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Research Article

Dual role of endogenous nitric oxide in tumor necrosis factor shock: induced NO tempers oxidative stress

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Abstract. Tumor necrosis factor (TNF) is involved in pathologies like septic shock, inflammatory bowel disease and rheumatoid arthritis. TNF and lipopolysaccharide can incite lethal shock, in which cardiovascular collapse is centrally orchestrated by the vasodilating free radical nitric oxide (NO). However, NO synthase (NOS) inhibition causes increased morbidity and/or mortality, suggesting a dual role for NO. To investigate the potential protective role of NO during TNF shock, we treated mice with TNF with or without NOS inhibition. Experiments in endothelial-NOS- and inducible NOS-deficient mice identified inducible NOS as the source of protective NO. Distinctive TNF-induced lipid peroxidation, especially in liver and kidney, was aggravated by NOS inhibition. In addition, various antioxidant treatments and a phospholipase A2 (PLA2) inhibitor prevented sensitization by NOS inhibition. Together, these in vivo results indicate that induced NO not only causes hemodynamic collapse, but is also essential for curbing TNF-induced oxidative stress, which appears to hinge on PLA2-dependent mechanisms.

Key words. Mice; TNF shock; nitric oxide; oxidative stress.

List of abbreviations: AA (arachidonic acid), BCG (*Bacillus Calmette-Guérin),* BHA (butylated hydroxyanisole), DPI (diphenyleneiodonium), FLAP (5-lipoxygenase activating protein), HNE (4-hydroxy-2-nonenal), L-NAME (N^G-nitro-L-arginine methyl ester), NO (nitric oxide), NOS (NO synthase), PAF (platelet activating factor), PLs (phospholipids), PLA2 (phospholipase A2), R (receptor), ROS (reactive oxygen species), sGC (soluble guanylate cyclase), (SOD) superoxide dismutase, (3-NP) 3-nitropropionic acid

Tumor necrosis factor (TNF) is a proinflammatory cytokine implicated in many different diseases including rheumatoid arthritis, Crohn's disease, diabetes and septic shock [1, 2]. In addition, more recent studies have even revealed a dual role for TNF in certain diseases, promoting ischemic injury on the one hand, while mediating the phenomenon of ischemic preconditioning on the other [3–5]. TNF was originally discovered as a factor with extraordinary antitumor activity, but its shock-inducing properties still prevent its systemic use in cancer treatment. Clinical trials revealed that hypotension is the major dose-limiting factor in TNF-based therapy [6]. The refractory hypotension observed in septic shock and in lipopolysaccharide (LPS) or TNF toxicity is mediated by the vasodilator nitric oxide (NO), as inhibition of NO synthases (NOS) prevents hemodynamic collapse [7–10]. However, many of these studies suggested a possible dual role for NO. In septic shock patients also, nonselective

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NOS inhibition was associated with an increased death rate, despite beneficial effects on systemic blood pressure and vascular resistance [11, 12]. To achieve selective inhibition of the deleterious effects of NO, most research focuses on specific inhibitors of the inducible (i)NOS (NOS-2), as the NO released by constitutive endothelial (e)NOS (NOS-3) is considered beneficial. Whereas selective iNOS inhibition successfully restored systemic blood pressure in most studies [13, 14], peripheral organ failure was attenuated only partially or not at all [14–16]. Intravenous injection of mouse (m)TNF in mice induces NO production and cardiovascular collapse. We have previously shown that both shock and mortality can be prevented by inhibitors of soluble guanylate cyclase (sGC) activation, but not by NOS inhibitors [17]. In addition, sGC inhibition cannot protect against TNF toxicity in the absence of induced NO (using NOS inhibitors or iNOS-deficient animals), suggesting a protective function for induced NO.

Lipid peroxidation is a characteristic aspect of many inflammatory diseases and is causally involved in many of the pathological effects associated with oxidative stress, which may be defined as an excess of reactive oxygen species (ROS) generation relative to antioxidant defenses. ROS production can be the result of NADPH oxidase or xanthine oxidase activity, mitochondrial respiration, arachidonic acid (AA) metabolism, or 'uncoupled' NOS activity under conditions of substrate or cofactor deficiency [18, 19]. Natural antioxidant defenses include superoxide dismutase (SOD), catalase, glutathione peroxidase, iron storage and transport proteins, and the antioxidant vitamins E and C. NO is a free radical with an unpaired electron in the highest orbital, enabling its rapid reaction with other free radicals, leading to either pro- or antioxidant effects. When equimolar concentrations of nitric oxide and superoxide are present, peroxynitrite is formed, a powerful oxidant [20, 21]. Nevertheless, NO can protect against oxidative stress as well. By itself, NO can neutralize superoxide and lipid peroxyl radicals [21, 22]. In this context, NO is actually a more potent inhibitor of lipid peroxidation than vitamin E and even protects vitamin E from oxidation [23]. Furthermore, vitamin E and NO cooperatively inhibit lipid peroxidation, exhibiting greater antioxidant capacity than vitamin E and C combined. Therefore, an excess of superoxide or equimolar concentrations of NO and superoxide promotes lipid peroxidation, whereas an excess of NO neutralizes superoxide and inhibits lipid peroxidation [22]. The anti-adhesive properties of (iNOS-derived) NO also depend on its ability to inactivate superoxide [24, 25]. Next to the direct scavenging of superoxide or lipid peroxyl radicals, NO can also indirectly reduce oxidative stress by inhibiting NADPH oxidase [26], upregulating SOD [27], or inhibiting potential initiators of lipid peroxidation such as peroxidase and lipoxygenase enzymes [28]. However, most of the studies designed to examine the role of NO in oxidative stress have used cellular systems and/or exogenous NO donors, instead of inflammatory molecules, as a source of NO. Hence, we decided to investigate whether antioxidative activities of endogenous NO might be protective during TNF-induced shock in vivo. Theoretically, NO can interfere with oxidative stress on many different levels, and so we tried to pinpoint at what level NO might be acting by comparing various antioxidant treatments and pharmacological inhibitors of different ROS-producing systems. In summary, our experiments indicate that endogenous NO is indeed necessary to temper TNF-induced oxidative stress in vivo, which seems to be largely dependent on PLA2-mediated effects. TNF-induced lipid peroxidation in liver and kidney was aggravated by NOS inhibition, and the comparison of various antioxidative treatments suggests that the protective antioxidant effect of NO is situated intracellularly within a lipophilic context. We therefore propose that attenuation of lipid peroxidation is the major chemical mechanism by which endogenous NO may limit TNF-induced oxidative injury in vivo.

Materials and methods

Mice

Female C57BL/6 mice were purchased from Janvier (Le Genest-St-Isle, France), gp91-deficient animals [29] from The Jackson Laboratory (Bar Harbor), from which we also bought iNOS-deficient [30] and eNOS-deficient [31] breeding couples on a C57BL/6 background, which were bred as homozygotes in our facilities. Female offspring were used for all the experiments described. Mice were housed in temperature-controlled, air-conditioned facilities with 14–10 h light/dark cycles and food and water ad libitum, and used at the age of 8–12 weeks All experiments were approved by and performed according to the guidelines of the animal ethics committee from Ghent University, Belgium.

Cytokines, reagents and injections

Recombinant mTNF was produced in and purified from *Escherichia coli*. The endotoxin content was <0.02 ng/mg, as assessed by a chromogenic *Limulus* amebocyte lysate assay (NODIA BV, Antwerp, Belgium). Injections with mTNF were intravenous (i.v.). (diluted in 200 µl endotoxin-free PBS); the LD_{100} was tested before each individual experiment and ranged from 15 to 25 µg, depending on the batch. Lethality was always scored up to 7 days after challenge. The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from Novabiochem (Bierges, Belgium) and injected i.v. at a dose of 100 mg/kg (together with TNF). Butylated hydroxyanisole (BHA; 550 mg/kg; Sigma, St. Louis, Mo.) was given 1 h before TNF, either orally (via gavage) or subcuta-

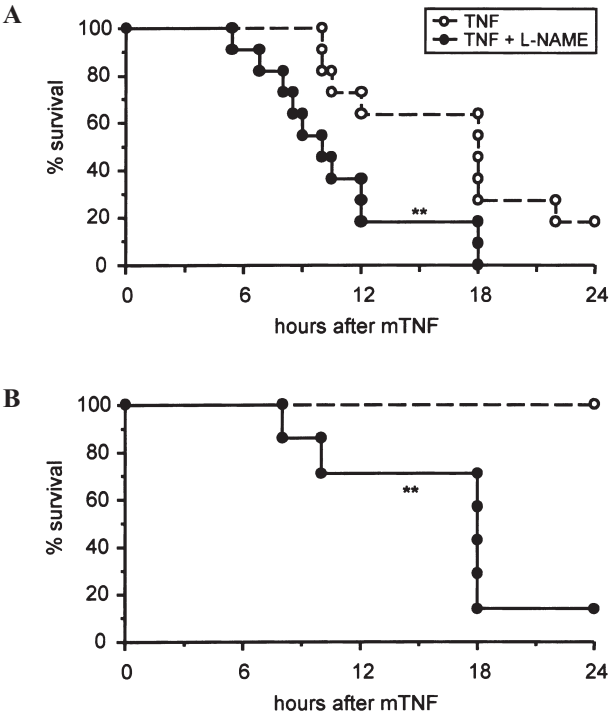

Figure 1. L-NAME sensitizes for TNF shock. (*A*) C57BL/6 mice were injected i.v. with 15 µg mTNF without or with L-NAME. Survival is presented as a combined Kaplan-Meyer plot of two independent, representative experiments. **p = 0.0091, n = 11. (*B*) C57BL/6 mice were injected i.v. with 10 µg mTNF without or with L-NAME. Survival is presented as a Kaplan-Meyer plot of a representative experiment. Lethality was recorded for 7 days, no further deaths occurred. **p = 0.021, n = 7.

neously (s.c.) in 50 µl DMSO. Tempol (Sigma) was injected twice intraperitoneally (i.p.) (265 mg/kg 40 min before and 110 mg/kg 2.5 h after TNF). SOD (155000 U/kg; ICN/MB Biomedicals Europe, Asse, Belgium) and catalase (Sigma; 80,000 U/kg) were also given twice i.p. (40 min before and 2.5 h after TNF). In some experiments, the tempol + catalase treatment was combined with 50 µl DMSO, given orally via gavage 1 h before TNF. Allopurinol (Sigma; 200 mg/kg) was injected s.c. 1 h before TNF in 100 µl 40% 1 N NaOH. Diphenyleneiodonium (DPI; ICN; 2.25 mg/kg), rotenone (Sigma, 5.3 mg/kg) and aristolochic acid (Sigma; 5.3 mg/kg) were administered s.c. in 50 µl DMSO about 80 min before TNF. 3-Nitropropionic acid (3-NP, Sigma; 20 mg/kg, –1.5 h) and indomethacin (Sigma; 5.5 mg/kg; –1 h) were injected i.p. in PBS. The FLAP inhibitor MK-886 (Biomol/Tebu-Bio, Boechout, Belgium; 22 mg/kg, –2 h) and the PAF-R antagonist CV-3988 (Biomol; 8.85 mg/kg, –1 h) were inoculated i.p. in 50 or 40 µl DMSO, respectively. The PAF-R antagonist BN-52021 (Biomol; 17.7 mg/kg, –45 min) was injected i.p. in 400 µl 0.9% NaCl. All treatment routes, schedules and doses were based on other mouse and/or rat studies where the given treatment had been reported to have a clearly positive or protective effect.

Immunohistochemical staining

Organs were dissected and immediately fixed in 4% paraformaldehyde at room temperature. After dehydration through baths of 50%, 70%, 95% and 100% ethanol and 100% histoclear (National Diagnostics, Atlanta, Ga.), tissues were embedded in paraffin (Sigma, St. Louis, Mo). Sections (4 µm) were prepared, followed by an overnight incubation at 37 °C. Sections were deparaffinized in xylene for 15 min, rehydrated in 100%, 96% and 70% ethanol each time for 7 min, and then washed with PBS for 5 min. Before 4-hydroxy-2-nonenal (HNE) staining, endogenous peroxidase activity was blocked with peroxidase blocking reagent (DAKO Diagnostics, Leuven, Belgium) for 5 minutes and then washed with PBS for 5 minutes. After incubation with pig serum (DAKO, Denmark) for 20 min, sections were incubated for 2 h at 37 °C with a rabbit anti-HNE antibody (1/500; Alpha Diagnostic International, San Antonio, Tex.). After washing, 'Envision' (horseradish peroxidase-conjugated anti-rabbit antibody; DAKO) was used as a secondary antibody for 45 min at room temperature. Immune complexes were detected with an aminoethylcarbazole substrate (DAKO). Sections were counterstained with hematoxylin (Sigma) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.).

Statistics

Survival curves were compared using a logrank test, total % lethality via a χ^2 test. To indicate statistical significance in the figures, we used *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Result

L-NAME sensitizes for TNF-induced lethality

Our previous results suggested a protective function for induced NO during TNF-induced shock [17]. To investigate the molecular target for this beneficial NO, we set up and studied an 'L-NAME sensitization' model. L-NAME injection alone did not cause any toxicity in mice, not even when 500 mg/kg was injected (data not shown). When L-NAME (100 mg/kg) was combined with a dose of mTNF approximating the LD_{100}, mice died significantly earlier (fig. 1A), while a sublethal or even nonlethal dose of mTNF became 90–100% lethal in combination with L-NAME (fig. 1B). L-NAME sensitization was comparable in wild-type (fig. 2A) and eNOS-deficient mice (fig. 2B). In contrast, iNOS-deficient animals were much more sensitive to mTNF per se (a nonlethal dose for wild-type or eNOS-deficient mice was 100% lethal in iNOS-deficient mice), and extra L-NAME treatment could not sensitize them further (fig. 2C), corroborating the protective capacity of NO in TNF shock and identifying iNOS as its source.

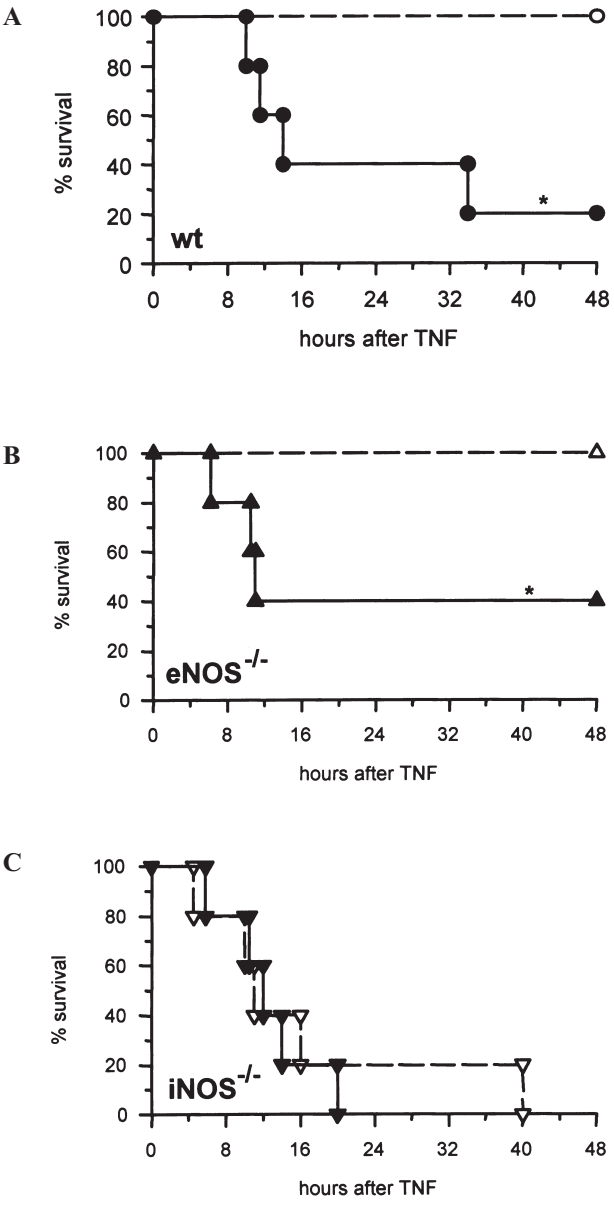

Figure 2. L-NAME sensitizes for TNF in eNOS-deficient but not in iNOS-deficient mice. Wild-type C57BL/6 (*A*), eNOS$^{-/-}$ (*B*) and iNOS$^{-/-}$ (*C*) mice were injected i.v. with 10 µg mTNF without or with L-NAME. Lethality was recorded for 7 days, no further deaths occurred. *p = 0.0133 (*A*) or 0.0494 (*B*), n=5.

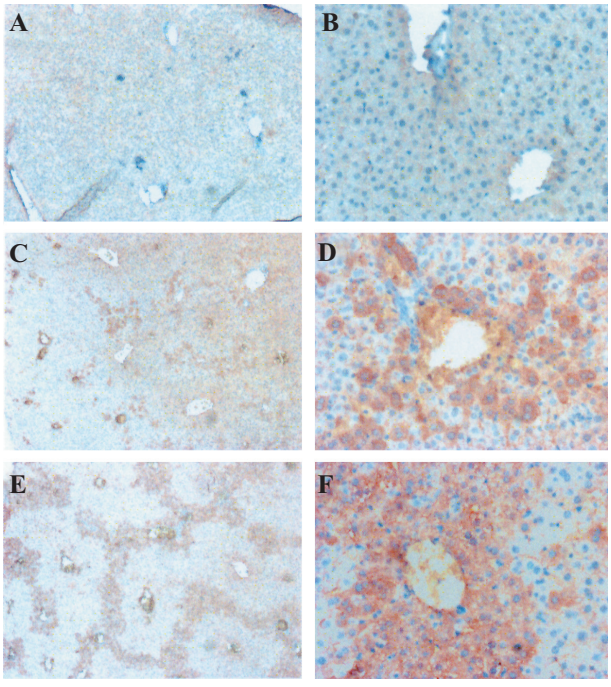

Figure 3. HNE immunolabeling in hepatocytes. HNE immunohistochemistry of livers collected 6 h after i.v. injection with PBS (*A*, *B*), a lethal dose of TNF (*C*, *D*) or TNF + L-NAME (*E*, *F*); magnification ×10 (*A*, *C*, *E*) or ×50 (*B*, *D*, *F*).

Immunohistochemical detection of HNE adducts in various organs

In vitro, both pro- and antioxidant activities have been attributed to NO, triggering a debate as to whether NO plays a harmful or protective role in tissue damage. Lipid peroxidation, for which the aldehydic metabolite HNE is considered one of the most reliable markers [32, 33], contributes significantly to oxidative injury to mammalian cells [34]. To study lipid peroxidation, we performed HNE immunohistochemistry on the kidney, liver, spleen, heart, lung and jejunum of PBS control-treated and TNF ± L-NAME challenged mice. The most striking differences were found in hepatocytes (fig. 3) and renal tubular cells (not shown) where we observed TNF-induced HNE immunoreactivity, aggravated by L-NAME. In the liver, TNF caused lipid peroxidation in hepatocytes, most notably in the vicinity of the hepatic vasculature (fig. 3C, D). After TNF challenge, HNE immunostaining was restricted to discrete clusters of hepatocytes (fig. 3C), whereas the combination of TNF with L-NAME caused lipid peroxidation in broad bands of hepatocytes, which seemed to match with the irregular hexagonal boundary of the hepatic lobules (fig. 3E). In contrast, no HNE immunolabeling was observed in control livers (fig. 3A, B).

Effect of antioxidant therapies on L-NAME sensitization

Several possible molecular mechanisms by which NO could counteract oxidative stress and membrane peroxidation in vivo can be envisioned. To establish which antioxidant activity specifically accounts for the protective effect of NO during TNF shock, we treated TNF + L-NAME-challenged mice with various antioxidant therapies, including the lipophilic food antioxidant BHA, catalase, SOD, or the cell-permeable SOD-mimetic tempol, known to increase the bioavailability of NO in vitro [35] and in vivo [36]. SOD and catalase could not reverse L-NAME sensitization, not even when coadministered (fig. 4A). Tempol was either ineffective or only marginally prevented

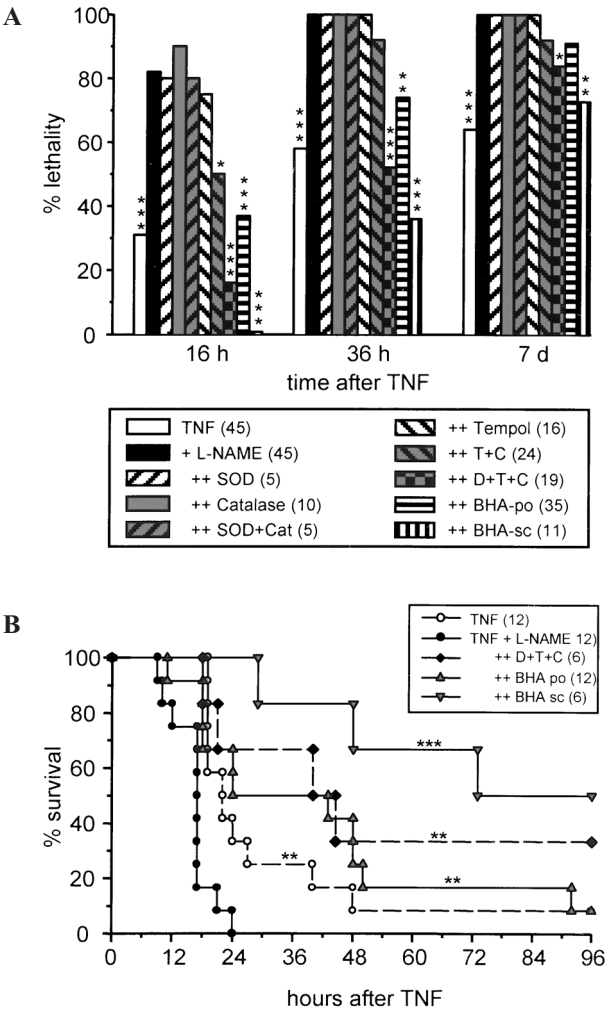

Figure 4. L-NAME sensitization and antioxidant treatment. (*A*) Plotted is the total % lethality of mice used in up to eight independent experiments; the total number of mice is indicated between brackets (five or six mice per treatment group in each individual experiment). TNF (LD_{60} to LD_{80}) was injected without or with L-NAME; antioxidant treatments used in combination with TNF + L-NAME were SOD, catalase, SOD + catalase, tempol, tempol + catalase, DMSO + tempol + catalase, oral or subcutaneous BHA. ***p ≤ 0.0002, **p = 0.0015, *p ≤ 0.0241, always compared with TNF + L-NAME. (*B*) Mice were injected i.v. with a lethal dose of mTNF without or with L-NAME. Survival is presented as a combined Kaplan-Meyer plot of two independent experiments (n indicated between brackets). In addition, TNF + L-NAME challenge was combined with antioxidant pretreatments, including DMSO + tempol + catalase (D+T+C), BHA ovally (po) or BHA s.c. ***p < 0.0001, ** $P \leq 0.0046$, versus ●.

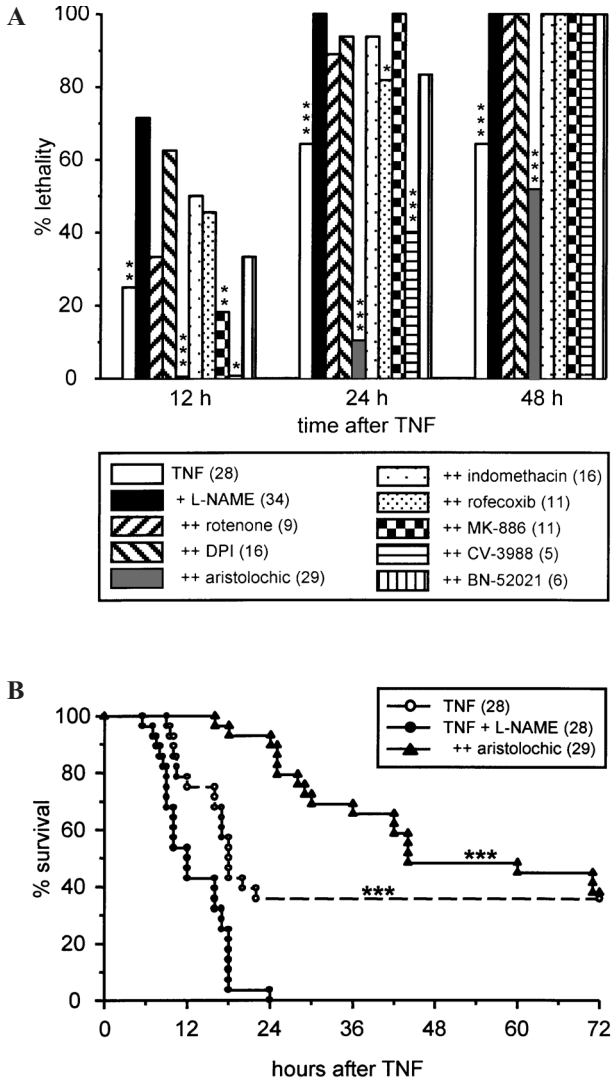

Figure 5. L-NAME sensitization in the absence of ROS-generating systems. (*A*) Plotted is the total % lethality (till 48 h after challenge) of mice used in up to five independent experiments; the total number of mice is indicated between brackets (five or six mice per treatment group in each individual experiment). TNF (LD_{60} to LD_{80}) was injected i.v. without or with L-NAME. Inhibitors used to block specific ROS production in combination with TNF + L-NAME include rotenone, DPI, aristolochic acid, indomethacin, rofecoxib, MK-886, CV-3988 and BN-52021. ***p ≤ 0.0007, **p ≤ 0.0093, *p ≤ 0.0394, always compared with TNF + L-NAME. (B) PLA2 inhibition by aristolochic acid prevents L-NAME sensitization. Survival is presented as a combined Kaplan-Meyer plot of four independent experiments (n=28–29 in total), ***p ≤ 0.0001 versus ●.

L-NAME sensitization, depending on the individual experiment. When tempol was combined with catalase, sensitization was significantly reduced (fig. 4A). This partial protection could be considerably improved by additional treatment with DMSO, a scavenger of hydroxyl radicals [37] (fig. 4A, B). Also orally (po) or subcutaneously (sc.) administered BHA prevented L-NAME sensitization (fig. 4A, B). In addition to preventing L-NAME-induced early TNF death (defined as 'sensitization'), the BHA and the DMSO + tempol + catalase treatments also significantly improved survival (fig. 4A, B). Taken together, these results suggest the involvement of intracellular superoxide. The protective efficacy of BHA may indicate that the ROS involved are generated and/or act in a lipophilic, hydrophobic environment, such as the cellular and/or organellar membranes.

L-NAME sensitization in the absence of specific ROS-producing systems

As the various antioxidant treatments indicated a role for NO in limiting TNF-induced oxidative stress in vivo, we decided to analyze L-NAME sensitization in the absence of specific ROS-producing systems. Mice with a disrupted gp91 phagocytic oxidase gene, encoding a subunit of NADPH oxidase [29], were as susceptible to L-NAME sensitization as wild-type animals (data not shown). Allopurinol, an inhibitor of the cytoplasmic xanthine oxidase, could not prevent L-NAME sensitization (not shown). The majority of intracellular ROS is often suggested to derive from mitochondrial respiration. In vitro, inhibition of mitochondrial complex I or II protects against TNF toxicity, especially when combined, whereas inhibition of complex III sensitizes the cells [38]. We therefore decided to block complex I and/or II in mice using rotenone, DPI and 3-NP. The results suggested that rotenone could prevent L-NAME sensitization, but were not statistically significant (fig. 5A). DPI is mostly used to inhibit NADPH oxidase, but as a general inhibitor of all NAD(P)H-dependent flavoenzymes it is as potent as rotenone in inhibiting the mitochondrial production of ROS [39]. Nevertheless, DPI could not prevent L-NAME sensitization (fig. 5A) and neither did inhibition of complex II by 3-NP (not shown).

Phosholipase A2 (PLA2)-dependent signaling constitutes another possible intracellular source of ROS. PLA2 liberates AA from phospholipids (PLs) and the subsequent conversion of AA into eicosanoids involves the generation of radical intermediates. To specifically inhibit PLA2 we used aristolochic acid [40, 41], which very significantly prevented L-NAME sensitization and prolonged survival (fig. 5A, B). We also evaluated the effects of aristolochic acid alone or in combination with rotenone or DPI, but the extra treatment did not result in extra protection (not shown). PLA2 activity generates AA, which is further metabolized via cyclooxygenase and lipoxygenase activities, and lysoPL, which is converted into platelet-activating factor (PAF). However, general cyclooxygenase inhibition by indomethacin, or cyclooxygenase-2 inhibition by rofecoxib, could not prevent L-NAME sensitization effectively (fig. 5A). When 5-lipoxygenase-activating protein (FLAP) was inhibited using MK-886, L-NAME sensitization was prevented more efficiently (fig. 5A). Cytosolic PLA2 is also essential for the production of PAF [42], a potent vasoactive and inflammatory phospholipid that causes superoxide production in activated leukocytes. Two different PAF-R antagonists prevented L-NAME sensitization, but only one (CV-3988) did so significantly and also prolonged survival, although less effectively than aristolochic acid (fig. 5A).

Table 1. Antioxidant treatments in wild-type and iNOS$^{-/-}$ mice

		Survival [c]		
Treatment [a]	Mice [b]	24 h	48 h	72 h
Solvent	wt	0/12		
BHA	wt	3/6 $^{++}$	1/6	0/6
D+T+C	wt	0/6		
Aristolochic acid	wt	0/6		
Indomethacin	wt	2/5 $^{+}$	1/5	1/5
Rofecoxib	wt	0/5		
MK-886	wt	0/5		
Solvent	–/–	0/12		
BHA	–/–	9/12 °°°	5/12 °	3/12 °
D+T+C	–/–	*** 9/9 °°°	** 7/9 °°°	**6/9 °°°
Aristolochic acid	–/–	0/12		
Indomethacin	–/–	5/8 °°	3/8 °	3/8 °
Rofecoxib	–/–	0/5		
MK-886	–/–	0/5		

[a] All mice were challenged with TNF (15 µg) and pretreated with solvent (PBS or DMSO), or BHA (s.c.), DMSO + tempol + catalase (D+T+C), aristolochic acid (PLA2 inhibitor), indomethacin (inhibits cyclooxygenase-1/2), rofecoxib (cyclooxygenase-2 inhibitor) or MK-886 (FLAP inhibitor).

[b] Age-matched female wt or iNOS$^{-/-}$ mice.

[c] Number of living mice/total number of mice after 24, 48 or 72 h; no futher deaths occurred after 72 h.

$^{+}$ Significantly different ($^{++}p = 0.0073$, $^{+}p = 0.0197$) from wt mice receiving control pretreatment (solvent), as determined by a two-tailed χ^2 test.

° Significantly different ($°°°p < 0.0008$, $°°p = 0.0016$, $°p < 0.022$) from iNOS$^{-/-}$ mice receiving control pretreatment (solvent), as determined by a two-tailed χ^2 test

* Significantly different ($***p = 0.0001$, $*p < 0.01$) from wt mice treated and challenged in exactly the same way, as determined by a two-tailed χ^2 test.

Effect of antioxidants on TNF toxicity in iNOS-deficient mice

Mice were sensitized to TNF by L-NAME, but also by iNOS-deficiency (fig. 2). When mice were challenged with a dose of TNF lethal for both iNOS-deficient and wild-type animals, treatment with BHA or DMSO + tempol + catalase significantly improved the survival of iNOS-deficient mice (table 1). BHA prolonged the survival of about half the wild-type and 75% of iNOS$^{-/-}$ mice, with 25% of the iNOS$^{-/-}$ animals completely surviving the experiment. The results were even more straightforward when DMSO + tempol + catalase was used; survival was prolonged in none of the wild-type mice, compared to 100% of the iNOS$^{-/-}$ mice, 67% of which eventually survived the experiment. When PLA2, cyclooxygenase-2 or FLAP were inhibited, no improvement in survival rate was noted in wild-type or iNOS-deficient mice. The inhibition of cyclooxygenases by indomethacin partially protected both iNOS$^{-/-}$ and wild-type mice against TNF challenge.

Discussion

Several superoxide-generating systems have been described, including NADPH oxidase, cytosolic xanthine oxidase, the mitochondrial electron transport chain at NADH dehydrogenase (complex I) and ubiquinone-cytochrome c reductase (complex III) where electrons may leak out to oxygen molecules, and the PLA2/AA pathway [18, 19]. Under normal conditions, superoxide generation is kept under tight control by the endogenous SOD enzymes, resulting in the formation of hydrogen peroxide, which is detoxified by catalase or glutathione peroxidase enzymes. However, in a spontaneous reaction catalyzed by free iron, hydrogen peroxide can be transformed into the highly reactive hydroxyl radical. Among the more susceptible targets are polyunsaturated fatty acids, in which the autocatalytic process of lipid peroxidation is initiated, leading to oxidative membrane destruction. In this study, we show that inhibition of NO production by L-NAME significantly aggravates TNF-induced lipid peroxidation in vivo. Both the lipophilic antioxidant BHA and a combination of tempol + catalase + DMSO efficiently prevented L-NAME sensitization and even increased survival. Furthermore, a combination of tempol + catalase (without DMSO) was also effective in avoiding L-NAME sensitization, while SOD, even in combination with catalase, was not. We therefore propose the involvement of intracellular rather than extracellular superoxide radicals. In addition, the efficacy of BHA in counteracting NOS inhibition might indicate that NO plays a protective role against ROS generated and/or acting in a lipophilic, hydrophobic environment, such as the cellular and/or organellar lipid membranes. Taken together, these results suggest that endogenous NO plays an important role in curbing TNF-induced lipid peroxidation in vivo.

We recently reported that the caspase inhibitor zVAD-fmk dramatically sensitizes mice to TNF-induced toxicity and hemodynamic shock by enhancing oxidative stress and mitochondrial damage [43]. Sensitization was completely reversed by aristolochic acid and to a lesser extent by inhibition of mitochondrial complex I, FLAP or PAF. Taken together, we proposed a pivotal role for PLA2 in TNF-induced oxidative stress and toxicity, for which caspase-mediated proteolytic breakdown might provide a negative feedback. In the present study, when agents inhibiting the various ROS generating systems were used to prevent L-NAME sensitization, the PLA2 inhibitor aristolochic acid was clearly the most effective. Not only did aristolochid acid completely prevent L-NAME sensitization, it also prolonged survival. Downstream from PLA2 activity, we found no involvement of cyclooxygenases and partial involvement of 5-lipoxygenase and PAF. The use of rotenone as an inhibitor of mitochondrial complex I suggested that mitochondrial respiration could be marginally involved as well. Hydrolysis of membrane PLs by PLA2 may cause mitochondrial membrane damage, which may result in impaired ATP synthesis and excessive superoxide formation. Next to its radical scavenging effect, BHA has also been shown to inhibit mitochondrial respiration [44, 45] and to block TNF-R1-induced AA release [46]. Therefore, the efficacy of BHA in preventing L-NAME sensitization could be due not only to its lipophilic radical-scavenging potency, but also to its inhibition of TNF-induced ROS formation at the level of PLA2 and/or mitochondrial complex I.

Other than L-NAME injection, the absence of iNOS also sensitized mice to TNF toxicity. Treatment with BHA or DMSO + tempol + catalase prevented L-NAME sensitization and partially protected iNOS-deficient mice against TNF shock. However, when aristolochic acid was used, the significant prevention of L-NAME sensitization could not be extended to protection in iNOS-deficient animals, implying that sensitization via NOS inhibition by exogenous L-NAME is somehow 'inferior' to endogenous iNOS deficiency. Two different explanations can be envisioned. On the one hand, L-NAME may be incapable of inhibiting a particular beneficial function of iNOS other than NO production. In conditions of reduced substrate or cofactor availability, NOS enzymes can generate superoxide. However, not only is this 'uncoupled' superoxide production unlikely to be beneficial, L-NAME has been proven to prevent superoxide production by iNOS as well [47]. On the other hand, L-NAME might not be able to reach and/or penetrate certain (intra)cellular or organellar compartments in the mouse. Restricted and differential permeability of isolated mitochondria to various NOS inhibitors has been described [48]. Moreover, we have previously observed the inability of L-NAME to prevent systemic NO production induced by Bacillus Calmette-Guérin (BCG) or interleukin (IL)-12 [17]. Even higher L-NAME doses (500 mg/kg) and/or repetitive injections (i.p. and i.v.) could not inhibit the BCG- or IL-12-induced NO release in the circulation, further indicating that L-NAME cannot penetrate into certain NO-producing compartments in the mouse.

In conclusion, the ability of L-NAME or iNOS deficiency to sensitize mice to TNF-induced toxicity, and the ability of certain antioxidants to prevent this sensitization, suggest an important role for induced NO in limiting TNF-induced oxidative stress and lipid peroxidation. In addition, the L-NAME sensitization model clearly corroborates the central, pivotal role of PLA2 in TNF-induced oxidative stress in vivo, with the possible involvement of downstream 5-lipoxygenase and/or PAF-mediated pathways. Pharmacological inhibition of complex I suggests a possible minor involvement of mitochondria, which could be indirect, resulting from PLA2-mediated loss of mitochondrial membrane integrity. Hence, excessive NO generated endogenously during TNF shock exerts a dual role: provoking hemodynamic collapse on the one hand, but interfering with damaging lipid peroxidation on the other.

Consequently, in the absence of endogenous NO induction, self-amplifying detrimental oxidative effects may prevail. Lipid peroxidation chain reactions can propagate unrestrained, and the increased oxidative stress may result in oxidation of the NOS cofactor tetrahydrobiopterin, causing eNOS to generate superoxide by 'uncoupled' electron transfer [49, 50]. In addition, aldehydic molecules derived by lipid peroxidation, such as HNE, which are often used to detect oxidative stress in vivo, can also act as additional mediators of oxidative stress [51], for example via the inhibition of NF-κB-regulated iNOS expression [52, 53].

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