Nitrite regulation of shock

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Abstract

Severe sepsis and septic shock, which are among the most common causes of death in intensive care units worldwide, cause high morbidity, mortality, and social and economic costs. Therefore, developing successful therapies against sepsis is one of the most important challenges in critical care medicine. Death from septic shock is caused by refractory hypotension and multiple organ failure (MOF). Although excessive systemic vasodilation triggered by nitric oxide (NO) is believed to mediate the hypotension, several endogenous factors and phenomena are responsible for MOF, including tissue hypoperfusion and ischaemia, mitochondrial dysfunction, and other cytotoxic effects, all of which might be directly or indirectly antagonized by local NO. Hence, selective inhibition of the production of hypotension-causing NO in the macrocirculation and/or selective treatment with microvasculature-specific NO donors could theoretically constitute a successful therapy. Recently, the NO metabolite nitrite was recognized as an NO donor specifically in hypoxic/acidic conditions, which can be expected in the septic microvasculature. We recently showed that treatment with nitrite can protect mice against progressive hypothermia, mitochondrial dysfunction, organ damage, and even death induced by tumour necrosis factor or lipopolysaccharide. In this review, we discuss the rationale for using nitrite for the treatment of shock, the possible mechanisms of nitrite-mediated protection, and the lessons that can be drawn for possible future translation of the results from mouse models to the clinic.

Keywords

Nitrite • Shock • TNF • LPS • Mitochondria

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1. Introduction

Despite many decades of extensive research and the development of various therapeutic approaches, the incidence of sepsis and the number of sepsis-related deaths are rising. Epidemiological studies show that the frequency is increasing by nearly 9% per year, which may be attributed to the spread of antibiotic resistance and the increase in the number of invasive medical techniques and procedures, immunologically compromised individuals, and elderly patients. Consequently, sepsis and septic shock have become the main causes of death in intensive care. Worldwide, 18 million people are affected each year, with 1400 people dying daily. Depending on the standards of medical care, worldwide mortality rates range from 30 to 70% (with an aggregate rate of ~50%). Even in survivors, the effects of sepsis can be long-lasting, with a substantially reduced quality of life and increased risk of death.

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in the presence of microbial infection. The most frequent sources of sepsis are infections of the respiratory tract (50–70%), the abdomen (20–25%), and the urinary tract (7–10%). Recently, Gram-positive infections have become the predominant cause, followed by Gram-negative bacteria, fungi, and parasites. In addition to infections, sepsis can also result from acute pancreatitis and major trauma, including burns.

Despite many studies in a variety of experimental sepsis models, very few, if any, of the findings that showed promise in preclinical animal studies were confirmed in human clinical trials. The idea that sepsis is caused by an exaggerated and overwhelming inflammatory reaction of the body to the invading microorganisms led to the development of many different anti-inflammatory therapeutics. The failure of these numerous studies became known as the ‘graveyard of pharmaceutical companies’. As a consequence, the current management of septic shock relies on immediate treatment (preferably within the first hour of diagnosis) with broad-spectrum antibiotics and supportive measures to control hypotension and cardiac output and to maintain organ function. The only mediator-targeted therapy currently available is recombinant human activated protein C and/or low-dose glucocorticoids, but these treatments are for selected patients only and their use remains controversial and much debated.

During the last decades, it has become increasingly clear from both animal and clinical studies that no single endogenous inflammatory mediator or pathway accounts for the pathophysiology of sepsis. The inability to treat sepsis by targeting a single pro-inflammatory mediator is probably due to the heterogeneity of the clinical response.
syndrome (because of different triggers and medical background), the existence of pro-inflammatory and immune paralysed forms of sepsis, the redundant effects of the different inflammatory molecules, and the Janus-faced character of many of these mediators, which exert both detrimental and beneficial effects. Therefore, it might be worthwhile to consider alternative approaches and to focus on the downstream effects of these mediators and on possible therapeutic ways to manipulate them. One possible strategy could be to exploit and manipulate endogenous self-defence mechanisms, such as the recently identified vagus nerve-mediated cholinergic anti-inflammatory pathway, which was shown to modulate cytokine and nitric oxide (NO) production and leucocyte trafficking, and to improve survival in endotoxaemia, sepsis, and hypovolaemic shock. Other approaches could be to prevent organ damage and multiple organ failure (MOF) by restoring or improving peripheral microvascular perfusion and/or mitochondrial respiration.

2. Sepsis and MOF: the problem of (metabolic) hypoxia

By definition, severe sepsis is sepsis accompanied by signs of failure of at least one organ. Although pulmonary dysfunction is the most common organ failure at the onset of sepsis, worsening neurological, coagulation, renal, hepatic, and cardiovascular dysfunction over the first 3 days seems to be associated with increased mortality. In general, as the number of failing organs increases, the mortality rates also increase, and the quality of life of survivors is inversely related to the severity of MOF. The causes of organ failure are multiple: redistribution of blood flow as well as microvascular failure, constriction, obstruction, and permeability changes cause tissue hypoxia and ischaemia (failure of oxygen delivery); mitochondrial damage and dysfunction cause ‘metabolic’ hypoxia (failure of oxygen use); and reactive nitrogen and oxygen species exert direct cytotoxic effects as well, damaging membranes, lipids, nucleic acids, and proteins. In addition, splanchic hypoperfusion and subsequent mucosal ischaemia result in increased inflammation, gut permeability, and bacterial translocation, further exacerbating SIRS and MOF. Also, hepatic dysfunction might lead to the systemic release of inflammatory toxins, which further worsen tissue injury and organ dysfunction. The importance of tissue oxygenation in sepsis has been receiving greater recognition lately, and it has been suggested that vasodilators could be used therapeutically to open the microcirculation (arterioles, capillaries, and venules <100 μm diameter). However, vasodilation, improvement of peripheral flow, and oxygenation might not be the only solution, as the problem might lie in cellular oxygen utilization rather than in oxygen delivery. Impaired oxygen utilization, also called ‘cytopathic’ or ‘metabolic’ hypoxia, is mainly due to reduced activities of mitochondrial respiratory enzyme complexes. Interestingly, mitochondrial dysfunction and reduced concentration or activity of complex I seem to be directly associated with organ failure and mortality in septic patients, which implies that treatments that protect mitochondrial function or stimulate mitochondrial biogenesis and recovery might be useful for preventing organ failure and morbidity in sepsis.

3. Septic shock: inhibition of NO?

When sepsis is associated with refractory hypotension (systolic blood pressure <90 mmHg or a reduction of 40 mmHg from baseline), despite adequate fluid resuscitation, the syndrome is defined as septic shock. The main reason for persistent and irreversible hypotension is excessive arterial vasodilation. Back in 1980, the release of a labile but powerful vasodilating factor from endothelial cells was described; 6 years later, this endothelium-derived relaxing factor was identified as NO. Soon after its discovery, NO was claimed to be the most important endogenous vasodilator and regulator of blood pressure, both physiologically and pathologically. Inhibitors of NO synthesis were used in animal models to successfully prevent, revert, or reduce hypotension induced by inflammatory challenges, such as lipopolysaccharide (LPS) or tumour necrosis factor (TNF). In addition to its effects on vascular tone and blood pressure, other harmful properties of NO were suggested, including cytotoxic and inflammatory effects and the ability to inhibit mitochondrial enzymes important for respiration. This created high hopes for inhibition of NO synthases (NOS) as a therapy in septic patients. However, animal studies with NOS inhibitors were not uniformly successful, and some even reported worsening of the immediate or delayed LPS-induced hypotension, which indicates that NO might not be the major vasorelaxing/hypotensive factor after all, and that other factor(s) involved might even be antagonized by endogenous NO. In addition, even if haemodynamic parameters are improved by NOS inhibition, most studies also described serious adverse effects, such as decreased cardiac output and decreased regional circulation and oxygenation (mainly hepatosplanchnic), as well as increased acidification, leucocyte adhesion, platelet aggregation, microthrombosis, organ damage, and even mortality. Unfortunately, harmful effects were also noted in a large Phase III clinical trial in which the treatment of septic patients with an NOS inhibitor had to be prematurely terminated because of increased mortality, which seemed to be caused by cardiac failure, despite positive effects on blood pressure and systemic vascular resistance.

4. Septic shock: treatment with NO donors?

Although it has been suggested that the increased mortality in the Phase III trial might be related to high doses of the NOS inhibitor and participation of patients with a lower-range cardiac index, the detrimental effects of NOS inhibition in animal models underscore the importance of NO to protect our organs from failure during sepsis and septic shock. Reasons for this protection are probably multiple, because NO is a vasodilator necessary for maintaining proper perfusion and oxygenation in the microcirculation, an inhibitor of platelet aggregation, and an important endogenous anti-oxidant and anti-adhesive molecule. Although the anti-oxidant and anti-adhesive effects are thought to be mainly due to NO’s reactive chemistry (scavenging toxic oxygen radicals), the platelet and vasodilator responses are predominantly mediated by its prototype ‘receptor’, soluble guanylate cyclase (sGC), and the generation of cGMP. In addition, as sGCα1-deficient mice experience increased cardiac dysfunction and mortality in inflammatory shock models, NO might also have important cardioprotective sGC-dependent effects. Interestingly, in some studies, NOS inhibitors exacerbated immediate and/or delayed hypotension, which suggests that NO might not be the only or primary cause of septic hypotension and that other factor(s) exerting a decisive hypotensive function are actually antagonized by NO. Whether this antagonizing effect is sGC-mediated has not been investigated yet.

Although administering NO to septic animals or patients seems paradoxical given its potential harmful effects on the macrocirculation, several studies with various NO donors have already been performed,
and most of them showed a significant improvement of microcirculatory blood flow and oxygenation in organs such as the liver, kidney, and gut, associated with a reduction in morbidity and mortality (Table 1 and references therein). Also in septic patients, nitroglycerin infusion markedly increased sublingual microvascular flow.27 However, because systemic treatment with general ‘classical’ NO donors could cause or exacerbate hypotension,28–32 also in septic patients,27 a clinical trial would require an NO donor that does not affect the macrocirculation.

5. Septic shock: treatment with nitrite?

To avoid the systemic side effects of treatment with an NO donor, we decided to use nitrite, which was recently shown to behave as a carrier of NO bioactivity in circulation.33,34 Specifically in hypoxic and acidic conditions, which can be found in the microcirculation during shock, nitrite might be reduced to NO, which ensures its availability in areas where blood flow should be increased but where NOS-derived and thus oxygen-dependent NO production is compromised.

We recently reported that acute systemic nitrite treatment might protect against both TNF- and LPS-induced toxicity in mice.35 Protection, measured as reduced hypothermia and mortality, was strongest when nitrite was administered as a pre-treatment, but therapeutic treatment could also provide significant protection if administered when hypothermia was not yet too serious. Interestingly, when nitrite was given chronically in the drinking water, there also seemed to be a protective tendency. This tendency could be significantly improved by the combination with therapeutic nitrite, which emphasizes the benefit of sufficient dietary intake of nitrates and nitrites, which are found in fruits and leafy vegetables.36 Although nitrite provided significant protection when given 2 h before the shock-inducing challenge, at the time of the challenge there was very little left of the injected nitrite in the general circulation, the heart or the liver.35 This might indicate that either another form of circulating, ‘protected’ NO serves as the systemic transport and hypoxia donor molecule of NO, such as 3-nitrosothiol (RSNO),37 or that the protective nitrite effect might be due to some kind of preconditioning.38 However, the effect of therapeutic nitrite (given after the shock-inducing challenge) could be directly due to the effect of nitrite itself serving as a hypoxic/acidic NO donor in the appropriate hypoxic/acidic organs.

As mentioned above, the protective effect of nitrite was manifested in reduced peripheral hypothermia (reflecting both systemic and peripheral perfusion, with hypotension and hypoperfusion leading to decreased peripheral body temperature) and lower mortality rate. Circulating alanine aminotransferase, creatinine, and creatine kinase levels were also significantly reduced, indicating decreased damage to the liver, kidney, and muscle tissue.35 Interestingly, nitrite completely prevented the decrease in mitochondrial complex I activity in the liver, whereas complex IV activity was significantly rescued. In addition, nitrite increased liver ATP production and aconitase activity, indicative of reduced mitochondrial dysfunction and oxidative stress, respectively.

6. How does NOa affect shock-induced mitochondrial dysfunction?

We specifically studied the effect of nitrite on mitochondrial complex activities and function, to determine whether protection by nitrite is correlated with its inhibition of mitochondrial complex I (the proposed protective mechanism of nitrite in liver ischaemia/reperfusion injury39), or rather with its positive effect on mitochondrial function and thus on ‘metabolic’ hypoxia, which is increasingly recognized as an organ-damaging and morbidity-determining factor in sepsis and septic shock.3,40 In patients with septic shock, increased NO production is not only associated with decreased complex I activity, but is also inversely correlated with ATP levels and survival.41 Furthermore, addition of serum of septic shock patients to endothelial cells in vitro impairs their respiration in an NO-dependent way.42 Together, these findings suggest that mitochondrial dysfunction is an important mechanism underlying MOD and mortality in sepsis and that NO, and its derivatives (such as peroxynitrite), might play a major role in this context as inhibitors of complexes I and IV.42 Hence, despite the proven positive effects of NO donors on regional blood flow (Table 1), translation to human clinical trials is severely hampered by concern over the possible detrimental effects of NO on mitochondrial activity and ‘metabolic’ hypoxia. In our study, we found that mitochondrial dysfunction (in terms of aconitase activity, necessary for the Krebs cycle, and ATP generation) and complex I and IV damage in TNF-induced shock were not at all prevented by NOS inhibition but were significantly rescued by nitrite treatment, which indicates that NO has a protective rather than a detrimental effect on mitochondria in an in vivo shock situation.35 This contrasts strongly with the general view on NO-induced mitochondrial damage, which is based on the following findings: (i) long-term exposure of cells (in vitro) to NO leads to a gradual and persistent inhibition of complex I;43 (ii) increased NO production is inversely correlated with mitochondrial function and survival in septic patients;14 and (iii) decreased mitochondrial respiration in cultured endothelial cells, caused by adding septic sera, can be rescued by inhibiting NO.41 However, NO is not the only potential danger to mitochondria; also carbon monoxide and hydrogen sulfide (H2S) can competitively inhibit complex IV and thus cause mitochondrial superoxide production and damage.44,45 In addition, hypoxia, reactive oxygen species (ROS), hyperglycaemia, hormones, and genetic down-regulation of mitochondrial protein turnover may cause severe damage and dysfunction.46 Furthermore, not only NO production but also endothelial ROS production (induced in vitro by septic sera) is directly correlated with septic shock mortality,46 and mitochondrial swelling or dysfunction in livers of animals in (septic) shock is reversed by antioxidants, but not by NOS inhibition.35,47 Also, in a hyperacute caspase-independent TNF shock model, mitochondrial swelling and membrane disruption in the liver and kidney were completely prevented by anti-oxidant treatment.38 Therefore, we hypothesize that in an in vivo inflammatory shock situation, the net effects of NO, at least in the liver, do not result in mitochondrial dysfunction, but rather in protection of mitochondrial enzyme complexes and respiration from inflammation-induced oxidative damage, as documented by the nitrite-induced restoration of the activities of complex I, complex IV, and aconitase, and of the production of ATP, and the inability of the NOS inhibitor L-NAME to do the same.35 This congruous hypothesis has already been postulated,48 based on the observations that NOS inhibition did not alter intestinal nor whole-body oxygen extraction capabilities in a canine model of endotox shock, whereas the NO donor SIN-1 increased oxygen extraction.49,50
Table 1 NO donors used in experimental animal and human septic shock models

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal</th>
<th>Challenge</th>
<th>NO donor</th>
<th>Timing</th>
<th>MAP</th>
<th>Regional flow/injury</th>
<th>Acidosis</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boughton-Smith et al.</td>
<td>Rat</td>
<td>LPS + L-NMMA (at −15 min)</td>
<td>SNAP</td>
<td>Pre (−10 min→end)</td>
<td>↓</td>
<td>Jejunal damage ↓ and vascular leakage ↓</td>
<td></td>
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</tr>
<tr>
<td>Wright et al.</td>
<td>Rabbit</td>
<td>LPS + L-NMMA (at −25 min)</td>
<td>SNAP</td>
<td>Pre (−10 min→3 h)</td>
<td>↔</td>
<td>Liver flow ↑ (PV + HA)</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Goto et al.</td>
<td>Rat (newborn)</td>
<td>LPS</td>
<td>SNP</td>
<td>Simultaneous (end)</td>
<td>↓</td>
<td>Hindquarter flow ↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Westberg et al.</td>
<td>Rat</td>
<td>LPS + L-NAME (start-30 min)</td>
<td>NTG</td>
<td>Pre (−1 h→end)</td>
<td>↓</td>
<td>Glomerular thrombosis ↓</td>
<td></td>
<td></td>
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<tr>
<td>Pastor and Payen</td>
<td>Rabbit</td>
<td>LPS</td>
<td>SIN-1</td>
<td>Post (+75 min)</td>
<td>↓</td>
<td>Liver flow ↑ (PV + HA)</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Pastor et al.</td>
<td>Rabbit</td>
<td>LPS</td>
<td>SIN-1</td>
<td>Post (for 3 h)</td>
<td>↓</td>
<td>Liver flow ↑ (PV + HA)</td>
<td>↓</td>
<td></td>
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<tr>
<td>Zingarelli et al.</td>
<td>Rat</td>
<td>LPS</td>
<td>MOL</td>
<td>Pre (−1 h)</td>
<td>↓</td>
<td>Intestinal vascular leakage ↓ (ileum and colon)</td>
<td></td>
<td></td>
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<tr>
<td>Laszlo et al.</td>
<td>Rat</td>
<td>LPS + L-NAME (at 0 h)</td>
<td>SNOG/SNAP</td>
<td>Simultaneous (end)</td>
<td>↓</td>
<td>Mesenteric flow ↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhang et al.</td>
<td>Dog</td>
<td>LPS</td>
<td>SIN-1</td>
<td>Post (±1 h)</td>
<td>↓</td>
<td>Liver injury (ALT) ↓</td>
<td></td>
<td></td>
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<tr>
<td>Zhang et al.</td>
<td>Dog</td>
<td>LPS</td>
<td>SIN-1</td>
<td>Post (±1 h)</td>
<td>↓</td>
<td>Liver (PV)/mesenteric flow =</td>
<td></td>
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<tr>
<td>Kumins et al.</td>
<td>Mouse</td>
<td>LPS</td>
<td>MOL</td>
<td>Pre (−30 min)</td>
<td>↓</td>
<td>Renal flow ↓</td>
<td></td>
<td></td>
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<tr>
<td>Gundersen et al.</td>
<td>Pig</td>
<td>LPS + L-NAME (at +3 h)</td>
<td>SNP</td>
<td>Post (±3.25 h)</td>
<td>↓</td>
<td>Liver flow ↑ (HA)</td>
<td>↓</td>
<td></td>
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<tr>
<td>Gundersen et al.</td>
<td>Rat</td>
<td>LPS</td>
<td>SNP</td>
<td>Simultaneous (end)</td>
<td>↓</td>
<td>Liver flow ↑ (sinusoids)</td>
<td>↑</td>
<td></td>
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<tr>
<td>Cochran et al.</td>
<td>Rat (newborn)</td>
<td>LPS</td>
<td>MOL</td>
<td>Pre (−1 h) and post (±4 h)</td>
<td>↓</td>
<td>Sublingual flow ↑</td>
<td>↓ (trend)</td>
<td></td>
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<td>Spronk et al.</td>
<td>Human</td>
<td>Septic shock</td>
<td>NTG</td>
<td>Pre (−1 h)</td>
<td>↓</td>
<td>Liver injury (ALT) ↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown et al.</td>
<td>Rat</td>
<td>BDL + LPS</td>
<td>MOL</td>
<td>Pre (−30 min)</td>
<td>↑</td>
<td>Liver flow ↑ =</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siegemund et al.</td>
<td>Pig</td>
<td>LPS</td>
<td>SIN-1</td>
<td>Post (±30 min→150 min)</td>
<td>↑</td>
<td>Mesenteric flow =</td>
<td></td>
<td></td>
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<tr>
<td>Liu et al.</td>
<td>Rat</td>
<td>LPS</td>
<td>SIN-1</td>
<td>Pre (−5 min)</td>
<td>↓</td>
<td>Liver injury/weight ↑</td>
<td></td>
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<tr>
<td>Tamandil et al.</td>
<td>Pig</td>
<td>LPS</td>
<td>SNP</td>
<td>Simultaneous (end)</td>
<td>↓</td>
<td>Liver flow ↑ (HA)</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Assadi et al.</td>
<td>Pig</td>
<td>Pseudomonas aeruginosa</td>
<td>SNP</td>
<td>Post (±2 h)</td>
<td>↓</td>
<td>Ileal mucosal flow ↑</td>
<td></td>
<td></td>
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<tr>
<td>Johannes et al.</td>
<td>Rat</td>
<td>LPS</td>
<td>NTG</td>
<td>Post (±2 h)</td>
<td>↓</td>
<td>Renal flow/oxygenation/function =</td>
<td></td>
<td></td>
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<tr>
<td>Bloomfield et al.</td>
<td>Pig</td>
<td>Pseudomonas aeruginosa</td>
<td>NO</td>
<td>Pre (−15 min→end) or post (±30 min/60 min→end)</td>
<td>↑</td>
<td>Lung injury/MPO ↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pedoto et al.</td>
<td>Rat</td>
<td>LPS + L-NA (at +1 h)</td>
<td>NO</td>
<td>Post (−50 min→end)</td>
<td>↓</td>
<td>Mesenteric flow = adhesion/permeability ↓</td>
<td></td>
<td></td>
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<tr>
<td>Neiveir et al.</td>
<td>Rat</td>
<td>LPS</td>
<td>NO</td>
<td>Simultaneous (0→4 h)</td>
<td>↓</td>
<td>Lung injury/edema/MPO ↓</td>
<td>Postponed</td>
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<td>Jakubowski et al.</td>
<td>Rat</td>
<td>LPS</td>
<td>SNO-HSA</td>
<td>Post (±2 h→6 h)</td>
<td>↓</td>
<td>Liver injury (ALT) ↓</td>
<td>↓</td>
<td></td>
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<tr>
<td>Cauwels et al.</td>
<td>Mouse</td>
<td>TNF/LPS</td>
<td>Nitrite</td>
<td>Pre (−2 h)</td>
<td>↓</td>
<td>Renal injury (creatinine) ↓</td>
<td>Creatine kinase ↓</td>
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</table>

Shock was induced mainly by endotoxin (LPS) or live Pseudomonas bacteria, sometimes in combination with NOS inhibitors such as L-NMMA, L-NAME, or i-NA, given either together with, before or after LPS (time point indicated between parentheses). NO donors used were S-nitroso-N-acetyl-penicillamine (SNAP), sodium nitroprusside (SNP), nitroglycerin (NTG), linsidomine (SIN-1), molsidomine (MOL), S-nitroso-glutathione (SNOG), inhaled NO (iNO), S-nitroso-human-serum-albumin (SNO-HSA), or nitrite. The table includes only reports of studies that described the effects on mean arterial pressure (MAP), and/or regional blood flow or injury to the liver, kidney, intestines, lung, or muscle, and/or mortality. Listed first are reports of ‘classical’ NO donors, followed by NO donors with ‘preferential’ delivery (iNO, SNO-HSA, and nitrite). BDL, bile duct ligation; ↔, stabilized; ↑, increased; ↓, decreased; ↓, prevented; =, no influence; PV, portal vein; HA, hepatic artery.
7. How does nitrite protect against mitochondrial dysfunction?

The most straightforward explanation for mitochondrial protection by NO is its direct anti-oxidant potential as an oxygen radical scavenger and a ‘sink’ for free ferrous iron (Figure 1). However, since the overall protection provided by nitrite against TNF-induced toxicity largely depended on sGC signalling, one might speculate that the protection of mitochondrial function could also be sGC-dependent. The simplest explanation would then be that sGC-mediated dilation of hypoxic microvessels leads to a decrease in hypoxia and hypoxia-induced mitochondrial ROS production and damage (Figure 1). Also the sGC-dependent inhibitory effect of NO on platelet aggregation and thus thrombus formation and microvascular obstruction could help improve the microcirculation. In addition, this could also influence platelet ATP release and downstream ATP-mediated inflammation, which might exacerbate mitochondrial damage (ATP being an important activator of the inflammasome and caspase-1, necessary for IL-1 production). Theoretically, the inhibitory effect of NO on caspases and apoptosis could also contribute to protection, either sGC-dependently or sGC-independently via S-nitrosation (SNO). NO might also have direct sGC-dependent effects on mitochondria via the opening of mitochondrial \( K_{\text{ATP}} \) channels located in the inner membrane, a protective process that has been implicated in both pre- and post-conditioning in the heart. In addition, we have recently shown that the ability of NO to prevent contractile cardiomyocyte dysfunction during endotoxic shock may be, at least partially, dependent on sGC. Other possible explanations are the involvement of mitochondrial biogenesis, which is critical for the metabolic recovery process necessary to survive, or reduced susceptibility of elongated mitochondrial networks to inflammation-induced damage. Both mitochondrial biogenesis and fission inhibition are influenced sGC-dependently by NO, and the latter was recently elegantly shown to provide cardioprotection in the mouse.

Overall, we expect that the protective effect of nitrite is most likely mediated by a combination of sGC- and non-sGC-dependent NO effects (Figure 1). The sGC-dependent effects seem to be essential (at least in TNF-induced shock) and might be related to increased hypoxic microvascular dilation and blood flow, reduced platelet aggregation and microvascular obstruction, as well as to cardiac protection and/or pre- or post-conditioning. These effects combined could be important for preventing or reverting ischaemia. By preventing platelet aggregation, inflammation too might be reduced. It has even been suggested that nitrite might act as a signalling molecule in its own right, activating sGC independently of its reduction into NO. In addition, although not documented yet, sGC might be necessary for the reduction of nitrite into bioactive NO, and nitrite or NO might have other hitherto undiscovered sGC-dependent protective effects on mitochondrial respiration. However, it is most likely that non-sGC-dependent mechanisms also contribute to nitrite protection, such as the direct anti-oxidant capacity of NO and its potential to inhibit free ferrous iron.

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**Figure 1** Potential mechanisms of nitrite-mediated protection against inflammation-induced mitochondrial damage, organ failure, and death. On the basis of the literature, the potent protection provided by nitrite against mitochondrial dysfunction could be most rationally explained by NO’s radical and powerful anti-oxidant capacities. However, the sGC-dependency of nitrite-mediated protection against TNF-induced mortality may reflect the critical involvement of sGC-dependent NO signalling as well. Listed in the scheme are all hypothetically possible direct and sGC-mediated beneficial effects of nitrite-derived NO, again based on literature. Alternatively, sGC might be involved in the reduction of nitrite into bioactive NO, although this has not been documented so far (hence the question mark). The possible effect of NO on white blood cell (WBC) recruitment and infiltration was put in italics, since our results obtained in the lung and liver did not indicate such involvement. See text for more detailed description and references.
effects of NO scavenging oxygen radicals and free ferrous iron.\textsuperscript{23} This might protect mitochondria from ROS-mediated damage and dysfunction, which is necessary for reverting and preventing ‘metabolic’ hypoxia. Further studies on the sGC-dependency of mitochondrial protection might give more insight into the mechanism of nitrite-mediated protection.

Various enzymatic systems reduce nitrite to NO. These include haemoglobin, mitochondrial respiratory complexes, cytochrome P450 enzymes, xanthine oxidase, aldehyde oxidase, and endothelial NOS (eNOS).\textsuperscript{39,60} So far, we have not been able to pinpoint which system mediates nitrite protection against mitochondrial and organ damage in shock, but eNOS does not seem to be crucial.\textsuperscript{35} It has been suggested that the reduction of mitochondrial nitrite, perhaps by complexes III and IV or by cytochrome c in a pentacoordinate form, might contribute more during pathological conditions associated with low pH, whereas other nitrite reductases might play a more dominant role in NO generation during physiological hypoxia.\textsuperscript{51} If this is true, it might explain the potent ability of nitrite to protect mitochondria in shock, because it is reduced to NO at the exact site where protection is necessary. On the other hand, if mitochondrial protection by nitrite treatment is primarily realized by a sGC-dependent microcirculatory improvement, one would expect vascular or red blood cell nitrite reductases to be predominantly involved.

8. Conclusion and perspectives

In conclusion, as sepsis is increasingly recognized as a ‘disease of the microcirculation’, in which enhanced vasoconstriction and mitochondrial dysfunction cause irreversible damage and organ failure, novel therapies to rescue the microcirculation and revert hypoxia and ischaemia might be better strategies than interfering with upstream inflammatory mediators to improve organ functions and survival in shock. However, they will almost certainly have to be combined with therapies to prevent mitochondrial dysfunction and ‘metabolic’ hypoxia as well. Nitrite treatment reduces mitochondrial dysfunction, organ damage, and mortality in shock.\textsuperscript{35} The sGC-dependency of this protection may indicate the necessity for (i) improvement of the microcirculatory flow by enhancing hypoxic vasodilation and/or reducing occlusion and (ii) cardioprotection. Intravital microscopy as well as haemodynamic studies will provide further insight into these possibilities. For successful translation to clinical settings, it is essential to elucidate the exact mechanism of nitrite protection. NO inhalation might be a better therapy than nitrite treatment, as it has a proven systemic effect that is probably mediated by hypoxic delivery of bioactive NO by circulating nitrite and/or RSNO molecules.\textsuperscript{62} However, at air–liquid interfaces, inhaled NO might be converted into toxic intermediates, such as peroxynitrite and nitrogen dioxide, which may enhance lung injury and thus limit its use.\textsuperscript{63} Furthermore, the sGC-dependency of nitrite protection could point to sGC activators or stimulators as alternative therapies, although in contrast to nitrite or other NO donors, they might not be able to prevent oxidative stress and ‘metabolic’ hypoxia. Other NO-donating drugs specific for certain conditions (e.g. hypoxia), cell types, or even subcellular environments might also prove to be better therapies with fewer and/or milder side effects. In addition, combined treatments, which might be given at different times during progression of sepsis, will presumably be necessary. For example, NOS inhibitors might be given to prevent macrocirculatory hypotension together with specific NO donors to microcirculatory flow and mitochondrial respiration. Such combined treatments might be consolidated even further by adding particular anti-oxidants to antagonize both ROS-dependent inflammatory hypotension\textsuperscript{64} and ROS-mediated microcirculatory vasoconstriction and mitochondrial dysfunction.\textsuperscript{65,66}

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