



# Beneficial effects of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a superoxide dismutase mimetic, in zymosan-induced shock

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**1** The therapeutic efficacy of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a novel superoxide dismutase mimetic which scavenges peroxynitrite, was investigated in rats subjected to shock induced by peritoneal injection of zymosan.

**2** Our data show that MnTBAP (given at 1, 3 and 10 mg kg<sup>-1</sup> intraperitoneally, 1 and 6 h after zymosan injection) significantly reduce in dose dependent manner the development of peritonitis (peritoneal exudation, high nitrate/nitrite and peroxynitrite plasma levels, leukocyte infiltration and histological examination).

**3** Furthermore, our data suggest that there is a reduction in the lung, small intestine and liver myeloperoxidase (MPO) activity and lipid peroxidation activity from MnTBAP-treated rats.

**4** MnTBAP also reduced the appearance of nitrotyrosine immunoreactivity in the inflamed tissues.

**5** Furthermore, a significant reduction of suppression of mitochondrial respiration, DNA strand breakage and reduction of cellular levels of NAD<sup>+</sup> was observed in *ex vivo* macrophages harvested from the peritoneal cavity of zymosan-treated rat.

**6** *In vivo* treatment with MnTBAP significantly reduced in a dose-dependent manner peroxynitrite formation and prevented the appearance of DNA damage, the decrease in mitochondrial respiration and the loss of cellular levels of NAD<sup>+</sup>.

**7** In conclusion our results showed that MnTBAP was effective in preventing the development of zymosan-induced shock.

**Keywords:** MnTBAP; peroxynitrite; zymosan-induced shock; multiple organ failure; nitric oxide; inflammation; myeloperoxidase; nitrotyrosine; SOD mimetic; cell injury

**Abbreviations:** iNOS, inducible nitric oxide synthase; MDA, malonaldehyde; MnTBAP, Mn(III)tetrakis (4-benzoic acid) porphyrin; MPO, myeloperoxidase; NO, nitric oxide; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte

## Introduction

Zymosan, a product consisting of purified baker's yeast ghost cells, has been reported to elicit an acute peritonitis and multiple organ failure in experimental animals characterized by functional and structural changes in liver, intestine, lung and kidneys (Deitch *et al.*, 1990; Demling *et al.*, 1994; Goris *et al.*, 1991; Cuzzocrea *et al.*, 1997a,b). The organ dysfunction in zymosan treated animals may be, in part, dependent on bacterial translocation (Demling *et al.*, 1994; Goris *et al.*, 1991; Mainous *et al.*, 1991). The role of the production of pro-inflammatory cytokines, such as tumour necrosis factor (Von Asmuth *et al.*, 1990) and interleukin-6 (IL-6) (Jansen *et al.*, 1996), and pro-inflammatory lipid mediators, such as platelet-activating factor (Damas *et al.*, 1993), and of prostaglandin metabolites (Cuzzocrea *et al.*, 1997b; Mainous *et al.*, 1991) is well established in the pathophysiology of zymosan-induced shock. The role of oxygen-derived free radicals (Demling *et al.*, 1994; Mainous *et al.*, 1991) and, more recently, the role of NO (Cuzzocrea *et al.*, 1997a,b) or a related species such as peroxynitrite have also been demonstrated in various models

of zymosan-induced shock and inflammation (Cuzzocrea *et al.*, 1998a, 1999a). Recently we showed that the onset of peritonitis and the maximal end-organ damage occurred at 18 h after zymosan administration, i.e. at the same time of the onset of inflammatory response in the peritoneal cavity and was associated with systemic hypotension, high peritoneal and plasma levels of NO, maximal cellular infiltration, exudate formation and cyclooxygenase activity (Cuzzocrea *et al.*, 1997a,b).

Recent data have demonstrated the protective effects of NOS inhibitors in zymosan-induced shock, suggesting the involvement of NO, or related species, such as peroxynitrite, in the zymosan-induced local or systemic inflammatory process (Cuzzocrea *et al.*, 1997a,b; Teixeira *et al.*, 1993).

Recent data have demonstrated that Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a novel, stable and cell-permeable superoxide dismutase mimetic and related compounds may also inhibit the oxidation of dihydrorhodamine 123 elicited by authentic peroxynitrite (Zingarelli *et al.*, 1997; Faulkner *et al.*, 1994; Day *et al.*, 1995; Szabó *et al.*, 1996). However MnTBAP is not a scavenger of nitric oxide (Szabó *et al.*, 1996). In the present study we investigated whether MnTBAP provides a protective effect on shock induced by zymosan administration.

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## Methods

### Animals

Male Sprague-Dawley rats (300–350 g; Charles River; Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulation (D.M. 116192) on protection of animals used for experimental and other scientific purpose, as well as with the EEC regulations (O.J. of E.C.L 358/1 18/12/1986).

### Experimental groups

Animals were randomly divided into eight groups ( $n=10$  per group). The first group was intraperitoneally (i.p.) treated with 0.9% NaCl and served as sham group. The second group was treated with zymosan ( $500 \text{ mg kg}^{-1}$ , suspended in saline solution, i.p.). In the third and fourth groups, rats received MnTBAP ( $1 \text{ mg kg}^{-1}$ , i.p. 1 and 6 h after zymosan or saline administration). In the fifth and sixth groups rats received MnTBAP ( $3 \text{ mg kg}^{-1}$ , i.p. 1 and 6 h after zymosan or saline administration). In the seventh and eighth groups rats received MnTBAP ( $10 \text{ mg kg}^{-1}$ , i.p. 1 and 6 h after zymosan or saline administration). After zymosan or saline injection animals were monitored for evaluation of mortality for 72 h.

### Acute peritonitis assessment

In a second group of experiments at 18 h after zymosan injection, animals were sacrificed under ether anaesthesia in order to evaluate the development of acute inflammation in the peritoneum. Through an incision in the linea alba, 10 ml of

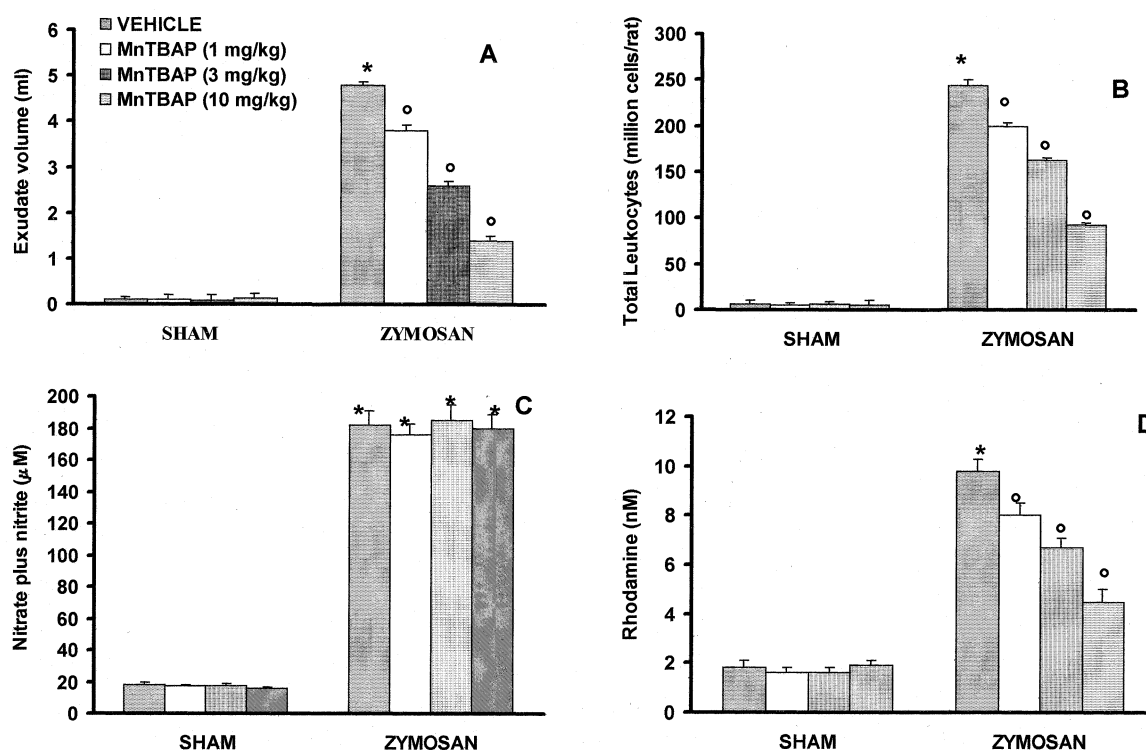
phosphate buffer ((mM) saline NaCl 137; KCl 2.7;  $\text{NaH}_2\text{PO}_4$  1.4;  $\text{Na}_2\text{HPO}_4$  4.3; pH 7.4) was injected into the abdominal cavity. Washing buffer was removed by a plastic pipette and was transferred into a 10 ml centrifuge tube. The total volume of peritoneal exudate was evaluated removing 10 ml of the buffer. Peritoneal exudate was centrifuged at  $7000 \times g$  for 10 min at room temperature, and the supernatant was utilized for  $\text{NO}_2^- + \text{NO}_3^-$  ( $\text{NO}_x$ ) assay. Cells were suspended in phosphate buffered saline and counted with optical microscope by Burker's chamber after vital Trypan Blue stain.

### Nitrate and nitrite levels in the supernatant

Nitrite/nitrate production, an indicator of NO synthesis, was measured in supernatant samples as previously described (Cuzzocrea *et al.*, 1998a). First, nitrate in the samples were reduced to nitrite by incubation with nitrate reductase ( $670 \text{ mU ml}^{-1}$ ) and NADPH ( $160 \mu\text{M}$ ) at room temperature for 3 h. After 3 h, the nitrite concentration in the samples was measured by Griess reaction. Briefly,  $100 \mu\text{l}$  of Griess reagent (0.1% naphthaethylenediamine dihydrochloride in  $\text{H}_2\text{O}$  and 1% sulphanimide in 5% concentrated  $\text{H}_2\text{PO}_4$ ; vol. 1:1) were added to  $100 \mu\text{l}$  of samples. The optical density at 550 nm ( $\text{OD}_{550}$ ) was measured using a ELISA microplate reader (SLT-Labinstruments Salzburg, Austria). Nitrate concentrations were calculated by comparison to standard solutions of sodium nitrate.

### Measurement of peroxynitrite production

The formation of peroxynitrite was measured by the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123, as previously described (Cuzzocrea *et al.*,



**Figure 1** Data represent values at 18 h after vehicle or zymosan administration ( $500 \text{ mg kg}^{-1}$  i.p.). (A) Exudate volumes; (B) leukocytes accumulation; (C) plasma  $\text{NO}_x$  levels; (D) plasma peroxynitrite production. MnTBAP ( $1, 3$  and  $10 \text{ mg kg}^{-1}$ , i.p. 1 and 6 h after zymosan) treatment induced in a dose dependent manner a significant decrease of peritoneal exudate, leukocyte migration and  $\text{NO}_x$  and peroxynitrite levels. Data are means  $\pm$  s.e.mean of 10 rats for each group. \* $P < 0.01$  versus sham. ° $P < 0.01$  versus zymosan.

1998a). Briefly, cells were rinsed with PBS and then replaced with PBS containing  $5 \mu\text{M}$  dihydrorhodamine 123. After a 60 min incubation at  $37^\circ\text{C}$ , 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). The formation of peroxynitrite was also estimated in plasma samples at 18 h after zymosan administration. In separate groups of experiment, animals were injected with dihydrorhodamine 123 ( $2 \mu\text{mol kg}^{-1}$  in 0.3 ml saline i.v.). Twenty minutes later rats were sacrificed and plasma samples taken for rhodamine fluorescence evaluation using a fluorimeter at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively). The rate of rhodamine formation, an index of peroxynitrite production, was calculated using a standard curve obtained with authentic rhodamine (1–30 nM) prepared in plasma obtained from untreated rats. Background plasma fluorescence was subtracted from all samples.

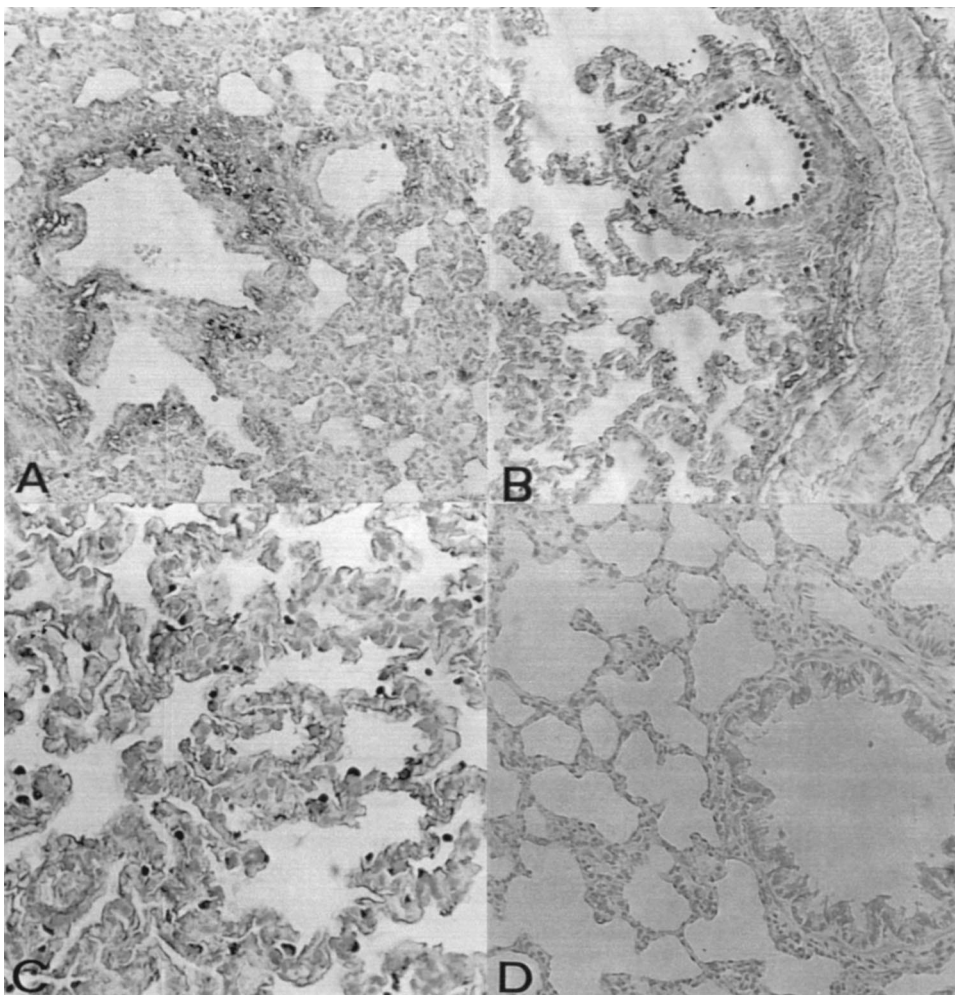
#### *Immunohistochemical localization of nitrotyrosine*

Tyrosine nitration was detected as previously described (Cuzzocrea *et al.*, 1997c) in lung and small intestine sections by immunohistochemistry. At 18 h after saline or zymosan injection, tissues were fixed in 10% buffered formalin and  $8 \mu\text{m}$  sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with

$0.3\% \text{H}_2\text{O}_2$  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 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 (DBA, Milan, Italy). The sections were then incubated overnight with 1 : 1000 dilution of primary anti-nitrotyrosine antibody (DBA, Milan, Italy) 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 (DBA, Milan, Italy).

#### *Myeloperoxidase activity*

The usefulness of measuring myeloperoxidase (MPO) activity to assess neutrophil infiltration has been previously reported (Mullane *et al.*, 1988). Briefly, after weighing, segments of various organs were suspended in 0.5% hexadecyl-trimethylammonium bromide (pH 6.5, 50 mg of tissue per ml) and were then homogenized. After freezing and thawing the homogenate three times, the tissue levels of MPO were determined by utilizing 0.0005% hydrogen peroxide as a substrate for the enzyme. One unit of MPO activity is defined as that degrading  $1 \mu\text{mol}$  of hydrogen peroxide per minute at  $25^\circ\text{C}$  and is expressed in units per gram weight ( $\text{Ug}^{-1}$ ) of wet tissue.



**Figure 2** Immunohistochemical localization of nitrotyrosine in the rat lung. Eighteen following zymosan injection, nitrotyrosine immunoreactivity was localized mainly in vascular wall (A), bronchial (B) and macrophages (C). There was no detectable immunostaining in the lungs of zymosan-treated rats when rats were treated with MnTBAP (D). Original magnification:  $\times 100$ .

### Malonaldehyde measurement

Levels of malonaldehyde (MDA) in the lung, intestinal and liver tissues were determined as an index of lipid peroxidation, as described by Ohkawa *et al.* (1979). Lung, intestine and liver tissues were homogenized in 1.15% KCl solution. An aliquot (100  $\mu$ l) of the homogenate was added to a reaction mixture containing 200  $\mu$ l of 8.1% SDS, 1500  $\mu$ l of 20% acetic acid (pH 3.5), 1500  $\mu$ l of 0.8% thiobarbituric acid and 700  $\mu$ l of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000  $\times g$  for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

### Light microscopy

Lung and small intestine samples were taken 18 h after saline or zymosan injection. The tissue slices were fixed in Dietric solution (14.2% ethanol, 1.8% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, U.S.A.). Section (thickness 7  $\mu$ m) were deparaffinized with xylene, stained with trichromic Van Gieson and observed in Dialux 22 Leitz microscope.

### Metabolic changes

Plasma samples were taken at 18 h after saline or zymosan injection and levels of bilirubin, lactate dehydrogenase, alkaline phosphatase and alanine aminotransferase were determined by a clinical laboratory.

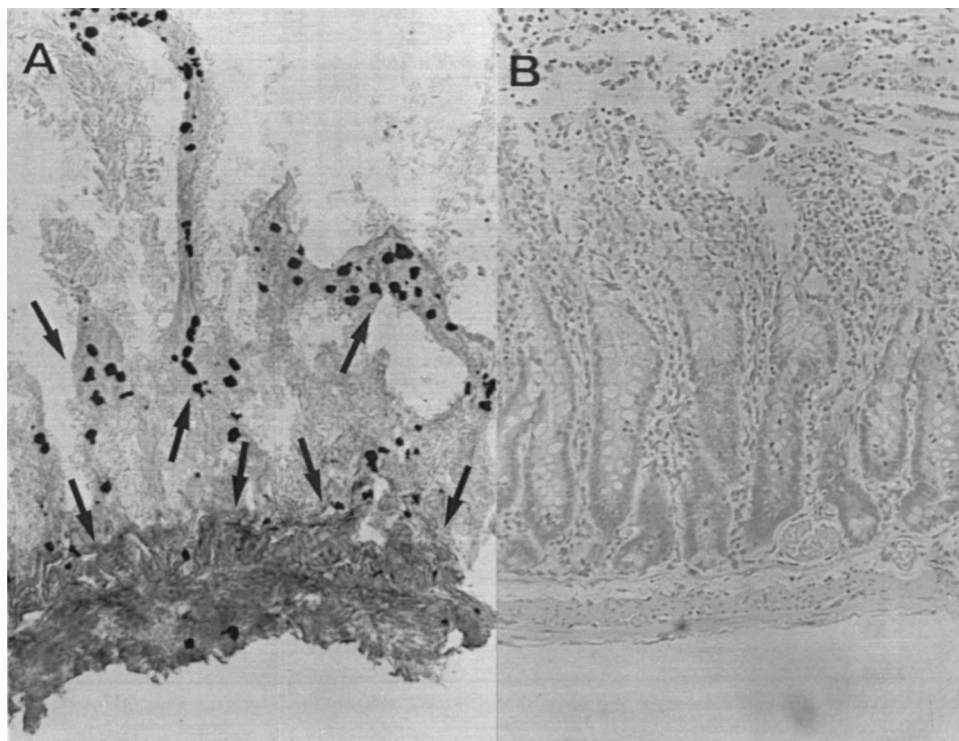
### Cell culture

Peritoneal macrophages from rats were harvested by peritoneum lavage with DMEM medium, supplemented with

L-glutamine (3.5 mM), penicillin (50 U ml<sup>-1</sup>), streptomycin (50  $\mu$ g ml<sup>-1</sup>) and heparin sodium (10 U ml<sup>-1</sup>). The cells were collected 18 h after the zymosan injection from rats treated with or without MnTBAP. The cells were plated on 12-well plates at 1 million cells ml<sup>-1</sup> and incubated for 2 h at 37°C in a humidified 5% CO<sub>2</sub> 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<sup>+</sup>. Mitochondrial respiration and peroxynitrite formation were measured in the adherent cells in the subsequent 1-h period.

### Measurement of cellular energetic status

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.*, 1998a). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg ml<sup>-1</sup>) for 1 h. Culture medium was removed by aspiration and the cells were solubilized in DMSO (100  $\mu$ l). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of OD<sub>550</sub>. As previously discussed (Darley-Usmar & 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 is useful to monitor changes in the general energetic status of the cells (Darley-Usmar & Halliwell, 1996).

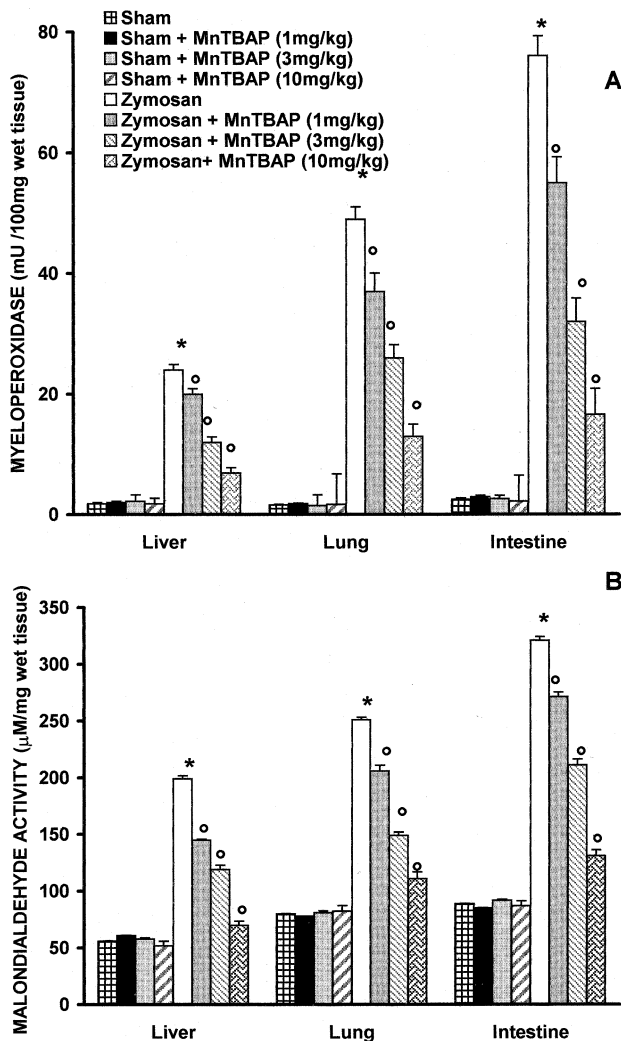


**Figure 3** Immunohistochemical localization of nitrotyrosine in the rat small intestine. Positive staining was found in the intestine from zymosan-treated rats (A). There was no detectable immunostaining in the intestine of zymosan-treated rats when they were treated with MnTBAP (10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) (B). Original magnification: A-B 100 $\times$ ; C-D 200 $\times$ .

### 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 (Cuzzocrea *et al.*, 1998a). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (mM): myo-inositol 250; NaH<sub>2</sub>PO<sub>3</sub> 10; MgCl<sub>2</sub> 1; 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: (mM): NaOH 10; urea 9 M; ethylenediamine-tetraacetic acid 2.5; sodium dodecyl sulphate 0.1%) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption. 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) were then added to the P and B tubes. 0.1 ml of solution E (neutralizing solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D were added. From this point

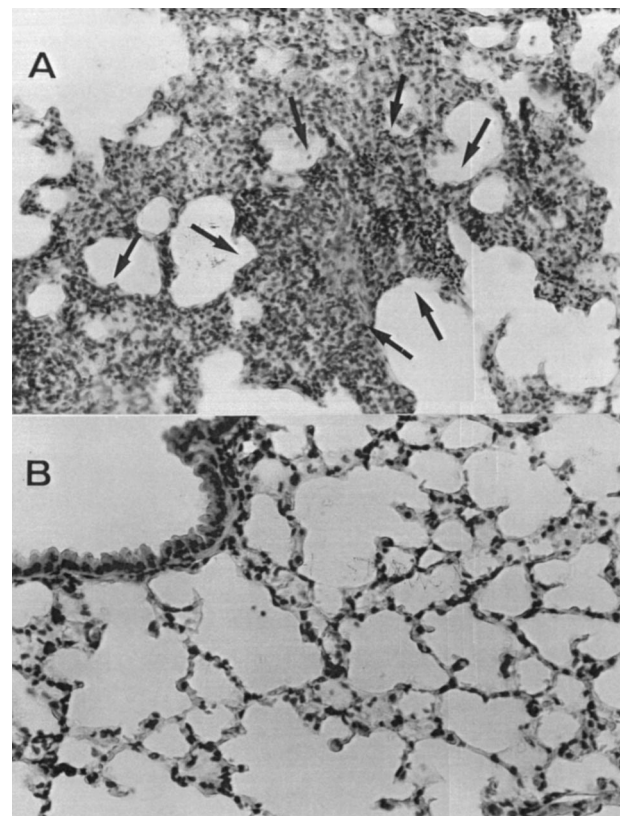
incubations were carried out in the dark. A 30-min incubation period at 0°C was then allowed during which the alkali diffused into the viscous lysate. Since the neutralizing solution, solution E, was added to the T tubes before addition of the alkaline solutions C and D, the DNA in the T tubes was never 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. 1.5 ml of solution F (ethidium bromide 6.7 µg ml<sup>-1</sup> 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 X [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.



**Figure 4** Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) (B) in the lungs, small intestine and liver of zymosan-treated rats. MPO activity and MDA levels were significantly increased in the lung, small intestine and liver of the zymosan-treated rats in comparison to sham rats. MnTBAP (1, 3 and 10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) treatment significantly reduce in dose dependent manner the zymosan-induced increase in MPO activity and MDA levels. Each value is the mean ± s.e. mean for n = 10. \*P < .01 versus sham; °P < .01 versus zymosan.

### Measurement of cellular NAD<sup>+</sup> levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 N HClO<sub>4</sub> scraped, neutralized with 3 M KOH, and centrifuged for 2 min at 10,000 × g. The supernatant was assayed for NAD<sup>+</sup> using a modification of the colorimetric method (Heller *et al.*, 1995) in which NADH produced by enzymatic



**Figure 5** Lung sections from a zymosan-treated rat revealed inflammatory infiltration of neutrophil, macrophage and plasma cells (A). Lung sections from zymosan-treated rat that received MnTBAP (10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) demonstrate reduced inflammatory infiltration (B). Sections are representative of n = 4 rats examined for each group. Original magnification: × 100.

cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine methosulfate. The rate of MTT reduction is proportional to the concentration of the co-enzyme. The reaction mixture contained 10  $\mu\text{l}$  of a solution of 2.5 mg ml MTT, 20  $\mu\text{l}$  of a solution of 4 mg ml<sup>-1</sup> phenazine methosulfate, 10  $\mu\text{l}$  of a solution of 0.6 mg ml alcohol dehydrogenase (300 U mg<sup>-1</sup>), and 190  $\mu\text{l}$  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 addition of 20  $\mu\text{l}$  of the sample. The rate of increase in absorbance was read immediately after the addition of NAD<sup>+</sup> samples and after 10- and 20-min incubation at 37°C against blank at 560 nm in the ELISA microplate reader (SLT-Labinstruments Salzburg, Austria).

### Reagents

MnTBAP was obtained from Calbiochem. Zymosan A from *Saccharomyces cerevisiae*, and all the other reagents and compounds used for the biochemical assays were obtained from Sigma Chemical (Milan, Italy). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, avidin-biotin peroxidase complex and primary anti-nitrotyrosine antibody were obtained from DBA (Milan, Italy).

### Data analysis

Data are expressed as means  $\pm$  s.e.mean analysis of variance test was used to compare means followed by Bonferroni's test for multiple comparison. Statistical analysis for survival data was calculated by Fisher's exact probability test. Differences were considered significant when the *P* value was less than 0.05.

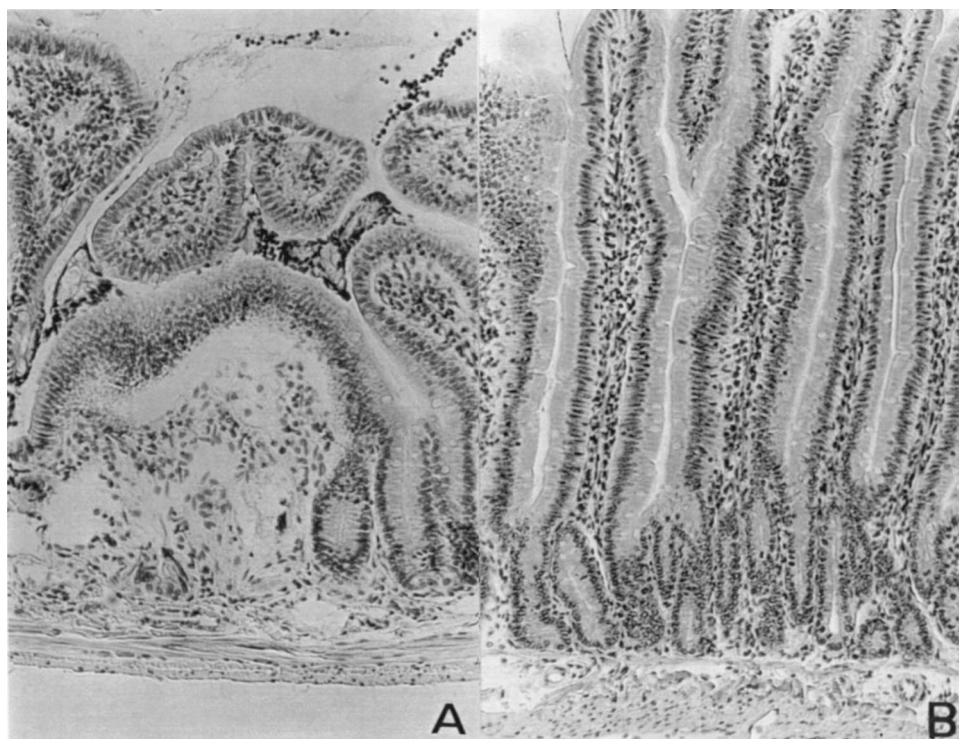
## Results

### *MnTBAP treatment reduces the development of zymosan-induced shock model*

All zymosan-injected rats developed an acute peritonitis, as indicated by the production of turbid exudate (Figure 1A). Trypan blue stains revealed a significant increase in the polymorphonuclear leukocytes in comparison to sham rats (Figure 1B). Sham animals demonstrated no abnormalities in the peritoneal cavity or fluid. The degree of peritoneal exudation and polymorphonuclear migration were significantly reduced in dose dependent manner in rats treated with MnTBAP (1, 3 and 10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) (Figure 1A,B). In sham rats MnTBAP treatment did not cause significant changes in these parameters (Figure 1A,B).

The biochemical and inflammatory changes observed in the peritoneal cavity of zymosan-treated rats were associated with a significant elevation of plasma NOx and peroxynitrite levels. Nitrate/nitrite levels were significantly elevated in zymosan treated rats ( $182 \pm 22 \mu\text{M}$ ) in comparison to sham rats ( $18 \pm 2 \mu\text{M}$ ,  $P < 0.01$ ) (Figure 1C). MnTBAP (1, 3 and 10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) treatment did not affect nitrite/nitrate levels (Figure 1C).

Plasma levels of peroxynitrite, as assessed by oxidation of dihydrorhodamine 123 to rhodamine 123, were also significantly increased in the zymosan-treated rats in comparison to sham animals (Figure 1D). MnTBAP (1, 3 and 10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) treatment reduced in dose dependent manner the peroxynitrite formation (Figure 1D). At 18 h lung, and small intestine were also analysed for the presence of nitrotyrosine, revealing a positive staining in lung and small intestine from zymosan-treated rats, (Figure 2A–C and 3A). A marked reduction of nitrotyrosine staining was



**Figure 6** Distal ileum section from a zymosan-treated rat showed inflammatory infiltration of neutrophil, lymphocytes and plasma cells extending through the wall and concentrated below the epithelial layer (A). Distal ileum from zymosan-treated rat that received MnTBAP (10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) showed reduced zymosan-induced inflammatory infiltration (B). Original magnification:  $\times 100$ .

found in the lung and small intestine of the zymosan-treated rats treated with MnTBAP (10 mg kg<sup>-1</sup> i.p. 1 and 6 h after zymosan) (Figure 2D3B). Staining was absent in tissue from sham rats (data not shown).

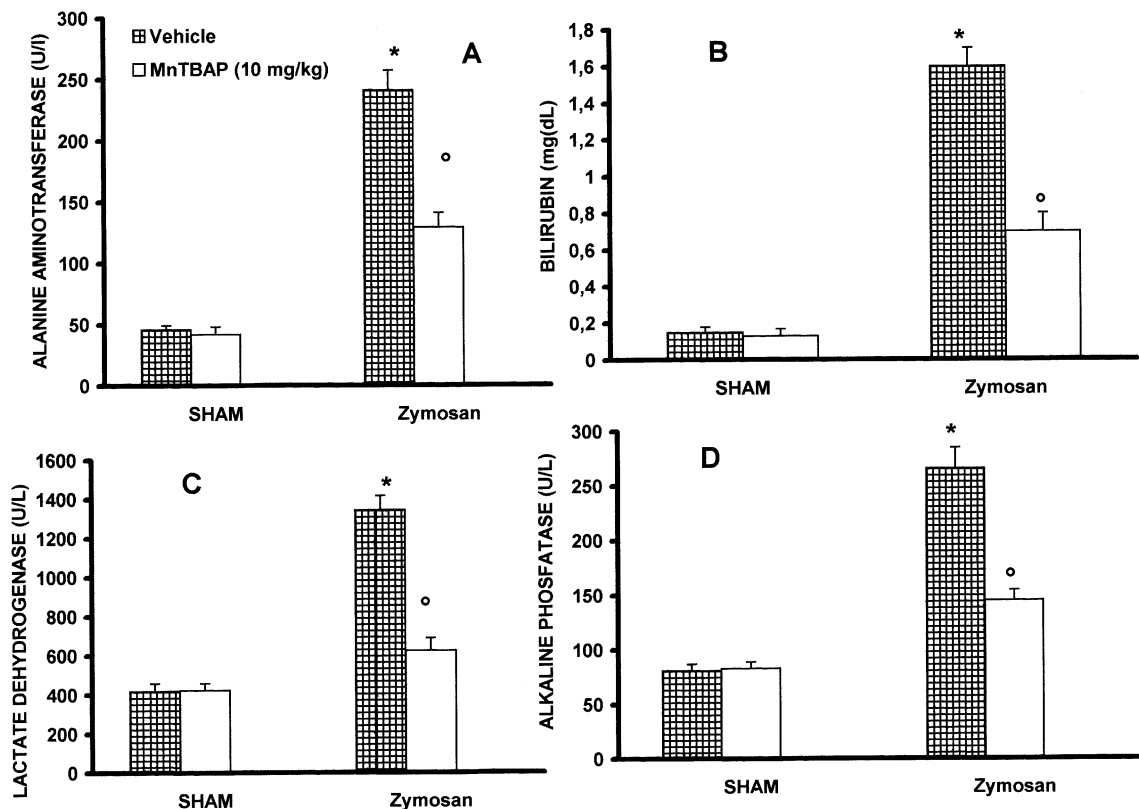
At 18 h after zymosan administration, lung, small intestine and liver were investigated for MDA levels in order to estimate lipid peroxidation and MPO activity, indicative of neutrophil infiltration. As shown in Figure 4A,B, MPO activity and MDA levels were significantly increased in the examined organs ( $P < 0.01$ ) at 18 h after zymosan injection. Histological examinations of lung and small intestine (see representative sections at Figures 5 and 6) revealed pathologic changes. In particular, lung biopsy examination revealed inflammatory infiltration by neutrophil, macrophage and plasma cells (Figure 5A). The histological examination of ileum section showed inflammatory infiltration by neutrophil, lymphocytes and plasma cells extending through the wall and concentrated below the epithelial layer; occasionally focal ulceration, sometimes extending through the muscularis mucosa were observed (Figure 6A). However, MPO activity and MDA levels were significantly ( $P < 0.01$ ) reduced in dose dependent manner by MnTBAP (3 and 10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) treatment (Figure 4A,B). In addition, treatment with MnTBAP (10 mg kg<sup>-1</sup> i.p. 1 and 6 h after zymosan) markedly reduced the zymosan-induced organ injury (Figures 5B and 6B).

Metabolic changes paralleled the organ morphological alterations. For example, blood levels of alanine aminotransferase increased from basal levels of  $46 \pm 2.6$  U l<sup>-1</sup> to  $241 \pm 12$  U l<sup>-1</sup> at 18 h after zymosan administration ( $P < 0.01$ ). Zymosan-treated rats exhibited also a marked hyperbilirubinemia from basal levels of  $0.15 \pm 0.04$  mg dl<sup>-1</sup> to

$1.6 \pm 0.13$  mg dl<sup>-1</sup> (Figure 7A,B). A significant increase of lactate dehydrogenase and alkaline phosphatase was also found at 18 h after zymosan injection (Figure 7C,D). Treatment with MnTBAP (10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) lowered the increase in alanine aminotransferase, bilirubin, lactate dehydrogenase and alkaline phosphatase blood levels in the zymosan-treated rats (Figure 7). In a separate set of experiment animals were observed for 72 h. At the end of observation period (72 h), 53% of zymosan-treated rats were dead (Figure 8). Sham animals injected only with saline appeared healthy and active through the observation period. MnTBAP (10 mg kg<sup>-1</sup>; i.p. 1 and 6 h after zymosan) reduced the mortality (Figure 8).

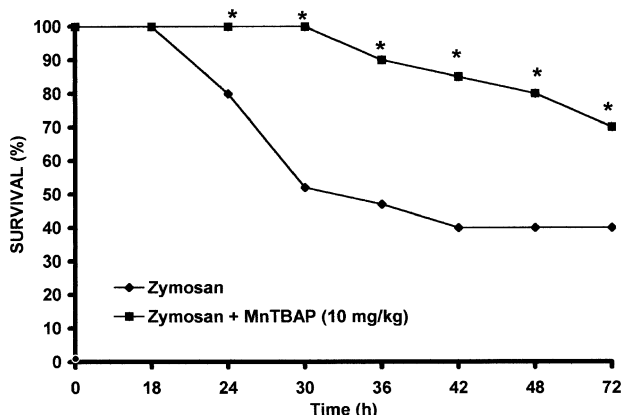
#### MnTBAP protects against the cellular energetic failure

In peritoneal macrophages obtained 18 h after zymosan injection, a significant nitrate/nitrite production was detectable ( $52 \pm 3.3$   $\mu$ M;  $n = 4$ ; Figure 9A). A rapid and sustained production of peroxynitrite ( $66 \pm 2.4$  pmoles/min/million cells) in comparison to sham ( $2.8 \pm 0.8$  pmoles/min/million cells) was also observed after zymosan-induced shock ( $n = 4$ ; Figure 9B). A marked increase in DNA strand breakage was also observed after zymosan-induced shock (Figure 9C). Zymosan-mediated disruption of cellular energetic pool was evidenced by a significant decrease in energetic status and intracellular concentration of NAD<sup>+</sup> (Figure 9D,E). The *in vivo* treatment of the animals with MnTBAP significantly inhibited in a dose dependent manner dihydrorhodamine 123 oxidation, prevented the -induced DNA single strand breakage (Figure 9B,C), significantly inhibited the decrease in cellular energetic status and partially restored the



**Figure 7** Plasma level of alanine aminotransferase (A), bilirubin (B), lactate dehydrogenase (C) and alkaline phosphatase (D). Zymosan-treated rats exhibited a dysmetabolic change. Administration of MnTBAP (10 mg kg<sup>-1</sup>, i.p. 1 and 6 h after zymosan) prevented the metabolic modification. Each value is the mean  $\pm$  s.e. mean for  $n = 10$ . \* $P < .01$  versus sham; ° $P < .01$  versus zymosan.





**Figure 8** Effect of MnTBAP treatment on zymosan-induced mortality.  $n = 10$  rats for each group. \* $P < 0.01$  versus zymosan.

depletion of intracellular levels of  $\text{NAD}^+$  (Figure 9D,E). Production of nitrite/nitrate was unaffected by MnTBAP treatment (Figure 9A).

## Discussion

The cellular and molecular mechanism of the zymosan-induced shock has been well characterized. In fact several pro-inflammatory cytokines, such as tumor necrosis factor (Von Asmuth *et al.*, 1990), and pro-inflammatory lipid mediators, such as platelet-activating factor (Damas *et al.*, 1993), and cyclo-oxygenase metabolites (Mainous *et al.*, 1991; Cuzzocrea *et al.*, 1997b; Doherty *et al.*, 1990; Rao *et al.*, 1993) have been proposed as responsible for the zymosan-induced shock. The role of oxygen-derived free radicals (Demling *et al.*, 1994; Van Bebber *et al.*, 1989; Mainous *et al.*, 1995) and, more recently, the role of NO (Cuzzocrea *et al.*, 1997a,b) has also been demonstrated in zymosan-induced shock and inflammation. It is well established that the treatment of plasma with zymosan activates the complement system and generates  $\text{C}_{5a}$ , which is a potent chemotactic factor for neutrophils (Fernandez *et al.*, 1978; Snyderman *et al.*, 1970). There is evidence that zymosan triggers the production of oxygen-derived free radicals in various cell types (Murohara *et al.*, 1993; Mehta *et al.*, 1991), and it also induces peroxynitrite generation (Cuzzocrea *et al.*, 1997c). In fact, potent novel, potent, cell-permeable superoxide dismutase mimetics prevent peroxynitrite formation and protect against the cellular injury in various models of inflammation (Zingarelli *et al.*, 1997; Szabó *et al.*, 1996).

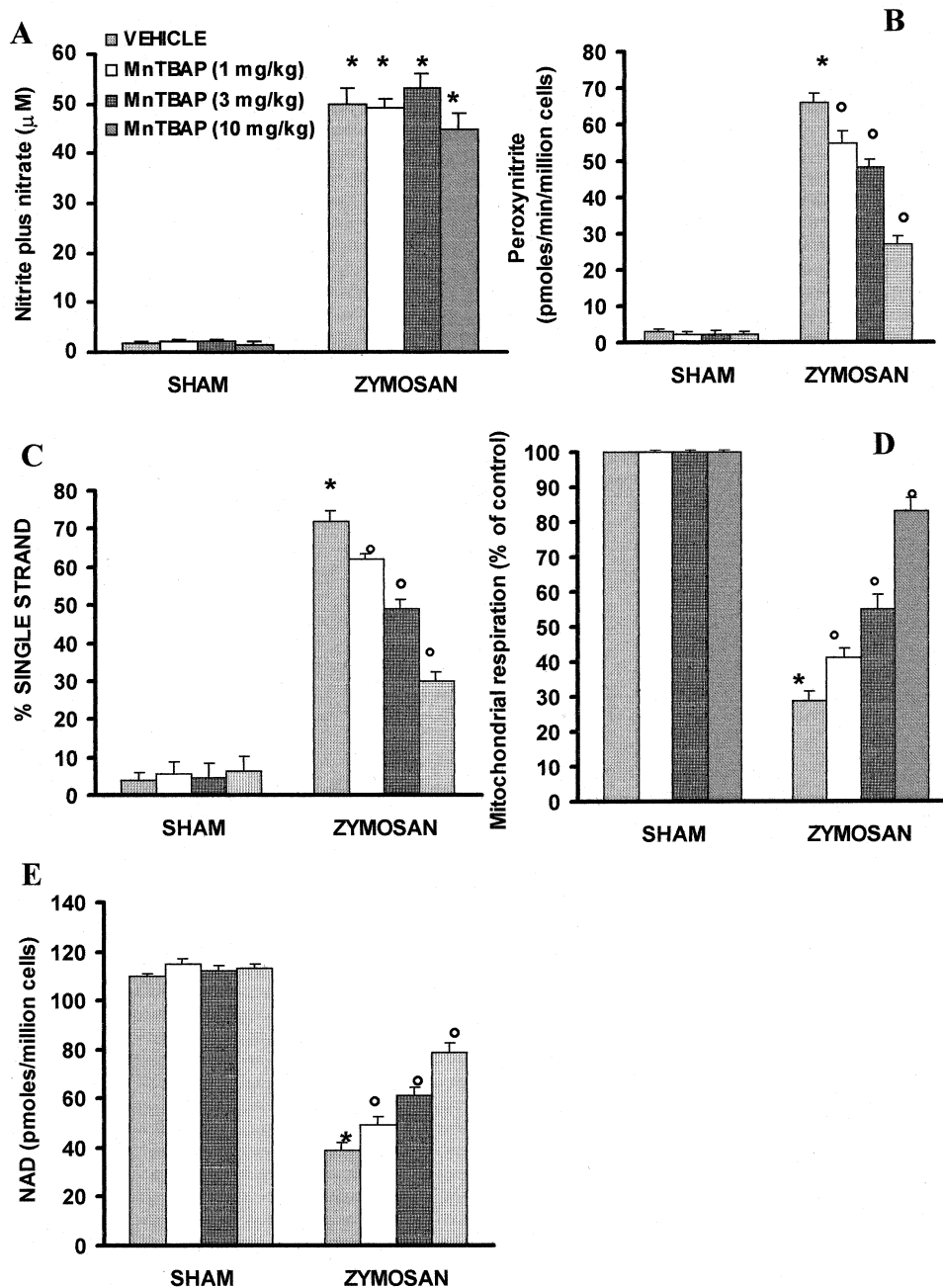
The main findings of the current study are the following: MnTBAP reduces (1) the development of zymosan-induced peritonitis; (2) morphological injury and neutrophil infiltration; (3)  $\text{ONOO}^-$  production and (4) MnTBAP reduces nitrotyrosine staining in the lung and small intestine from zymosan-treated rats.

The therapeutic efficacy of SOD itself in animals with systemic inflammation, haemorrhage and shock is controversial. The following reasons may explain the lack of effect of SOD against the tissue injury associated with local or systemic inflammation: (1) SOD scavenges superoxide, but without efficient removal of the hydrogen peroxide which is produced, levels of hydroxyl radicals may increase (Goode & Webster, 1983). Indeed, SOD may function as a pro-oxidant by catalysing the conversion of hydrogen peroxide to hydroxyl radicals (Yim *et al.*, 1990). (2) Neither SOD nor superoxide anions easily cross biological membranes. Thus, an increase in

the amounts of extracellular SOD does not attenuate the effects of superoxide anions generated by intracellular sources (Fridovich, 1995). In contrast to SOD, spin trapping nitrones, such as phenyl *N-tert-butyl* nitron (PBN), consistently improve outcome in rat models of endotoxic (McKechnie *et al.*, 1986) and traumatic shock (Novelli *et al.*, 1992).

What, then, is the mechanism by which MnTBAP protects the MOF injury associated with this model of shock. MnTBAP (Mn(III)tetrakis (4-benzoic acid) porphyrin) is a novel, stable and cell-permeable superoxide dismutase mimetic which permeates biological membranes and scavenges superoxide anions and peroxynitrite *in vitro* (Zingarelli *et al.*, 1997); Faulkner *et al.*, 1994; Day *et al.*, 1995; Szabó *et al.*, 1996). Thus, MnTBAP scavenges intracellular superoxide anions and prevents the formation of hydroxyl radicals. Unlike recombinant SOD, which is not able to cross biological membranes, hence, MnTBAP crosses biological membranes and functions as an intracellular scavenger of superoxide anions. In addition, we demonstrate that MnTBAP attenuates the nitrosylation of proteins in the lung and intestine of rats treated with zymosan. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation 'footprint' of peroxynitrite (Beckman, 1996). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich *et al.*, 1998). Increased nitrotyrosine staining is considered, therefore, as an indication of 'increased nitrosative stress' rather than a specific marker of the generation of peroxynitrite. Thus, it is possible that the inflammatory effects observed in response to zymosan represent the sum of a complex interaction between various oxygen- and nitrogen-derived radicals and oxidants. It is logical to assume that the pharmacological effects of MnTBAP arise from the combination of the following action: (1) by inactivating superoxide, and thus preventing the formation of peroxynitrite, with consequent protection against the development of peroxynitrite-induced cellular energetic failure, (2) by scavenging peroxynitrite, direct inhibition of peroxynitrite-induced oxidative process and (3) by reducing neutrophil recruitment into the inflammatory site. At present we cannot see a suitable pharmacological approach to separate the above factors. The reduced neutrophil infiltration into the inflamed tissue is not clear at present. It may be related to a prevention by MnTBAP of endothelial oxidant injury and to a preservation of endothelial barrier function. Clearly, the understanding of the relative contribution of MnTBAP's multiple mechanism of action to the anti-inflammatory effects observed in the current study requires further investigations. Recently a novel pathway of inflammation, governed by the nuclear enzyme poly (ADP-ribose) synthetase (PARS) has been proposed in relation to hydroxyl radical- and peroxynitrite-induced DNA single strand breakage (Szabó *et al.*, 1997a,b; Cochrane 1991; Cuzzocrea *et al.*, 1998b). This pathway plays an important role in various forms of shock and reperfusion injury (Cuzzocrea *et al.*, 1997d; Thiemeermann *et al.*, 1997). It is clear that MnTBAP has potent protective effects in experimental systems where the injury is likely to be mediated by superoxide alone (Zingarelli *et al.* 1997). Therefore, recent studies have demonstrated that peroxynitrite potently triggers DNA single strand breakage in different cell types, including thymocytes, macrophages and vascular smooth muscle cells by PARS activation with consequent reduction of mitochondrial respiration (Szabó *et al.*, 1997a; Cuzzocrea *et al.*, 1998b).





**Figure 9** Nitrate/nitrite production (A), peroxynitrite production (B), DNA single strand breakage (C), reduction of mitochondrial respiration (D) and cellular levels of  $\text{NAD}^+$  (E) in peritoneal macrophages harvested 18 h after zymosan administration. \* $P < 0.01$  versus macrophages from control rats; <sup>o</sup> $P < 0.01$  represents protective effects of MnTBAP.

Zingarelli *et al.* (1997) have recently demonstrated that this mesoporphyrin compound protects against the suppression of mitochondrial respiration induced by authentic peroxynitrite in vascular smooth muscle cells and on the cellular energetic failure in a rodents model of endotoxin shock.

Therefore the protection by MnTBAP against the development of DNA single strand breakage and the partially restore of the depletion of intracellular levels of  $\text{NAD}^+$  (as shown in Figure 7C), may be related to either superoxide and peroxynitrite and thus prevent the activation of PARS in inflammation (Cuzzocrea *et al.*, 1999b).

Taken together, the results of the present study, coupled with recent data by ours and several other groups support the view that MnTBAP can exert potent anti-inflammatory effects.

Based on the current results, we propose that the mode of MnTBAP's protective action is, at least in part, related to: (1) reduction of superoxide formation and (2) scavenging of peroxynitrite. Therefore the relative contribution of the two effects of MnTBAP during zymosan induced non-septic shock is considerable and worthy of further investigation.

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