Nitrite protects against morbidity and mortality associated with TNF- or LPS-induced shock in a soluble guanylate cyclase–dependent manner

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Nitrite (NO_2^-), previously viewed as a physiologically inert metabolite and biomarker of the endogenous vasodilator NO, was recently identified as an important biological NO reservoir in vasculature and tissues, where it contributes to hypoxic signaling, vasodilation, and cytoprotection after ischemia–reperfusion injury. Reduction of nitrite to NO may occur enzymatically at low pH and oxygen tension by deoxyhemoglobin, deoxymyoglobin, xanthine oxidase, mitochondrial complexes, or NO synthase (NOS). We show that nitrite treatment, in sharp contrast with the worsening effect of NOS inhibition, significantly attenuates hypothermia, mitochondrial damage, oxidative stress and dysfunction, tissue infarction, and mortality in a mouse shock model induced by a lethal tumor necrosis factor challenge. Mechanistically, nitrite-dependent protection was not associated with inhibition of mitochondrial complex I activity, as previously demonstrated for ischemia–reperfusion, but was largely abolished in mice deficient for the soluble guanylate cyclase (sGC) α1 subunit, one of the principal intracellular NO receptors and signal transducers in the cardiovasculature. Nitrite could also provide protection against toxicity induced by Gram-negative lipopolysaccharide, although higher doses were required. In conclusion, we show that nitrite can protect against toxicity in shock via sGC-dependent signaling, which may include hypoxic vasodilation necessary to maintain microcirculation and organ function, and cardioprotection.

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Abbreviations used: ALT, alanine amino transferase; eNOS, endothelial NOS; iNOS, inducible NOS; L-NAME, N^G-nitro–L-arginine methyl ester; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; NOS, NO synthase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; VASP, vasodilator-stimulated phosphoprotein.

Despite more than two decades of extensive research, sepsis and excessive systemic inflammatory response syndrome have recently become the leading cause of death in intensive care units (Nguyen et al., 2006). Severe sepsis, which is complicated by organ dysfunction, may rapidly progress into septic shock, characterized by refractory hypotension. Only 50% of patients with severe sepsis survive hospital admission; mortality rates of ≥70% have been reported for septic shock. In the first days after diagnosis, severe refractory hypotension is generally the cause of death. Afterward, deaths are caused by multiple organ failure resulting from prolonged hypotension and tissue ischemia because of injured and dysfunctional peripheral microvasculature, in combination with severe mitochondria- and organ-damaging cytotoxicity. These are collectively the result of exaggerated proinflammatory and procoagulant processes triggered by both pathogen-associated molecular patterns from the infecting microorganism, such as LPS, as well as endogenously induced cytokines, chemokines, danger-associated molecular patterns, reactive nitrogen species, and reactive oxygen species (ROS; Cinel and Opal, 2009).

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The circulating anion nitrite (NO_2^-) was previously considered a stable and inert oxidation product of NO metabolism, but recent data clearly indicate that nitrite is actually a bioactive product, serving as an endocrine transporter and storage pool of NO (Lundberg et al., 2008). The generation of bioactive NO, via its reduction from the circulating nitrite reservoir, may occur along a pH and oxygen gradient by several mechanisms, including enzymatic reduction by deoxygenated hemoglobin or myoglobin, components of the mitochondrial respiratory chain, xanthine oxidase, endothelial NO synthase (eNOS), and cytochrome P450, as well as nonenzymatically by acidic disproportionation (Lundberg et al., 2008; Raat et al., 2009). In this way, nitrite may function as an important hypoxic and/or acidic NO reservoir in both vasculature and tissues, contributing to hypoxic signaling, vasodilation, and cytoprotection. In a variety of animal models, the reduction of nitrite back to NO thus limits apoptosis and cytotoxicity at reperfusion in the mammalian heart, liver, kidney, and brain (Lundberg et al., 2008; Raat et al., 2009). The mechanism of this protection against ischemia–reperfusion injury appears to involve the S-nitrosation and subsequent inhibition of mitochondrial complex I, resulting in diminished mitochondrial generation of ROS at reperfusion (Shiva et al., 2007).

Until the recognition of nitrite as an endogenous source of NO, NO was known to be generated by three NOS enzymes: the constitutive eNOS and neuronal NOS isoforms, and the inducible NOS (iNOS) enzyme. During infection and inflammation, iNOS produces high levels of NO. Contrary to the general expectation, the major source of systemic iNOS-derived NO metabolites during inflammation or sepsis seems to be parenchymal cells rather than blood cells (Bultinck et al., 2006). Although excessive NO production by iNOS has long been implicated in the development of severe sepsis and shock (Cauwels, 2007), eNOS-derived NO has also been shown to contribute (Connelly et al., 2005). However, NOS inhibition in animal models and septic shock patients exacerbated organ damage and mortality despite circulatory improvement, suggesting additional protective effects of NO (Feihl et al., 2001; Cauwels, 2007). Historically, these protective effects were attributed to NO generated by the constitutive eNOS enzyme, and cardiomyocyte overexpression of eNOS could indeed attenuate myocardial dysfunction and improve survival in endotoxemia and experimental sepsis (Ichinose et al., 2007). On the other hand, iNOS-derived NO was also credited with protective effects in TNF-, LPS-, or sepsis-induced shock models (Cauwels, 2007).

As an uncharged diatomic gas, NO may diffuse to neighboring cells to activate its intracellular receptor soluble guanylate cyclase (sGC), a heterodimer consisting of an α1 or α2 subunit together with a β1 subunit, which has long been regarded the predominant target for the vasodilating effect of NO (Cauwels, 2007). Nevertheless, our studies with mice deficient for $sGC\alpha_1$, an important cardiovascular sGCα isoform (Mergia et al., 2003), revealed no protection against inflammatory shock at all, although hypotension was prevented, and actually indicated a protective role for $sGC\alpha_1$ against cardiac dysfunction and mortality (Buys et al., 2009).

When injected into mice, TNF causes inflammation, accompanied by oxidative and nitrosative stress, culminating in hypothermia, hypotension, multiple organ failure, and death, similar to the shock syndrome induced by LPS or bacteria (Cauwels et al., 2003; Bultinck et al., 2006; Cauwels and Brouckaert, 2007). Although NOS inhibition may revert TNF-induced hypotension (Kilbourn et al., 1990), NOS inhibition or iNOS deficiency also exacerbates TNF toxicity, implicating a protective role for NO that is at least partially attributable to its antioxidant capacities (Cauwels et al., 2003, 2005). In vitro, TNF-induced necrosis is largely caused by mitochondrial ROS (Fiers et al., 1999). Also in vivo, we have identified mitochondrial complex I as an important source of TNF-induced oxidative stress (Cauwels et al., 2003). Considering the fact that (a) both iNOS- and eNOS-derived NO may exert protective effects in inflammatory and septic shock, (b) circulating nitrite may function as an important source of NO specifically in hypoxic and/or acidic conditions that are present in the ischemic microvasculature of vital organs during shock, (c) mitochondrial complex I–derived ROS are involved in TNF-induced toxicity in vitro and in vivo, and (d) nitrite may inhibit mitochondrial complex I and mitochondrial oxidative damage during ischemia–reperfusion (Shiva et al., 2007), we decided to test the protective capacity of nitrite on TNF-induced toxicity. We found that, as hypothesized, nitrite is protective not only against TNF- but also against LPS-induced mortality. To evaluate the mechanism of this phenomenon, we studied the effect of nitrite on mitochondrial respiration and oxidative damage, on tissue damage and inflammation, and in $eNOS^{-/-}$ and $sGC\alpha_1^{-/-}$ mice.

RESULTS AND DISCUSSION

Nitrite protects against TNF-induced morbidity and mortality, in contrast to NOS inhibition

i.v. pretreatment of mice with sodium nitrite protected against progressive hypothermia and mortality induced by a lethal dose of TNF (Fig. 1, A and B). Although biological variability existed between experiments, as protection by nitrite pretreatment varied from 40% (Fig. 1, A and B) to 100% (Fig. 1, C and D), the significance of the protective capacity of nitrite increased dose dependently when protection was not absolute (Fig. 1 B). After TNF challenge, endogenous concentrations of nitrite (∼0.5 μM) were not significantly increased after 2 h, but reached ∼3 μM after 6 h (Fig. S1 A). In contrast, the protective effect of exogenous nitrite was observed at doses ≥0.17 mg/kg (50 nmol/mouse), which is known to increase endogenous nitrite concentration in circulation to >10 μM (Duranski et al., 2005). Nitrite did not accumulate systemically or locally, because at the time of the TNF challenge (2 h after nitrite injection), or 2 or 6 h later, there was no significant increase of nitrite levels in circulation or in tissues (Fig. S1, A–C). Nevertheless, nitrite increased phosphorylation of vasodilator-stimulated

phosphoprotein (VASP), an in vivo biomarker for activation of the NO–sGC–protein kinase G pathway, in naive mice as well as in TNF-challenged animals (Fig. S1 D), indicating its cGMP-mediated bioactivity.

Interestingly, therapeutic treatment with nitrite (injected 3 h after TNF challenge) resulted in an improvement of subsequent hypothermia and mortality (Fig. 1, E and F). The same was true for oral nitrite therapy administered via the drinking water (Fig. 1, G and H). Additional prophylactic or therapeutic nitrite injection in mice on increased dietary nitrite significantly improved survival (Fig. 1, G and H). However, treatment of mice with 60 mg/kg of the NO donor diethylenetriamine/NO adduct could not provide any protection (unpublished data).

In contrast to the protective effect of nitrite, NOS inhibition by N^G-nitro–L-arginine methyl ester (L-NAME), which was given either as a pretreatment (Fig. 2, A and B), posttreatment (Fig. 2, C and D), or oral treatment (Fig. 2, E and F), drastically exacerbated TNF-induced toxicity and mortality. In fact, the detrimental effect of NOS inhibition was so drastic that nitrite pretreatment could only moderately ameliorate morbidity for a couple of hours (Fig. 2 G) but had no effect on mortality (Fig. 2 H). These results might indicate the involvement of eNOS in the reduction of nitrite into bioactive

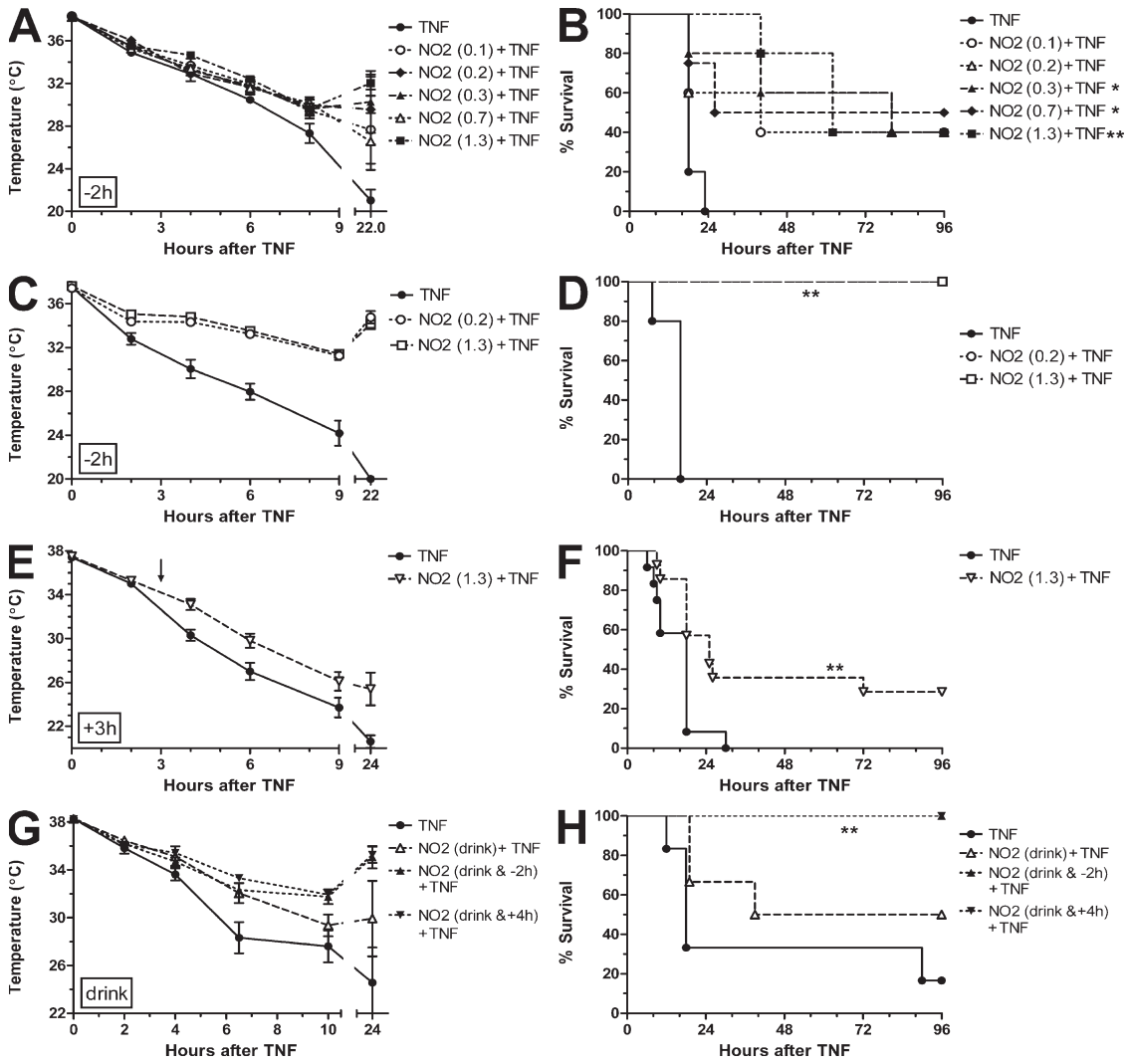

Figure 1. Nitrite protection against TNF-induced hypothermia and mortality. Results for protection against hypothermia (left) and mortality (right) are shown. (A and B) WT mice were injected i.v. with 6 µg TNF at t = 0 and were pretreated i.v. with PBS or 0.1, 0.2, 0.3, 0.7, or 1.3 mg/kg sodium nitrite 2 h earlier (n = 5 for each group, except for 0.7 mg/kg [n = 4]). (C and D) WT mice were injected i.v. with 8 µg TNF at t = 0 and were pretreated i.v. with PBS or 0.2 or 1.3 mg/kg sodium nitrite 2 h earlier (n = 5). (E and F) WT mice were injected i.v. with 6 µg TNF at t = 0 and were treated i.v. with PBS (n = 12) or 1.3 mg/kg sodium nitrite 3 h later (arrow). The combined results of two independently performed experiments are shown (n = 14). (G and H) WT mice were injected i.v. with 7 µg TNF. Control TNF-treated mice received normal drinking water, and the other groups received 1 wk of 500 mg/liter of nitrite drink. In addition, mice were injected with 1 mg/kg nitrite at −2 or +4 h (n = 6 for all groups; a representative experiment is shown, repeated twice). Data are means ± SEM. **, P < 0.01; and *, P < 0.05 compared with control TNF-challenged animals.

and protective NO, as previously suggested (van Faassen et al., 2009). However, eNOS deficiency could not prevent nitrite-dependent protection against TNF toxicity (Fig. 3).

Nitrite protection is not caused by decreased mitochondrial respiration

To elucidate the mechanism of nitrite-mediated protection against TNF-induced morbidity and mortality, we examined mitochondria isolated from the liver of mice 2 and/or 6 h after the lethal TNF challenge. As complex I is known to be an important site of ROS production after TNF (Fiers et al., 1999) and considering the protective inhibitory effect of nitrite on complex I activity in ischemia–reperfusion (Shiva et al., 2007), we first tested the effects of nitrite pretreatment on complex I respiration. Interestingly, and to our knowledge shown for the first time in vivo for a shock-inducing agent, TNF caused a drastic decrease in complex I activity (Fig. 4 A) as early as 2 h after the TNF challenge and persisting for at least 6 h. However, unlike its inhibitory effect on complex I activity in ischemia–reperfusion (Shiva et al., 2007), nitrite treatment did not further decrease complex I activity in our shock model (Fig. 4 A).

To determine whether nitrite-mediated protection was mediated through other components of the respiratory chain, we also determined complex II and complex IV activities in isolated liver mitochondria. As demonstrated in Fig. 4 (B and C),

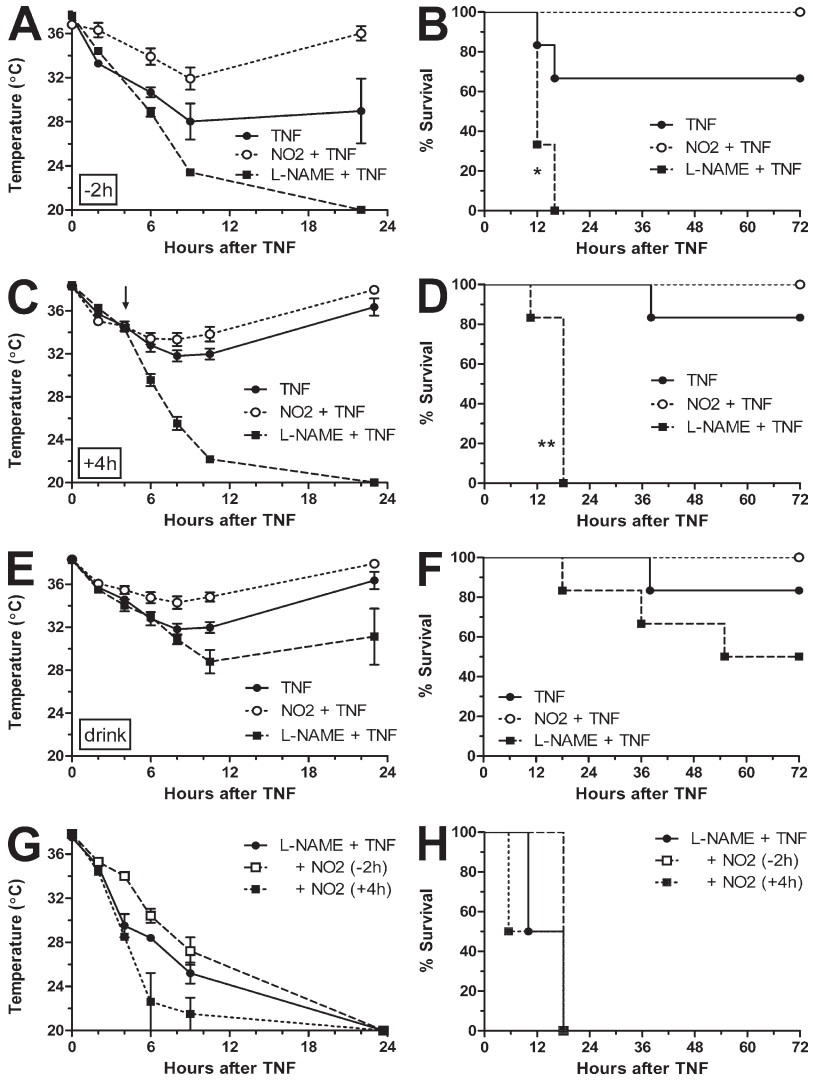

Figure 2. In contrast to nitrite, L-NAME worsens TNF-induced hypothermia and mortality. Results for TNF-induced hypothermia (left) and mortality (right) are shown. (A and B) WT mice were injected i.v. with 5 µg TNF at t = 0 and were pretreated i.v. with PBS, L-NAME, or 1.3 mg/kg sodium nitrite 2 h earlier ($n = 6$). (C and D) WT mice were injected i.v. with 4.6 µg TNF at t = 0 and were treated i.v. with PBS, L-NAME, or 1.3 mg/kg sodium nitrite 4 h later (arrow; $n = 6$). (E and F) WT mice received normal drinking water or 500 mg/liter nitrite or L-NAME for 1 wk and were then injected i.v. with 4.6 µg TNF ($n = 6$). (G and H). WT mice were injected i.v. with 5 µg TNF and 100 mg/kg L-NAME at t = 0 and were treated i.v. with PBS or 1.3 mg/kg sodium nitrite 2 h earlier or 4 h later ($n = 6$). Experiments were repeated at least once (representatives are shown). Data are means ± SEM. **, $P < 0.01$; and *, $P < 0.05$ compared with control TNF-challenged animals.

TNF did not affect complex II activity, whereas complex IV activity was severely inhibited. The subunits of the five mitochondrial enzyme complexes necessary for oxidative phosphorylation are encoded by both nuclear and mitochondrial DNA (mtDNA), with 13 out of the 82 structural subunits encoded by mtDNA and complex II the only complex in which all subunits are encoded by nuclear genes (Shoubridge, 2001). Although TNF significantly inhibits mitochondrial complexes I and IV, complex II activity is not altered at all (Fig. 4, A–C), even after 6 h (not depicted), making it interesting to speculate that selective damage of mtDNA by TNF contributes to TNF-induced toxicity and mortality in vivo. TNF-induced ROS and reactive nitrogen species may not only damage mtDNA, which is much more susceptible than nuclear DNA, but also mitochondrial enzymes and lipids (Bailey et al., 2005; Van Houten et al., 2006). Inhibition and destruction by oxidative/nitrosative stress of the mitochondrial respiration complexes would call for immediate turnover of the appropriate proteins, for which intact mtDNA and functional transcription/translation machinery is required. Interestingly, reactive species–induced damage to mtDNA, mRNA stability, protein synthesis, respiration, and ATP production have been shown as early as 1 h after various oxidative/nitrosative challenges (Ballinger et al., 2000). Consistent with the inhibition of mitochondrial respiration, a significant decrease in hepatic ATP levels was observed as early as 2 h after challenge (Fig. 4 D).

Nitrite protects mitochondria and organs from shock-induced damage

Although nitrite did not have inhibitory effects on complex I in this shock model, it was found that nitrite treatment could dose-dependently protect against TNF-mediated inhibition of complexes I and IV. In contrast, NOS inhibition had no protective effect at all (Fig. 4, A and C). Consistent with its protective effect on mitochondrial respiration, nitrite also improved cellular energetics, as indicated by the protection of ATP levels (Fig. 4 D). As complex I is an important site of ROS production in mitochondria, we evaluated the effect of nitrite on the activity of the Fe-S–containing mitochondrial matrix enzyme aconitase, which is highly susceptible to oxidation and an indicator of mitochondrial oxidative damage (Shiva et al., 2007). Consistent with its protection against respiratory damage, nitrite also preserved the activity of aconitase, in contrast with NOS inhibition (Fig. 4 E). These data demonstrate that nitrite is capable of protecting mitochondrial function and preventing TNF-induced oxidative damage in our model.

To assess the effect of nitrite on tissue infarction associated with shock, we measured systemic parameters for liver and kidney function and myocyte damage. Alanine amino transferase (ALT) and creatinine levels were much higher in the circulation of TNF-challenged mice but were restored to basal levels by nitrite pretreatment, indicating decreased liver damage as well as improved creatinine clearance (kidney function) in nitrite-treated animals (Fig. 4, F and G). As a measure for myocyte damage, circulating creatine kinase levels were assessed and revealed a striking increase because of the lethal TNF challenge, which was significantly prevented by nitrite (Fig. 4 H). In contrast to NO delivery by nitrite, NOS inhibition by L-NAME did not attenuate TNF-induced organ damage (Fig. 4, G and H).

The importance of NO as an antiinflammatory cytoprotective molecule has been well established. However, the

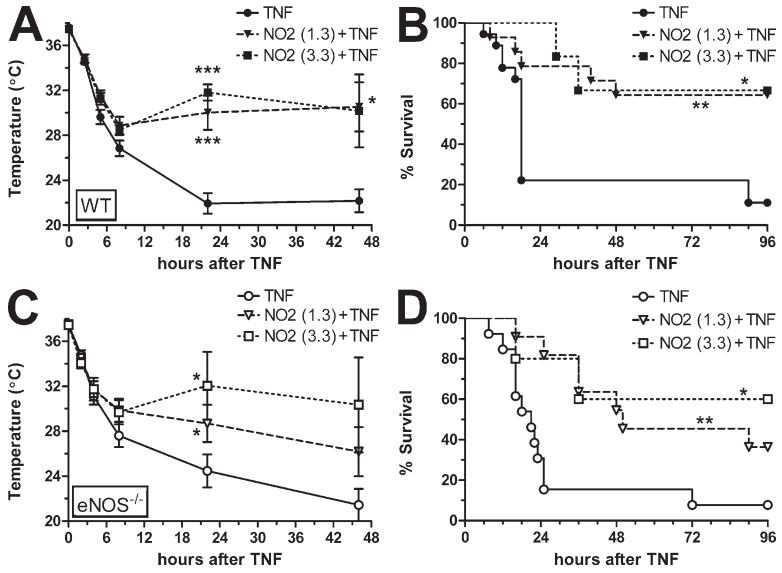

Figure 3. Nitrite protection is independent from eNOS. (A and B) WT or (C and D) eNOS$^{-/-}$ mice were injected i.v. with 8 µg TNF at t = 0 and were pretreated i.v. with PBS or 1.3 or 3.3 mg/kg sodium nitrite 2 h earlier. The combined results of three completely independent experiments are shown (WT mice: n = 18 [TNF alone], 14 [1.3 mg/kg $NaNO_2$], or 6 [3.3 mg/kg $NaNO_2$]; eNOS$^{-/-}$ mice: n = 13 [TNF alone], 11 [1.3 mg/kg $NaNO_2$], or 5 [3.3 mg/kg $NaNO_2$]). Data are means ± SEM. ***, P < 0.001; **, P < 0.01; and *, P < 0.05 compared with control TNF-challenged animals.

mechanism by which NO can prevent leukocyte adherence is still controversial, with data suggesting the involvement of its direct antioxidant properties (Granger and Kubes, 1994) as well as indirect sGC-dependent signaling (Ahluwalia et al., 2004). To evaluate the effect of nitrite on leukocyte adherence, myeloperoxidase (MPO) activity was determined in

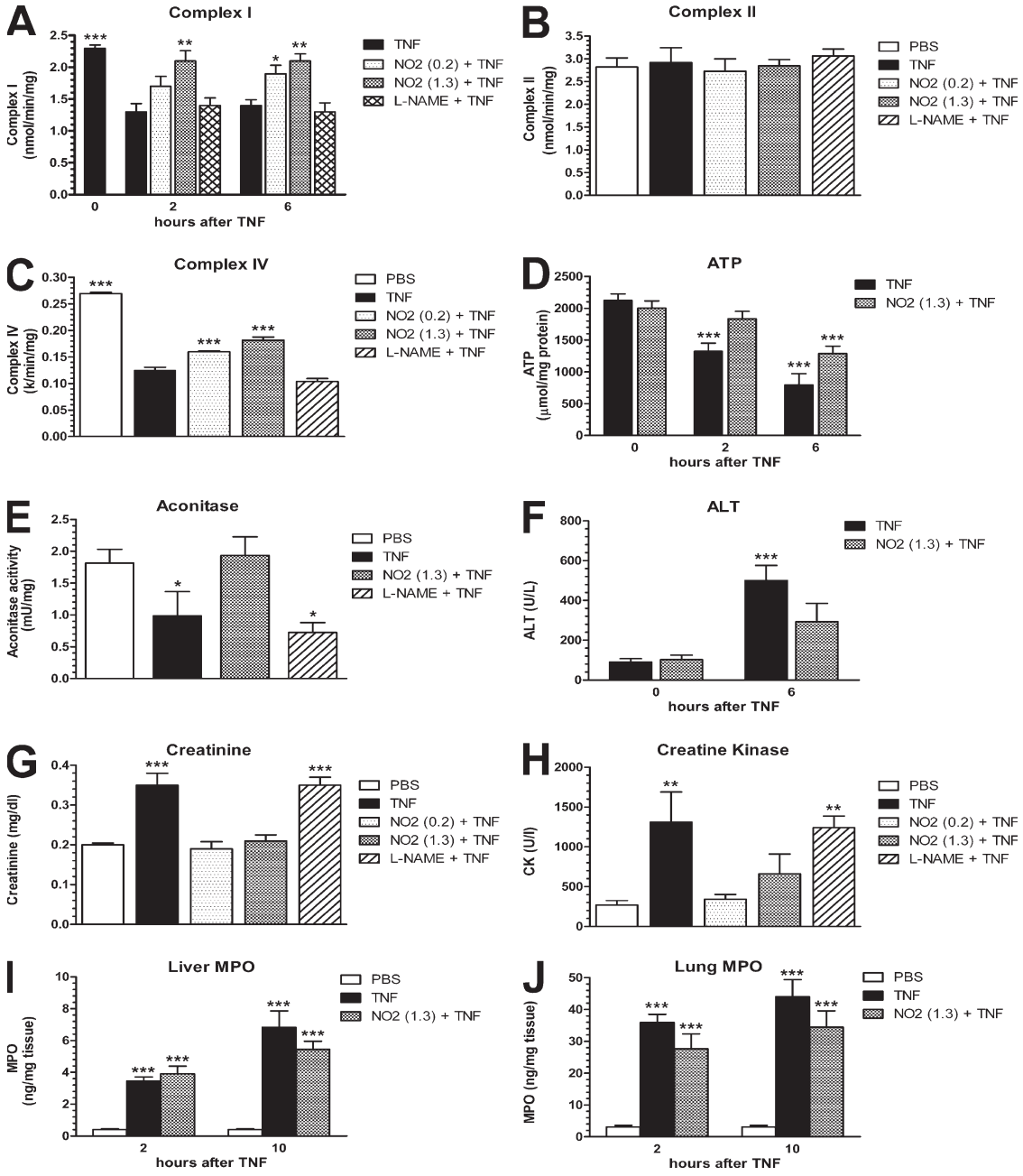

Figure 4. Nitrite protects mitochondria, liver, kidney, and myocytes from TNF-induced damage and oxidative stress. (A) Complex I activity of liver tissue from mice 0, 2, or 6 h after TNF challenge without or with nitrite (0.2 or 1.3 mg/kg) or L-NAME (100 mg/kg) pretreatment. (B) Complex II activity rates of liver tissue from mice 2 h after TNF without or with nitrite (0.2 or 1.3 mg/kg) or L-NAME (100 mg/kg) pretreatment. (C) Complex IV activity of liver tissue from mice 2 h after TNF without or with nitrite (0.2 or 1.3 mg/kg) or L-NAME (100 mg/kg) pretreatment. Data are means ± SEM. ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ compared with TNF-challenged mice for all mitochondrial complex data (A–C). (D) ATP production in liver tissue from mice 2 or 6 h after TNF challenge without or with nitrite (1.3 mg/kg) pretreatment. (E) Aconitase activity of mitochondria from liver tissue from mice 6 h after TNF challenge without or with nitrite (1.3 mg/kg) or L-NAME (100 mg/kg) pretreatment. (F–H) ALT (F), creatinine concentrations (G), and creatine kinase activities (H) in serum from mice 6 h after TNF challenge without or with nitrite (0.2 or 1.3 mg/kg) or L-NAME (100 mg/kg) pretreatment. (I and J) MPO activity in perfused liver (I) and lung (J) from mice 2 or 10 h after TNF challenge without or with nitrite (1.3 mg/kg) pretreatment. The combined results of two independently performed experiments are shown ($n = 8$). Data are means ± SEM. ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ compared with control mice (D–J).

organs from perfused mice, revealing no significant difference between sham- or nitrite-pretreated mice early or late after TNF challenge (Fig. 4, I and J).

Nitrite protection occurs predominantly via sGCα1

Besides its interaction with other radical species, NO is known to act mainly via two different signaling pathways, either modulating protein function by S-nitrosation or through binding to the heme of sGC, the principle NO receptor. Because the mechanism of nitrite-dependent protection against toxicity in TNF shock did not depend on the inhibition of mitochondrial complex I (Fig. 4 A), which is known to occur via S-nitrosation (Shiva et al., 2007), we focused our attention on the sGC enzyme. In addition, the NO–sGC–cGMP signaling cascade modulates several pathophysiological processes that may theoretically contribute to improved mitochondrial and organ function and survival in shock, including vasodilation and inhibition of platelet aggregation in the microvasculature, as well as protection against cardiac dysfunction (Buys et al., 2009). To evaluate the involvement of sGC in nitrite protection, we made use of animals deficient in sGCα1 (Buys et al., 2008), which is considered to be the principal cardiovascular isoform of sGC (Mergia et al., 2003). Although nitrite significantly protected against the progressive hypothermia and mortality induced by a lethal dose of TNF in WT mice (Fig. 5, A and B), it failed to do so in sGCα1$^{-/-}$ animals (Fig. 5, C and D). Although there was significant protection against hypothermia in sGCα1$^{-/-}$ very early (2–5 h) after challenge, after this period the body temperatures dropped as drastically as in the sham-treated TNF-challenged control sGCα1$^{-/-}$ mice (Fig. 5 C). Our data thus suggest that nitrite provides protection against shock via sGC-mediated vasodilation, which is necessary to maintain peripheral blood flow in the ischemic microcirculation of vital organs, and/or via sGC-mediated cardioprotection.

Nitrite protects against LPS-induced morbidity and mortality

To validate the potential of nitrite to protect against toxicity and mortality in shock, we tested the effects of nitrite in a model of LPS-induced shock. Although nitrite doses of 0.2–1.3 mg/kg provided up to 100% protection against TNF toxicity (Fig. 1 D), they failed to protect against LPS-induced shock (Fig. 6, A and B). However, increasing the dose to 3.3 mg/kg resulted in significant protection (Fig. 6, A and B). To investigate whether we could further increase the protective potential, we injected mice with even higher doses of nitrite, resulting in very significant protection (Fig. 6, C and D).

In conclusion, we have shown that nitrite significantly reduces hypothermia, mitochondrial damage and oxidative stress and dysfunction, tissue infarction, and even mortality in mouse shock models induced by lethal TNF or LPS challenges. Mechanistically, nitrite-dependent protection was not associated with inhibition of mitochondrial complexes I, II, or IV. In contrast, protection by nitrite clearly depended on the presence of a functional sGCα1 enzyme, one of the primary NO receptors and signal transducers in the cardiovascular system, suggesting that the protection involves sGC-dependent microvascular dilation and/or inhibition of platelet aggregation, which is necessary to maintain adequate circulation of vital organs, and/or sGC-dependent cardioprotection. Increased NO production has been held

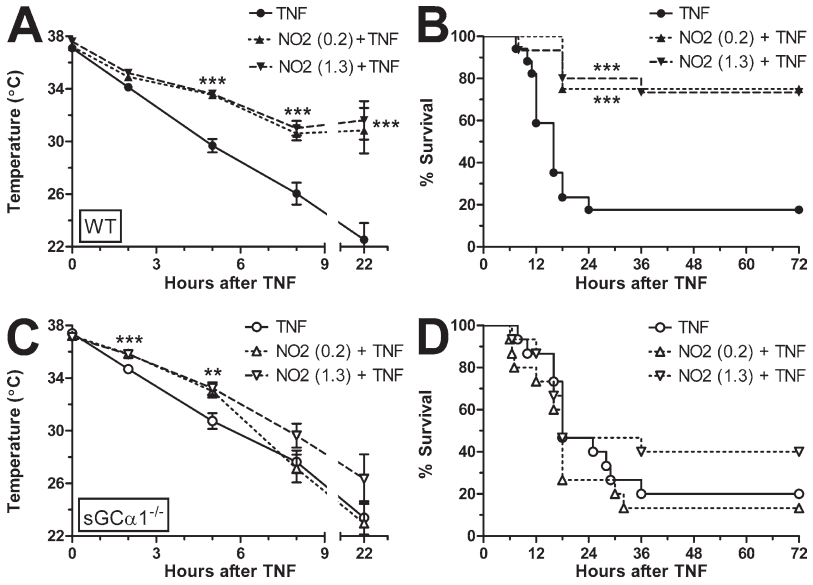

Figure 5. Nitrite protection depends on sGCα$_1$ signaling. (A and B) WT or (C and D) sGCα$_1^{-/-}$ mice were injected i.v. with 8 µg TNF at t = 0 and were pretreated i.v. with PBS or 0.2 or 1.3 mg/kg sodium nitrite 2 h earlier. The combined results of three completely independent experiments are shown (n = 15–17 for WT mice; n = 15 for each group of sGCα$_1^{-/-}$ mice). Data are means ± SEM. ***, P < 0.001; and **, P < 0.01 compared with control TNF-challenged animals.

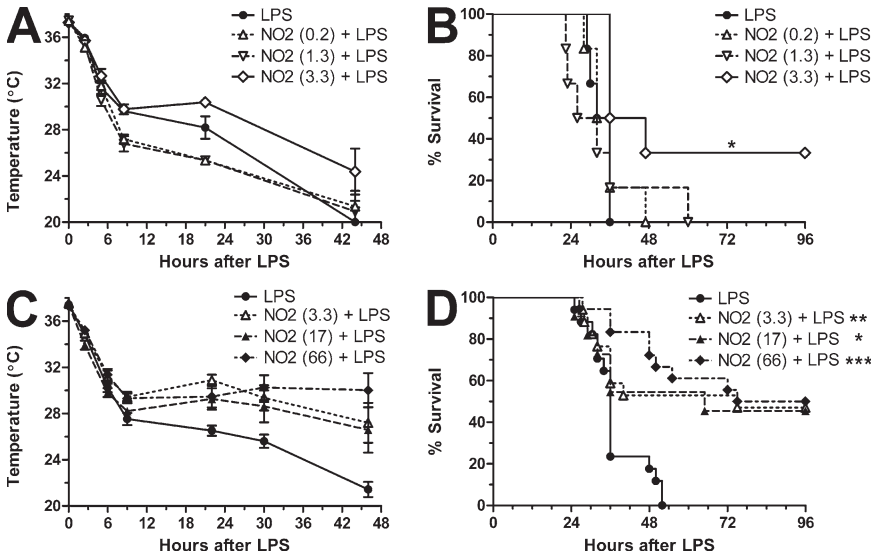

Figure 6. Nitrite protection against LPS-induced hypothermia and mortality. (A and B) WT mice were injected i.v. with 170 µg LPS at t = 0 and were pretreated i.v. with PBS or 0.2, 1.3, or 3.3 mg/kg sodium nitrite 2 h earlier (n = 6). (C and D) WT mice were injected i.v. with 160 µg LPS at t = 0 and were pretreated i.v. with PBS or 3.3, 17, or 66 mg/kg sodium nitrite 2 h earlier. The combined results of three completely independent experiments are shown (n = 11–17 for each group). Data are means ± SEM. ***, P < 0.0001; **, P = 0.0041; and *, P = 0.0118 compared with control LPS-challenged animals.

responsible for the progressive and refractory hypotension in septic shock, leading to the development of (selective) NOS inhibitors to treat patients. Nevertheless, several studies from the 1990s already suggested that treatment with various exogenous NO donors might be a useful therapy to maintain adequate organ blood flow and tissue perfusion during endotoxic shock (Westberg et al., 1994; Pastor et al., 1995; Zhang et al., 1996; Pedoto et al., 1998). Nitrite therapy, however, had only been used in a feline splanchnic ischemic shock model so far, where it improved survival time (Aoki et al., 1990). Our data provide an indication for nitrite therapy to prevent tissue damage, organ failure, and mortality in inflammatory circulatory shock. In addition, they again challenge the current paradigm that NO and sGC contribute to organ damage and death associated with shock.

MATERIALS AND METHODS

Mice. Female C57BL/6J mice were bought from Janvier, and $eNOS^{-/-}$ (Shesely et al., 1996) mice on a C57BL/6J background were bred in our own facilities. $sGC\alpha_1^{-/-}$ mice were generated on a mixed 129S6/Swiss background as previously described (Buys et al., 2008) and backcrossed for 10 generations to the C57BL/6J background. $sGC\alpha_1^{-/-}$ mice carry a targeted deletion of the sixth exon of the gene encoding $sGC\alpha_1$, resulting in the expression of a mutant, catalytically inactive protein. 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 10–14 wk. All experiments were approved by the animal ethics committee of Ghent University.

Reagents and injections. Recombinant mouse TNF was produced in and purified from *Escherichia coli*, and LPS content was <0.02 ng/mg (chromogenic Limulus amebocyte lysate assay; Kabivitrium). TNF or *E. coli* LPS (serotype 0111:B4; Sigma-Aldrich) were injected i.v. in LPS-free PBS. Mortality was scored up to 5 d. $NaNO_2$ (Sigma-Aldrich) was given either i.v. (2 h before or 3 or 4 h after the shock-inducing challenge) or in the drinking water (500 mg/liter for 1 wk). L-NAME (Novabiochem) was given i.v. at 100 mg/kg or in the drinking water (500 mg/liter for 1 wk). Diethylenetriamine/NO adduct (Sigma-Aldrich) was injected i.v. at 60 mg/kg.

Body temperature measurements. Rectal body temperature was recorded with an electronic thermometer (model 2001; Comark Electronics).

Plasma and tissue nitrite determination. The concentration of nitrite in plasma was determined by triiodine-based reductive chemiluminescence as previously described (MacArthur et al., 2007). Heart and liver tissue was harvested, immediately flash frozen, and homogenized in a buffer containing 1 mM potassium cyanide and 1 mM potassium hexacyanoferrate before being subjected to triiodide-based reductive chemiluminescence.

Immunoblot analysis. Lung tissue was isolated 2 or 6 h after challenge and snap frozen in liquid nitrogen. Tissue samples were homogenized in 1 ml RIPA buffer (Boston BioProducts) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phospatase inhibitor cocktail (Sigma-Aldrich), and were microcentrifuged for 20 min at 20,000 *g*. 15 µg of supernatant proteins were fractionated on 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h in 5% nonfat milk in TBS with 0.1% Tween 20 (TBST milk) and incubated overnight with a primary antibody directed against Phospho-VASP (Ser239; diluted 1:1,000; Cell Signaling Technology). Bound antibody was detected with a horseradish peroxidase–linked antibody directed against rabbit IgG (diluted 1:1,000; Cell Signaling Technology) in TBST milk and visualized using chemiluminescence with ECL Plus (GE Healthcare). Antibody directed against GAPDH (diluted 1:1,000; Cell Signaling Technology) was used to confirm that equal amounts of protein were loaded in each lane.

Mitochondrial isolation, respiration, and aconitase activity. Livers were isolated 2 or 6 h after challenge and snap frozen in liquid nitrogen. Whole tissue was used for the measurement of mitochondrial complexes I, II, and IV, and aconitase activities as follows: for complex I, the rotenone-sensitive rate of nicotinamide adenine dinucleotide oxidation was spectrophotometrically monitored at 340 nm in permeabilized tissue. Complex II activity was determined by measuring the reduction of dichloroindophenol at 600 nm, which was coupled to the oxidation of CoQ_2 using succinate as a substrate. Thenoyltrifluoroacetone (complex II inhibitor) was used to determine

specificity of the assay for complex II activity. Complex IV was measured by monitoring the oxidation of ferrocytochrome c at 550 nm. Potassium cyanide was used to determine specificity of oxidation by complex IV. For aconitase activity, tissue was lysed by three freeze/thaw cycles, and aconitase activity was measured by spectrophotometrically monitoring the formation of NADPH at 340 nm using the Bioxytech Aconitase-340 kit (Oxis Research).

ATP determination. ATP levels were measured in lysed snap-frozen tissue using a luciferin–luciferase–based kit (ATP determination kit; Invitrogen).

ALT, creatinine, and creatine kinase determination. 6 h after challenge, blood was collected by cardiac puncture, and serum was obtained after clotting at room temperature and cold centrifugation. Creatinine was assayed using a rate-blanked kinetic-compensated Jaffe method using isotope dilution mass spectrometry calibration (Wuyts et al., 2003). The activity of creatine kinase was determined at 37°C according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC; Schumann et al., 2006). ALT was assayed in plasma samples photometrically according to the IFCC method (Schumann et al., 2002), using a kinetic ALT kit (Infinity; Thermo Fisher Scientific) according to the manufacturer's specifications.

MPO activity. To minimize background MPO activity by the remaining nonadherent intravascular leukocytes, mice were perfused with at least 10 ml of 0.9% NaCl via insertion of a needle into the beating heart. Lungs and liver were snap frozen in liquid nitrogen and stored at −80°C. To determine tissue MPO activity, samples were weighed, thawed, and homogenized in a lysis buffer exactly as directed by the manufacturer. MPO activity was measured using an MPO ELISA kit (Cell Sciences).

Statistics. Statistics were performed using Prism software (GraphPad Software, Inc.). Body temperatures, mitochondrial complex and aconitase activities, and serum parameters are shown as means ± SEM; they were compared with a one-way analysis of variance test with either a Bonferroni posttest for comparison of all pairs, or a Dunnett posttest for comparison of all data with the control (PBS). Survival curves were compared with a log-rank Mantel-Cox test.

Online supplemental material. Fig. S1 shows endogenous nitrite concentrations in plasma, heart, and liver, as well as phosphorylated VASP in lungs from mice injected with PBS, nitrite, TNF, or nitrite plus TNF. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091236/DC1.

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