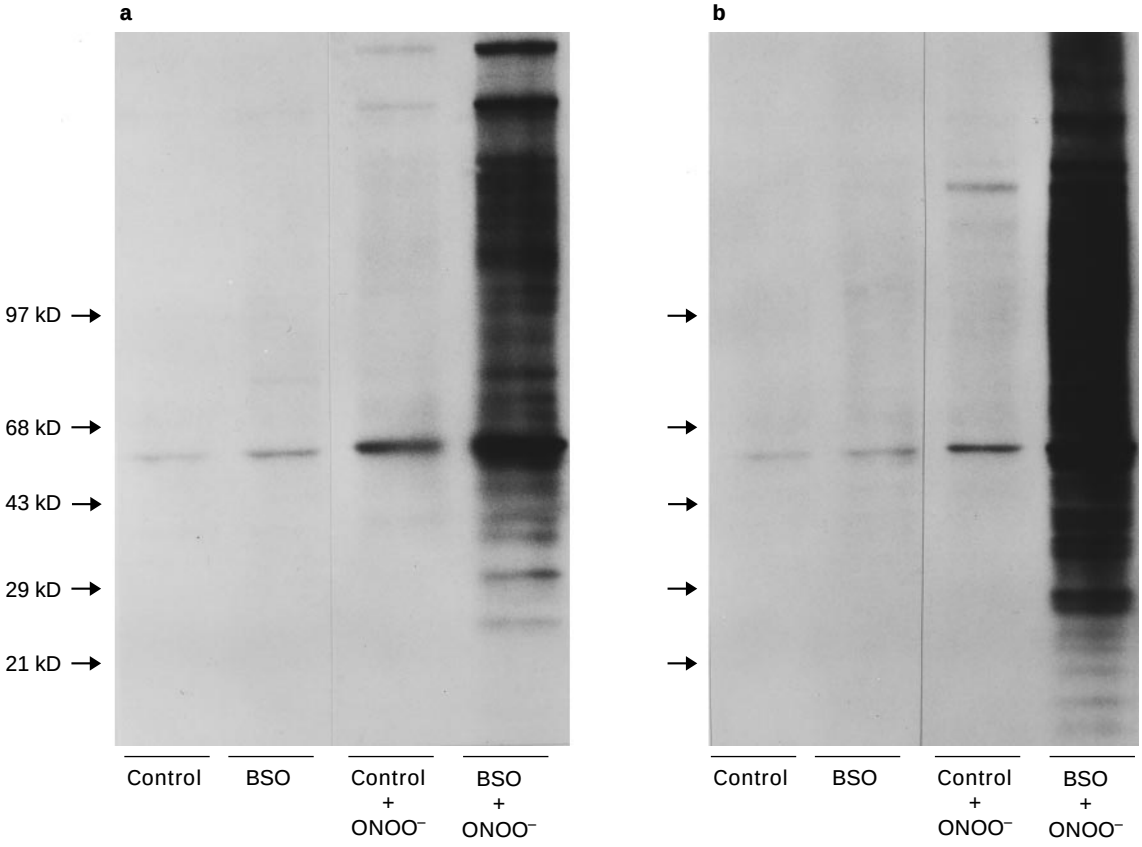

Figure 3 Effect of peroxynitrite ($ONOO^-$, 750 μM, 1 h) on protein oxidation in extracts of cultured (a) HUVEC and (b) RASM cells; effect of BSO.

breakage and PARS activation. We have observed that BSO pretreatment caused a significant enhancement of the ability of peroxynitrite to induce DNA single strand breakage in HUVECs and in RASM cells (Figure 2). In agreement with previous observations in RASM cells and macrophages (Szabó, 1996b), we have observed that peroxynitrite causes a dose-dependent activation of PARS (Figure 2). We observed a slight enhancement of the activation of PARS in response to peroxynitrite in the cells pretreated with BSO (Figure 2).

Peroxynitrite is also a potent inducer of protein oxidation and nitration *in vitro* and *in vivo* (Crow & Beckman, 1995; Szabó, 1996a). BSO pretreatment caused an enhancement of peroxynitrite-induced protein oxidation in both HUVECs and RASM cells (Figure 3). While we have detected a large number of oxidized proteins after peroxynitrite exposure in both cell types (including a protein of approximately 55 kD molecular weight), peroxynitrite-induced tyrosine nitration was mainly observed with a protein of approximately 55 kD (Figure 4). Pretreatment of the RASM cells with BSO caused an enhancement of the peroxynitrite-induced tyrosine nitration (Figure 4), whereas there was only a marginal enhancement of the tyrosine nitration after BSO pretreatment in the HUVECs (Figure 4).

In the RASM cells, expression of the inducible NO synthase (iNOS) was elicited by stimulation with LPS and IFN for 24 h. As demonstrated previously (Szabó *et al*., 1996b), the production of NO (as detected by the measurement of the accumulation of nitrite, a breakdown product of NO) was associated with a significant suppression of mitochondrial respiration. In cells which had been pretreated with BSO, there was a significant enhancement of the suppression of mitochondrial respiration in response to immunostimulation (Figure 5b). BSO pretreatment did not affect the amount of nitrite produced (Figure 5a). In unstimulated cells, in accordance with recent observations (Boese *et al*., 1996), a small amount of nitrite production was detected, which was inhibited by L-NMA (Figure 5). This basal nitrite production was also abolished in the absence of serum, and, therefore, may be related to a low level of iNOS induction due to a serum factor (Cuzzocrea *et al*., 1997).

In cells not treated with BSO, treatment with the NOS inhibitor L-NMA prevented the suppression of mitochondrial respiration in response to LPS/IFN. While L-NMA also caused a significant improvement of the mitochondrial respiration in the LPS/IFN treated cells after BSO pretreatment, it did not cause a complete restoration of the cellular respiration (Figure 5). Stimulation of the cells with LPS/IFN for 24 h also resulted in an increase in protein oxidation, which was significantly enhanced in the BSO-treated cells and was prevented by inhibition of NOS with L-NMA (data not shown).

Effect of BSO pretreatment on the peroxynitrite-mediated suppression of vascular contractility and endothelial dysfunction in thoracic aortic rings

Exposure of endothelium-intact vascular rings to peroxynitrite caused a simultaneous, marked depression of the noradrenaline-induced contractions (Figure 6a) and an impairment of the acetylcholine-induced, endothelium-dependent relaxations (Figure 6b). This latter effect appears to be related to a reduced ability of the vascular endothelium to produce NO in

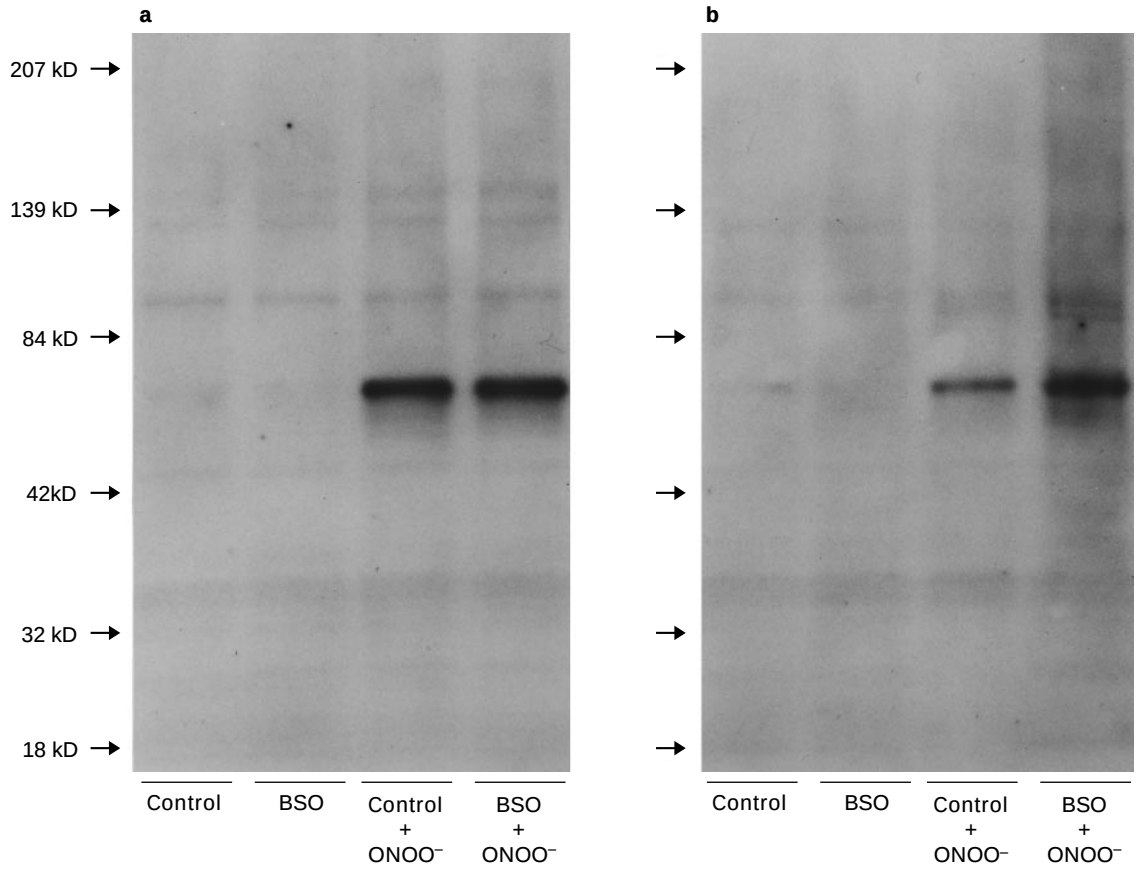

Figure 4 Effect of peroxynitrite ($ONOO^-$, 750 μM, 1 h) on tyrosine nitration in extracts of cultured (a) HUVEC and (b) RASM cells; effect of BSO.

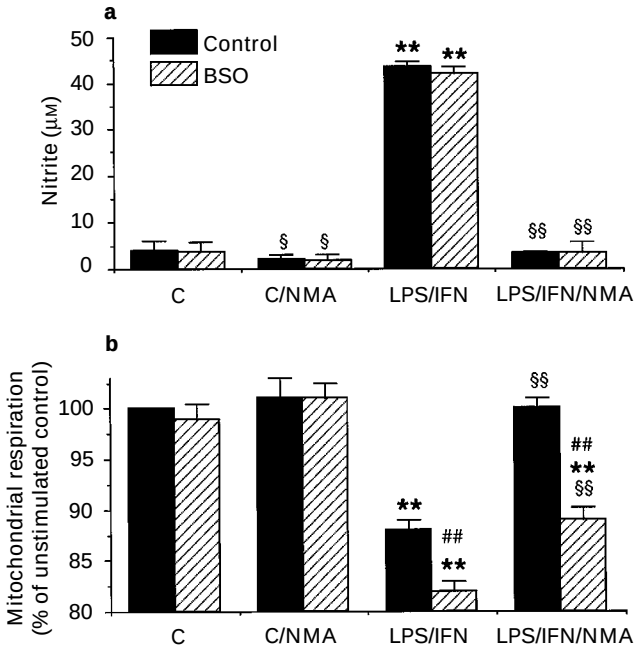

Figure 5 Effect of LPS and IFN on nitrite production (a) and mitochondrial respiration (b) in RASM cells; effect of BSO. **Represents a significant effect of LPS and IFN when compared to unstimulated controls ($P<0.01$); §,§§represent significant effects of the NOS inhibitor L-NMA when compared to results in the corresponding groups without L-NMA ($P<0.05$, $P<0.01$, respectively) and ##represents significant change in the effect of LPS/IFN after BSO treatment, when compared to results without BSO ($P<0.01$). Data represent means ± s.e.mean of $n=9-12$ observations.

response to acetylcholine, and not due to a reduced ability of the vascular smooth muscle to relax to NO, for the relaxant effect of the NO donor compound SNAP remained unaltered (Figure 6c).

In vascular rings taken from the rats which had been pretreated with BSO to deplete endogenous glutathione, there were no alterations in the contractility or relaxations in the absence of *in vitro* treatment with oxidants (Figure 6). However, in the vessels obtained from the BSO-pretreated animals, there was a significantly more pronounced suppression of the contractility and a significantly more (near-complete) inhibition of the endothelium-dependent relaxations after peroxynitriate exposure (Figure 6).

The presence of 3 mM glutathione in the incubation medium during peroxynitrite exposure significantly reduced the degree of vascular hyporeactivity and the degree of endothelial dysfunction (Figure 6).

Effect of in vivo *BSO treatment on the production of peroxynitrite, induction of iNOS and on the changes in vascular function in endotoxic shock*

There was no marked nitrotyrosine staining in the aorta of control animals (Figure 7a,b). In agreement with previous observations (Szabó *et al*., 1995), in vascular rings obtained from rats injected with LPS, a pronounced nitrotyrosine staining was detected in the vessel wall, indicative of peroxynitriate formation *in vivo* (Figure 7c). This staining was substantially more marked in the animals pretreated with BSO (Figure 7d).

In the BSO-pretreated rats, there was a reduction in the net production of NO in response to LPS treatment, as evidenced

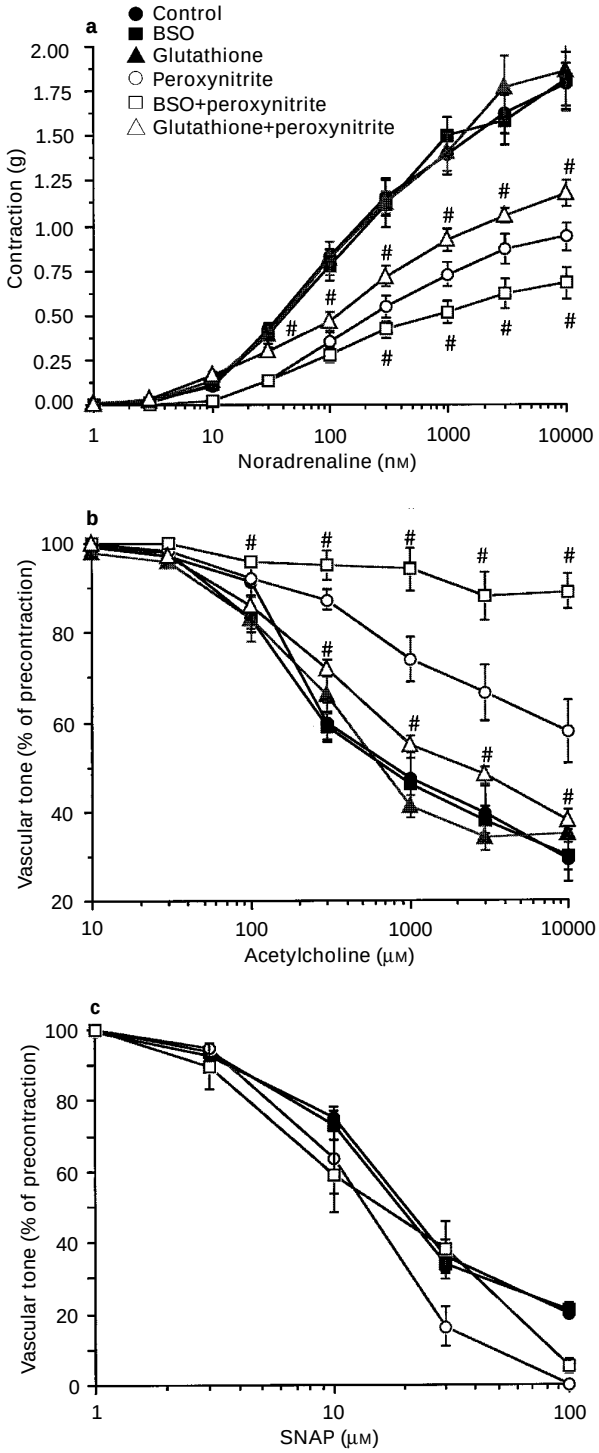

Figure 6 Effect of peroxynitrite (750 μM) on (a) vascular contractility to noradrenaline (1 nM – 10 μM); (b) endothelium-dependent relaxations to acetylcholine (10 nM – 10 μM) and (c) relaxations to the NO donor compound SNAP (1 – 100 μM) in rat aortic rings. Responses in rings from control animals, responses in rings from animals treated with BSO, and responses in control rings in the presence of glutathione (3 mM) are shown. #Represents significant effects of BSO or glutathione in the peroxynitrite-treated rings ($P<0.05$). For (b and c), the levels of precontraction were 1.5 ± 0.1 g; 1.3 ± 0.2 g and 1.8 ± 0.3 g in control rings without any pharmacological intervention, in rings taken from BSO-pretreated animals and in control rings in the presence of glutathione, respectively; and 0.72 ± 0.2; 0.27 ± 0.1 and 1.4 ± 0.2 g in peroxynitrite-treated rings without any pharmacological intervention, in peroxynitrite treated rings taken from BSO pretreated animals, and in rings treated with peroxynitrite in the presence of glutathione, respectively. Data are expressed as means of $n=9-12$ vascular rings; vertical lines show s.e.mean.

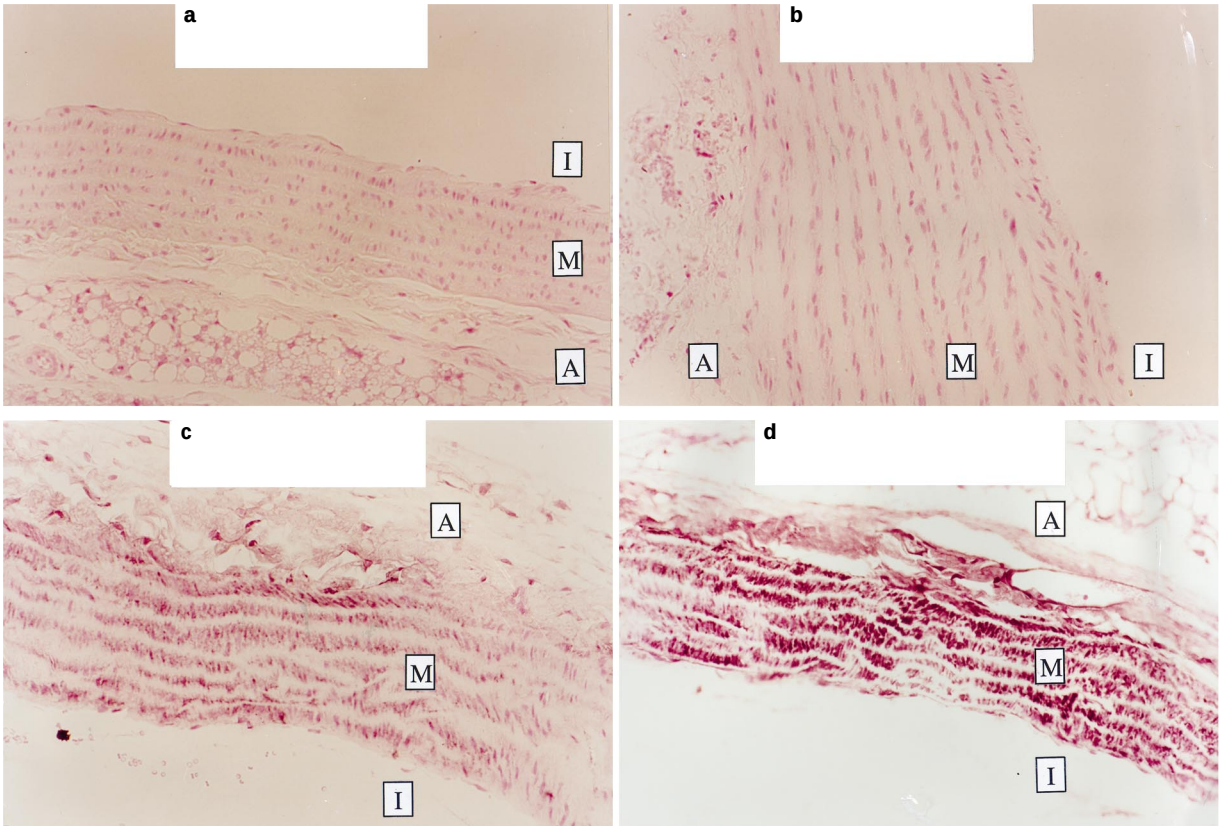

Figure 7 Immunohistochemical staining of nitrotyrosine, an indicator of peroxynitrite production, in a control rat thoracic aortic ring section (a), in a section of a ring of a control animal treated with BSO to deplete endogenous glutathione pools (b), in a section of a ring of an LPS-treated animal (c) and in a section of a ring of an LPS-treated animal which had been pretreated with BSO (d). I indicates intima; M indicates media; A indicates adventitia. Note the marked increase in the nitrotyrosine staining after LPS treatment, and the enhancement of the staining after BSO. Figures show representative immunohistochemical patterns. A similar pattern was seen in 3–4 different tissue sections in each group.

by reduced LPS-induced plasma nitrite/nitrate levels (Figure 8a). However, and in agreement with the *in vitro* results in LPS/IFN stimulated RASM cells (Figure 5), BSO pretreatment did not affect the degree of the expression of the calcium-independent NOS (iNOS) activity in the thoracic aorta in response to LPS treatment (Figure 8b).

In vascular rings obtained from rats injected with LPS, there was a significant reduction of the noradrenaline-induced contractions and an impairment of the endothelium-dependent relaxations to acetylcholine at 6 h *ex vivo* (Figure 9a). Pretreatment of the animals with BSO exacerbated the LPS-induced development of both components of the vascular dysfunction (hyporeactivity and endothelial dysfunction) (Figure 9b). Treatment of the rats with LPS also caused a modest reduction in the endothelium-independent relaxant effect of SNAP, an effect which was not substantially affected by BSO pretreatment (Figure 9c).

Discussion

By use of BSO as a specific inhibitor of γ-glutamylcysteine synthetase, a number of studies have proposed the importance of endogenous glutathione in the protection against shock, especially in relation to end-organ injury (Keller *et al*., 1985; Nemeth & Boda, 1989; Stein *et al*., 1990; Liu *et al*., 1993; Gatti *et al*., 1993; Zhang *et al*., 1994; Lee *et al*., 1995). Moreover, BSO was shown to exacerbate ischaemia/reperfusion injury in some (Singh *et al*., 1989; Liu *et al*., 1994) but not other (Verbunt *et al*., 1996) experimental systems. However, to our knowledge, the present study represents the first investigation focusing on the potential importance of endogenous glutathione in the development of vascular failure (contractile or endothelial-dependent relaxant responses) both in response to peroxynitrite, and in endotoxic shock.

Role of glutathione in the peroxynitrite-induced cellular injury

The *in vitro* studies demonstrated a significant enhancement of the peroxynitrite-induced suppression of mitochondrial respiration, DNA injury, tyrosine nitration and protein oxidation by BSO pretreatment. On the other hand, glutathione, and glutathione ethyl ester elicited protective effects. Interestingly, at higher concentrations of peroxynitrite, the difference in the degree of suppression of mitochondrial respiration between control and BSO-pretreated cells diminished (Figure 1). One possible explanation for this phenomenon may be that, at high concentrations, peroxynitrite depletes reduced thiols in various cells (Szabó *et al*., 1996b; Vatassery, 1996), and the remaining reactive peroxynitrite is still capable of causing overwhelming oxidative injury.

In addition studies, the potential reversal of the effects of BSO by glutathione or glutathione ethyl ester was investigated. Although both glutathione and its ethyl ester protected against peroxynitrite-induced cell injury in the absence of BSO, in BSO-treated cells only glutathione ethyl ester, but not glutathione exerted protective effects. This latter finding may be due to the fact that in the BSO-treated cells, the enhancement of the cellular injury is due to the lack of intracellular glutathione. Thus, glutathione, due to its weak cell uptake, cannot substitute for it, whereas glutathione ethyl

ester can. On the other hand, in the absence of BSO, glutathione may react with peroxynitrite extracellularly and thus exert protective effects. Taken together, the present results are consistent with the proposal, but do not prove, that the enhancement by BSO of the peroxynitrite-induced cellular injury is a consequence of intracellular glutathione depletion.

In a study by Kang (1994), the BSO-induced inhibition of cell proliferation was found to be reversed by glutathione, but this effect did not correlate with repletion of intracellular glutathione levels. Kang proposed that the effect of glutathione may be related to recovery of BSO-inhibited cystine uptake (the latter phenomenon is thought to be responsible for the antiproliferative effect of BSO). It is not known, at present, whether the effect of BSO on cellular cystine uptake (or other, unrelated pharmacological effects of BSO) on cellular cystine uptake (or other, unrelated pharmacological effects of BSO) also contribute to the enhancement by BSO of the peroxynitrite-induced cellular injury or vascular failure *in vitro* or in endotoxic shock.

Role of glutathione in the peroxynitrite-induced vascular dysfunction

Our findings, demonstrating enhanced vascular failure in response to peroxynitrite and in the vascular rings of BSO-treated animals are in agreement with previous suggestions that glutathione has an important protective role against the oxidant-induced injury. Moreover, our data directly demonstrate the protective effect of excess glutathione against the peroxynitrite-induced vascular changes. Since glutathione only has a limited cellular uptake (Hiraishi *et al*., 1994; Deneke *et al*., 1995), it appears likely that extracellular neutralization by glutathione of peroxynitrite contributes to the observed effects.

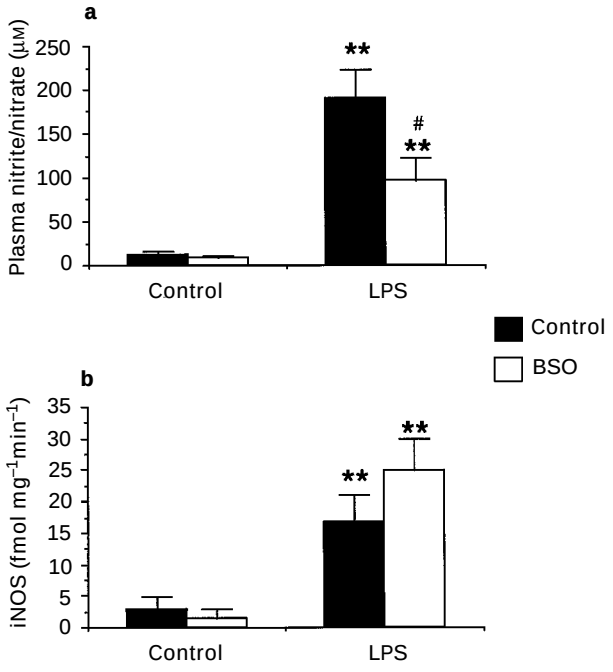

Figure 8 Plasma nitrite/nitrate levels (a) and iNOS activity (b) in rat thoracic aortae. Columns represent responses in control animals and in animals treated with LPS (15 mg kg^{-1} for 6 h i.p.). Values in rings from control animals and those in rings from animals treated with BSO, in order to deplete endogenous glutathione pools, are shown **Represents a significant effect of LPS when compared to unstimulated controls ($P<0.01$); #represents significant differences between the respective values in the control response versus the response of BSO-treated animals ($P<0.05$). Data are expressed as means ± s.e.mean of $n=6-9$ determinations.

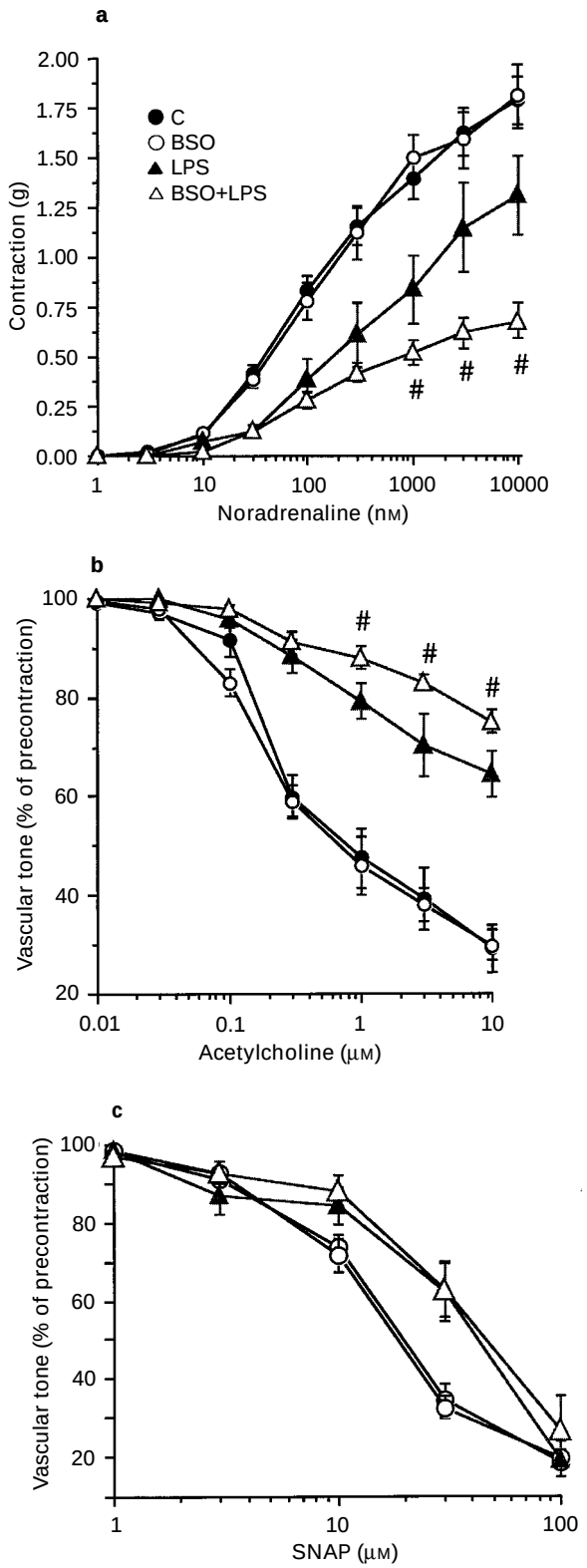

Figure 9 Effect of exposure to vehicle or LPS treatment (15 mg kg^{-1} for 6 h i.p.) on (a) vascular contractility to noradrenaline (1 nM–10 μM), (b) endothelium-dependent relaxations to acetylcholine (10 nM–10 μM) and (c) relaxations to the NO donor compounds SNAP (1–100 μM) in rat aortic rings from control animals and from animals treated with BSO. #Represents significant differences between the respective responses in the control rings and rings from BSO-treated animals ($P<0.05$). In (b and c), the levels of precontraction were 1.5 ± 0.1 and 1.4 ± 0.2 in control rings without any pharmacological intervention and in control rings prepared from BSO pretreated animals, respectively; and 1.1 ± 0.1 and 0.8 ± 0.2 g in rings from LPS-treated animals without any pharmacological intervention and in rings from BSO pretreated and LPS-treated animals, respectively. Data are expressed means of $n=9-12$ vascular rings; vertical lines show s.e.mean.

There are a variety of additive or synergistic cytotoxic processes triggered by peroxynitrite, the combination of which leads to acute and delayed cytotoxicity (see Introduction). It is conceivable that the depletion of glutathione enhances the effects of peroxynitrite towards many (if not all) of these pathways.

Our finding that BSO pretreatment markedly affected the degree of endothelial dysfunction in response to peroxynitrite exposure merits further discussions. There is a substantial body of work implicating the role of oxyradicals in the pathogenesis of endothelial injury in ischaemia-reperfusion, shock and atherosclerosis (White *et al*., 1994; 1996; Darley-Usmar *et al*., 1996). More recently, it has been suggested, that NO, derived from the endothelium, or from other sources, may combined with superoxide to form peroxynitrite, which, in turn, exerts cytotoxic effects towards the endothelial cells themselves (White *et al*., 1996; Az-Ma *et al*., 1996; Zingarelli *et al*., 1997). Regarding the potential mechanisms of peroxynitrite-induced endothelial dysfunction, limited information is available. In bovine cultured endothelial cells, exposure to peroxynitrite caused a reduced ability of the cells to mobilize calcium in response to endothelium-dependent vasodilator agonists (Elliott, 1996), a finding which would be in agreement with published data demonstrating inactivation of calcium pumps after peroxynitrite exposure (Viner *et al*., 1996). In the current scenario, it appears likely that the inhibition of endothelium-dependent relaxations is secondary to cytotoxic processes triggered by peroxynitrite, such as inhibition of cellular metabolic pathways, calcium mobilization, alterations in cellular signalling, activation of PARS and/or reduction of the activity of membrane pumps. Based on the present observations, we propose that glutathione plays a crucial role in modulating peroxynitrite-related endothelial injury *in vitro* and also, possibly, in the other, above mentioned, pathophysiological conditions.

Effect of BSO on the vascular reactivity in endotoxaemia

The vascular hypocontractility and the endothelial dysfunction in response to LPS are important, distinct features of the vascular changes in endotoxaemia. The vascular hyporeactivity in endotoxic shock was suggested to be the consequence of the expression of iNOS and the related overproduction of NO, since inhibitors of NOS protect against these changes (Julou-Schaeffer *et al*., 1990; Szabó *et al*., 1993; Paya *et al*., 1995; Rees, 1995; Szabó, 1995). However, more recent work has put forward the possibility of an additional pathway, whereby peroxynitrite production during endotoxic shock contributes to the loss of contractile tone (Szabó *et al*., 1996b; Zingarelli *et al*., 1997). Similarly, endothelial dysfunction in endotoxic shock may be related to activated neutrophil granulocytes and superoxide production (Parker & Adams, 1993; Yaku *et al*., 1994; Myers *et al*., 1995), and, possibly, the formation of peroxynitrite in the vicinity of the endothelium (Zingarelli *et al*., 1997). Based on the present findings, demonstrating a more pronounced hyporeactivity and endothelial dysfunction in the BSO-treated animals after LPS challenge, we propose that endogenous glutathione has an important protective role against the development of vascular failure. In addition to reduced relaxations to the endothelium-dependent relaxant acetylcholine, during endotoxic shock, was also found a reduced relaxation to SNAP. A similar reduction in the relaxations in response to NO donors have been previously observed after endotoxic shock in some (Schneider *et al*., 1994; Fullerton *et al*., 1995) but not other (Parker & Adams, 1993; Yaku *et al*., 1994) studies. It is conceivable that iNOS expression, and an increased 'basal' production of NO in the vascular rings obtained from endotoxaemic animals causes an increased activation of guanylyl cyclase in the smooth muscle, which renders the system less responsive to exogenous NO. The more pronounced impairment of the relaxations after BSO pretreatment in the LPS-treated animals is more likely to be related to changes in the endothelial rather than smooth muscle function, since BSO pretreatment had little effect on the SNAP-induced relaxations during endotoxaemia (Figure 9c).

Endogenous glutathione and iNOS expression

In the present study we found that in RASM cells, depletion of glutathione enhanced the LPS and IFN-induced suppression of mitochondrial respiration, despite its lack of effect on nitrite production. The suppression of mitochondrial respiration in the response to LPS and IFN was completely prevented by L-NMA, confirming the role of NO or a related species, such as peroxynitrite, in the process. However, the more pronounced cytotoxic action of LPS and IFN, observed after reduction of endogenous glutathione levels by BSO, was not completely prevented by L-NMA. The most likely explanation for these findings is that oxygen-derived oxidants (such as superoxide or hydroxyl radical) are responsible for the residual cytotoxicity after inhibition of NOS in these glutathione-depleted cells. Indeed, a prolonged superoxide production after LPS and IFN stimulation has been demonstrated in RASM cells (Szabó *et al*., 1996b).

In vivo, LPS triggers the expression of iNOS in many cell types, including macrophages and the vascular smooth muscle (Nathan, 1992; Rees, 1995; Szabó, 1995). Simultaneous production of NO and superoxide in LPS-treated animals results in the production of peroxynitrite, with subsequent nitrotyrosine formation in the vasculature (Wizemann *et al*., 1994; Szabó *et al*., 1995). In the present study, we have observed a much more pronounced nitrotyrosine staining after LPS challenge in the BSO-treated animals, suggesting the presence of more biologically active peroxynitrite within the blood vessel wall. This more pronounced nitrotyrosine staining was not related to the increased production of NO within the vasculature, as demonstrated by the measurement of vascular tissue iNOS activity. In fact, we observed a reduction in the degree of plasma nitrite/nitrate levels in response to LPS in BSO-pretreated animals. From our results, we cannot exclude the possibility that this effect may be related to an altered disposition of nitrate in the BSO-treated rats. Alternatively, it is possible that, in accordance with data by Buchmuller-Rouiller *et al.* (1996), BSO pretreatment reduces nitrite production in macrophages. Since the majority of NO produced in LPS-treated rodents derives from macrophages and not from vascular tissues (Salkowski *et al*., 1997), a reduction of macrophage- but not smooth muscle-derived NO production by BSO in the LPS-treated rats would explain our present *in vivo* findings. The elucidation of the mechanism of inhibition of iNOS expression by depletion of endogenous glutathione pools in some, but not other cell types requires further investigations and is beyond the scope of the present study.

Relative importance of reactions of glutathione with NO, oxyradicals and peroxynitrite in the vascular changes during endotoxic shock

Although BSO is considered to be a specific inhibitor of γ-glutamylcysteine synthetase, and BSO-depleted cells or animals are widely used for the elucidation of the role of

glutathione in biological systems (e.g. Keller *et al*., 1985; Nemeth & Boda, 1989; Singh *et al*., 1989; Stein *et al*., 1990; Liu *et al*., 1993; 1994; Gatti *et al*., 1993; Zhang *et al*., 1994; Lee *et al*., 1995), we cannot exclude the possibility that some independent pharmacological effects of BSO also contributed to the observed changes. Nevertheless, based on our data demonstrating that excess glutathione protects against peroxynitrite-induced cytotoxicity, and that BSO exacerbates peroxynitrite-induced cytotoxicity, we proposed a role for glutathione as an important endogenous factor in modulating peroxynitrite-related cellular injury.

What, then, is the mode of glutathione's protective action in endotoxic shock? Glutathione is a known oxyradical scavenger (Packer, 1994). Moreover, glutathione can react with NO to form S-nitrosoglutathione, a vasodilator compound (Simon *et al*., 1993; Stamler, 1995), although the reaction is likely to involve NO_2 or NO_2O_3, rather than NO *per se* (Butler *et al*., 1995). Thus, theoretically, the mechanism of the observed vascular alterations in the BSO-pretreated cells and tissues may be related to a diminished neutralization by glutathione of peroxynitrite, oxyradicals, NO or the combination of these. From the available data in the literature, it appears unlikely that a glutathione-NO reaction plays a major role in the observed changes. This conclusion is based on the following considerations: (1) in accordance with previous studies (Sakanashi *et al*., 1991; Jia & Furchgott, 1993), we found no differences in the endothelium-independent relaxant effect of SNAP in the aortae from control and BSO-treated animals, suggesting that the reduction in endogenous glutathione does not affect NO-induced relaxant responses; and (2) *in vitro* studies in macrophages and other cell types have established that endogenous glutathione only protects against very high fluxes of NO, but not against lower levels of NO production, such as the ones which are relevant to the *in vitro* or *in vivo* conditions in our experiments (Walker *et al*., 1995; Petit *et al*., 1996). On the other hand, the possibility that the enhancement of cytotoxicity in BSO-treated cells or tissues after LPS treatment is related, in part, to increased oxyradical-induced cytotoxic effects can be considered. Indeed, there are existing data showing that depletion of endogenous glutathione enhances the cytotoxic effects of hydrogen peroxide and of oxyradicals (see above). In the experiments which involve LPS stimulation, it is conceivable that a more pronounced inhibition of mitochondrial respiration by peroxynitrite, NO and/or oxygen-derived free radicals and oxidants can lead to a dysfunctional electron transfer, with increased superoxide production from the mitochondria. This positive feedback cycle would also lead to an enhancement of peroxynitrite production and may increase cytotoxicity.

In immunostimulated cells and tissues, the production of various oxygen- and nitrogen-derived free radicals and oxidants occurs in a simultaneous fashion. Therefore, it is conceivable that important interactions exist between these reactive species in terms of oxidative potential and cytotoxicity. For instance, with respect to peroxynitrite-induced oxidative injury it is well established that the ratio of NO and superoxide determines the oxidant capacity, and excess NO reduces peroxynitrite-induced oxidative processes (Rubbo *et al*., 1994; Miles *et al*., 1996). Hydrogen peroxide, on the other hand, prolongs the half-life of peroxynitrite (Alvarez *et al*., 1995), and has a synergistic effect with peroxynitrite in terms of cytotoxicity (Szabó *et al*., 1997b). Thus, it is possible that the cytotoxic effects we observed in response to immunostimulation represent the sum of complex interactions between various oxygen- and nitrogen-derived radicals and oxidants. Nevertheless, we propose that the reaction of glutathione with peroxynitrite, or with a peroxynitrite-derived oxidant importantly contributes to the protective effects of endogenous glutathione in immunostimulated cells and in endotoxic shock.

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