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The protective role of endogenous glutathione in carrageenan-induced pleurisy in the rat

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Abstract

In the present study we investigated the protective role of endogenous glutathione, a known free radical scavenger, in rats subjected to carrageenan-induced pleurisy. In vivo depletion of endogenous glutathione pools with L-buthionine-(*S,R*)-sulfoximine (BSO, 1 g/kg for 24 h, intraperitoneally) enhances the carrageenan-induced degree of pleural exudation and polymorphonuclear leukocyte migration in rats subjected to carrageenan-induced pleurisy. Lung myeloperoxidase activity and lipid peroxidation were significantly increased in BSO pretreated rats. However, the inducible nitric oxide (NO) synthase in lung samples was unaffected by BSO pretreatment. Immunohistochemical analysis for nitrotyrosine revealed a positive staining in lungs from carrageenan-treated rats, which was massively enhanced by BSO pretreatment. Furthermore, in vivo BSO pretreatment significantly increased peroxynitrite formation as measured by the oxidation of the fluorescent dye dihydrorhodamine 123, enhanced the appearance of DNA damage, the decrease in mitochondrial respiration and partially decreased the cellular level of NAD^+ in ex vivo macrophages harvested from the pleural cavity of rats subjected to carrageenan-induced pleurisy. In vivo treatment with exogenous glutathione (50 mg/kg i.p.) significantly reverts the effects of BSO and exerts anti-inflammatory effects. Thus, endogenous glutathione plays an important protective role against carrageenan-induced local inflammation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Inflammation; Nitric oxide (NO); Peroxynitrite; Glutathione; Superoxide; Hydroxyl radical; Carrageenan

1. Introduction

The expression of the inducible isoform of nitric oxide (NO) synthase has been proposed as an important mediator of inflammation (Nathan, 1996; Cuzzocrea et al., 1997a). The systemic inflammatory response is also associated with the production of oxygen-derived free radicals (Youn et al., 1991; McCord, 1993), and 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, a toxic oxidant formed from the reaction of NO and superoxide (Beckman et al., 1990). The formation of peroxynitrite has been demonstrated in immunostimulated macrophage (Ischiropoulos et al., 1992), in various inflammatory disorders (Halliwell, 1995; Cuzzocrea et al., 1998a) and in circulatory shock (Wiseman and Halliwell, 1996).

The biological activity and decomposition of peroxynitrite is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, carbon dioxide levels and other factors), and these factors influence its toxic potential (Beckman et al., 1990; Rubbo et al., 1994; Villa et al., 1994). In a number of pathophysiological conditions, peroxynitrite has been proposed as an important mediator of cell damage under conditions of inflammation and oxidant stress (Miles et al., 1996; Cuzzocrea et al., 1998a,b).

Peroxynitrite is cytotoxic via a number of independent mechanisms. Its cytotoxic effects include initiation of lipid peroxidation, inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane

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pumps) (Radi et al., 1994), glutathione depletion (Phelps et al., 1995) and DNA damage (Cuzzocrea et al., 1998b, 1999) with subsequent activation of poly ADP ribose synthetase and concomitant cellular energy depletion (Cuzzocrea et al., 1998b).

Another mechanism of peroxynitrite-induced delayed toxicity may be related to interference by peroxynitrite of membrane signal transduction pathways (Berlett et al., 1996; Gow et al., 1996).

Peroxynitrite is a potent oxidant, and, therefore, it is conceivable that endogenous antioxidant mechanisms counteract with its toxicity. In in vitro studies, it has been established that antioxidants such as glutathione, ascorbic acid and alpha-tocopherol are scavengers of peroxynitrite and inhibitors of its oxidant capacity (Radi et al., 1991; Karoui et al., 1996). There is a marked depletion of cellular glutathione in endothelial cells and smooth muscle cells after exposure of endogenously produced or exogenously applied peroxynitrite (Van der Vliet et al., 1994; Cuzzocrea et al., 1998c) and depletion of all of the above antioxidants in the plasma after exposure to peroxynitrite (Phelps et al., 1995). The rate limiting enzyme in the intracellular regulation of glutathione is γ-glutamylcysteine synthase (Meister, 1992). This enzyme is readily inhibited by the drug L-buthionine-(*S,R*)-sulfoximine (BSO) and therefore administering BSO quickly depletes cells of their store of glutathione (Martensson et al., 1989; Reddy, 1990).

In the current study, we investigated the role of endogenous glutathione against the peroxynitrite-induced injury in a model of local inflammation by carrageenan. In specific, we have investigated whether depletion of endogenous glutathione pools with BSO affects the inflammatory response (pleural exudate formation, cellular infiltration) and cellular injury in ex vivo macrophages harvested from the pleural cavity of the rats subjected to carrageenan-induced pleurisy.

2. Materials and methods

2.1. Carrageenan-induced pleurisy

Rats were lightly anaesthetised under isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscles were dissected and 0.2 ml saline alone or containing 1% λ-carrageenan were injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were sacrificed under CO_2 vapour. The chest was carefully opened and the pleural cavity washed with 2 ml of saline solution with heparin (5 U/ml) and indomethacin 10 μg/ml). The exudate and washing were removed by aspiration and the total volume measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (2 ml) from the total volume recovered. The number of leucocytes in the exudate were suspended in phosphate buffer saline and counted with optical microscope by Burker's chamber after vital Trypan Blue stain. Injection of carrageenan (or vehicle) was given to groups of animals pretreated with BSO (1 g/kg for 24 h, i.p.) to deplete glutathione, and to animals pretreated with BSO vehicle (saline). Thus, the following groups of animals were used: control, control + BSO, carrageenan and carrageenan + BSO ($n = 10$ rats in each group). In a second group and third group of experiments glutathione (GSH, 50 mg/kg i.p.) was administered to BSO pretreated rat and to animals pretreated with BSO vehicle (saline) 15 min before carrageenan administration. In a fourth group of experiments, the inactive isomer L-buthionine-*R*-sulfoximine (1 g/kg for 24 h, i.p.) was administered to the rats ($n = 10$).

2.2. Cell culture

Pleural macrophages from rats were harvested by pleural lavage with Dulbecco's Modified Eagle Medium (DMEM) medium, supplemented with L-glutamine (3.5 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) and heparin sodium (10 U/ml). The cells were collected 4 h after the carrageenan injection from rats treated with or without BSO. The cells were plated on 12-well plates at 1 million cells/ml and incubated for 2 h at 37°C in a humidified 5% CO_2 incubator. After incubation, supernatant was collected for the measurement of nitrite and nitrate. Nonadherent cells were removed by rinsing the plates three times with 5% dextrose water. After removing nonadherent cells, adherent macrophages were scraped for the measurement of DNA strand breaks and cellular NAD^+. Mitochondrial respiration and peroxynitrite formation were measured in the adherent cells in the subsequent 1-h period.

2.3. Measurement of nitrite / nitrate

Nitrite + nitrate production, an indicator of NO synthesis, was measured in the supernatant samples as previously described (Cuzzocrea et al., 1998b). First, nitrate in the exudate was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μM) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4, vol. 1:1) to 100 μl samples. The optical density at 550 nm (OD_{550}) was measured using ELISA microplate reader (SLT-Labinstruments, Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

2.4. Measurement of peroxynitrite-induced oxidation of dihydrorhodamine 123

The formation of peroxynitrite was measured by the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123, as previously described (Cuzzocrea et al., 1998b). Cells were rinsed with phosphate-buffered saline and then medium was replaced with phosphate-buffered saline containing 5 μM dihydrorhodamine 123. After a 60 min incubation at 37°C, the fluorescence of rhodamine 123 was measured using a fluorimeter at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively).

2.5. Measurement of mitochondrial respiration

Cell respiration was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Cuzzocrea et al., 1998b). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of OD_{550}. As previously discussed (Darley-Usmar and Halliwell, 1996), the measurement of reduction of MTT appears to be mainly by the mitochondrial complexes I and II, it also may involve NADH- and NADPH-dependent energetic processes that occur outside the mitochondrial inner membrane. Thus, this method cannot be used to separate the effect of free radicals, oxidants or other factors on the individual enzymes in the mitochondrial respiratory chain, but it may be used as an indicator of cells dysfunction (Darley-Usmar and Halliwell, 1996).

2.6. Determination of DNA single-strand breaks

The formation of DNA strand breaks in double-stranded DNA was determined by the alkaline unwinding methods as previously described (Schraufstatter et al., 1986; Cuzzocrea et al., 1998b). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (myoinositol 250 mM, NaH_2PO_3 10 mM, $MgCl_2$ 1 mM, pH 7.2). The cell lysate was then transferred into plastic tubes designated T (maximum fluorescence), P (fluorescence in sample used to estimate extent of DNA unwinding), or B (background fluorescence). To each tube, 0.2 ml of solution B (alkaline lysis solution: NaOH 10 mM, urea 9 M, ethylenediaminetetraacetic acid 2.5 mM, sodium dodecyl sulphate 0.1%) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption. A quantity of 0.1 ml each of solutions C (0.45 volume solution B in 0.2 N NaOH) and D (0.4 volume solution B in 0.2 N NaOH) was then added to the P and B tubes. Another 0.1 ml of solution E (neutralising solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D were added. From this point incubations were carried out in the dark. A 30-min incubation period at 0°C was then allowed during which the alkali diffused into the viscous lysate. Since the neutralising solution, solution E, was added to the T tubes before addition of the alkaline solutions C and D, the DNA in the T tubes was never exposed to a denaturing pH. At the end of the 30 min incubation, the contents of the B tubes were sonicated for 30 s to ensure rapid denaturation of DNA in the alkaline solution. All tubes were then incubated at 15°C for 10 min. Denaturation was stopped by chilling to 0°C and adding 0.4 ml of solution E to the P and B tubes. A 1.5 ml solution of F (ethidium bromide 6.7 μg/ml in 13.3 mM NaOH) was added to all the tubes and fluorescence (excitation: 520 nm, emission: 590 nm) was measured by a fluorimeter. Under the conditions used, in which ethidium bromide binds preferentially to double stranded DNA, the percentage of double stranded DNA (D) may be determined using the equation: $\%D = 100 \times [F(P) - F(B)]/[F(T) - F(B)]$, where F(P) is the fluorescence of the sample, F(B) the background fluorescence, i.e., fluorescence due to all cell components other than double stranded DNA, and F(T) the maximum fluorescence.

2.7. Measurement of cellular NAD^+ levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 N $HClO_4$ scraped, neutralised with 3 M KOH, and centrifuged for 2 min at $10{,}000 \times g$. The supernatant was assayed for NAD^+ using a modification of the colorimetric method (Heller et al., 1995) in which NADH produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine methasulfate. The rate of MTT reduction is proportional to the concentration of the co-enzyme. The reaction mixture contained 10 μl of a solution of 2.5 mg/ml MTT, 20 μl of a solution of 4 mg/ml phenazine methosulfate, 10 μl of a solution of 0.6 mg/ml alcohol dehydrogenase (300 U/mg), and 190 μl of 0.065 M glycyl–glycine buffer, pH 7.4, that contained 0.1 M nicotinamide and 0.5 M ethanol. The mixture was warmed to 37°C for 10 min, and the reaction was started by the addiction of 20 μl of the sample. The rate of increase in absorbance was read immediately after the addition of NAD^+ samples and after 10- and 20-min incubation at 37°C against blank at 560 nm in the ELISA microplate reader (SLT-Labinstruments).

2.8. Lipid peroxidation measurement

Determination of the lung malondialdehyde was carried out in order to estimate lipid peroxidation in the damaged tissue. Sample of 0.2 ml of tissue homogenate obtained 4 h after the induction of pleurisy by carrageenan injection, were frozen at −70°C until the analysis. The assay was carried out by using a colorimetric commercial kit (Lipid peroxidation assay kit, Calbaiochem-Novabiochem, USA).

2.9. Immunohistochemical localisation of nitrotyrosine

Tyrosine nitration was detected as previously described (Cuzzocrea et al., 1998a) in lung sections by immunohistochemistry. At the specified time following the carrageenan injection, tissues were fixed in 10% buffered formalin and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, 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 minimised by incubating the section in 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (biotin blocking kit, DBA, Milan, Italy). The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine antibody (DBA) or with 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, DBA).

2.10. Myeloperoxidase activity

Myeloperoxidase activity, an index of polymorphonuclear leukocyte accumulation, was determined as previously described (Mullane et al., 1985). Lungs tissues, collected at the specified time, were homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at $20{,}000 \times g$ at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured by spectrophotometer at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C and was expressed in milliunits per gram weight of wet tissue.

2.11. Nitric oxide synthase assay

Ca^{2+}-independent conversion of L-arginine to L-citrulline in homogenates of the pleural macrophage cells and of the lungs (obtained 4 h after carrageenan treatment in the presence or the absence of BSO) served as an indicator of inducible NO synthase activity (Szabó et al., 1994). Cells were scraped into a homogenisation buffer composed of 50 mM Tris–HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) and homogenised in the buffer on ice using a tissue homogenizer. Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates as described (Szabó et al., 1997). 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 M) 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 50 W ($Na+$ form) columns and the eluted [^{3}H]L-citrulline activity was measured by a Beckman scintillation counter.

2.12. Nitrotyrosine western blotting

Cells cultured in 6-well plates were then washed with 1.0 ml cold phosphate-buffered saline (PBS) and scraped in 1.0 ml cold PBS and transferred to microfuge tubes. Tubes were spun in Eppendorf microfuge for 20 s. PBS was then removed and 150 μl RIPA (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.5 mM PMSF) was added to each tube and DNA sheared by repeated passage through 22 gauge needle. Cells were incubated on ice for 30 min, then centrifuged at $14{,}000 \times g$ for 20 min at 4°C. Thirty micrograms of protein from each sample was diluted in an equal volume of treatment buffer and heated to 95°C for 3 min. Samples were then loaded into 8–16% Tris–Glycine. Gels were run at 120 V for 2 h, then transferred to 0.45 μm nitro-cellulose at 70 V for 60 min using 1/2 × Towbin buffer system (1.45 g Tris, 7.2 g 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 (DBA) 1 μg/ml in PBS-T (phosphate-buffered saline with 0.05% Tween 20) 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-HRP (1:3000). The blot was washed three times with PBS-T, once with di H_2O, then 1.5 ml mixed ECL chemiluminescence reagent (Amersham) was added for 1 min. The blot was then exposed to X-ray film for 60 s.

2.13. Glutathione measurement

Total GSH levels were assessed in the lungs and in the pleural macrophages. Lungs and cells were collected from control and from BSO pretreated rats. The assay was carried out by using a colorimetric commercial kit (Glutathione assay kit, Calbaiochem-Novabiochem).

2.14. Densitometry evaluation

Immunocytochemistry photographs ($n = 10$) and western blot analysis were assessed for densitometry. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266).

2.15. Materials

Cell culture medium, heparin and fetal calf serum were obtained from Sigma (Milan, Italy). Perchloric acid was

Table 1
Effect of BSO treatment on the total GSH levels

	Total GSH level	
	Lung (μmol/g tissue)	Pleural Macrophage (μg/ml protein)
Control	1.49 ± 0.041	2.96 ± 0.14
BSO	0.32 ± 0.012[a]	0.68 ± 0.16[a]

[a] $P < 0.01$ vs. control.

obtained from Aldrich (Milan, Italy). Primary anti-nitrotyrosine antibody was from Upstate Biotech (DBA). All other reagents and compounds used were obtained from Sigma.

2.16. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of *n* observations. For the in vitro studies, data represent the number of wells studied (6–9 wells from 2–3 independent experiments). For the in vivo studies *n* represents the number of animals studied. In the experiments involving immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by ANOVA test and by one- and two-way analysis of variance and individual group means were then compared with Student's unpaired *t*-test. A *P*-value less than 0.05 was considered significant.

3. Results

In preliminary experiments we established that BSO treatment significantly reduces the total GSH levels both in the lung and in the pleural macrophages (Table 1).

3.1. Endogenous glutathione protects against carrageenan-induced pleurisy

All carrageenan-injected rats developed an acute pleurisy, producing 1.43 ± 0.1 ml of turbid exudate (Fig. 1A). Trypan blue stain revealed $80 \pm 1.8 \times 10^6$/rat polymorphonuclear leukocyte in comparison to sham rat ($2.4 \pm 0.4 \times 10^6$/rat) (Fig. 1B). NO_x levels were also signifi-

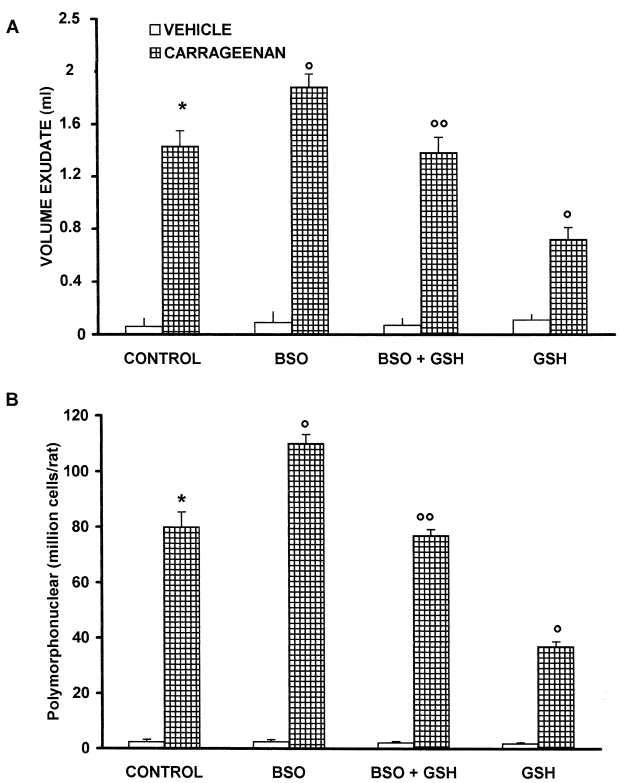

Fig. 1. Effect of endogenous GSH on Carragenan-induced inflammation. Volume exudate (A) and polymorphonuclear accumulation (B) in pleural cavity at 4 h after carrageenan injection. BSO (1 g/kg for 24 h, i.p.) pretreatment significantly increased pleural exudation and leukocyte migration. GSH (50 mg/kg, i.p.) treatment significantly reverted the effect of BSO. Data are means ± SEM of 10 rats for each group. * $P < 0.01$ vs. sham. ° $P < 0.01$ vs. carrageenan. °° $P < 0.01$ vs. BSO.

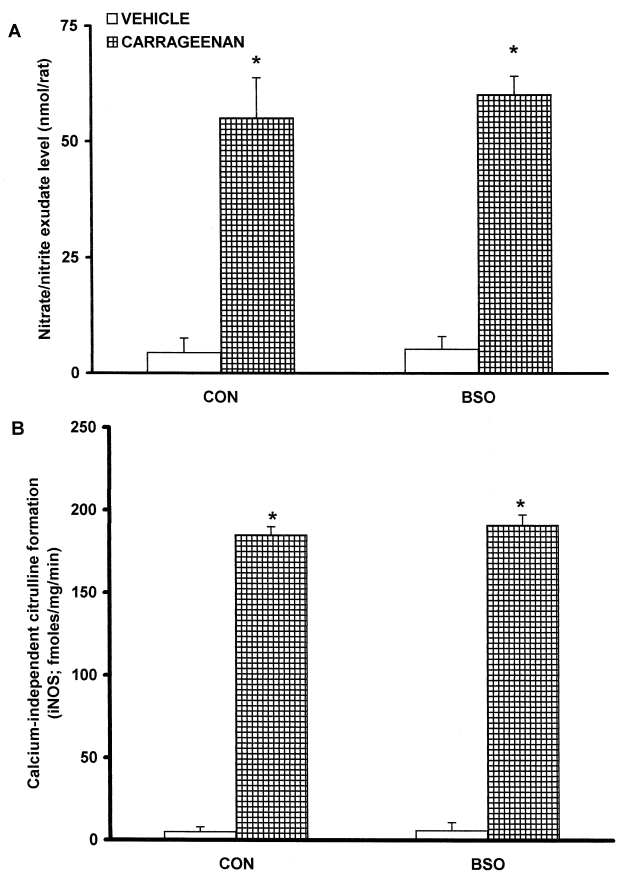

Fig. 2. Effect of endogenous GSH on NO production. Nitrite and nitrate concentrations in pleural exudate (A) and inducible NO synthase activity in lungs (B) at 4 h after carrageenan administration. Inducible NO synthase activity in the carrageenan-treated rats was significantly increased vs. sham group. BSO (1 g/kg for 24 h) did not affect the NO production. Value are means ± SEM of 10 rats for each group. * $P < 0.01$ vs. sham.

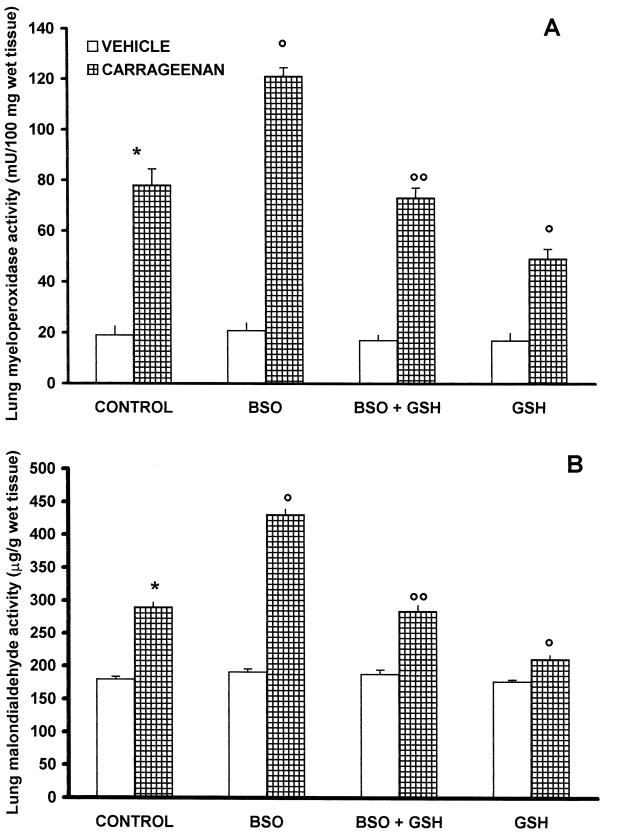

Fig. 3. Effect of endogenous GSH on polymorphonuclear leukocyte migration and lipid peroxidation. Myeloperoxidase activity (A) and malondialdehyde (B) in the lungs of carrageenan-treated rats sacrificed at 4 h. Myeloperoxidase activity and malondialdehyde levels were significantly increased in the lungs of the carrageenan-treated rats in comparison to sham rats. BSO (1 g/kg for 24 h, i.p.) pretreatment significantly enhanced the carrageenan-induced increase in myeloperoxidase activity and malondialdehyde levels. GSH (50 mg/kg, i.p.) treatment significantly revert the effect of BSO. Values are means ± SEM of 10 rats for each group. $^{*}P < 0.01$ vs. sham, $^{\circ}P < 0.01$ vs. carrageenan. $^{\circ\circ}P < 0.01$ vs. BSO.

cantly ($P < 0.01$) increased in the exudate after carrageenan challenge (50 ± 4.8 nmol/rat vs. 5.6 ± 0.4 nmol/sham rat) (Fig. 2A). Sham animals demonstrated no abnormalities in the pleural cavity or fluid. The degree of peritoneal exudation and polymorphonuclear migration were significantly enhanced in rats pretreated with BSO (Fig. 1A,B). BSO pretreatment did not cause significant changes in these parameters in sham rats (Fig. 1A,B). Glutathione treatment significantly reverted the effect of BSO and significantly reduced the degree of peritoneal exudation and polymorphonuclear migration in carrageenan-treated rats (Fig. 1A,B). In the BSO-pretreated rats, the carrageenan-induced exudate nitrate/nitrite was unaffected (Fig. 2A).

In the lungs obtained from animals subjected to carrageenan-induced pleurisy, a significant increase of inducible NO synthase activity was detected at 4 h (185 ± 17 fmol/mg/min) (Fig. 2B). Inducible NO synthase activity was unaffected by BSO pretreatment (Fig. 2B).

At 4 h after carrageenan administration, lungs were examined for the measurement of myeloperoxidase activity, the latter being indicative of neutrophil infiltration and for malondialdehyde, in order to estimate lipid peroxidation. As shown in Fig. 3A,B, myeloperoxidase activity and malondialdehyde levels (78 ± 5 mU/100 mg wet tissue, 290 ± 7 μg/g wet tissue, respectively) were significantly ($P < 0.01$) increased in the lung at 4 h after carrageenan injection when compared to sham rats (19 ± 3.5 mU/100 mg wet tissue, 180 ± 4 μg/g wet tissue, respectively). Myeloperoxidase activity and malondialdehyde levels were significantly ($P < 0.01$) enhanced to 121 ± 3 mU/100 mg wet tissue and 431 ± 8 μg/g wet tissue, respectively, by

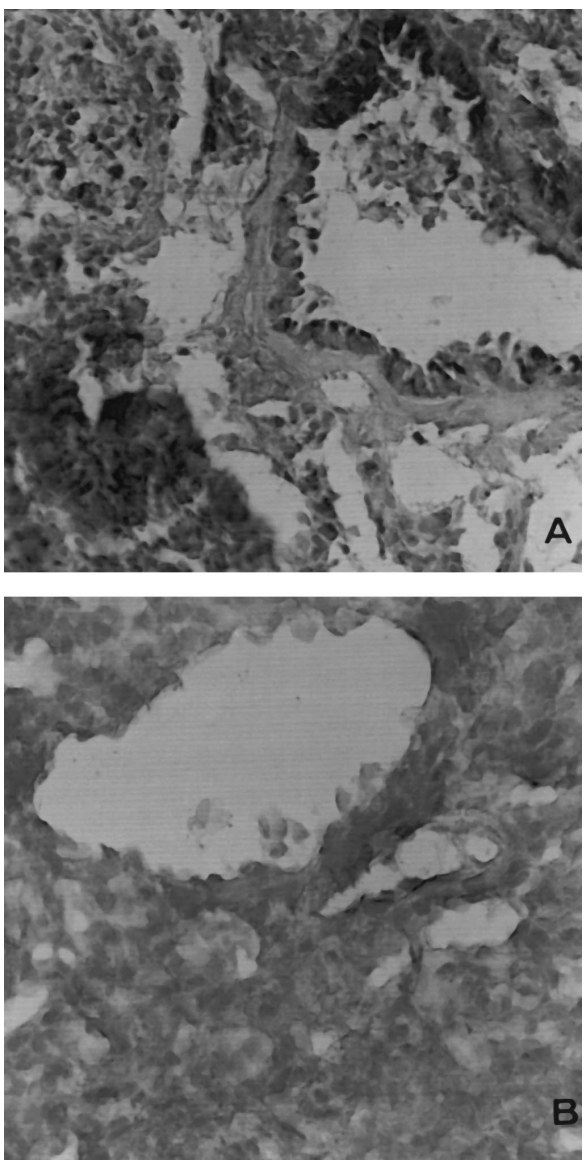

Fig. 4. Effect of endogenous GSH on nitrotyrosine formation. Immunohistochemical localization of nitrotyrosine in the rat lung. Positive nitrotyrosine immunoreactivity was localized in the lungs of carrageenan-treated rats (B). Nitrotyrosine staining was substantially more pronounced in the lungs of the carrageenan-treated rats when they were pretreated with BSO (A). Original magnification: ×125.

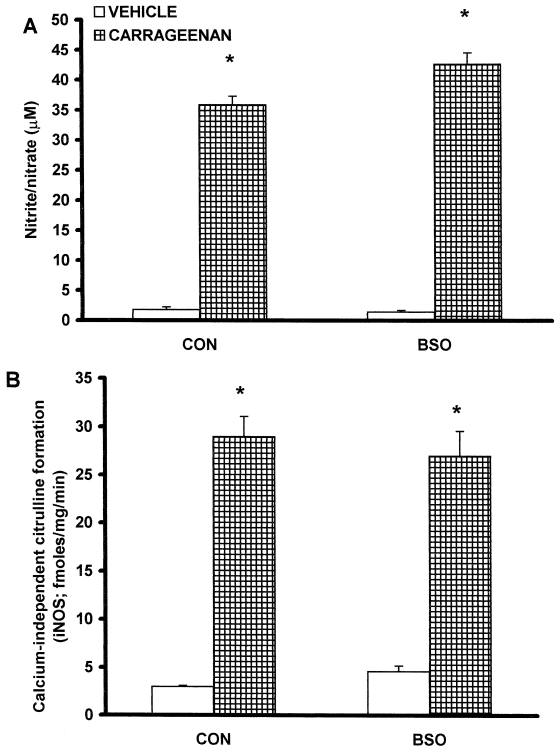

Fig. 5. Effect of endogenous GSH on NO production in pleural macrophages. Nitrate/nitrite production (A), inducible NO synthase activity (B) in pleural macrophages harvested from control and carrageenan-treated rats. NO production was unaffected by BSO pretreatment. $^{*}P < 0.01$ vs. macrophages from control rats.

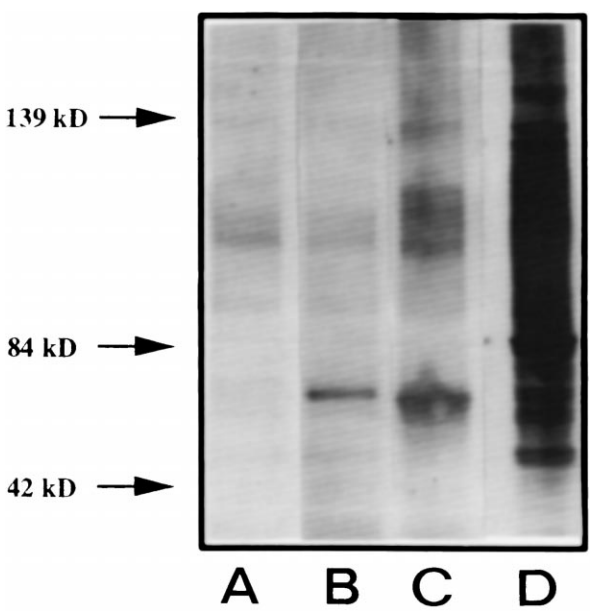

Fig. 6. Effect of endogenous GSH on tyrosine nitration in pleural macrophages. Using Western blotting, immunoreactivity for nitrotyrosine a nitrosilated product, was detected. In pleural cells from control animals (Lane A) and from BSO-treated rat (Lane B) a weak immunoreaction was found. However, there was a marked increase in the nitrotyrosine immunoreactivity in pleural cells from carrageenin-treated rats (Lane C). Nitrotyrosine immunoreactivity was substantially more pronounced in the pleural cell of the carrageenan-treated rats when they were pretreated with BSO (Lane D).

BSO pretreatment (Fig. 3A,B). Glutathione treatment significantly reverted the effects of BSO and significantly reduced myeloperoxidase activity and malondialdehyde levels (Fig. 3A,B).

At 4 h following the intrapleural injection of carrageenan, lung sections were analysed for the presence of nitrotyrosine. Immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, revealed a positive staining ($0.30 \pm 0.09\%$ of total tissue area) in lungs from carrageenan-treated rats (Fig. 4B). Nitrotyrosine staining was significantly more pronounced ($5.12 \pm 0.1\%$ of total tissue area) in the lungs of the carrageenan-treated rats when they were pretreated with BSO (Fig. 4A). Staining was absent in control tissue (data not shown). In another set of experiments, pretreatment with the isomer L-buthionine-*R*-sulfoximine, which does not reduce glutathione levels, did not induced any modification which were comparable to those seen in vehicle-pretreated rats subjected to carrageenan-induced pleurisy (data not shown).

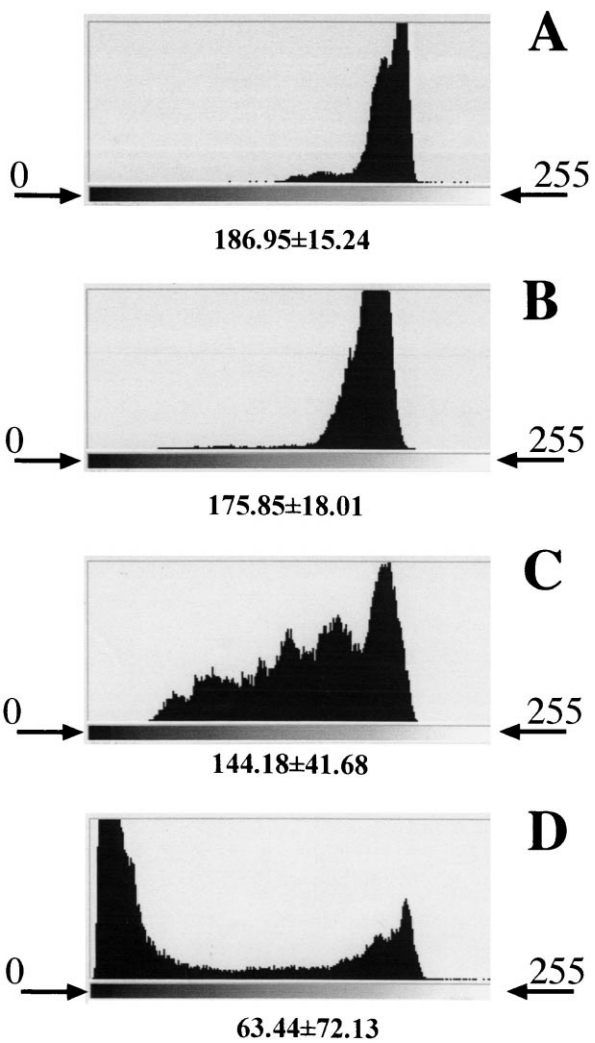

Fig. 7. Typical Densitometry evaluation. Densitometry analysis of Western Blot for tyrosine nitration were carried out. Lane A (A), lane B (B), lane C (C) and lane D (D). Data are expressed in pixel. Value are means ± SD.

3.2. Endogenous glutathione protects against the cellular energetic failure

In pleural macrophages obtained from rats at 4 h after carrageenan injection, a significant nitrate/nitrite production was detectable (36 ± 3 μM) and it was correlated with a significant inducible NO synthase activity increase (29 ± 1.7 fmol mg/min, Fig. 5A,B). Using Western blotting, immunoreactivity for nitrotyrosine a nitrosilated product, was also detected. In pleural cells from control animals (Lane A) and from BSO-treated rat (Lane B) a weak immunoreaction was found (Figs. 6 and 7). However, there was a significant increase in the nitrotyrosine immunoreactivity in pleural cells from carrageenan-treated rats (Lane C, Figs. 6 and 7). Nitrotyrosine immunoreactivity was significantly more pronounced in the pleural cell of the carrageenan-treated rats when they were pretreated with BSO (Lane D, Figs. 6 and 7). A rapid and sustained production of peroxynitrite (48 ± 1.6 pmol/min/million cells) was also observed after carrageenan-induced pleurisy (Fig. 8A).

There was a marked increase in DNA strand breakage and depression of mitochondrial respiration in pleural macrophages cells from carrageenan-treated rats (Figs. 8B and 9A). Carrageenan-mediated disruption of cellular energetic pool was evidenced by a significant decreased in cellular respiration and intracellular concentration of NAD^+ (Fig. 9B). In vivo pretreatment of the animals with BSO significantly increased dihydrorhodamine 123 oxidation, enhanced the carrageenan-induced DNA single strand breakage (Fig. 8A,B), the decrease in cellular respiration and partially the depletion of intracellular levels of NAD^+ (Fig. 9A,B). Glutathione treatment significantly reverted the effect of BSO and reduced peroxynitrite formation and prevented the appearance of DNA damage, the decrease in mitochondrial respiration and the loss of cellular levels of NAD^+ (Figs. 8A,B and 9A,B). We have observed that depletion of endogenous glutathione store with BSO did not effect NO production (Fig. 5A,B). In vivo pretreatment with the isomer L-buthionine-*R*-sulfoximine, which does not reduce glutathione levels, did not cause any further increase in cellular energetic failure observed after carrageenan-induced pleurisy (data not shown).

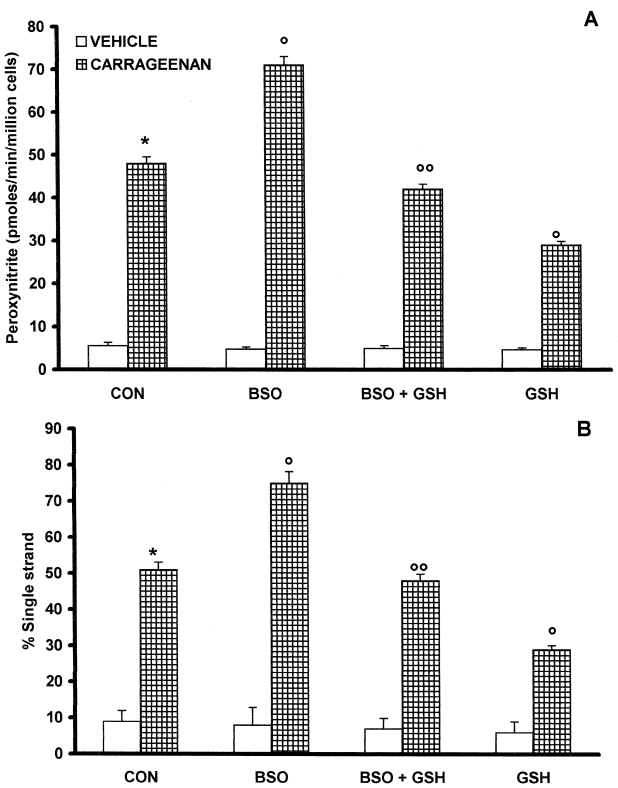

Fig. 8. Effect of endogenous GSH on peroxynitrite formation and DNA damage. Peroxynitrite production (A) and development of DNA single strand breakage (B), in macrophages from control and carrageenan-treated rats. $^*P < 0.01$ vs. macrophages from control rats, $^\circ P < 0.01$ vs. macrophages from carrageenan-treated rats. $^{\circ\circ}P < 0.01$ vs. macrophages from carrageenan-BSO-treated rats.

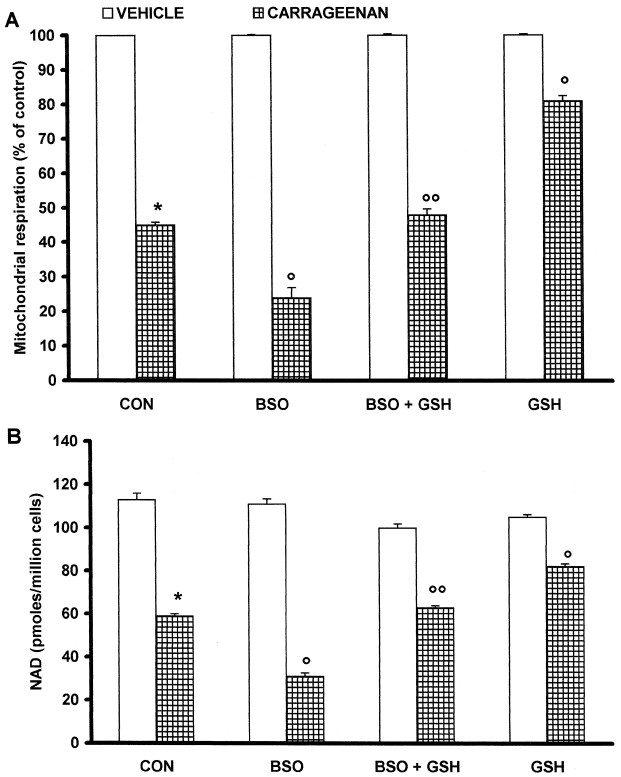

Fig. 9. Effect of endogenous GSH on cellular dysfunction. Reduction of mitochondrial respiration (A) and cellular levels of NAD^+ (B) in macrophages from control and carrageenan-treated rats. $^*P < 0.01$ vs. macrophages from control rats, $^\circ P < 0.01$ vs. macrophages from carrageenan-treated rats. $^{\circ\circ}P < 0.01$ vs. macrophages from carrageenan-BSO-treated rat.

4. Discussion

The results of the present study indicate that endogenous glutathione plays a crucial role as a protective factor against the carrageenan-induced development of acute in-

flammation. Although there are available data that depletion of endogenous glutathione can increase mortality in various forms of shock (Nemeth and Boda, 1989; Gatti et al., 1993) and can exacerbate ischemia/reperfusion injury in some (Stein et al., 1990; Lee et al., 1995) but not other (Singh et al., 1989) experimental models, to our knowledge, this is the first investigation to study the importance of endogenous glutathione in the development of acute inflammation (carrageenan-induced pleurisy).

4.1. Effect of in vivo depletion of endogenous glutathione synthesis on nitrotyrosine formation in carrageenan-induced acute inflammation

In recent years, the role of the L-arginine-NO pathway has been proposed to play an important role in the carrageenan-induced inflammatory response. Pharmacological inhibitors of NO synthase, and also ablation of the gene for inducible NO synthase has been shown to reduce the development of the carrageenan-induced inflammatory response (Wei et al., 1995; Salvemini et al., 1996; Cuzzocrea et al., 1998a). More recent studies have shown the formation of peroxynitrite in this model of inflammation (Cuzzocrea et al., 1997b, 1998a, 1999). Using nitrotyrosine immunohistochemistry, we have confirmed here the production of peroxynitrite in the lung of rats subjected to carrageenan-induced pleurisy. Moreover, we have observed that in the BSO-treated animals, a much more pronounced nitrotyrosine staining was present, suggesting the presence of more, biologically active peroxynitrite in the alveolar macrophage and in the airway epithelial cells. The more pronounced nitrotyrosine staining was not due to increased production of NO, as demonstrated by the measurement of lung inducible NO synthase activity.

4.2. Endogenous glutathione protects against pleural macrophages dysfunction

It is well known that in acute inflammatory process, in which vascular permeability increases and leukocyte migration occurs, there is an involvement of several mediators including neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical (Salvemini et al., 1996; Cuzzocrea et al., 1998a). It is proposed that reactive oxygen species, including oxygen radicals, and non-radicals that are either oxidising agent and/or are easily converted into radicals, such as HOCl, ozone, peroxynitrite, single oxygen and H_2O_2 can cause structural alteration in DNA (Wiseman and Halliwell, 1996) with consequent cellular dysfunction (Szabó et al., 1997). In ex vivo macrophages harvested from the pleural cavity of rats subjected to carrageenan-induced pleurisy, we have recently reported the production of NO, superoxide and peroxynitrite, concomitant with inhibition of suppression of mitochondrial respiration, DNA single strand breakage, NAD depletion, and ATP depletion (Cuzzocrea et al., 1998b). Using pharmacological inhibitors and scavengers, it appears that the most important cytotoxic species, under these conditions is peroxynitrite, and not NO or superoxide per se. This conclusion is based on the simultaneous protective effects of NO synthase inhibitors (Salvemini et al., 1996; Cuzzocrea et al., 1997b, 1998a) and a cell-permeable superoxide dismutase scavenger compound (Cuzzocrea et al., 1999) against the suppression of mitochondrial respiration, and by the protective effects of various peroxynitrite scavengers (Cuzzocrea et al., 1997b, 1998a). Our data are in agreement with previous reports demonstrating that BSO pretreatment increased macrophage migration and exudate production in murine silica induced inflammation (Lombard-Gillooly and Hubbard, 1993), while it did not effect NO production during hepatic inflammation (Walker et al., 1995).

Although a variety of endogenous antioxidant systems in the cell are actively involved during the inflammatory process, it is remarkable that depletion of glutathione alone exerted a marked potentiating effect of peroxynitrite-induced cytotoxicity, both in endothelial cells in macrophages. These findings are in agreement with previous suggestions that glutathione plays an important role against the oxidant-induced injury and, specifically, against the peroxynitrite-induced injury (Karoui et al., 1996; Cuzzocrea et al., 1999). Several data support this hypothesis: (1) the enhancement of the appearance of DNA strand breaks; (2) the demonstration of a further decrease in the conversion of MTT to formazan; (3) the partially enhanced reduction of the intracellular levels of NAD^+. A variety of additive or synergistic cytotoxic processes triggered by peroxynitrite may contribute to acute and delayed cytotoxicity and depletion of glutathione may interfere also with these pathways.

4.3. Role of glutathione on NO, oxyradicals and peroxynitrite formation in carrageenan-induced acute inflammation

Glutathione is a known oxyradical scavenger (Darley-Usmar and Halliwell, 1996). Thus, theoretically, the mechanism of the observed inflammatory alterations in the BSO-pretreated animals may be related to peroxynitrite, oxyradicals, NO, or the combination of these. In vitro studies in macrophages and other cell types have established that endogenous glutathione only protects against very high (pharmacologically relevant) fluxes of NO, but not against lower levels of NO production (Walker et al., 1995; Petit et al., 1996), such as the ones which are relevant to the ex vivo or in vivo conditions in our experiments. There also are data that depletion of endogenous glutathione enhances the cytotoxic effects of hydrogen peroxide and oxyradicals (Laskin et al., 1994; Darley-Usmar and Halliwell, 1996). In our experiments it is conceivable that a more pronounced inhibition of mitochondrial respiration by oxygen-derived free radicals and oxidants can lead to a dysfunctional electron transfer, with

more superoxide production from the mitochondria. This effect would also lead to an enhancement of peroxynitrite production, with subsequent increased cytotoxicity. It is noteworthy in this context, that the production of superoxide, not the production of NO is the rate-limiting factor in peroxynitrite formation during endotoxemia (Walker et al., 1995). Regarding the conclusion that the production of superoxide is the rate-limiting factor in peroxynitrite formation during carrageenan-induced inflammation, we have recently obtained similar data with Mn(III)tetrakis (4-benzoic acid) porphyrin, a superoxide dismutase mimetic, demonstrating a suppression of peroxynitrite formation, suggesting the role of superoxide activation in the process (Cuzzocrea et al., 1999).

Furthermore, hydrogen peroxide prolongs the half-life of peroxynitrite (Alvarez et al., 1995). In addition, recent reports have shown that nitrotyrosine formation may also result from reaction between nitrite and myeloperoxidase (Eiserich et al., 1998). Thus, it is possible that the cytotoxic effects observed in response to carrageenan represent the sum of a complex interaction between various oxygen- and nitrogen-derived radicals and oxidants.

In conclusion, the current study demonstrated that endogenous glutathione plays an important role against the carrageenan-induced inflammation and strategies to improve cellular glutathione store may be effective pharmacological tools in inflammatory disease.

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