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Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger

Csaba Szabó[a,*], Brian J. Day[b], Andrew L. Salzman[a]

[a]*Children's Hospital Medical Center, Division of Critical Care, 3333 Burnet Avenue, Cincinnati, OH 45229, USA*
[b]*Duke University Medical Center, Department of Medicine, Division of Pulmonary and Critical Care Medicine, Durham, NC 27710, USA*

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Abstract Here we report that the cell-permeable superoxide dismutase mimetic Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) inhibits the oxidation of dihydrorhodamine-123 by peroxynitrite, but does not scavenge nitric oxide (NO). MnTBAP protects against the suppression of mitochondrial respiration in J774 cells exposed to peroxynitrite or to NO donors. MnTBAP and N^G-methyl-L-arginine provide additive protective effect against the suppression of respiration in immunostimulated cells. Our data suggest separate contributions of NO and peroxynitrite to the suppression of mitochondrial respiration and support the role of oxidative stress in the expression of the inducible isoform of NO synthase.

Key words: Peroxynitrite; Nitric oxide; Endotoxin; Shock; Inflammation; Nitric oxide synthase; Superoxide; Mn(III) porphyrins; Superoxide dismutase

1. Introduction

Superoxide (O_2^-), nitric oxide ($^{\bullet}NO$), and peroxynitrite all have been proposed to mediate cellular damage under conditions of shock, inflammation and oxidative stress [1–3]. Multiple interactions of NO and superoxide have been described. Initially, it was proposed that superoxide acts as an inactivator of NO, since superoxide dismutase prolongs the biological half-life of NO [4,5]. Along the lines of this concept, it has been demonstrated that NO can limit the cytotoxicity of superoxide [6,7]. On the other hand, the reaction of NO and superoxide has been shown to yield peroxynitrite, a reactive oxidant species, and an important mediator of cell damage under conditions of inflammation and oxidant stress [3,8,9]. Current data suggest that NO can act as an inactivator of the biological activity of peroxynitrite [10,11], and that the biological activity and decomposition of peroxynitrite is very much dependent on cellular or chemical environment (presence of proteins, thiols, glucose, carbon dioxide and other factors) [12–15].

Another level of regulation of NO production by superoxide exists at the level of the regulation of the expression of the inducible isoform of NO synthase (iNOS). Oxidant stress upregulates iNOS mRNA [16] and is involved in the activation of nuclear factor kappa B expression [17], a key intracellular pathway in the process of iNOS induction in response to bacterial lipopolysaccharide [18].

*Corresponding author. Fax: (1) (513) 559-4892.

The direct investigation of the role of endogenously produced superoxide in the regulation of the expression of iNOS and in biological activity of NO action has been hampered by the lack of potent, biologically useful, cell-permeable superoxide dismutase mimetics. Previously, a number of such compounds have been synthesized and characterized [19]. However, many of these agents are not stable in the presence of metal chelators, present in biological systems. Recently, a new class of superoxide dismutase mimetics (metal complexes of substituted mesoporphyrins) has been described which overcomes this limitation [20], and protects endothelial cells against oxidant stress due to paraquat exposure [21].

In the present study we characterized the actions of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) [20,21] on peroxynitrite and NO and show that MnTBAP is a potent inhibitor of a peroxynitrite-induced oxidative reaction, but is not a scavenger of NO. Here we investigate in the J774 monocytic-macrophage cell line in vitro: (1) the potential protective effect of MnTBAP against the cell damage in response to authentic peroxynitrite; (2) the relative importance of endogenously produced peroxynitrite in the cytotoxicity in response to NO donor compounds; (3) the role of oxidant stress to the induction of iNOS in response to various forms of immunostimulation; and (4) the relative contribution of NO vs. superoxide or peroxynitrite to the depression of cellular respiration in immunostimulated macrophages.

2. Materials and methods

2.1. Materials

Bacterial lipopolysaccharide (LPS, *E. coli*, serotype No. 0127:B8), L-glutamine and MTT were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Gibco (Grand Island, NY). N^G-methyl-L-arginine (L-NMA) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) were purchased from Calbiochem (La Jolla, CA). Diethylamine:NO NONOate (DNO) was purchased from RBI (Natick, MA). Dihydrorhodamine-123 was from Molecular Probes Inc. (Eugene, OR). Murine γ-interferon (IFN) was obtained from Genzyme (Cambridge, MA). Mn(III)-tetrakis (4-benzoic acid) porphyrin (MnTBAP) was kindly provided by Dr. James Crapo (Duke University Medical Center, Durham, NC). Peroxynitrite was kindly provided by Dr. Harry Ischiropoulos (University of Pennsylvania, Philadelphia, PA).

2.2. Cell culture

J774 macrophages were cultured in DMEM medium, supplemented with L-glutamine (3.5 mmol/l) and 10% fetal calf serum, as described [22]. Cells were cultured in 96-well plates (200 μl medium/well) until confluence. To induce iNOS, fresh culture medium containing *E. coli* LPS (011:B4; 10 μg/ml) alone or in combination with murine γ-interferon (γ-IFN; 10 U/ml) was added in the presence or absence of the

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NOS inhibitor, the SOD mimetic or the combination of the two compounds for 24 h. Moreover, cells were exposed to the NO donor compounds SNAP (3 mM) and DNO (3 mM) for 24 h or to authentic peroxynitrite (1 mM) for 1 h. Nitrite/nitrate concentration in the medium and mitochondrial respiration were then measured as described below.

2.3. Measurement of nitritenitrate production

Nitrite/nitrate production, an indicator of NO synthesis, was measured in the supernatant, as previously described [22]. First, nitrate in the culture medium was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μM) at room temperature for 2 h. After 2 h, nitrite concentration in the samples was measured by the Griess reaction, 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 using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in culture medium. All measurements were corrected for the interference of MnTBAP at this wavelength. MnTBAP (up to 300 μM) did not scavenge nitrite or nitrate and did not interfere with the activity of nitrate reductase.

2.4. 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 [22]. 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}. All measurements were corrected for the interference of MnTBAP at this wavelength.

2.5. Measurement of peroxynitrite-induced oxidation of dihydrorhodamine-123

The peroxynitrite-dependent oxidation of dihydrorhodamine-123 to rhodamine-123, was measured based on the principles of the method described [22,23]. Briefly, peroxynitrite at 5 μM was added into phosphate-buffered saline containing 10 μM dihydrorhodamine 123, in the absence or presence of MnTBAP (3–100 μM). After a 10 min incubation at 22°C, the fluorescence of rhodamine 123 was measured using 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).

2.6. Measurement of NO-induced vascular relaxations

New Zealand white rabbits weighing 3–4 kg were anesthetized with pentobarbital (30 mg/kg). The descending thoracic aorta was isolated, removed, cleaned and placed in Krebs buffer (pH 7.5). Vessels were cut into 5 mm rings and hung on stirrups connected to force transducers. Rings were suspended in 20 ml jacketed baths which were kept at 37°C and bubbled with 95% O_2 and 5% CO_2. Rings were equilibrated with 2 g resting tension for 1 h before use and contracted with phenylephrine. Saturated NO solution was prepared by bubbling compressed NO gas through a NaOH trap and then into anaerobic deionized water. Aliquots of the nitric oxide solutions (final concentration: 75–300 nM) were added to the rings (in the presence or absence of 100 μM MnTBAP) and relaxant responses were recorded.

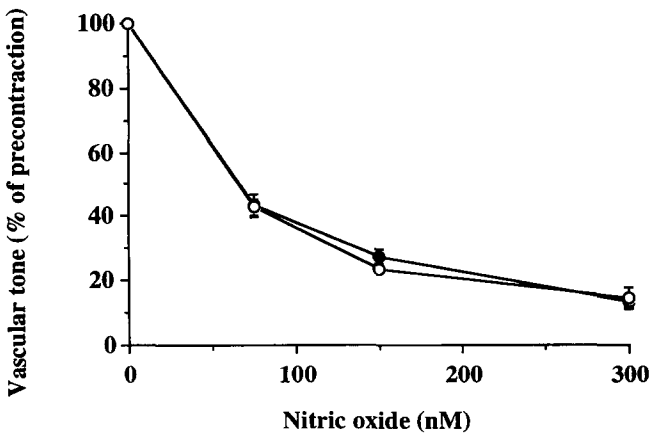

Fig. 1. Relaxation in response to authentic nitric oxide in precontracted rabbit aortic rings in the absence (closed circles) and presence (open circles) of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (100 μM). Data are expressed as means ± S.E.M. of $n = 4$–8 rings.

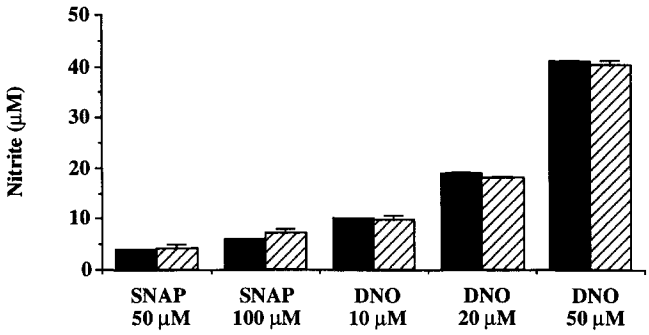

Fig. 2. Accumulation of nitrite at 2 h after *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; 50 and 100 μM) or diethylamine:NO NONOate (DNO; 10, 20 and 50 μM), absence (closed bars) and presence (hatched bars) of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (300 μM). Data are expressed as means ± S.E.M. of $n = 6$ wells.

2.7. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of *n* observations, where *n* represents the number of wells studied (12 wells from 2–3 independent experiments). Data sets were examined by 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

3.1. MnTBAP is not a scavenger of nitric oxide

MnTBAP did not inhibit the relaxations in vascular rings in response to authentic NO (Fig. 1). Moreover, MnTBAP, at 300 μM did not inhibit nitrite/nitrate accumulation in the culture medium in response to the NO donor compound *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), and caused a slight inhibition in response to diethylamine:NO NONOate (DNO) (Fig. 2). These data, and the finding that NO does not affect the spectral changes of MnTBAP soret band (not shown), suggest that NO does not complex with the manganese in MnTBAP (unlike the known reaction of NO with the iron in Fe-protoporphyrin complexes).

3.2. MnTBAP inhibits peroxynitrite-mediated oxidation

Peroxynitrite induced significant oxidation of dihydrorhodamine-123 to rhodamine-123, which was dose-dependently inhibited by MnTBAP with 50% inhibition at 30 μM (Fig. 3). This suggests that MnTBAP, similar to a number of free radical scavengers (cysteine, urate, ascorbate, α-tocopherol), inhibits the oxidations caused by peroxynitrite.

3.3. MnTBAP inhibits the suppression of mitochondrial respiration by authentic peroxynitrite in J774 cells

Mitochondrial respiration was profoundly inhibited by exposure to 1 mM peroxynitrite at 1 h (Fig. 4a). This effect was partially and dose dependently prevented by MnTBAP (Fig. 4a).

3.4. *MnTBAP inhibits the suppression of mitochondrial respiration by NO donors in J774 cells*

Mitochondrial respiration was also inhibited by exposure to the NO donor compound *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (Fig. 4b) and diethylamine:NO NONOate (DNO) for 24 h (Fig. 4c). This effect was partially and dose dependently prevented by MnTBAP (Fig. 4b,c)

3.5. *Effects of MnTBAP on NO production and suppression of mitochondrial respiration in immunostimulated J774 macrophages*

Immunostimulation of the cells by lipopolysaccharide (LPS; 10 μg/ml) alone, and, more pronouncedly, in the presence of γ-interferon (IFN; 10 U/ml), resulted in nitrite/nitrate formation and a pronounced inhibition of the mitochondrial respiration (Fig. 5). Administration of IFN alone did not induce detectable nitrite/nitrate production and only caused a mild (<15%) suppression of respiration ($n = 12$, not shown).

MnTBAP caused a dose-dependent inhibition of nitrite/nitrate production in cells stimulated with LPS (Fig. 5a). However, in cells immunostimulated with the combination of LPS and IFN, MnTBAP caused a less pronounced inhibition of nitrite/nitrate accumulation (300 μM) (Fig. 5a). For instance, at 100 μM and 300 μM, MnTBAP caused a significant, 63 and 86% inhibition of the LPS-induced nitrite/nitrate accumulation. When given in the combined presence of LPS and IFN, 100 μM MnTBAP only caused a 25% inhibition of nitrite/nitrate accumulation and pronounced inhibition (61%) was only observed at the highest concentration of MnTBAP tested (300 μM). L-NMA caused a near-complete inhibition of the production of NO in cells stimulated with LPS or LPS and IFN; and MnTBAP had no additional effect on nitrite/nitrate formation in the presence of L-NMA (Fig. 5a). The inhibition of nitrite/nitrate accumulation by MnTBAP in LPS-stimulated macrophages diminished by more than 50% when the agent was applied 6 h *after* LPS, whereas in the case of the inhibition seen with L-NMA the extent of inhibition seen was similar when the compound was given together with LPS or at 6 h thereafter ($n = 6$).

MnTBAP caused a dose-dependent, partial restoration of the immunostimulation-induced suppression of the mitochondrial respiration in both LPS-treated and (less potently) LPS and IFN-treated cells (Fig. 5b). Inhibition of NOS with L-NMA caused a restoration of the respiration to an extent comparable with that of 300 μM MnTBAP (Fig. 2b). The combined administration of 300 μM MnTBAP and 3 mM L-NMA caused an additional restoration of the mitochondrial respiration (Fig. 5b). In the presence of L-NMA and MnTBAP, respiration was restored to initial levels in the LPS-stimulated cells, but remained below normal in cells stimulated with LPS and IFN (Fig. 5b).

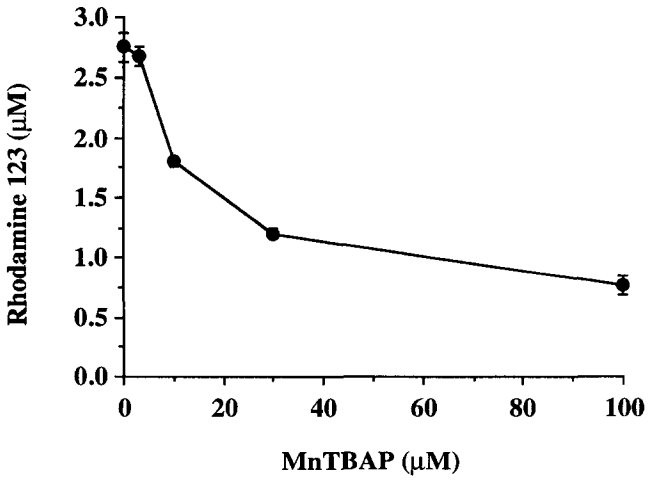

Fig. 3. Effect of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (3–100 μM) on the oxidation of dihydrorhodamine-123 to rhodamine-123 in response to peroxynitrite (5 μM). Data are expressed as means ± S.E.M. of triplicate determinations.

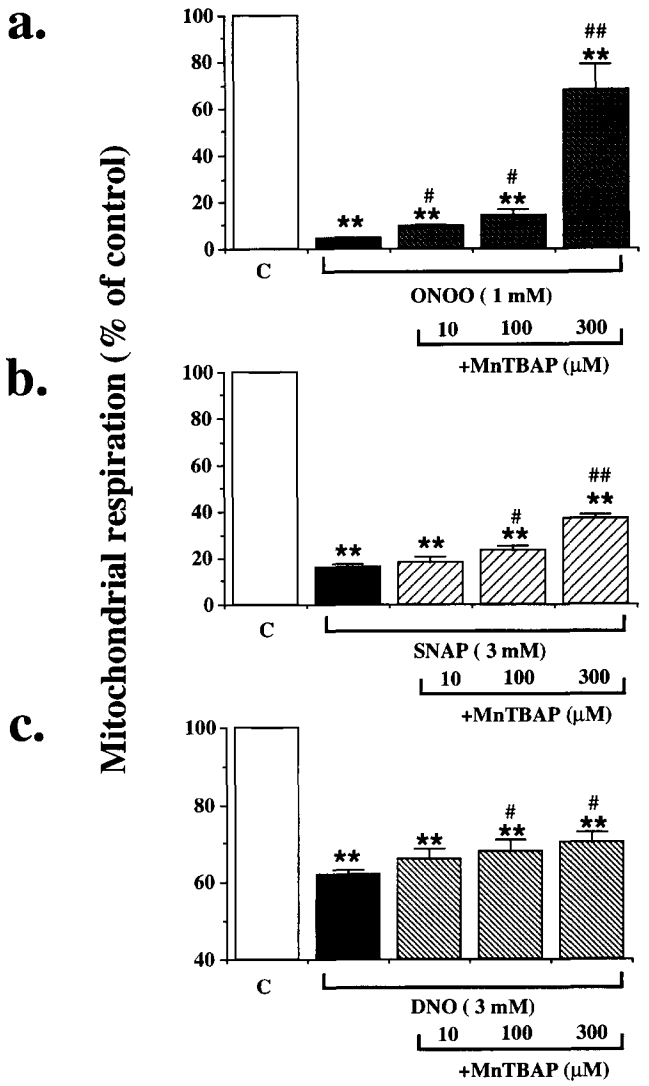

Fig. 4. Suppression by the peroxynitrite (a) and the NO donor compounds *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; 3 mM) (b) and diethylamine:NO NONOate (DNO) (c) on mitochondrial respiration (expressed as percent of respiration of unstimulated cells) in J774 macrophages, and the protective effect of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (10–300 μM) against this suppression. Data are expressed as means ± S.E.M. of $n = 12$ wells. $^{**}P < 0.01$ represents significant effect of SNAP when compared to control (C) values; · represent significant protective effects of MnTBAP ($P < 0.05$ and $P < 0.01$, respectively).

4. Discussion

The present data demonstrate that MnTBAP, a compound that has been described as a potent and stable superoxide

dismutase mimetic [20,21], is also a potent inhibitor of the peroxynitrite-mediated oxidation of dihydrorhodamine-123 to rhodamine-123. This property of the drug is likely to explain the pronounced protective effect of the drug against the suppression of the mitochondrial respiration in response to authentic peroxynitrite. Many oxygen radical scavengers, including ascorbate, cysteine, α-tocopherol and Trolox have been described as inhibitors of peroxynitrite-mediated oxidative reactions. It is noteworthy that a recent report demonstrates that (5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphinato)manganese III (MnTMPyP) reacts with peroxynitrite and catalyzes its decomposition [24].

The protective effects of MnTBAP (as a superoxide dismutase mimetic *and* peroxynitrite scavenger) on SNAP- and DNO-induced suppression of mitochondrial respiration suggests that part of the NO-mediated depression of mitochondrial respiration is dependent on basal superoxide and peroxynitrite formation. Superoxide presumably derives from the mitochondria. Previous work has demonstrated that peroxynitrite is a more potent inhibitor of aconitase [25,26] and a more potent antimicrobial agent [27] than NO. However, the protection by MnTBAP against these fairly high concentrations of the NO donor compounds (3 mM) was only partial, so part of the effect of NO on the mitochondrial respiration in J774 cells is peroxynitrite-independent. The observation that MnTBAP protects against NO toxicity is in line with recent findings showing that oxygen radical neutralization by vitamin E protects islet cells against SNAP [28], that Trolox protects cells against NO- or peroxynitrite-mediated injury [29,30], and that SNAP toxicity in the gut can be prevented by superoxide dismutase [31].

Less data are available regarding the *endogenous* formation

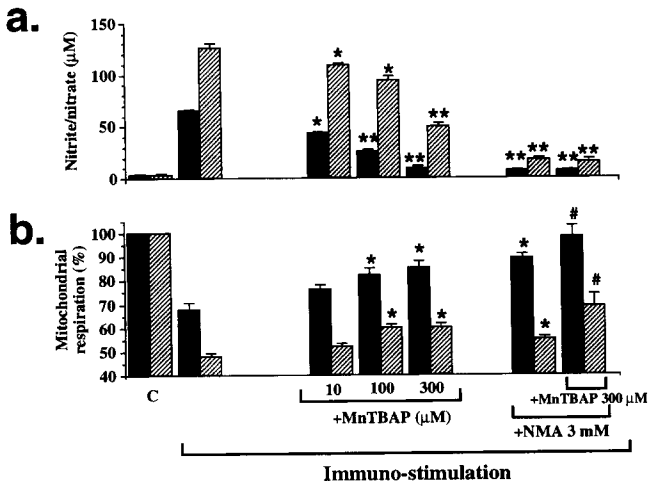

Fig. 5. Effect of bacterial lipopolysaccharide (10 μg/ml) alone (closed bars) or together with murine γ-interferon (10 U/ml) (hatched bars) on nitrite/nitrate production (expressed as μM; upper panels) and mitochondrial respiration (expressed as percentage of unstimulated control, C; lower panels) at 24 h after immunostimulation, and the effect of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (10–300 μM), the nitric oxide synthase inhibitor N^G-methyl-L-arginine (L-NMA; 3 mM) or the combination of 3 mM L-NMA and 300 μM MnTBAP on these changes. Data are expressed as means ± S.E.M. of $n = 12$ wells. $^{**}P<0.01$ represents significant effect of L-NMA or MnTBAP alone, represents significant additional protective effects of combined administration of L-NMA and MnTBAP, when compared to the effect of L-NMA or MnTBAP alone ($P<0.05$).

of superoxide or peroxynitrite in the cellular toxicity in response to immunostimulation. We have previously studied the time course of superoxide production, nitrite/nitrate, and peroxynitrite formation in J774 cells and found that superoxide is produced rapidly within 1 h after stimulus and its production is sustained over 24 h, whereas nitrite and nitrate is formation begins at 6 h after LPS, and peroxynitrite is produced parallel with nitrite/nitrate [22]. Here we demonstrate that inactivation of endogenous superoxide or peroxynitrite protects cells from the suppression of mitochondrial respiration after immunostimulation, supporting the previous proposition [32] that endogenous peroxynitrite is involved in the suppression of mitochondrial respiration in immunostimulated macrophages. However, the results derived from the experiments with the simultaneous administration of L-NMA and MnTBAP suggest that NO may also contribute to the suppression of mitochondrial respiration in a peroxynitrite-independent fashion in immunostimulated cells. The alternative explanation, that small amounts of NO that may be produced despite the presence of 3 mM L-NMA, would be sufficient to form cytotoxic amounts of peroxynitrite, appears unlikely.

The protection with L-NMA and MnTBAP was complete in cells stimulated with LPS. This suggests that a model proposing the involvement of oxygen and nitrogen free radicals and related oxidants is sufficient to explain the observed changes in this particular experimental system. However, with LPS and IFN used as a stimulation, the protection was not complete even with the combined application of L-NMA and MnTBAP, suggesting the existence of additional pathways contributing to the cytotoxic effect of immunostimulation.

MnTBAP inhibited nitrite formation when given together with LPS, but the inhibition diminished when the agent was added several hours after the stimulus of induction. This suggests (see [2]) supports the role of oxidant stress in the process of iNOS induction. (Since there is no significant nitrite/nitrate production before 6 h under the present experimental conditions [22], it must be an effect of MnTBAP on superoxide and not on peroxynitrite that is involved in modulating the induction process.) It is noteworthy that the inhibition of nitrite/nitrate formation was near-complete in response to LPS but was less effective when both LPS and IFN were used for stimulation. There are separate promoter regions of iNOS that are responsible for initiation of iNOS mRNA transcription in response to LPS and IFN, respectively [18]. Our data are in agreement with the proposal that the LPS-mediated iNOS induction is largely dependent on the activation of NF-kB activation (regulated by oxidant stress), whereas iNOS induction by IFN activates separate pathways [18].

There are distinct intracellular pathways responsible for the cytotoxic effects of NO, superoxide and peroxynitrite, and dissection of them is of importance in various experimental and pathophysiological conditions. For instance, DNA single strand breakage, and activation of an energy-depleting repair cycle governed by the DNA repair enzyme poly-ADP ribosyltransferase can be initiated by peroxynitrite, but not superoxide or NO in J774 cells [22,33]. The present data provide direct evidence for the role of endogenous peroxynitrite in the suppression of respiration in immunostimulated cells. Thus, it may be of therapeutic importance to develop therapeutic strategies aimed at interfering with the production or actions of peroxynitrite. Future studies, using MnTBAP, as a superoxide

dismutase mimetic and peroxynitrite scavenger are likely to provide useful information on the role of peroxynitrite in various pathophysiological conditions.

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