



NADPH oxidase is a primary target for antioxidant effects by inorganic nitrite in lipopolysaccharide-induced oxidative stress in mice and in macrophage cells



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ABSTRACT

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and oxidative stress is usually considered as an important factor to the pathogenesis of various diseases. Inorganic nitrite, previously viewed as a harmful substance in our diet or inert metabolites of endogenous NO, is recently identified as an important biological NO reservoir in vasculature and tissues. Stimulation of a nitrite-NO pathway shows organ-protective effects on oxidative stress and inflammation, but the mechanisms or target are not clear. In this study, the hypothesis that inorganic nitrite attenuated lipopolysaccharide (LPS)-induced oxidative stress in mice and in macrophage cells by modulating NADPH oxidase activity and NO bioavailability were investigated. We showed that nitrite treatment, in sharp contrast with the worsening effect of NO synthases inhibition, significantly attenuated aortic oxidative stress, endothelial dysfunction and mortality in LPS-induced shock in mice. Mechanistically, protective effects of nitrite were abolished by NO scavenger and xanthine oxidase inhibitor, and inhibition of NADPH oxidase with apocynin attenuated LPS-induced oxidative stress similar to that of nitrite. In the presence of nitrite, no further effect of apocynin was observed, suggesting NADPH oxidase as a possible target. In LPS-activated macrophage cells, nitrite reduced NADPH oxidase activity and oxidative stress and these effects of nitrite were also abolished by NO scavenger and xanthine oxidase inhibitor, where xanthine oxidase-mediated reduction of nitrite attenuated NADPH oxidase activity in activated macrophages via a NO-dependent mechanism. In conclusion, these novel findings position NADPH oxidase in the inflammatory vasculature as a prime target for the antioxidant effects of inorganic nitrite, and open a new direction to modulate the inflammatory response.

1. Introduction

NADPH oxidases are major sources of superoxide anion ($O_2^{\cdot-}$) [1,2]. $O_2^{\cdot-}$ is important for the generation of other reactive oxygen species (ROS) and is a component of the cellular redox signaling. However, overgeneration of NADPH oxidase-derived $O_2^{\cdot-}$ in the vasculature can become detrimental, and therefore oxidative stress significantly contributes to enhanced endothelial dysfunction and susceptibility to severe tissue injuries [1,3]. Nitric oxide (NO) is a key regulator of vascular function, and emerging evidence shows that vascular oxidative stress and NO deficiency are critically associated with the onset and development of cardiovascular disease [4,5]. Therefore, treatments that limit oxidative stress and increase NO production in the vasculature may have important implications for vessel and cardiovascular homeostasis.

Nitrite is one of the major end products of NO metabolism, closely

reflecting nitric oxide synthases (NOS) activity, but may also derive from the daily consumption of vegetables, fruits and processed or cured meats [6–8]. It has been found that endogenous concentrations of nitrite in human tissues and blood may reach the micromolar range [7,9], and levels of nitrite are significantly lower in the brains of Alzheimer's disease (AD) patients ($\sim 1.7 \mu\text{M}$ in AD, $\sim 2.7 \mu\text{M}$ in healthy group) [9]. The decrease in the levels of plasma nitrite may reflect the endothelial dysfunction and be correlated with increased cardiovascular risk [10]. As an endogenous signaling molecule, nitrite has emerged the potential therapeutic implications for various cardiovascular disease [11–15]. Nitrite treatment protects against tumor necrosis factor (TNF)-triggered morbidity and mortality in mice as well as tissue injury in endotoxemic rats [16,17]. Recently, inorganic nitrite was shown to attenuate ROS generation such as $O_2^{\cdot-}$, peroxynitrite ($ONOO^-$) and hypochlorite (OCl^-) in lipopolysaccharide (LPS)-activated mice peritoneal macrophages or human neutrophils [7,18,19]. It has been proven that these

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protective effects are mainly related to the decrease of oxidative stress via enhanced NO generation [7,18–20].

It has been revealed that the endogenous nitrate and nitrite are used to produce NO and that stimulation of this nitrate–nitrite–NO pathway could compensate for the disturbances in NO production from classic NOS pathway [21,22]. Oral commensal bacteria are obligatory for the reduction of nitrate to nitrite, while several mammalian enzymes can catalyze the subsequent reduction of nitrite to NO in tissues and blood [21,22]. Previous clinical and experimental studies have been shown that supplementation with nitrate can reduce blood pressure in healthy individuals [5,13,23,24]. Through restoring NO homeostasis, nitrite can reduce hypertension and biomarkers of oxidative stress, and ameliorate organ injuries in some models of cardiovascular diseases, and is cardioprotective in NOS-knockout mice [11–15,21,22]. The detailed mechanisms for nitrate- and nitrite-mediated antioxidant properties along with organ protection are not yet clear. In the present study, we tested the hypothesis that nitrite attenuated LPS-induced oxidative stress in mice and in macrophages by modulating NADPH oxidase activity and NO bioavailability. Conceptually, maintaining a suitable nitrite level in the human body may be convenient to a significant reduction of risks associated with inflammatory diseases.

2. Materials and methods

2.1. Animals and treatment

Male Kunming mice were bought from the Jiangxi Research Center for Laboratory Animals. Animals were maintained on standard rat chow and water ad libitum, and used at 10–14 weeks. All animal experiments described conformed to the NIH Guide for the Care and Use of Laboratory Animals. A schematic representation of the experimental design was shown in Scheme 1.

LPS and sodium nitrite were respectively dissolved in PBS for iv administration. LPS (150 µg) from *Escherichia coli* O111:B4 (Sigma-Aldrich) were injected i.v. in PBS. Mortality was scored up to 24 h. Sodium nitrite was given either i.v. (3.0, or 20 mg/kg, 2 h before or 3 h after the LPS challenge) or in the drinking water (400 mg/L for 1 week). Sodium nitrate was also given in the drinking water (2 g/L) for 1 week before LPS challenge. *N*_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma-Aldrich) was given i.v. (2 h before the LPS challenge) at 80 mg/kg. The doses selected for these drugs were adopted according to previous studies [16,17].

2.2. Vascular endothelial function and oxidative stress

After 6 h LPS treatment, normal and LPS-treated mice were anesthetized and killed. Aortas from these animals were isolated and cut into individual ring segments (about 2–3 mm) and bathed in a bicarbonate-buffered, Krebs-Heinseleit solution and equilibrated with 95% O₂–5% CO₂. Aortic rings were submaximally constricted with phenylephrine. Increasing doses of acetylcholine (ACh) was added to the bath to induce endothelium-dependent relaxation (vascular endothelial function), as previously described [25,26].

As a widely used biomarker of oxidative stress, protein carbonyls were measured by the spectrophotometric method [27–29]. Briefly, the

homogenate supernatant fraction was reacted with 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl, and then proteins were precipitated with trichloroacetic acid and washed with the ethanol/ethyl acetate mixture. Finally, the precipitates were dissolved in 6 M guanidine-HCl solution and the absorbances were measured at 370 nm ($\epsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Moreover, chemiluminescence technique was used to determine the NADPH oxidase activity as described previously [5,18,19]. Aortic rings were placed in PBS containing lucigenin and NADPH and O₂^{•-} level was determined by measuring lucigenin chemiluminescence every 10 s for 5 min with the Luminescence detector. Photon emission was then averaged over the 5 min period and normalized per mg tissue weight.

In the presence of LPS treatment, oxidative stress and endothelial function to sodium nitrite treatment were constructed in mouse aortas in the absence or presence (30 min pretreatment) of the NADPH oxidase inhibitor (apocynin, 20 mg/kg), NO scavenger (carboxy-PTIO potassium salt, PTIO, 50 mg/kg), xanthine oxidase inhibitor (allopurinol, 10 mg/kg), NOS inhibitor (L-NAME, 80 mg/kg), selective soluble guanylate cyclase inhibitor (1H-[1,2,4]Oxadiazolo [4,3-a]quinoxalin-1-one, ODC, 10 mg/kg) [5,31].

2.3. Assessment of ROS generation and oxidative stress in macrophages

RAW 264.7 macrophage cells were suspended and cultured in DMEM. In the presence of LPS treatment, cells were cultured with NO donor (diethylenetriamine/nitric oxide adduct, DETA, 0.1 mM), NO scavenger (PTIO, 50 µM), NOS inhibitor (L-NAME, 1 mM), NADPH oxidase inhibitor (apocynin, 10 µM) or xanthine oxidase inhibitor (allopurinol, 5 µM) [7,18,19]. Sodium nitrite and DETA were administered simultaneously with LPS, while other treatments were administered 20 min prior to LPS. Cells were then treated for 12 h [18,19].

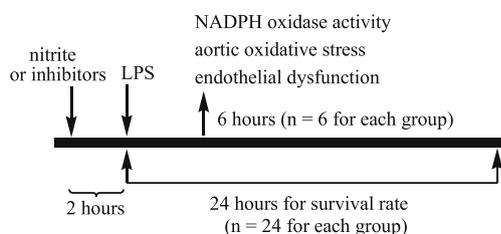
Cellular ROS generation was measured by 2',7'-dichlorofluorescein diacetate fluorescence with a commercial kit (Beyotime Institute of Biotechnology, China). The SOD-inhibitable cytochrome c reduction assay and 4-amino-5-methylamino-2', 7'-difluorescein were used for the detection of O₂^{•-} and NO production in macrophages, respectively [6]. Dihydroethidine (DHE, Beyotime Institute of Biotechnology, China) staining was applied to detect the formation of O₂^{•-} in both aortic segments and viable cells as described in previous study [29].

2.4. Western blot analysis

For detection of specific proteins, equal amounts of proteins were subjected to SDS–PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and then immunoblotted with primary antibodies against NADPH oxidase subunits (Nox2 (sc-130543, 1:500 dilution, Santa Cruz), p22phox (sc-271968, 1:1000 dilution, Santa Cruz), p47^{phox} (sc-17845, 1:500 dilution, Santa Cruz), p67^{phox} (sc-374510, 1:500 dilution, Santa Cruz)). These primary antibodies were detected using a secondary antibody conjugated to horseradish peroxidase (sc-2357, 1:2000 dilution, Santa Cruz). Chemiluminescence was used to identify specific proteins according to the ECL system (Pierce).

2.5. Statistical analysis

The results were reported as the means ± SD of the number of experiments indicated in the legends. Statistical analyses were performed using Origin software and determined using a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. P < 0.05 was considered significant.



Scheme 1. Scheme of animal treatment.

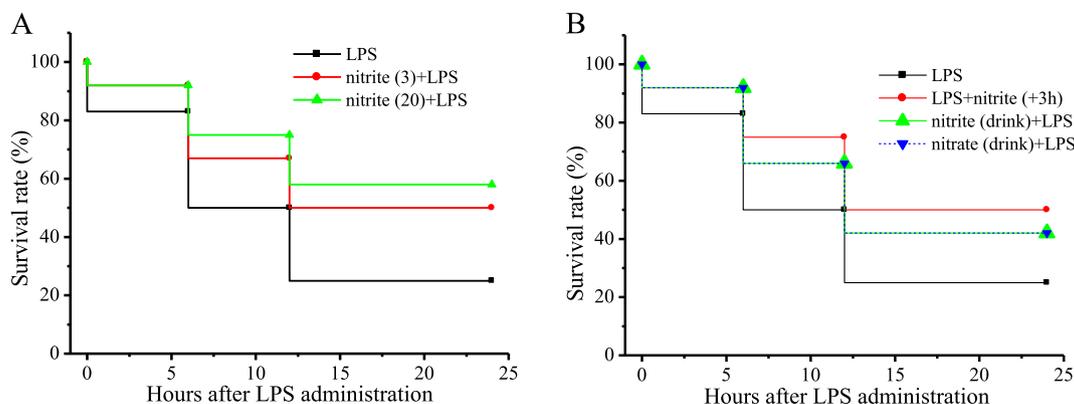


Fig. 1. Nitrite protected against LPS-induced mortality. (A) Protective effects of nitrite with different concentrations. Mice were injected i.v. with 150 μ g LPS at $t = 0$ and were pretreated i.v. with PBS or 3.0, or 20 mg/kg sodium nitrite 2 h earlier. (B) Protective effects of nitrite administered through iv injection or in drinking water as a nitrate solution. Mice were injected i.v. with 150 μ g LPS at $t = 0$ and were pretreated i.v. with PBS or 20 mg/kg sodium nitrite 3 h later or dietary nitrite. In addition, LPS-treated mice received normal drinking water or 1 week of 400 mg/L of nitrite (or 2 g/L nitrate) drink before LPS injection. Each group consisted of 24 animals.

3. Results

3.1. Nitrite protected against LPS-induced mortality, aortic oxidative stress and endothelial dysfunction, in contrast to NOS inhibition

To validate the potential of nitrite to protect against toxicity in a model of LPS-induced shock, we tested the effects of nitrite on the mortality induced by LPS injection. Consistent with previous studies [16,30], i.v. treatment of mice with a lethal dose of LPS induced progressive mortality. However, pretreatment of mice with sodium nitrite (2 h before LPS injection) effectively protected against LPS-induced mortality (Fig. 1A). In addition, increasing the dose of nitrite resulted in more significant protection, demonstrating that the protective capacity of nitrite increased dose dependently when protection was not absolute. Interestingly, therapeutic treatment with nitrite (injected 3 h after LPS challenge) also resulted in an improvement of the survival rate, and the same was true for oral nitrite (or nitrate) therapy administered via the drinking water (Fig. 1B). Moreover, the protective effects of nitrite on LPS-induced mortality were blocked in the presence of allopurinol (xanthine oxidase inhibitor) (Fig. S1), demonstrating the vital role of xanthine oxidase in nitrite response.

In contrast to the protective effect of nitrite, NOS inhibition by the pretreatment of L-NAME (NOS inhibitor) drastically exacerbated LPS-induced mortality (Fig. 2). Oral treatment of L-NAME to mice also decreased survival (data not shown), implying that the presence of endogenous NO was vital to the inhibition of LPS-induced mortality. Moreover, nitrite pretreatment could moderately ameliorate the detrimental effect of NOS inhibition, and the increased mortality by NOS

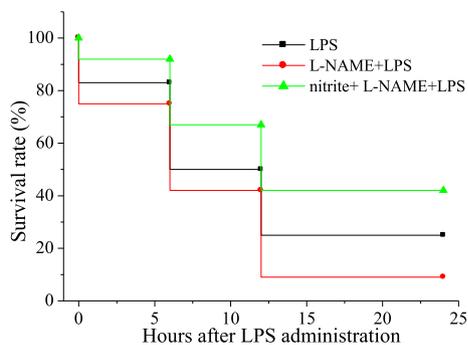


Fig. 2. In contrast to nitrite, L-NAME worsened LPS-induced mortality. Mice were injected i.v. with 150 μ g LPS at $t = 0$ and were pretreated i.v. with PBS, L-NAME, or 20 mg/kg sodium nitrite 2 h earlier. Each group consisted of 24 animals.

inhibition was inhibited by the pretreatment of nitrite (Fig. 2). In other words, NOS inhibition could not prevent nitrite-dependent protection against LPS toxicity, indicating that nitrite protection was independent from NOS.

To elucidate the mechanism of nitrite-mediated protection against LPS-induced mortality, we examined oxidative stress and endothelial function in the aortas of mice 6 h after the lethal LPS challenge. As oxidative stress is known to be an important factor to aortic endothelial dysfunction after LPS injection [26] and considering the inhibitory effect of nitrite on oxidative stress in TNF-induced shock [16], we tested the effects of nitrite pretreatment on aortic oxidative stress (reflected by the contents of protein carbonyls) and endothelial dysfunction. As shown in Fig. 3A and B and Fig. S2, consistent with the effects on mortality, nitrite treatment could significantly protect against LPS-induced aortic oxidative stress and endothelial dysfunction. In contrast, NOS inhibition by L-NAME worsened LPS-induced toxicity (Fig. 3C). These results further demonstrated that the protective potential of nitrite was closely related to the inhibition of oxidative stress and the formation of NO. Control experiments demonstrated that nitrite did not show significant effects on aortic oxidative stress and endothelial function from normal mice (no LPS injection) (Fig. 3A, B and Fig. S2), while NOS inhibition by L-NAME induced toxicity to normal mice (Fig. 3C).

3.2. Protective effects of nitrite on LPS-induced oxidative stress were mediated by xanthine oxidase-catalyzed NO formation

Next, we aimed to investigate the mechanism(s) for nitrite-mediated inhibition on LPS-induced oxidative stress. To further investigate whether the nitrite-mediated attenuation of aortic oxidative stress was dependent on generation of NO, a NO scavenger (PTIO), xanthine oxidase inhibitor (allopurinol) or soluble guanylate cyclase inhibitor (ODQ) was administered together with L-NAME and nitrite. In all cases, the presence of L-NAME was used to exclude the involvement of classic NOS–NO pathway. In the presence of PTIO, the protective effect of nitrite on LPS-induced oxidative stress was abolished, and the content of protein carbonyls was indistinguishable from that of LPS + L-NAME (Fig. 3C). Although allopurinol (xanthine oxidase inhibitor) alone did not significantly alter the oxidative stress to LPS + L-NAME (data not shown), it abrogated the protective effects of nitrite on oxidative stress (Fig. 3C), suggesting that xanthine oxidase was a key enzyme in the conversion of nitrite to NO [13,31]. Interestingly, the effect of nitrite was only partially attenuated during simultaneous inhibition of soluble guanylyl cyclase (sGC) with ODQ (Fig. 3C). Taken together, these findings demonstrated that nitrite could attenuate LPS-induced aortic

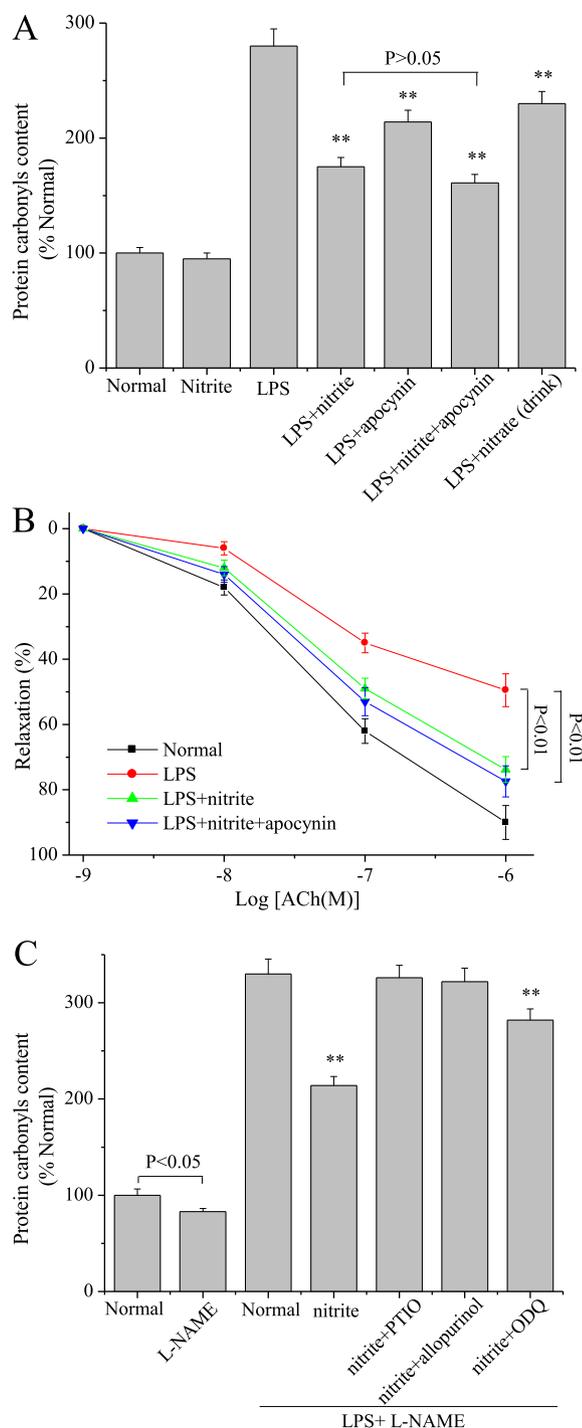


Fig. 3. Nitrite protected against LPS-induced oxidative stress in the aorta, and endothelial dysfunction (determined as ACh-mediated vascular relaxations) of aortic rings. Mice were injected i.v. with 150 µg LPS at t = 0 and were pre-treated i.v. with sodium nitrite 2 h earlier in the presence and absence of NADPH oxidase inhibitor (apocynin for A and B), or other inhibitors(C: NO scavenger (PTIO), xanthine oxidase inhibitor (allopurinol), NOS inhibitor (L-NAME) or selective sGC inhibitor (ODQ)). Endothelium-dependent function was assessed via relaxation in response to increasing doses of ACh. Values are means ± S.D. of six independent determinations (aortic strips from six different animals), A–B: **P < 0.01 compared with LPS-treated group; C: **P < 0.01 compared with LPS/L-NAME-treated group.

oxidative stress via xanthine oxidase-dependent NO generation. Moreover, the subsequent events were partially independent of the NO/sGC/cGMP pathway.

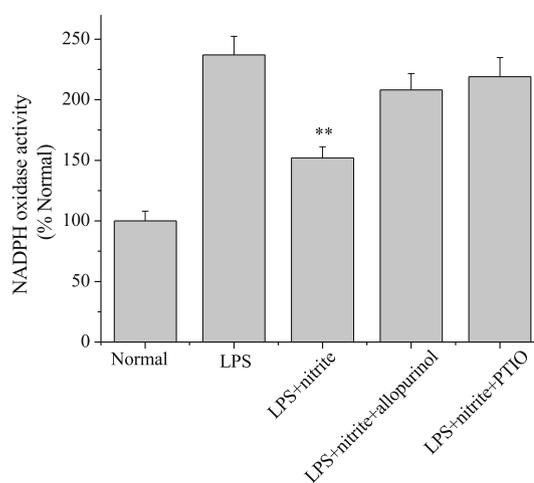


Fig. 4. Nitrite attenuated NADPH oxidase activity via xanthine oxidase-dependent NO formation in vivo. Mice were injected i.v. with LPS at t = 0 and were pre-treated i.v. with sodium nitrite 2 h earlier in the presence and absence of NO scavenger (PTIO) or xanthine oxidase inhibitor (allopurinol). Values are means ± S.D. of six independent determinations (aortic strips from six different animals), **P < 0.01 compared with LPS-treated group.

3.3. Nitrite modulated NADPH oxidase activity in vivo

Recent reports have suggested that nitrite may reduce oxidative stress in models of cardiovascular disease [5,6,12,15–19]. Here, inhibition of NADPH oxidases with apocynin markedly attenuated the aortic oxidative stress to LPS (Fig. 3A). This supported the notion that LPS-induced aortic oxidative stress was linked to increased NADPH oxidase activity. Simultaneous administration of nitrite and apocynin had no further attenuating effect compared with that of nitrite alone (Fig. 3A), suggesting that nitrite reduced LPS-induced aortic oxidative stress mainly by modulating NADPH oxidase function. Next, we investigated whether nitrite modulated NADPH oxidase activity in LPS-treated mouse aortas. Injection with LPS significantly increased NADPH oxidase activity, and this effect was significantly attenuated in the presence of nitrite (Fig. 4). To confirm the effects of nitrite treatment on oxidative stress, we evaluated O₂⁻ production by using the sensitive probe DHE in aortic slices from the animals. Consistently, aortas from LPS-treated mice exhibited increased O₂⁻ production compared with aortas from normal mice, and this change was markedly reversed by nitrite (Fig. S3A). As described in Fig. 3C, the nitrite-mediated reduction in aortic oxidative stress was dependent on functional xanthine oxidase. In LPS-treated mice, inhibition of xanthine oxidase with allopurinol abolished the ability of nitrite to reduce NADPH oxidase activity (Fig. 4). Therefore, these results suggested that nitrite modulated NADPH oxidase function in the vessels from LPS-treated mice, and this effect required functional xanthine oxidase.

3.4. NADPH oxidase was a possible target for the anti-oxidant effect by nitrite in LPS-activated macrophage cells

The activated immune cells are also the major site for the massive formation of ROS in the inflammatory response [7,18,19]. Therefore, we investigated if nitrite-mediated reduction in oxidative stress was present in LPS-activated macrophage cells. Similar to previous studies [7,18,19], the formation of protein carbonyls from LPS-activated macrophages was significantly higher compared with nonactivated cells, and nitrite (but not nitrate) significantly decreased oxidative stress in LPS-activated cells (Fig. 5A). Consistently, nitrite also attenuated LPS-induced cellular ROS generation in macrophages, which was measured by 2',7'-dichlorofluorescein diacetate fluorescence with a commercial kit (data not shown). Then, we explored whether nitrite-

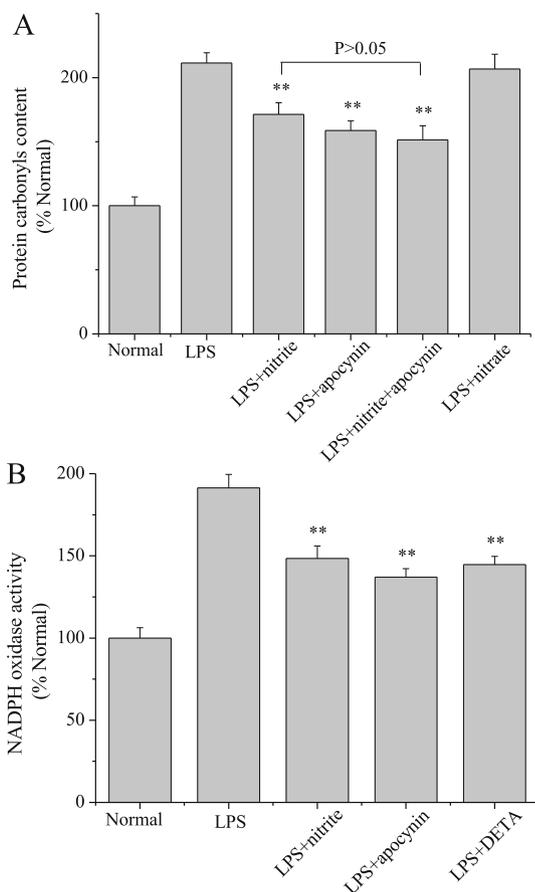


Fig. 5. Nitrite protected against LPS-induced oxidative stress (A) and attenuated NADPH oxidase activity (B) in macrophages. In the presence or absence of LPS, effects of nitrite on generation of protein oxidation and NADPH oxidase-derived $O_2^{\cdot-}$ were assessed in macrophages. Macrophages were pre-treated with NADPH oxidase inhibitor (apocynin) or NO donor (DETA) for 20 min prior to the addition of LPS. The respective normal values were set to 100%, to which other values were compared. Values are means \pm S.D. of three independent determinations, **P < 0.01 compared with LPS-treated group.

mediated reduction in oxidative stress was dependent on NADPH oxidase. Although inhibition of NADPH oxidase with apocynin significantly attenuated LPS-induced oxidative stress, no further effect of apocynin was observed in the presence of nitrite (Fig. 5A), suggesting NADPH oxidase as a possible target for the anti-oxidant effect by nitrite.

3.5. Nitrite inhibited NADPH oxidase activity in LPS-activated macrophage cells

NADPH oxidase is generally considered to be the primary source in phagocytes responsible for $O_2^{\cdot-}$ [1,2]. Macrophage cells stimulated with LPS produced higher levels of NADPH oxidase-derived $O_2^{\cdot-}$ compared with nonactivated cells, and nitrite treatment significantly reduced NADPH oxidase activity in LPS-activated cells similar to that of apocynin (NADPH oxidase inhibitor) (Fig. 5B). Consistently, LPS-treated macrophage exhibited increased $O_2^{\cdot-}$ production compared with non-activated cells, and this change was markedly reversed by nitrite (Fig. S3B). In resting cells, nitrite had no effect on $O_2^{\cdot-}$ production (data not shown). Similar to the effect of nitrite, the classical NO donor (DETA) also attenuated NADPH oxidase activity during LPS activation, suggesting that the effects of nitrite on NADPH oxidase activity were linked to NO generation.

Further, a NO scavenger (PTIO) or a NOS inhibitor (L-NAME) was administered to investigate the effects of NO on NADPH oxidase activity (Fig. 6A). PTIO or L-NAME alone increased NADPH oxidase

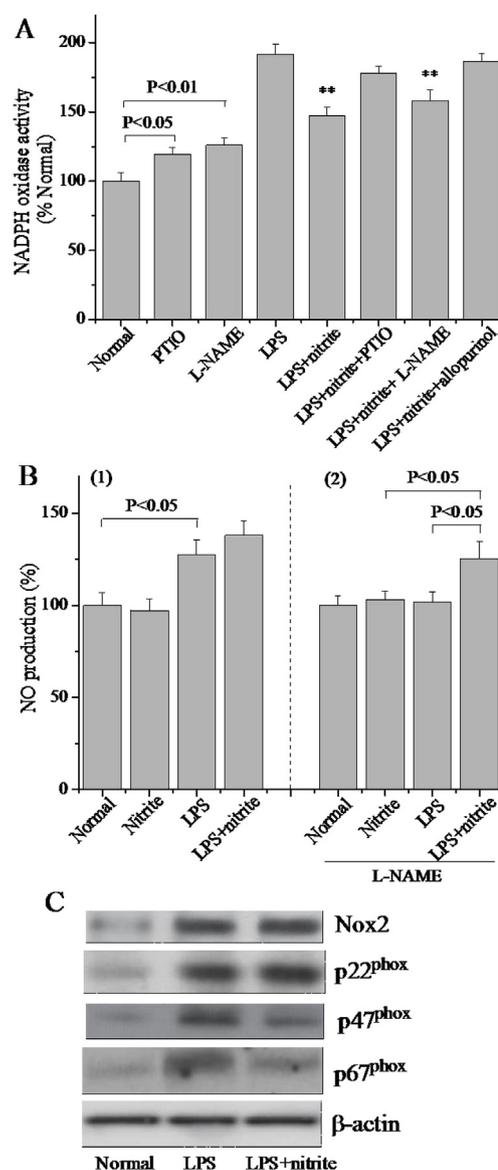


Fig. 6. Nitrite attenuated NADPH oxidase activity in LPS-treated macrophages via xanthine oxidase-dependent NO formation (A–B), and change in the protein expression of NADPH oxidase subunits (C). Nitrite reduced NADPH oxidase-derived $O_2^{\cdot-}$ generation (A) and increased NO generation (B) in LPS-activated macrophages independent of NOS. In the presence or absence of LPS, effects of nitrite on the generation of NO and NADPH oxidase-derived $O_2^{\cdot-}$, and protein expression of NADPH oxidase subunits were assessed in macrophages. Macrophages were pre-treated with NO scavenger (PTIO), NOS inhibitor (L-NAME) or xanthine oxidase inhibitor (allopurinol) for 20 min prior to the addition of LPS. The respective normal values were set to 100%, to which other values were compared. Values are means \pm S.D. of three independent determinations, **P < 0.01 compared with LPS-treated group. (C) Effects of nitrite on protein expression of NADPH oxidase subunits (Nox2, p22^{phox}, p47^{phox}, p67^{phox}). The protein expressions of NADPH oxidase subunits in macrophages were analyzed by Western blot. Representative results from four independent experiments were shown.

activity in nonactivated cells, suggesting that scavenging or reduction of NO was favor for NADPH oxidase-derived $O_2^{\cdot-}$ formation. In addition, nitrite-mediated reduction in NADPH oxidase-derived $O_2^{\cdot-}$ generation during LPS activation was abolished by PTIO rather than by L-NAME (Fig. 6A), implying that the effects of nitrite on NADPH oxidase activity was dependent on NO formation and independent on NOS. Moreover, the inhibitive effects of nitrite on NADPH oxidase activity were also abolished by xanthine oxidase inhibitor (allopurinol), suggesting a

crucial role of xanthine oxidase in reducing nitrite to NO in LPS-activated cells. We also found that LPS activation resulted in the increases in xanthine oxidase expression and activity compared with non-activated cells. However, simultaneous treatment with nitrite induced no changes in xanthine oxidase expression and activity (Fig. S4).

In addition, cocubation with LPS and nitrite also increased NO generation, but this was not significantly different compared with LPS alone (Fig. 6B-(1)). Simultaneous nitrite treatment increased NO generation in LPS-activated cells, and this effect was not significantly affected by L-NAME (Fig. 6B-(2)). Thus, nitrite increased NO generation in activated cells independent of NOS, again suggesting NOS-independent NO formation. Taken together, these results demonstrated that NO generation in resting cells could modulate NADPH oxidase-derived $O_2^{\cdot-}$ generation, and suggested that nitrite-mediated reduction in NADPH oxidase activity was associated with a NOS-independent but xanthine oxidase-dependent NO formation in activated cells.

One possible mechanism how nitrite might mediate this attenuation of NADPH oxidase-derived $O_2^{\cdot-}$ formation was through changes in enzyme expression. Therefore, we investigated protein expression levels of the NADPH oxidase subunits in nonactivated and activated macrophages. Consistent with previous studies [7,19], activation of macrophages with LPS significantly increased the protein expressions of NADPH oxidase subunits (Nox2, p22^{phox}, p47^{phox}, p67^{phox}). Nitrite did not influence the levels of the membrane-bound subunits Nox2 and p22^{phox}, whereas the expression of the cytosolic subunits p47^{phox} and p67^{phox} in LPS-treated cells was significantly reduced in the presence of nitrite (Fig. 6C). Nitrite had no effects in nonactivated cells (data not shown). Therefore, nitrite-mediated inhibition of NADPH oxidase activity was associated with the changes in protein expression of NADPH oxidase subunits.

4. Discussion

The present study provided novel evidence for an antioxidant role of nitrite in oxidative stress via its effects on the ROS-rich vasculature and macrophage. Together, our data showed that nitrite attenuated LPS-induced mortality, aortic oxidative stress and endothelial dysfunction in vivo by generating NO-like bioactivity and reducing NADPH oxidase activity. Moreover, this inorganic nitrite reduced NADPH oxidase-derived $O_2^{\cdot-}$ generation in activated macrophages via a NO-dependent mechanism. NADPH oxidase has been already shown as a target for antioxidant effects by nitrite in LPS-treated mouse macrophages [18,19] and in vivo studies in a mouse model of oxidative stress (superoxide dismutase-1 knockouts) have also suggested NADPH oxidase in the renal microvasculature as a primary target for the blood pressure-lowering effects of nitrite [5]. However, these mechanistic details in our research are important to further characterize inorganic nitrite as potential therapeutics in ROS-mediated cardiovascular and metabolic diseases. These results in the present study may explain the observed anti-inflammatory and tissue-protective properties of inorganic nitrite in experimental disease models and therefore be useful to reinforce the current evidence on nitrite protective effects.

Dietary nitrate has been shown to have blood pressure-lowering effect in healthy humans and nitrite can act as a potential therapeutic agent to ameliorate organ injuries in some models of cardiovascular diseases [5,11–15,23,24], but the definite mechanisms remain uncertain. Systemic vasodilatation by the activation of classic NO/sGC/cGMP pathway has been suggested and is likely important in the acute effects of nitrate and nitrite [5,21,22,32]. Many of the biological effects of nitrate and nitrite are thought to proceed via its reduction to NO. Several enzymes and proteins including xanthine oxidase and heme proteins have been implicated in nitrite reduction in the vasculature [21,22]. Our findings emphasized xanthine oxidase as a major nitrite reducer in the vasculature, because inhibitor of the enzyme completely inhibited the nitrite response (Figs. 3C and 6A) and xanthine oxidase has been shown to be highly expressed in the vasculature [5,21]. The

generated NO operated partly by stimulating a NO/sGC/cGMP pathway, but more importantly by inhibiting vascular NADPH oxidase-dependent $O_2^{\cdot-}$ signaling. Evidence for the involvement of NO was supported by the similar effects of NO donor (Fig. 5B) and more importantly by the functional inhibition of the nitrite response with the NO scavenger PTIO (Fig. 6A). In all cases, the nitrite response was also evident in the presence of L-NAME (Figs. 3C and 6A), thereby excluding the involvement of NOS. Therefore, nitrite treatment increased NO generation in activated cells by a NOS-independent mechanism and nitrite-dependent protection in oxidative stress were mediated by xanthine oxidase-catalyzed NO formation. In agreement with this study, the enhanced NO generation from xanthine oxidase-dependent reduction of nitrite was one of the main vasodilator mechanisms for nitrite in hypertension [5,13,15,32]. Moreover, the reduction of iNOS expression by nitrite may be due to a negative feedback through nitrite-derived NO generation and has been also suggested to contribute to the protective effects of nitrite [18]. It is possible that the increased levels of NO or reduced levels of $O_2^{\cdot-}$, during nitrite treatment, can reduce iNOS activation.

In the present study, it was shown that NADPH oxidase was a primary target for antioxidant effects by inorganic nitrite in LPS-induced oxidative stress in mice and in macrophage cells. This interpretation is supported by the following lines of evidence. First, in this study, LPS induced ROS production and oxidative stress due to the activation of NADPH oxidase (Figs. 3A and 5A). Pharmacological inhibition of NADPH oxidase by apocynin significantly suppressed the oxidative stress and ameliorated the aortic endothelial dysfunction in LPS-treated mice (Fig. 3A, B and Fig. S2), and apocynin also markedly attenuated the LPS-induced oxidative stress in macrophages (Fig. 5A). Therefore, NADPH oxidase was the major source of ROS and oxidative stress in vivo and in vitro after LPS treatment. Furthermore, nitrite also attenuated LPS-induced oxidative stress similar to that of inhibition of NADPH oxidase with apocynin in vivo and in vitro (Figs. 3A and 5A). No further effect of apocynin was observed in the presence of nitrite, suggesting NADPH oxidase as a possible target. Third, compared with LPS-treated mice, the pretreatment of nitrite improved the vascular endothelial function (Fig. 3B and Fig. S2) and attenuated the increase of NADPH oxidase activity (Fig. 4) and oxidative stress (Fig. 3A) in inflammatory vasculature. Consistently, the development of aortic endothelial function could be predicted by a reduced oxidative stress or NADPH oxidase in the inflammatory state. The protection of nitrite was also observed in LPS-induced oxidative stress in parallel with the decrease of NADPH oxidase activity in vitro (Figs. 5 and 6). These results further demonstrated that nitrite attenuated vascular oxidative stress and endothelial dysfunction mainly through inhibiting NADPH oxidase activity in vivo.

A remaining question that need further investigations was the exact mechanism for nitrite-mediated reduction in NADPH oxidase-dependent $O_2^{\cdot-}$ formation. Phagocytic NADPH oxidase, which is the main isoform expressed in macrophages, consists of two membrane-bound subunits Nox2 (or gp91^{phox}) and p22^{phox}, the cytosolic subunits p47^{phox}, p67^{phox}, p40^{phox} and a small GTPase Rac. The assembly of the active enzyme complex would result in a burst of $O_2^{\cdot-}$ production [1,2]. It was shown in previous study that nitrite was unable to exert direct $O_2^{\cdot-}$ scavenging properties [15]. Direct scavenging of $O_2^{\cdot-}$ by the NO was possible [4,5,7], but inhibition of NADPH oxidase activity by nitrite-derived NO was also plausible, for example, through NO-mediated S-nitrosylation of critical thiols in the catalytic sites of the enzyme [33,34] or via HO-1 activation [35,36]. Mechanistically, reduction of NADPH oxidase expression by nitrite would represent a third alternative, and our data of NADPH oxidase components did support this. The protein levels of all four investigated subunits were significantly increased after LPS stimulation. Interestingly, the simultaneous treatment with nitrite only reduced protein expression levels of cytosolic subunits (p47^{phox} and p67^{phox}), but not membrane-bound subunits (Nox2 and p22^{phox}) (Fig. 6C). Therefore, nitrite-mediated inhibition of

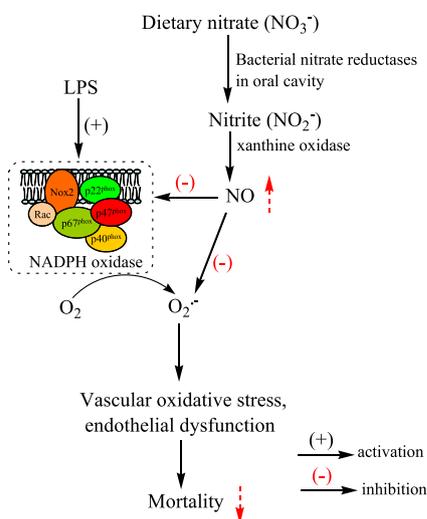


Fig. 7. Proposed mechanism for nitrate- and nitrite-mediated effects on vascular oxidative stress and NO regulation. Through mediating NADPH oxidase activity, the increase of NO bioavailability by nitrite-NO pathway will reduce vascular oxidative stress and endothelial dysfunction, resulting in higher survival during acute inflammation.

NADPH oxidase activity was partially explained by the protein reduction of NADPH oxidase subunits. In addition to expression levels, the intracellular distribution of these subunits also played an important role for NADPH oxidase activity and might be altered by nitrite. NO can also inhibit the translocation of cytosolic compounds of NADPH oxidase to plasma membrane in neutrophils [37–39]. Future studies are warranted to investigate if nitrite may influence these translocation processes, and hence contribute to the reduced NADPH oxidase activity. Several NADPH oxidase homologs (Nox1, Nox2, Nox4) and different activation mechanisms for these isoforms have been described in the vascular systems [1, 2, 20], the question that which isoform or subunit in vasculature is the primary target is still unclear and need further investigation.

Under normal physiological condition (without LPS stimulation), suitable levels of ROS production are crucial for immune cell function [3,7], and nitrite treatment showed no effect on the formation of these important ROS in non-activated mice and cells (Fig. 3A), indicating that nitrite did not interfere with physiological redox-dependent ROS signaling. However, under inflammatory condition (with LPS stimulation), excessive formation of ROS are detrimental and lead to oxidative stress and severe tissue injuries [3,4,18,19,27], and nitrite treatment showed inhibitive effect on NADPH oxidase activity in activated groups (Figs. 4 and 5B). Therefore, nitrite was probably inactive against normal levels of NADPH oxidase and oxidative stress during physiology, and should only inhibit excessive production of NADPH oxidase-derived $\text{O}_2^{\cdot-}$ associated with vascular inflammation. These data that nitrite inhibited NADPH oxidase activity and oxidative stress only in LPS-activated mice and cells, would open a new direction to modulate the inflammatory response in vivo.

Herein, dietary nitrate also protected against LPS-induced mortality, aortic oxidative stress and endothelial dysfunction in vivo (Figs. 1B and 3A), while nitrate did not attenuate oxidative stress in activated macrophages (Fig. 5A). These results further support the notion that oral bacteria seem obligatory in the bioreduction of dietary nitrate to nitrite, and nitrite is further reduced to NO in tissues and blood [21,22]. In addition to NO generation via the classic NOS pathway, the alternative nitrate-nitrite-NO pathway has also shown protective effects in many disease models via restoring NO bioavailability [5,21,22]. Therefore, dietary nitrate from various nitrate-rich vegetables (e.g. a rocket salad beverage, concentrated beetroot juice, and a spinach beverage) is receiving increased attention due to its reported cardioprotective

properties [40,41].

In conclusion, we showed that nitrite significantly reduced aortic oxidative stress and endothelial dysfunction, and even mortality in mouse shock models induced by LPS. Moreover, we demonstrated that NADPH oxidase was a primary target for antioxidant effects by nitrite in LPS-induced oxidative stress in mouse aortas and in macrophage cells. Mechanistically, nitrite-dependent protection was linked to NO generation and inhibition of NADPH oxidase in vivo and in vitro, and nitrite regulated NADPH oxidase-derived $\text{O}_2^{\cdot-}$ formation in the vasculature through xanthine oxidase-mediated NO formation (Fig. 7). A reduced protein expression of the cytosolic NADPH oxidase subunits might also contribute to the favorable effect of nitrite. Our data provide a therapeutic role of nitrite to prevent tissue damage and mortality in inflammatory circulatory shock. In addition, these results also indicates a physiological role for dietary nitrate and nitrite in regulation of vascular inflammatory response and in reduction of oxidative stress.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.niox.2019.05.002>.

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