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The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock

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1 Peroxynitrite is a toxic oxidant species produced from nitric oxide (NO) and superoxide. We have recently observed that the cell-permeable superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP) inhibits the suppression of mitochondrial respiration elicited by authentic peroxynitrite *in vitro*. Here we have investigated the relative potency of MnTBAP and a range of related compounds in terms of inhibition of peroxynitrite-induced oxidation and cytotoxicity. In addition, we tested the effects of MnTBAP on the vascular and the cellular energetic failure in rodent models of endotoxic shock.

2 We observed a dose-related inhibition of the peroxynitrite-induced oxidation of dihydrorhodamine 123 to rhodamine by MnTBAP, ZnTBAP and FeTBAP, but not by MnTMPyP [(5,10,15,20-tetrakis(N-methyl-4′-pirydyl)porphinato)-manganese (III)]. In addition, MnTBAP, ZnTBAP and FeTBAP, but not MnTMPyP prevented the suppression of mitochondrial respiration by authentic peroxynitrite in cultured J774 macrophages.

3 In rat cultured aortic smooth muscle cells, MnTBAP protected against the suppression of mitochondrial respiration in response to authentic peroxynitrite, immunostimulation and nitric oxide (NO) donor compounds. MnTBAP slightly reduced the amount of nitrite/nitrate produced in response to immunostimulation in these cells.

4 Administration of MnTBAP, 15 mg kg^{-1} i.v., before the administration of endotoxin (15 mg kg^{-1}, i.v.) to rats ameliorated the development of vascular hyporeactivity and the development of endothelial dysfunction in the thoracic aorta *ex vivo*.

5 MnTBAP also prevented the endotoxin-induced decrease in mitochondrial respiration, the development of DNA single strand breaks, and the depletion of intracellular NAD^{+} in peritoneal macrophages *ex vivo*.

6 MnTBAP did not inhibit the expression by endotoxin of the inducible NO synthase in lung samples.

7 MnTBAP did not alter survival rate in mice challenged with high dose endotoxin.

8 Our findings, taken together with previous data demonstrating protective effects of NO synthase inhibitors against the endotoxin-induced contractile and energetic failure in the models of shock used in the current study, and with the known ability of peroxynitrite to cause cellular energy depletion, suggest a role for peroxynitrite in the pathogenesis of cellular energetic failure and contractile dysfunction in endotoxin shock.

Keywords: Nitric oxide; endothelium; DNA strand breaks; DNA repair; polyADP ribose polymerase; mitochondrial respiration

Introduction

The inducible isoform of nitric oxide (NO) synthase (iNOS), expressed in macrophages and vascular smooth muscle cells, has been implicated in the development of cellular energetic and vascular contractile failure during conditions of immunostimulation, inflammation and various forms of circulatory shock (Nathan, 1992; Kilbourn & Griffith, 1992; Szabó, 1995). In endotoxic shock, for instance, iNOS is expressed in various tissues and organs, including lung, spleen, liver, heart and blood vessels. NO produced by iNOS results in a fall in blood pressure, a decrease in peripheral vascular resistance, and a hyporesponsiveness of arteries and veins to endogenous and exogenous vasoconstrictors (Kilbourn & Griffith, 1992; Szabó, 1995).

The reaction of NO and superoxide has been shown to yield peroxynitrite, a highly reactive oxidant species (Beckman *et al.*, 1990; Pryor & Squadrito, 1995; Szabó, 1996). The formation of peroxynitrite has been demonstrated in immunostimulated macrophages (Ischiropoulos *et al.*, 1992), in various inflammatory disorders (Halliwell, 1995; Miller *et al.*, 1995) and in circulatory shock (Wizemann *et al.*, 1994; Szabó, 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, and other factors), and these factors influence its toxic potential (Beckman *et al.*, 1990; Rubbo *et al.*, 1994; Villa *et al.*, 1994; Moro *et al.*, 1994; 1995; Pryor & Squadrito, 1995; Szabó, 1996). Under certain experimental conditions, peroxynitrite can form NO donors and can transfer NO to SH targets, whereas under other conditions, peroxynitrite can act as a more toxic species than NO or superoxide anion alone (Hausladen & Fridovich, 1994; Brunelli *et al.*, 1995; reviewed in: Szabó, 1996). In a number of pathophysiological conditions, peroxynitrite has been proposed as an important mediator of cell damage under conditions of inflammation and oxidant stress (Miller *et al.*, 1995; Crow & Beckman, 1995; Szabó *et al.*, 1996b; Zingarelli *et al.*, 1996; Szabó, 1996).

The mechanism of the cellular injury caused by peroxynitrite involves multiple factors, including initiation of lipid peroxidation (Rubbo *et al.*, 1994), inhibition of mitochondrial respiration (Hausladen & Fridovich, 1994; Radi *et al.*, 1994), inhibition of membrane pumps (Hu *et al.*, 1994), glutathione depletion (Phelps *et al.*, 1995) and DNA damage (Inoue & Kawanishi, 1995; Salgo *et al.*, 1995; Szabó *et al.*, 1996b) with subsequent activation of polyADP ribose synthetase (PARS) and concomitant cellular energy depletion (Szabó *et al.*, 1996b, c; Zingarelli *et al.*, 1996).

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There are putative scavengers of peroxynitrite, such as uric acid or glutathione, that can be of limited use for *in vitro* investigations (Phelps *et al*., 1995). However, the direct investigation of the contribution of peroxynitrite to cellular injury under various pathophysiological conditions is hampered by the fact that there are no potent and specific peroxynitrite scavengers suitable for *in vivo* use. Thus, a similar protective effect achieved by either the neutralization of superoxide or by inhibition of NO biosynthesis in a given experimental disease model can be interpreted as compelling evidence for the involvement of peroxynitrite, when coupled with the direct demonstration of the ability of peroxynitrite to cause the particular type of injury (Crow & Beckman, 1995; Szabó, 1996).

Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) is a novel, stable and cell-permeable superoxide dismutase mimetic (Faulkner *et al*., 1994; Day *et al*., 1995). We have recently observed that MnTBAP inhibits the oxidation of dihydrorhodamine 123 elicited by authentic peroxynitrite (Szabó *et al*., 1996a). However, MnTBAP is not a scavenger of nitric oxide (Szabó *et al*., 1996a). In the present studies, we have investigated the potency of MnTBAP, in comparison with a series of related compounds, to inhibit the peroxynitrite-induced oxidative and cytotoxic processes *in vitro*. Moreover, by using MnTBAP, we investigated the contribution of superoxide anion or peroxynitrite to the suppression of mitochondrial respiration in rat aortic smooth muscle cells in response to NO donors or immunostimulation and to the development of vascular contractile and cellular energetic failure in rats treated with endotoxin.

Methods

Cell culture

J774 macrophages were cultured in DMEM medium, supplemented with L-glutamine (3.5 mmol l^{-1}) and 10% foetal calf serum, and rat aortic smooth muscle cells were cultured in RPMI medium, supplemented with L-glutamine (3.5 mmol l^{-1}) and 10% foetal calf serum, as described (Szabó *et al*., 1996b). Cells were cultured in 96-well plates (200 μl medium/well) or in 12-well plates (3 ml medium/well) until confluence. Cells were pretreated with MnTBAP and related compounds for 10 min, followed by the addition of authentic peroxynitrite (1 mM). Mitochondrial respiration was measured at 1 h.

To induce iNOS in the rat aortic smooth muscle cells, fresh culture medium containing *E. coli* lipopolysaccharide (LPS) (011:B4; 10 μg ml^{-1}) in the presence or absence of murine γ-interferon (γ-IFN, 10 u ml^{-1}) was added in the presence or absence of MnTBAP for 24 h. In addition, smooth muscle cells were exposed to the NO compound S-nitroso-N-acetyl-DL-penicillamine (SNAP, 3 mM) or the peroxynitrite donor compound 3-morpholino-sidnonimine (SIN-1, 3 mM) in the presence or absence of MnTBAP for 24 h. After 24 h, supernatants were analysed for nitrite production and mitochondrial respiration was measured. All spectrophotometric measurements were corrected for the interference of the test compounds.

Measurement of peroxynitrite-induced oxidation of dihydrorhodamine 123

The peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123, was measured as described previously (Szabó *et al*., 1996a). Briefly, peroxynitrite at 5 μM was added into phosphate-buffered saline containing 10 μM dihydrorhodamine 123, in the absence or presence of MnTBAP and related compounds (3–100 μM). After a 10 min incubation at 22°C, the fluorescence of rhodamine 123 was measured by a Perkin-Elmer fluorimeter (Model LS50B; Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively).

Measurement of nitrite production

Nitrite production, was measured as previously described (Szabó *et al*., 1996b), by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of conditioned medium. 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 OD_{550} of standard solutions of sodium nitrite prepared in culture medium. For the measurement of total nitrite/nitrate concentrations, nitrate was first converted to nitrite as described (see below).

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 (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 dimethyl sulphoxide (DMSO; 100 μl). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of OD_{550}.

Endotoxic shock

The animal experiments have been performed in accordance with NIH guidelines and with the approval of the Institutional Review Board of the Children's Hospital Research Foundation. Male Wistar rats (Charles River Laboratories, Wilmington, MA) were anaesthetized with sodium thiopentone (120 mg kg^{-1}, i.p.) and instrumented as described (Szabó *et al*., 1996c). The trachea was cannulated to facilitate respiration and temperature was maintained at 37°C with a homeothermic blanket. The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, animals were allowed to stabilize for 10 min. In 16 rats, endotoxic shock was then induced by injection of *E. coli* LPS (15 mg kg^{-1}, i.v.) at time 0. Fifteen minutes before LPS administration, one subgroup of these rats ($n=8$) was treated with MnTBAP (15 mg kg^{-1}, i.v. bolus), whereas the remaining animals ($n=8$, respectively) received saline. At 180 min after LPS or vehicle injection, plasma samples, lung samples, thoracic aortae and peritoneal macrophages were taken for the various measurements (see below). Control (sham-shocked) animals ($n=9$) received vehicle instead of LPS treatment, in the absence ($n=5$) or presence ($n=4$) of MnTBAP treatment for 180 min and were treated the same as LPS injected rats.

Organ bath experiments

Thoracic aortae from rats were cleared of adhering periadventitial fat and cut into rings of 3–4 mm width. Endothelium was removed from some of the rings by gently rubbing the intimal surface with a thin wooden stick. The 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, in the presence of indomethacin (10 μM). Isometric force was measured with isometric transducers (Kent Scientific Corp. Litchfield, CT, U.S.A.), digitalized by a Maclab A/D converter (AD Instruments, Milford, MA, U.S.A.) and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied and the rings were equilibrated for 60 min; the Krebs solution was changed every 15 min (Szabó *et al*., 1996c). Indomethacin was used to prevent the production of cyclo-oxygenase metabolites.

In endothelium-denuded vessels, after incubation and washouts, first the vessels were precontracted with a medium concentration of noradrenaline (100 nM), and then the effect of acetylcholine (1 nM–10 μM) was tested. The lack of a detectable acetylcholine-induced relaxation was taken as evidence

that endothelial cells had been removed. Concentration-response curves to noradrenaline ($10^{-9}-10^{-5}$ M) were then obtained in endothelium-denuded aortic rings taken from either control rats or rats injected with LPS (with or without MnTBAP treatment) and killed 180 min later.

In endothelium-intact rings, relaxation responses to acetylcholine (10 nM – 10 μM) were tested in rings obtained from the various groups of animals (control; control + MnTBAP, LPS, LPS + MnTBAP).

Nitric oxide synthase assay

Calcium-independent conversion of L-arginine to L-citrulline in homogenates of lungs obtained from rats treated with *E. coli* LPS (15 mg kg^{-1}, i.v., for 180 min) served as an indicator of iNOS activity. Lungs were taken from the animals and stored at -70°C. Lungs were homogenized on ice in a buffer composed of 50 mM. Tris-HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) on ice with a Tissue Tearor 985-370 homogenizer (Biospec Products, Racine, WI, U.S.A.). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described previously (Szabó *et al*., 1994). 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 a Wallac scintillation counter (Wallac, Gaithersburg, MD, U.S.A.).

Plasma nitrite/nitrate production

In plasma samples, nitrate is the major degradation product of NO. Nitrate was converted to nitrite as described by Zingarelli *et al.* (1996), by incubation with 60 mu nitrate reductase and 25 μM NADPH for 180 min. Nitrite was then measured as described above by the Griess reaction. Nitrite/nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite and sodium nitrate. The measurements of nitrite/nitrate were performed with reagents free of nitrite and nitrate; no basal or background nitrite or nitrate levels were detected.

Preparation of peritoneal macrophages

Peritoneal macrophages from rats were harvested by peritoneal lavage with DMEM medium containing L-glutamine (3.5 mM), penicillin (50 u ml^{-1}), streptomycin (50 μg ml^{-1}) and heparin sodium (10 u ml^{-1}). The cells were collected at 3 h after after i.v. injection of *E. coli* endotoxin (15 mg kg^{-1}) (Zingarelli *et al*., 1996). The cells were plated on 12-well plastic plates at 1×10^6 cells ml^{-1} 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/nitrate (as above). Non-adherent cells were removed by rinsing the plates three times with 5% dextrose in phosphate-buffered saline. In some of the 12-well plates, after removal of non-adherent cells, adherent macrophages were scraped for the measurement of DNA strand breaks and cellular NAD^+ content. In other 12-well plates, after removal of the non-adherent cells, a medium containing MTT (see above) was added to the cells and mitochondrial respiration was measured in the adherent cells in the subsequent 1 h time period.

Determination of DNA single strand breaks

The formation of strand breaks in double-stranded DNA was determined by the alkaline unwinding method as previously described (Szabó *et al*., 1996b; Zingarelli *et al*., 1996). 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; 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. Solution E (0.1 ml; neutralizing solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D. From this point incubations were carried out in the dark. A 30 min incubation period at 0°C was then allowed during which time 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, then 1.5 ml of solution F (ethidium bromide 6.7 μg ml^{-1} in 13.3 mM NaOH) was added to all the tubes and fluorescence (excitation: 520 nm, emission: 590 nm) was measured by a Perkin-Elmer 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 from 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.

Measurement of cellular NAD^+ levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 N $HClO_4$, scraped, neutralized with 3 M KOH and centrifuged for 2 min at 10,000 *g*. The supernatant was assayed for NAD^+, by a modification of the colorimetric method (Hinz *et al*., 1973; Szabó *et al*., 1996b), in which NADH, produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine methosulphate. 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^{-1} MTT, 20 μl of a solution of 4 mg ml^{-1} phenazine methosulphate, 10 μl of a solution of 0.6 mg ml^{-1} alcohol dehydrogenase (300 u mg^{-1}) and 190 μl 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 started by addition of 20 μl of the sample. The rate of increase in the absorbance was read immediately after addition of the NAD^+ samples and after 10 and 20 min incubation at 37°C against a blank at 560 nm in the Spectramax spectrophotometer.

Survival studies

Swiss albino mice (26 – 30 g, Charles River Laboratories, Wilmington, MA) were injected with *E. coli* LPS (120 mg kg^{-1}, i.p.) (Szabó *et al*., 1996c). Fifteen minutes before the injection of LPS and every 6 h thereafter, animals were treated with MnTBAP i.p. or with vehicle.

Materials

DMEM, RPMI and foetal calf serum were obtained from Gibco (Grand Island, NY). Perchloric acid was obtained from Aldrich (St. Louis, MO). S-nitroso-N-acetyl-DL-penicillamine (SNAP) were purchased from Calbiochem (San Diego, CA). Murine γ-IFN was obtained from Genzyme (Cambridge, MA).

3-Morpholino-sidnonimine (SIN-1) was obtained from Cassella AG (Frankfurt, Germany). [^{3}H]-NAD$^+$ was purchased from DuPont/NEN (Boston, MA, U.S.A.). Alcohol dehydrogenase and NAD$^+$ were obtained from Boehringer Mannheim (Indianapolis, IN). Bacterial lipopolysaccharide (LPS, *E. coli*, serotype No. 0127:B8), and all other chemicals were from Sigma (St. Louis, MO). Peroxynitrite was a kind gift of Dr H. Ischiropoulos (University of Pennsylvania, PA). MnTBAP and related compounds were prepared and purified as previously described (Faulkner *et al*., 1994; Day *et al*., 1995; Day & Crapo, 1996).

Statistical evaluation

EC_{50} values (concentration of agonist causing half-maximal contraction) were calculated by linear regression after logit-log transformation of concentration-response curves. All values in the figures and text are expressed as mean ± s.e.mean of n observations. For the *in vitro* studies, data represent pooled data from $n=12$ wells obtained on at least 3 different experimental days. For the vascular studies, $n=4-10$ rings, for the studies with peritoneal macrophages, plasma nitrate and pulmonary iNOS determinations, 4–5 rats were used in each group. Student's unpaired t test was used to compare means between groups, with the Bonferroni correction for multiple comparisons. For comparisons in the survival rate, the Chi-square test was used. A P value less than 0.05 was considered statistically significant.

Results

MnTBAP and related compounds reduce the peroxynitrite-induced oxidation and protect against the inhibition of mitochondrial respiration in response to peroxynitrite in J774 macrophages

As we have previously shown (Szabó *et al*., 1996a), peroxynitrite induced significant oxidation of dihydrorhodamine 123 to rhodamine 123, which was dose-dependently inhibited by MnTBAP, ZnTBAP and FeTBAP, with FeTBAP being slightly more potent than the other two compounds (Figure 1). In contrast, we found no inhibition of dihydrorhodamine 123 oxidation by MnTBPyP ((5,10,15,20-tetrakis(N-methyl-4′-pirydyl)porphinato)-manganese(III) (Figure 1).

In agreement with the studies on dihydrorhodamine 123 oxidation, we found that MnTBAP, FeTBAP and ZnTBAP, but not MnTMPyP inhibited the suppression of mitochondrial respiration in response to authentic peroxynitrite in J774 cells (Figure 2). Also in this assay, FeTBAP appeared to be more potent and maximal protection was already achieved at 10 μM (Figure 2).

Thus, the relative potency of the various compounds on the dihydrorhodamine oxidation by peroxynitrite did not follow the order of potency for superoxide dismutase mimetic activity which is MnTMPyP > FeTBAP > MnTBAP > > > ZnTBAP (Faulkner *et al*., 1994; Day *et al*., 1995; Day, unpublished observations).

MnTBAP protects against the inhibition of mitochondrial respiration in response to NO donors or immunostimulation in cultured smooth muscle cells

Next, we studied the effects of MnTBAP in rat cultured aortic smooth muscle cells. Mitochondrial respiration was inhibited by exposure to peroxynitrite, to the NO donor compound SNAP and by SIN-1, a compound that simultaneously releases NO and superoxide, as well as by immunostimulation (Figure 3). These effects were dose-dependently prevented by MnTBAP treatment (Figure 3). MnTBAP slightly reduced nitrite/nitrate formation

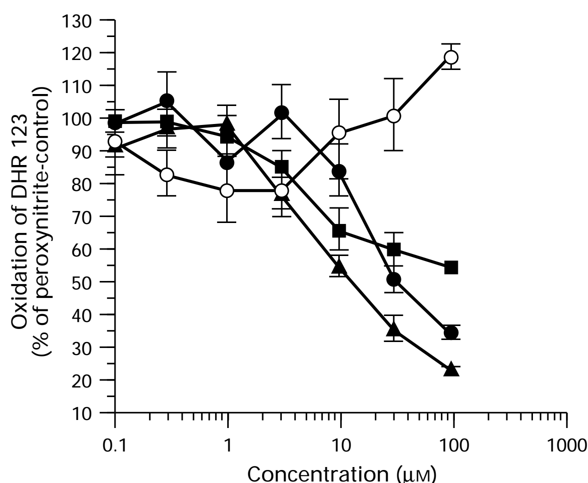

Figure 1 Effect of MnTBAP (●), ZnTBAP (■), FeTBAP (▲) and MnTMPyP (○) (all at 0.1–100 μM) on the peroxynitrite-induced oxidation of dihydrorhodamine 123 (DHR 123); 100% represents rhodamine 123 formation in the presence of peroxynitrite, but in the absence of inhibitors. Data are expressed as means of $n=4-6$ determinations; vertical lines show s.e.mean.

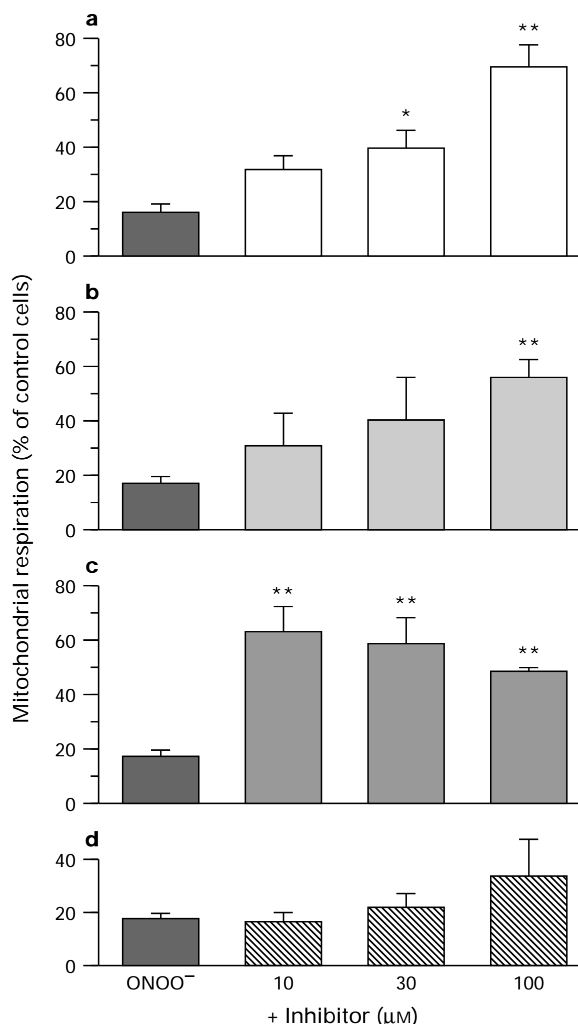

Figure 2 Effect of (a) MnTBAP, (b) ZnTBAP, (c) FeTBAP and (d) MnTMPyP (10,30 and 100 μM) on the peroxynitrite (1 mM)-induced suppression of mitochondrial respiration in J774 cells. Data are expressed as % of mitochondrial respiration in untreated (control) cells and are means ± s.e.mean of $n=6-9$ wells. There was a significant ($P<0.01$) suppression of mitochondrial respiration in response to peroxynitrite. Asterisks represent significant protective effect of MnTBAP, ZnTBAP, FeTBAP against peroxynitrite-induced suppression of mitochondrial respiration *$P<0.05$ and **$P<0.01$).

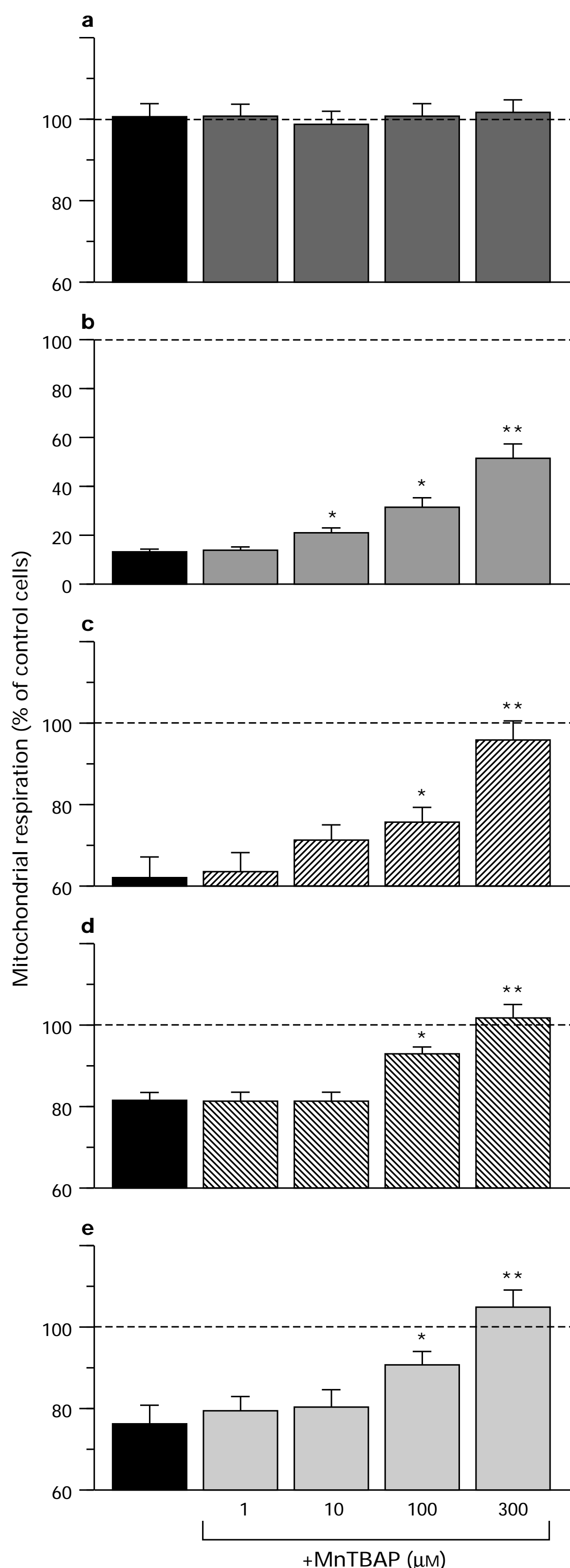

Figure 3 Suppression by (b) peroxynitrite (1 mM), (c) SNAP (3 mM), (d) SIN-1 (3 mM) and (e) by immunostimulation (bacterial lipopolysaccharide at $10\,\mu g\,ml^{-1}$, together with murine γ-interferon at $10\,u\,ml^{-1}$) of mitochondrial respiration (expressed as % of respiration of unstimulated cells) in rat aortic smooth muscle cells, and the protective effect of MnTBAP ($1-300\,\mu M$) against this suppression. (a) Control respiration in presence of vehicle. Mitochondrial respiration was measured at 1 h in the case of peroxynitrite, and at 24 h in case of the other stimuli. Data are expressed as means $\pm$ s.e.mean of $n=12$ wells. SNAP, SIN-1 and immunostimulation all significantly decreased mitochondrial respiration ($P<0.01$). Asterisks represent significant protective effect of MnTBAP when compared to values in the absence of MnTBAP (*$P<0.05$ and **$P<0.01$).

in immunostimulated cells. For instance, nitrite/nitrate concentration was $88 \pm 2\ \mu M$ at 24 h after stimulation with LPS and IFN, whereas in the presence of 300 μM MnTBAP, LPS and IFN, nitrite/nitrate concentration was $62 \pm 1\ \mu M$ ($P<0.05$; $n=6$). In the absence of MnTBAP but in the presence of immunostimulation, 12% of the total nitrite/nitrate ratio was detected as nitrate, whereas in the presence of MnTBAP and immunostimulation, only 2% of the total nitrite/nitrate was detected as nitrate ($n=6$).

Effects of MnTBAP on ex vivo *vascular reactivity in endotoxic shock*

LPS caused a significant depression of the ability of the thoracic aorta to contract to noradrenaline (1 nM – 10 μM) *ex vivo*, an effect which was ameliorated by *in vivo* MnTBAP treatment (Figure 4). EC_{50} values for the contractions to noradrenaline were 51 ± 6, 49 ± 17, 212 ± 52, and 221 ± 36 nM for control, MnTBAP + control, LPS and MnTBAP + LPS groups, respectively. The EC_{50} values for the LPS groups were significantly higher ($P<0.05$) than controls. Thus, MnTBAP caused a partial protection against the LPS-induced reduction of contractility in endotoxemia, without improving the sensitivity of the rings to noradrenaline.

There was an impairment of the endothelium-dependent relaxations after endotoxin treatment, as evidenced by the shift in the EC_{50} values for the relaxations to acetylcholine from $0.7 \pm 0.1\ \mu M$ to $3.2 \pm 1.4\ \mu M$ ($P<0.05$), which was prevented by MnTBAP treatment (EC_{50} value in LPS-rings from rats treated with MnTBAP: $0.56 \pm 0.11\ \mu M$). MnTBAP treatment in sham-shocked animals did not alter the EC_{50} value to acetylcholine (not shown). The maximal relaxation (to 10 μM acetylcholine) was 78 ± 4% of precontractile tone and was not altered by endotoxemia or by MnTBAP treatment ($n=4-10$; not shown).

Effects of MnTBAP on the development of cellular energetic deficit in macrophages ex vivo

LPS treatment of the rats caused a significant increase in the production of nitrite/nitrate, a significant suppression of the

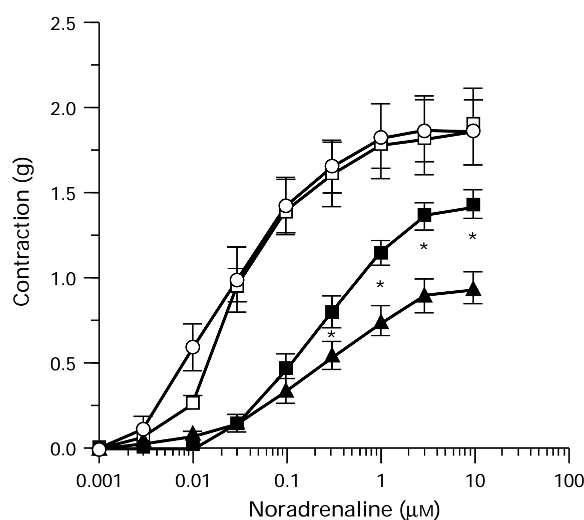

Figure 4 Effect of endotoxic shock ($15\,mg\,kg^{-1}$ LPS injected at time 0 for 3 h) on the contractile activity to noradrenaline (1 nM – 10 μM) in the thoracic aortic rings *ex vivo* (▲) when compared to sham-operated controls (□), and the effect of *in vivo* treatment with MnTBAP in sham-shocked animals (○) and in animals subjected to endotoxin shock (■). Endotoxemia significantly decreased contractility at 0.1 – 10 μM ($P<0.01$). *$P<0.05$ represents significant protective effect of MnTBAP against the LPS-induced vascular hyporeactivity. Data are expressed as means $\pm$ s.e.mean (vertical lines) of $n=4-10$ vascular rings.

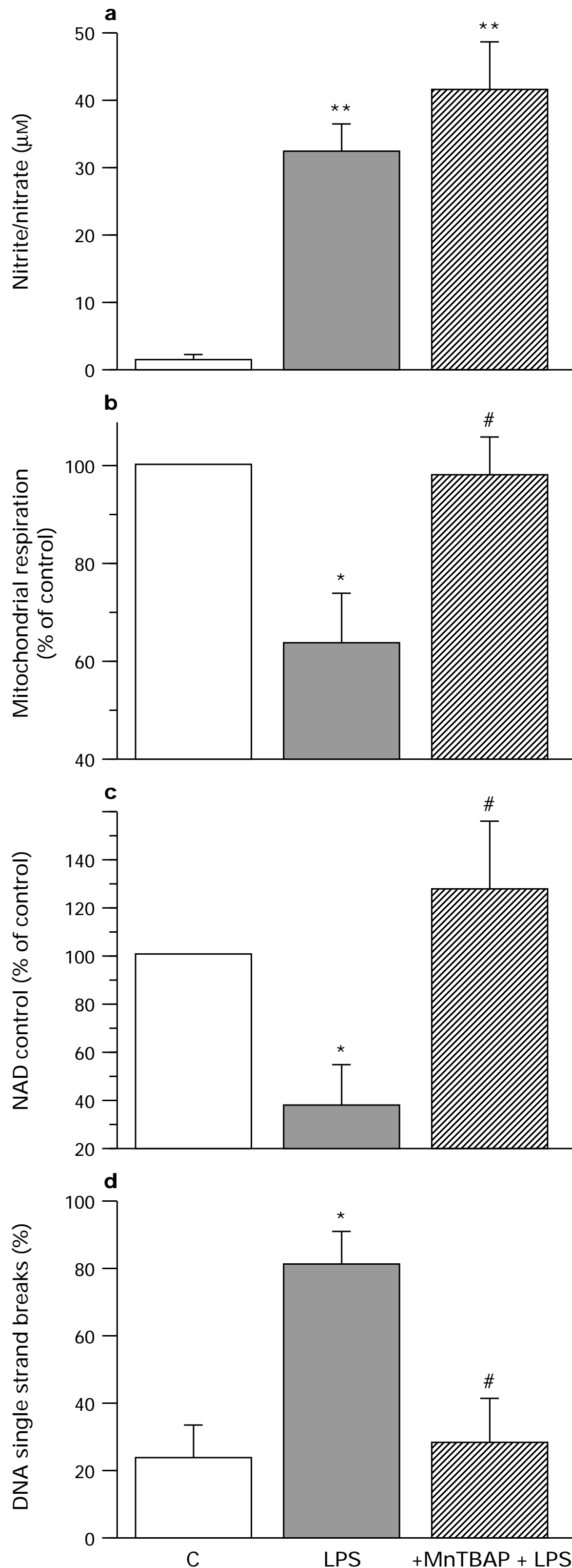

Figure 5 Effect of administration of MnTBAP *in vivo* on cellular energetics in peritoneal macrophages harvested from endotoxin-shocked rats. Depicted are nitrite/nitrate production (a), mitochondrial respiration (b), intracellular NAD^+ content (b) and DNA strand breakage (d) in peritoneal macrophages obtained from control rats (C), from rats treated with *E. coli* LPS ($15\,mg\,kg^{-1}$) for 3 h (LPS) and from rats treated with MnTBAP before LPS challenge (MnTBAP + LPS). Asterisks represent a significant increase in nitrite/nitrate or decrease in the respiration or NAD^+ content in response to LPS. $*P<0.05$, $**P<0.01$), #represents significant protective effects of MnTBAP in the presence of LPS when compared to LPS alone ($P<0.05$); $n=4-5$.

mitochondrial respiration and the intracellular NAD content, and an increase in the DNA strand breakage in peritoneal macrophages obtained 180 min after the injection of LPS *ex vivo* (Figure 5a–d). Nitrite/nitrate production was unaffected by *in vivo* MnTBAP treatment (Figure 5a). MnTBAP, however, caused a significant amelioration of the LPS-induced decrease in the mitochondrial respiration (Figure 5b), prevented the decrease in the intracellular NAD^+ levels (Figure 5c), and prevented the LPS-induced DNA single strand breakage (Figure 5d). MnTBAP treatment in the absence of LPS did not significantly alter any of the above parameters ($n=4$, not shown).

Effects of MnTBAP on NO production and iNOS induction in endotoxic shock

LPS caused a significant increase in pulmonary iNOS activity and in the plasma nitrite/nitrate levels at 180 min (Figure 6). This was not significantly altered by MnTBAP treatment (Figure 6).

Effect of MnTBAP on endotoxin-induced mortality in mice

Intraperitoneal injection of LPS ($120\,mg\,kg^{-1}$) resulted in approximately 75% mortality over 30 h. *In vivo* administration of MnTBAP (bolus injections 15 min before LPS and every 6 h thereafter) at the doses of $0.1-3\,mg\,kg^{-1}$ tended to reduce LPS-induced mortality, but the effect did not reach statistical

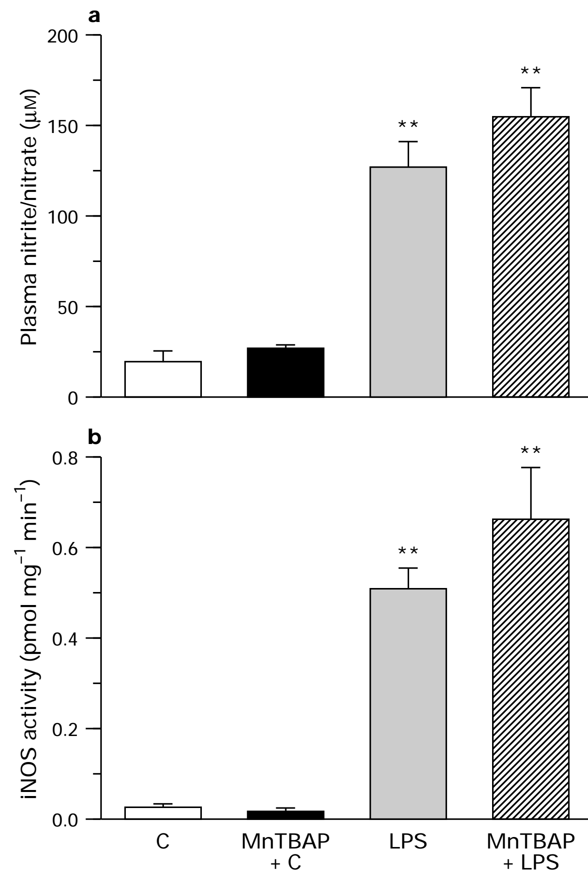

Figure 6 MnTBAP does not affect NO production in endotoxic shock. Depicted are (a) plasma nitrite/nitrate levels and (b) pulmonary iNOS activities *ex vivo* in control rats (C), in control rats treated with MnTBAP (MnTBAP + C), and in rats treated with LPS in the absence or presence of MnTBAP (LPS and MnTBAP + LPS, respectively). Data are expressed as mean ± s.e.-mean of $n=4-5$ animals. $**P<0.05$ represents significant increase in plasma nitrite/nitrate or pulmonary iNOS activity at 180 min after LPS when compared to control.

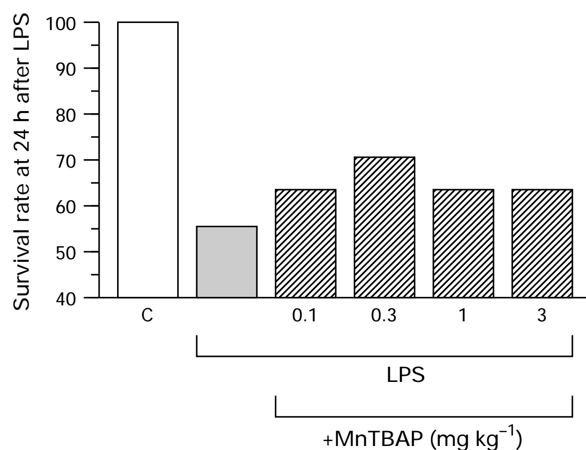

Figure 7 Effect of endotoxic shock (120 mg kg^{-1} LPS i.p. at time 0) on 30 h survival rate in vehicle-treated mice (solid column) and in mice treated with various doses of MnTBAP (0.1–3 mg kg^{-1}) prior to LPS and every 6 h thereafter. There were no statistically significant improvements in survival by MnTBAP. Data are expressed as % of initial survival rate (100%); 16–24 animals were used in the individual groups.

significance (Figure 7). Increasing the dose of MnTBAP to 10–20 mg kg^{-1} did not result in a significant improvement in the survival rate, either ($n=7$, not shown).

Discussion

In vitro *effects of MnTBAP and related compounds*

The present results show that a range of mesoporphyrin compounds inhibit peroxynitrite-induced oxidative and cytotoxic processes. Manganese tetrakis(4-benzoic acid) porphyrin as well as its zinc or iron analogue had potent inhibitory effects in this respect. On the other hand, MnTMPyP did not inhibit peroxynitrite-induced oxidations or protect the cells against peroxynitrite-induced toxicity. The exact mechanism of the inactivation of scavenging of peroxynitrite by these compounds requires further investigations, but the order of potency of peroxynitrite scavenging does not correlate with the order of potencies of these compounds as superoxide dismutase mimetics. Groves and Marla (1995) have recently demonstrated the reaction of peroxynitrite with MnTMPyP, with the concomitant formation of an oxomanganese intermediate. These authors found that this latter reactive species potentiated the oxidative injury triggered by peroxynitrite (Groves & Marla, 1995). In contrast to this, we found no enhancement of the peroxynitrite-induced cytotoxicity by MnTMPyP in the J774 cells (Figure 2). Although the understanding of the interaction of TBAPs with peroxynitrite requires further studies, it is possible that the reaction of peroxynitrite with TBAPs also results in the formation of secondary intermediates (R. Radi, Universidad de la Republica, Uruguay, personal communication). Thus, the protective efficacy of TBAPs may well be dependent on the molecular targets of peroxynitrite in the experimental system in question.

In the rat aortic smooth muscle cells, we found clear protection by MnTBAP against the suppression of mitochondrial respiration by authentic peroxynitrite and SIN-1, a compound that simultaneously generates NO and superoxide, which combine to form peroxynitrite (Figure 3). In these experiments, the decreased mitochondrial respiration may have reflected direct cytotoxic effects of peroxynitrite. Alternatively, peroxynitrite may have reacted with glucose or other constituents of the culture medium or the cells and subsequently may have formed NO donor species. Since (i) MnTBAP is not a scavenger of NO, but is a scavenger of peroxynitrite (Szabó *et al*., 1996b), and (ii) scavenging NO does not protect against the cytotoxicity elicited by authentic peroxynitrite (Szabó *et al*., 1996b), we speculate that the cytotoxicity seen in the present study is related to peroxynitrite rather than NO, and MnTBAP reduced the toxicity by neutralizing peroxynitrite and/or enhancing its decomposition. It is noteworthy, that, similar to other studies, extremely high initial concentrations of peroxynitrite were needed to obtain cytotoxic effects in the present study. As discussed previously (Szabó *et al*., 1996b), it is evident that much of the peroxynitrite added to the culture medium may have decomposed before reaching the cells, due to its reaction with scavengers in the culture medium. Further amounts of peroxynitrite may then become inactivated when entering the cells, due to a reaction with cell membrane constituents (lipids, proteins). Another reason for the necessity of high concentrations of peroxynitrite to elicit cytotoxic effects is that the reaction of peroxynitrite may have resulted in the formation of a NO donor species, which reduces the oxidant capacity of peroxynitrite (Rubbo *et al*., 1994; Miles *et al*., 1996).

We have also found that there was a marked protection by MnTBAP against the suppression of mitochondrial respiration in response to the 'pure' NO donor, SNAP. Since MnTBAP is not a NO scavenger, a potential explanation may be that, in the vicinity of the mitochondria, endogenously produced superoxide and SNAP-derived NO combine to form cytotoxic amounts of peroxynitrite. It is hypothesized that the initial, reversible inhibition of mitochondrial respiratory enzymes by NO leads to enhanced superoxide formation by the mitochondria, and superoxide, when combining with NO (derived from SNAP, for example), produces cytotoxic amounts of peroxynitrite, leading to irreversible inhibition of mitochondrial respiration (see also: Szabó, 1996). The degree of such an effect may well be cell type-dependent, and may be determined by the extent of endogenous superoxide formation and by the capacity of the cell to scavenge peroxynitrite. For instance, in the J774 cells, MnTBAP provided an incomplete protection against SNAP-induced suppression of the respiration (Szabó *et al*., 1996a), whereas no significant protective effect was seen in human umbilical vein endothelial cells or in human DLD-1 cells (Kennedy, Krafte-Jacobs, Salzman & Szabó, unpublished observations).

Similar to our previous findings in the J774 cells (Szabó *et al*., 1996a), MnTBAP inhibited the suppression of mitochondrial respiration in response to immunostimulation in the rat aortic smooth muscle cells. This effect is likely to be due to two effects of MnTBAP: (1) reduction of superoxide formation due to the superoxide dismutase-like activity of the compound and (2) scavenging of peroxynitrite. The relative contribution of the two effects needs to be determined in further studies. In contrast to the J774 cells, here we found that MnTBAP only slightly reduced the induction of iNOS in the smooth muscle cells. We proposed that the suppression of iNOS induction by MnTBAP in these cells is due to the prevention of the activation of the transcription factor nuclear factor κB, which is regulated by an oxidant-sensitive process and is involved in the induction of iNOS (Szabó *et al*., 1996a). There are distinct differences in the promoter regulation between murine macrophages and rat aortic smooth muscle cells, especially in respect of nuclear factor κB (Spink *et al*., 1995), which may account for this difference.

Effects of MnTBAP in rodent models of endotoxin shock

Various forms of shock and inflammation are associated with a generalized simultaneous increase in the production of oxygen and nitrogen centered free radicals. When produced in large quantities, both superoxide and NO have been implicated in various forms of inflammatory tissue injury. In rodent models of endotoxic shock, it has been widely assumed that NO *per se* is responsible for the development of vascular contractile failure (Fleming *et al*., 1991; Kilbourn & Griffith, 1992; Szabó, 1995) and cellular injury, resulting in multiple organ failure

and mortality (Nava *et al*., 1994; Wei *et al*., 1995; Thiemermann *et al*., 1995; Szabó, 1995). It is known, for instance, that iNOS, the enzyme which produces NO in great abundance during inflammatory states, is expressed throughout various tissues and in the vascular smooth muscle after several hours of endotoxic shock. Moreover, pharmacological agents which inhibit the induction or the activity of iNOS prevent or reverse the delayed tissue injury and *ex vivo* vascular hyporeactivity (Szabo, 1995). A variety of *in vitro* studies also confirm that inhibition of NO biosynthesis in immunostimulated cells or organs that express iNOS can prevent potent, toxic autocrine effects.

However, recent data have challenged the prevailing wisdom that NO is independently toxic (Pryor & Squadrito, 1995; Szabó, 1996, see also: Introduction). In the present study, we obtained data which suggest that peroxynitrite, rather than superoxide contributes to the loss of vascular contractility and cellular energetics in a rodent model of endotoxic shock. This evidence is necessarily indirect, inasmuch as there is currently no existing specific and effective direct scavenger of peroxynitrite. From the range of TBAP analogue presently studied, we have selected MnTBAP for *in vivo* studies, despite, the fact that FeTBAP was more potent in the *in vitro* assays. The reason for this was two fold: (1) stability issues: the stability of MnTBAP is higher than that of FeTBAP (Day, unpublished observations) and (2) practical reasons (availability of the compounds for *in vivo* use). We found that MnTBAP partially blocked the loss of vascular contractility and fully inhibited the loss of mitochondrial respiration and the depletion of intracellular NAD in cells obtained from endotoxin-challenged animals. These protective effects were apparent despite the fact that another pharmacological action of superoxide dismutase or its analogue is the prolongation of the biological half-life of NO (Gryglewski *et al*., 1986; Rubanyi & Vanhoutte, 1986), by which MnTBAP would have enhanced 'purely' NO-mediated vasodilator or cytotoxic responses.

It is logical to assume that the pharmacological effects of MnTBAP arise from the combination of the following actions: (1) by inactivating superoxide, and thus preventing the formation of peroxynitrite, with consequent protection against the development of peroxynitrite-induced cellular energetic failure and consequent hypocontractility, (2) by scavenging peroxynitrite, direct inhibition of peroxynitrite-induced oxidative processes and (3) by scavenging superoxide, increasing the half-life of NO, resulting in the prolongation of vasodilatation. At present, we cannot see a suitable pharmacological approach to separate the above factors. While the partial protection by MnTBAP against the vascular hyporeactivity points towards the contribution of additional (such as NO- and cyclic GMP-mediated) factors in this response, the near-complete protection by MnTBAP against the suppression of cellular energetic status in the macrophages may suggest the central role of peroxynitrite as opposed to NO in these latter changes (see also below).

What, then, is the mechanism of peroxynitrite-mediated toxicity during endotoxic shock? We have recently identified polyadenosine 5′-diphosphate (ADP) ribosyltransferase (PARS) as a pathway contributing to peroxynitrite toxicity in immunostimulated macrophages *in vitro* (Szabó *et al*., 1996b; Zingarelli *et al*., 1996) and in endotoxic shock *ex vivo* (Szabó *et al*., 1996c). Peroxynitrite (but not NO) is a potent trigger of DNA single strand breaks in macrophages, smooth muscle cells and epithelial cells *in vitro*, and that DNA single strand breaks activate the nuclear enzyme PARS, which causes excessive ADP ribosylation of nuclear proteins, with consequent intracellular NAD and ATP depletion and cellular injury (Szabó *et al*., 1996b). Thus, it is conceivable that the important pathway in endotoxic shock involves production of NO and superoxide >> generation of peroxynitrite >> genesis of DNA single strand breaks >> activation of PARS >> cellular energetic dysfunction >> contractile dysfunction and organ failure. Peroxynitrite may also induce PARS-independent toxic effects, such as lipid peroxidation, direct effects on the mitochondrial respiratory enzymes (e.g. aconitase), and other actions. In addition to peroxynitrite-mediated pathophysiological mechanisms, the importance of solely NO-mediated (e.g. cyclic GMP-dependent) vasodilator mechanisms in the pathogenesis of endotoxin shock cannot be disputed. Superoxide may also have numerous, cytotoxic effects independent of NO and peroxynitrite, and it is clear that MnTBAP has potent protective effects in experimental systems where the injury is likely to be mediated by superoxide alone (Day *et al*., 1995; Patel *et al*., 1996). The protection by MnTBAP against the development of endothelial dysfunction in the present study may be related to either superoxide or peroxynitrite. Both superoxide and peroxynitrite have been implicated in the pathogenesis of endothelial dysfunction in various pathophysiological conditions (Villa *et al*., 1994; White *et al*., 1994; Szabó *et al*., 1996a). Moreover, the lack of improvement in survival rate by MnTBAP in mice challenged with a high dose of LPS suggests the importance of additional pathways (not related to superoxide or peroxynitrite) in the toxic effects of LPS. The extent of contribution of these pathways remains to be further established.

The present data, demonstrating protective effects of a superoxide dismutase mimetic in experimental models of endotoxic shock that are known to be related to NO overproduction (Szabó, 1996; 1995), support the role for peroxynitrite in the pathogenesis of this condition. The protective effects of MnTBAP are likely to be due to two independent pharmacological actions: (1) reduction of superoxide formation due to the superoxide dismutase-like activity of the compound and (2) scavenging of peroxynitrite. Based on the complex chemistry and interactions of peroxynitrite, in pathophysiological states other than circulatory shock, the role of peroxynitrite may be quite different. While the specific role of peroxynitrite remains to be further investigated in various pathophysiological states, our findings support the view that it is of importance to develop and test therapeutic strategies based around the generation or actions of peroxynitrite for the experimental therapy of circulatory shock, and, possibly, other inflammatory conditions.

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