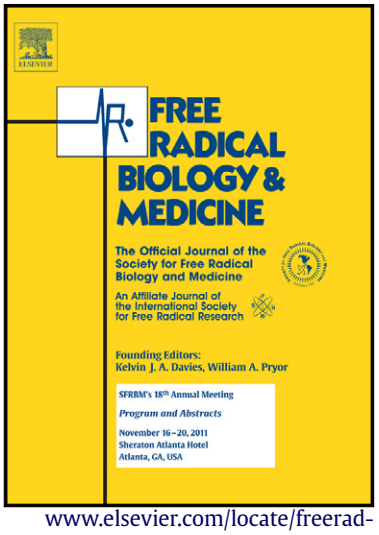

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Inorganic nitrite attenuates NADPH oxidase-derived superoxide generation in activated macrophages via a nitric oxide-dependent mechanism

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Abstract

Oxidative stress contributes to the pathogenesis of many disorders, including diabetes and cardiovascular disease. Immune cells are major sources of superoxide ($O_2^{\bullet-}$) as part of the innate host defense system, but exaggerated and sustained $O_2^{\bullet-}$ generation may lead to progressive inflammation and organ injuries. Previous studies have proven organ-protective effects of inorganic nitrite, a precursor of nitric oxide (NO), in conditions manifested by oxidative stress and inflammation. However, the mechanisms are still not clear. This study aimed at investigating the potential role of nitrite in modulating NADPH oxidase (NOX) activity in immune cells. Mice peritoneal macrophages or human monocytes were activated by lipopolysaccharide (LPS), with or without co-incubation with nitrite. $O_2^{\bullet-}$ and peroxynitrite ($ONOO^-$) formation were detected by lucigenin-based chemiluminescence and fluorescence techniques, respectively. The intracellular NO production was measured by DAF-FM DA fluorescence. NOX isoforms and inducible NO synthase (iNOS) expression were detected by qPCR. LPS increased both $O_2^{\bullet-}$ and $ONOO^-$ production in macrophages, which was significantly reduced by nitrite (10 μmol/L). Mechanistically, the effects of nitrite are *1)* linked to increased NO generation, *2)* similar to that observed with the NO donor DETA-NONOate, and *3)* can be abolished by the NO scavenger carboxy-PTIO or by the xanthine oxidase (XO) inhibitor febuxostat. Nox2 expression was increased in activated macrophages, but was not influenced by nitrite. However, nitrite attenuated LPS-induced upregulation of iNOS expression. Similar to that observed in mice macrophages, nitrite also reduced $O_2^{\bullet-}$ generation in LPS-activated human monocytes. In conclusion, XO-mediated reduction of nitrite attenuates NOX activity in activated macrophages, which may modulate the inflammatory response.

Key words: iNOS; lipopolysaccharide; macrophage; NADPH oxidase; nitrate; nitrite; nitric oxide; oxidative stress; reactive oxygen species

Abbreviations

cPTIO	carboxy-PTIO potassium salt
DAF-FM DA	4-Amino-5-methylamino-2′,7′-difluorescein diacetate
DETA-NONOate	Diethylenetriamine/NO adduct
iNOS	inducible nitric oxide synthase
L-NAME	Nω-nitro-L-arginine methylester hydrochloride
LPS	lipopolysaccharide
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
$O_2^{\bullet-}$	superoxide
$ONOO^-$	peroxynitrite
ROS	reactive oxygen species
XO	xanthine oxidase

Introduction

Oxidative stress refers to a disruption of normal redox signaling by overproduction of reactive oxygen species (ROS), which may lead to damage of cellular components including membranes and DNA [1, 2]. Increased production of ROS is considered a major contributing factor to the development and progression of various diseases, including kidney diseases, type 2 diabetes, cardiovascular diseases and neurodegenerative diseases [3-6]. Immune cells like macrophages, are the primary sources of ROS, in particular superoxide anion ($O_2^{\bullet-}$) generated through NADPH oxidase (NOX). $O_2^{\bullet-}$ is crucial for immune cell function and effective host defense but on the other hand, it is also an important element of the cytotoxic effects of immune cells. When overproduction of $O_2^{\bullet-}$ persists it may become detrimental and lead to severe organ injury [7].

The inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) were long considered to be inert end products of nitric oxide (NO) metabolism, or unwanted and potentially toxic residues in the food chain. However, several lines of evidence show the existence of a reverse pathway where these anions may undergo bioconversion to form NO and other bioactive nitrogen oxides [8-10]. Nitrate is converted to nitrite by oral bacteria, and subsequent reduction of nitrite to NO can be achieved by a variety of proteins and enzymes in blood and tissues, including xanthine oxidase (XO) [11-13]. Intervention studies have demonstrated that a diet rich in vegetables, which contain high levels of inorganic nitrate, has cardioprotective effects and lower the risk of type 2 diabetes [14, 15]. In pre-clinic studies, we previously showed that dietary nitrate supplementation has organ protective effects in models of renal and cardiovascular disease [12, 16] and reverses features of the metabolic syndrome [17]. Mechanistically, nitrate restored bioactive nitrogen oxides in the kidney, reduced renal

inflammation and markers of oxidative stress [16, 18]. Moreover, nitrate and nitrite supplementation decreased leukocyte recruitment in microvascular inflammation and NSAID induced intestinal injury [19]. Treatment with nitrite was also shown to protect against lipopolysaccharide (LPS)-induced morbidity and mortality in mice [20] as well as tissue injuries in rats [21]. Together, these studies demonstrate therapeutic effects of inorganic nitrate and nitrite on disorders characterized by oxidative stress and inflammation. However, it is still unknown if these anions can directly modulate the function of immune cells.

In this study we used mouse peritoneal macrophages and human monocytes to investigate the potential effect of nitrite on $O_2^{\bullet-}$ generation in these cells. We hypothesized that nitrite, at concentrations resembling those achieved after intake of nitrate-rich vegetables in humans, may reduce NOX-mediated $O_2^{\bullet-}$ production in phagocytes via NO-mediated mechanism.

Material and Methods

Mouse Macrophages

Peritoneal macrophages (IC-21, ATCC TIB-186; American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (Life Technologies, Grand Island, NY, USA). Cells were grown in 75ml flasks (Sigma-Aldrich, St. Louis, MO, USA) in humidified air with 5% CO_2 at 37°C until reaching 80% confluence. Macrophages were seeded in 6-well plates at a density of 0.5×10^6 ml^{-1} and grown overnight in medium without fetal bovine serum before starting experiments.

Human Monocytes

Monocytes were isolated from peripheral venous blood. Whole blood from healthy donors was provided by the Karolinska Hospital Blood Bank. Monocytes were separated from blood buffy coat with Ficoll under density gradient centrifugation, by using monocyte-specific anti-CD14-coated magnetic beads [22]. Isolated cells were then seeded for following experiments with the same protocol described above.

Substances used in the experiments

LPS from *Escherichia coli endotoxin* (0111:B4, 10 ng/ml), Sodium Nitrite ($NaNO_2$, 0.1, 10 and 1000 μM, Sigma), Diethylenetriamine/NO adduct (DETA-NONOate, 0.5 mM, Sigma), Nω-nitro-L-arginine methylester hydrochloride (L-NAME, 1 mM, Sigma), Febuxostat (30 nM, Selleckchem), Raloxifene Hydrochloride (5 nM, Sigma) and carboxy-PTIO potassium salt (cPTIO, 50 μM, Sigma). The substances were applied into the culture medium following

overnight seeding, cells were then treated for 24h. Nitrite and DETA-NONOate were administered simultaneously with LPS, whereas all other treatments were administered 30 min prior LPS. An additional dose of cPTIO (50 μM) was added 8h after the primary administration. 4-Amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM DA) was used for fluorescence detection of NO. Cellular viability was evaluated with Trypan Blue Solution (0.4%, Gibco®) exclusion test.

Superoxide Generation

NADPH mediated $O_2^{\bullet-}$ generation from macrophage and their precursors, monocytes, was detected by chemiluminescence as previously described [16, 23]. In brief, cells were collected with 300 μl/well dissociation buffer (Invitrogen) and 500 μl/well DPBS and transferred into reaction tubes. Corresponding concentration of L-NAME, Febuxostat, Raloxifene and cPTIO were added once again during harvesting. NADPH (100 μM, Sigma) and lucigenin (5 μM, Sigma) were added to the cell suspension, and $O_2^{\bullet-}$ level was determined by measuring lucigenin chemiluminescence every 3 sec for 3 min with the AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany).

Nitric Oxide Generation

A highly sensitive, photo-stable cell-permeable fluorescent probe, 4-Amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM DA) (Sigma), was used for the detection of NO production in macrophages. DAF-FM DA is diacetylated intracellularly by esterases to DAF-FM. It reacts with NO in the presence of oxygen to form green-fluorescent triazolofluoresceins. The fluorescent signal provides a temporally integrated estimate of

intracellular NO bioavailability. The cells were plated in a 96 well plate ($5x10^4$ cells/well) and treated as previously described. By the end of the 24 hour treatment, DAF-FM DA solution was added to the medium (final concentration 10μM) for 45 min. Cells were then washed and harvested in PBS containing L-Arginine (1.15 mM) with or without L-NAME (1 mM). Subsequently, cells were transferred to a black 96 well microplate (Nunc™, Thermo Scientific) and DAF-FM was excited with light at 495 nm from a fluorometer (Spectra Max Gemini, Molecular Devices Corporation, Downington, PA, USA) and the emission at 515 nm was detected (at 37°C). Change in fluorescence intensity of DAF-FM, as an indicator of NO production was quantified as the percent change from the respective control values as previously described [12].

Peroxynitrite Generation

Cells were seeded in black 96-well plate at $3x10^5$ cells/well. The generation of highly reactive oxygen species (hydroxyl radical (OH•) and peroxynitrite ($ONOO^-$)) was measured using hydroxyphenyl fluorescein (HPF, Sigma Aldrich) [24] regarding to the manufacture's protocol. Uric acid (1mM) was then used to scavenge $ONOO^-$. The fluorescence signal was measured at an excitation wavelength of 490 nm and an emission wavelength of 515 nm (at 37°C). The level of $ONOO^-$ was calculated by subtracting the HPF signal from the HPF + uric acid signal. The result was expressed as relative changes compared to control.

Real Time quantitative Reverse Transcription PCR

Total RNA was isolated from macrophages after treatments using RNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was synthesized with High Capacity cDNA Reverse

transcription kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative PCR analysis was performed regarding to the Applied Biosystems 7500 protocol**.** Power SYBR Green Master mix (Applied Biosystems) was used for amplification and detection of DNA. PCR reaction was performed in 96-well plates with 20 µl mixer/well (0.25 pmol/µl of each primer and 5 µl of cDNA corresponding to 25 ng of RNA, Applied Biosystems). The efficiency of PCR was calibrated according to the standard curve and the mRNA level was normalized with β-actin by the ΔCt method. Primer sequences and amplification profiles used for NOX isoforms and regulatory subunits (Nox2, p22phox, p47phox, p67phox) and iNOS as well as β-actin are described in **Table 1**.

Cellular Viability measurement

Cellular viability was estimated by Trypan Blue method. Briefly, after each experiment, freshly isolated macrophages (50µl) was incubated in 0.08% Trypan Blue dye (50µl, Gibco®) for 5 min and cells were counted with hematocytometer. Cell counting was repeated for 4 times per well, n=5 per group.

Statistical Analysis

Results are expressed as means ± SEM of at least 3 independent rounds of experiments. Chemiluminescence unit (CLU) was presented as percentage of each individual control group. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls *post hoc* test (GraphPad v6.0, San Diego, CA). A p-value less than 0.05 was considered statistically significant.

Results

Nitrite reduces superoxide generation in LPS activated macrophages

After 24h of LPS activation, the $O_2^{\bullet-}$ generation from macrophages was significantly higher compared to non-activated cells (155±7 vs 100±3%) **(Fig. 1A-B)**. Treatment with nitrite in a low (0.1 μM), intermediate (10 μM) or high (1000 μM) concentration in non-activated cells did not significantly change the $O_2^{\bullet-}$ production compared with control. Simultaneous administration of nitrite (10 μM) and LPS for 24 hours markedly reduced $O_2^{\bullet-}$ formation (116±4%) compared with LPS alone, and the levels were not significantly different from those observed in non-activated macrophages **(Fig. 1A-B)**. The higher or lower doses of nitrite tested in this study did not significantly alter LPS-induced $O_2^{\bullet-}$ formation (140±8% and 144±5%, respectively). Interestingly, similar effects as those observed with nitrite (10 μM) during LPS activation, were also seen with the classical NO donor DETA-NONOate (103±3%) **(Fig. 1C)**, suggesting that the effects of nitrite are linked to NO generation. In subsequent experiments we used the intermediate concentration of nitrite, which represents a rather high dose but still within the physiological range.

Nitrite decreases superoxide production via NOS-independent NO formation

To further investigate if the nitrite-mediated reduction in $O_2^{\bullet-}$ formation was dependent on NO generation, an NO scavenger (cPTIO) or an NO synthase inhibitor (L-NAME) was administered. cPTIO increased $O_2^{\bullet-}$ formation in non-activated macrophages compared with control (116±6 vs 100±3%), suggesting scavenging of endogenous NO. Simultaneous LPS and cPTIO treatment also elevated the $O_2^{\bullet-}$ levels compared with LPS group **(Fig. 2A)**. In the presence of cPTIO, the nitrite-mediated reduction in $O_2^{\bullet-}$ generation was abolished in LPS

activated macrophages. L-NAME markedly elevated $O_2^{\bullet-}$ generation in both control and LPS activated macrophages (141±10% and 242±15% respectively) **(Fig. 2B)**. However, simultaneous treatment with L-NAME did not abolish the effect of nitrite during activation with LPS (139±5%). Taken together, this demonstrates endogenous NO generation in resting macrophages that modulates $O_2^{\bullet-}$ generation, and suggests that nitrite treatment is associated with a NOS independent NO formation in activated macrophages.

Activation of macrophages with LPS was associated with increased NO formation, as measured with DAF-FM fluorescence, and this was completely inhibited by L-NAME **(Fig. 3 A-B)**. Co-incubation with inorganic nitrite and LPS also increased NO generation, but this was not different compared with LPS alone. In activated macrophages with simultaneous nitrite treatment the increased NO generation was not significantly affected by L-NAME **(Fig. 3B)**, again suggesting NOS independent NO formation.

Nitrite reduces superoxide generation in activated macrophages by a xanthine oxidase dependent mechanism without the involvement of aldehyde oxidase

A variety of proteins and enzymes may catalyze the reduction of nitrite to NO in blood and tissues, including NOS, aldehyde oxidase and XO [10]. Inhibition of aldehyde oxidase with raloxifene did not change $O_2^{\bullet-}$ generation in control or LPS activated macrophages, nor did it influence the nitrite-mediated effect on $O_2^{\bullet-}$ formation in activated macrophages **(Fig. 4A)**. Interestingly, inhibition of XO with febuxostat slightly increased $O_2^{\bullet-}$ formation in resting macrophages (121±7%), but did not significantly alter $O_2^{\bullet-}$ formation in LPS activated macrophages (157±6%). However, simultaneous administration of febuxostat abolished the

ability of nitrite to reduce $O_2^{\bullet-}$ levels during LPS activation (180±10%) **(Fig. 4B)**, suggesting a crucial role of XO in reducing nitrite to NO in activated macrophages.

Nitrite does not influence NOX expression in LPS activated macrophages

Nox 2 is generally considered to be the primary NOX isoform in phagocytes responsible for $O_2^{\bullet-}$ production [25]. We found that LPS activation significantly elevated macrophage Nox2 gene expression **(Fig. 5A)**, but this was not affected by simultaneous nitrite treatment. LPS and nitrite did not significantly influence the expression of p22phox, p67phox and p47phox *(data not shown)*.

Nitrite reduces iNOS expression & peroxynitrite production in LPS activated macrophages

Nitrite significantly reduced the iNOS mRNA expression in LPS activated macrophages, but did not change iNOS expression in non-activated cells **(Fig. 5B).** Under proinflammatory stimulation iNOS produces large quantities of NO, which may react with $O_2^{\bullet-}$ to form peroxynitrite. In our experimental conditions, macrophages stimulated with LPS showed significant higher peroxynitrite generation compared to control cells (278±12 vs 0±24 RFU). Nitrite significantly reduced the peroxynitrite production in LPS-activated macrophages (162±21 RFU), and these levels were not different from those observed in nitrite-treated non-activated macrophages (156±35 RFU) **(Fig. 5C)**.

Nitrite reduces superoxide levels in LPS activated human monocytes

To further validate the effects of nitrite on $O_2^{\bullet-}$ production in humans we used monocytes isolated from healthy volunteers. Similar effects as in mice macrophages were revealed **(Fig.**

6); LPS significantly increased the $O_2^{\bullet-}$ production in monocytes compared with non-activated cells (187±9% vs 100±5%). The increased $O_2^{\bullet-}$ levels observed under LPS activation were markedly attenuated with simultaneous nitrite administration (121±9%), which was not different from control.

Cell viability

Cellular viability was not affected by LPS and nitrite (Control: 98.8±0.1%; Nitrite: 99.2±0.1%; LPS: 98.3±0.7%; LPS+Nitrite: 98.7±0.2%), or by other treatments used *(data not shown)*.

Discussion

During oxidative stress, ROS can cause cellular damage that promote inflammatory responses and further contribute to tissue damage [5, 26-28]. Our previous studies demonstrate that a dietary dose of nitrate, selected to resemble the intake of nitrate-rich vegetables in humans, has therapeutic effects in several different disease models where oxidative stress and inflammation are considered important [16, 17, 29, 30]. In addition, two independent studies have demonstrated that treatment with inorganic nitrite can ameliorate tissue injury and reduce morbidity and mortality in models of LPS-induced shock [20, 21]. We and other groups have shown that attenuation of oxidative stress via enhanced NO generation is one of the main mechanisms by which nitrate and nitrite modulate disease progress [12, 16, 31, 32]. Although immune cells are one of the most important sources of free radicals it still remains unclear whether nitrate and nitrite have direct effects on these cells.

Phagocytes, like macrophages and monocytes, respond to various "danger signals" by generating reactive nitrogen- and oxygen species as a part of the host defense response. However when $O_2^{\bullet-}$ generation from phagocytes overwhelms the cellular antioxidants systems, it can induce DNA damage and microvascular permeability, which will aggravate the pro-inflammatory response and further contribute to disease development [33]. In this study, we observed a significant increase in $O_2^{\bullet-}$ production in IC-21 macrophages under LPS stimulation. Interestingly, this increase was attenuated by nitrite. Similar effects of nitrite were also discovered in human peripheral monocytes, suggesting a potential mechanism by which inorganic nitrate and nitrite protect against renal and cardiovascular disease as previously demonstrated [12, 16]. Interestingly, nitrite alone did not affect $O_2^{\bullet-}$ production in resting macrophages. From a therapeutic perspective this is important as physiological generation of

$O_2^{\bullet-}$ is essentially involved in multiple intracellular signaling pathways and homeostasis control [34].

It has been shown that the biological functions of inorganic nitrate and nitrite are mediated, at least in part, by the formation of NO [9, 10, 13]. Our data shows that the effect of nitrite in reducing $O_2^{\bullet-}$ production in LPS activated macrophages is mimicked by the NO donor DETA-NONOate, and could be abolished by cPTIO but not by L-NAME. In agreement, we also showed that nitrite increases NO production (estimated by DAF-FM fluorescence) in activated macrophages by a NOS independent mechanism. Pretreatment with cPTIO in non-activated macrophages resulted in elevated $O_2^{\bullet-}$ production compared to control levels. Moreover, cPTIO reversed the effects of NO_2 on NOX activity in LPS treated macrophages. Thus, although cPTIO may interact not only with NO, but under certain conditions also with $O_2^{\bullet-}$ or nitrogen dioxide ($^{\bullet}NO_2$) [35], our findings indicate that the effect of nitrite in modulating $O_2^{\bullet-}$ level is primarily NO mediated and NOSs independent. Surprisingly, nitrite alone did not change the intracellular levels of NO, suggesting that nitrite exerts its biological effects mainly on activated macrophages.

NO has been suggested to inhibit $O_2^{\bullet-}$ production in neutrophils [36] by a direct action on the membrane components of the NOX. We found that Nox2 mRNA was significantly increased following stimulation with LPS, while gene expression of other subunits (p22phox, p67phox and p47phox) were unchanged. In addition, nitrite did not alter the mRNA levels of these NOX components. Thus, the effect of nitrite on macrophage $O_2^{\bullet-}$ generation does not seem to be related to reduced NOX expression but more likely to reduced activity. Further studies are warranted to fully elucidate the details underlying this effect, which may be linked to NO-mediated S-nitrosylation of NOX or Nrf2 activation [37-39].

There are several proteins like hemoglobin, myoglobin, mitochondrial complexes, aldehyde oxidase and XO that can catalyze the reduction of nitrite to NO in blood and tissues [8]. Among these, XO is an enzyme involved in purine catabolism and elevated ROS production and this enzyme is significantly upregulated under conditions like ischemia [40, 41]. The effect of nitrite on LPS activated macrophages was completely abolished after specific inhibition of XO with febuxostat, suggesting that XO is a central enzyme mediating reduction of nitrite to NO in activated macrophages.

In resting macrophages the physiological "low-output" NO production is mainly carried out by constitutive NOSs, and is indispensible for cells to maintain fundamental functions. However after stimulation, iNOS in macrophages will respond to this "danger stimulus" and produce large quantities of NO [42, 43]. Furthermore, it has been shown that when cytosolic L-arginine availability is reduced [44], which often occurs during inflammation, iNOS *per se* can generate both $O_2^{\bullet-}$ and NO and thereby further form the potent oxidant $ONOO^-$. Thus, appropriate modulation of iNOS function may provide beneficial effects in inflammatory diseases. We confirmed that LPS stimulation significantly enhanced iNOS mRNA expression and $ONOO^-$ production in macrophages, both of which were attenuated by simultaneous administration of nitrite. These data further suggested a protective effect of nitrite under noxious stimulation. As proposed in **Fig. 7**, the inhibition on iNOS mRNA expression by nitrite may be due to a negative feedback through nitrite-derived NO production [45, 46]. Alternatively, it is possible that the reduced NOX activity and $O_2^{\bullet-}$ generation, during nitrite treatment, leads to less activation of iNOS. Importantly, nitrite did not completely abolish the increase in iNOS in response to LPS, which may be important for a preserved immune function in response to microorganisms. Indeed, similar finding have been

seen *in vivo* where dietary nitrate and nitrite reduced leukocyte infiltration in inflamed mouse tissue without affecting bacterial clearance [29].

Conclusion

Our study demonstrates that inorganic nitrite attenuates NOX-dependent $O_2^{\bullet-}$ formation in LPS-stimulated murine macrophages and in human monocytes. These effects are dependent on XO-catalyzed nitrite reduction and generation of NO. Intrinsic $O_2^{\bullet-}$ generation in resting macrophages was not affected by nitrite indicating that this anion does not interfere with physiological redox-dependent ROS signaling. These results may, at least in part, explain the observed organ-protective and anti-inflammatory properties of inorganic nitrate and nitrite in experimental disease models.

Conflict of interest statement

The authors have no relevant potential conflicts of interest to declare.

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Table 1. Sequences of Primers for RT-PCR

	Forward Primers (5'→3')	Reverse Primers (5'→3')
Nox2	GCA CCTGCAGCCTGCCTGAATT	TTGTGTGGATGGCGGTGTGCA
p22phox	TTGTGTGGATGGCGGTGTGCA	TTGTGTGGATGGCGGTGTGCA
p47phox	TTGTGTGGATGGCGGTGTGCA	GCCTCAATGGGGAACATCTCCTTCA
p67phox	AAGACCTTAAAGAGCCTTGACGCA	TCGGACT TCATGTTGGTTGCCAA
iNOS	TGACGGCACATGACTTCAG	GCCATCGGGCATCTGGTA
ß-actin	GCTCCTCCTGAGCGCAAT	GTGGACAGTGAGGCCAGGAT

Figure 1. ***Nitrite reduces superoxide generation in LPS-activated macrophages similar to that of a classical NO donor.***

NOX activity was measured as lucigenin-dependent chemiluminescence of superoxide every 3 s for 3 min, 24h after treatment (Panel A). Macrophages stimulated with LPS showed significant higher superoxide generation compared to control cells. Co-incubation with an intermediate concentration of inorganic nitrite reduced superoxide production to baseline levels, whereas the higher or lower doses did not significantly change LPS-induced superoxide generation. Nitrite alone did not significantly influence superoxide level in non-stimulated macrophage (Panel B). Co-incubation with inorganic nitrite (10 μM) or DETA-NONOate reduced superoxide production in LPS activated macrophages to the same extent (Panel C). Data are means±SEM (n=12-20). *, P<0.05 vs indicated group. LPS=lipopolysaccharide (10 ng/ml), NO_2=sodium nitrite (0.1, 100 or 1000 μM), DETA-NONOate=NO donor (0.5 mM).

Figure 2. ***Nitrite reduces superoxide generation in LPS-activated macrophages via NOS-independent NO production.***

NOX activity was measured as lucigenin-dependent chemiluminescence of superoxide (similar to that described for Fig. 1). Incubation with cPTIO alone increased superoxide level in non-stimulated macrophage, and elevated superoxide formation also in macrophages activated with LPS. Simultaneous treatment with cPTIO abolished the effect of nitrite during activation with LPS, showing no differences compared with macrophages treated with LPS+cPTIO (Panel A). Incubation with L-NAME alone increased superoxide levels in control macrophages, and markedly increased superoxide generation in LPS-activated macrophages. However, simultaneous treatment with L-NAME did not abolish the effect of nitrite during activation with LPS (Panel B). Data are means±SEM (n=10-12). *, P<0.05 vs indicated group,

#, P<0.05 vs untreated controls. LPS=lipopolysaccharide (10 ng/ml), NO_2=sodium nitrite (10 µM), cPTIO=NO scavenger (10 µM), L-NAME=non-selective NOS inhibitor (1 mM).

Figure 3. ***Nitrite increases NO generation in activated macrophages independent of iNOS***

DAF-FM fluorescence was used as an indicator of NO generation. Macrophages stimulated with LPS showed significantly higher NO generation compared to control cells. Co-incubation with inorganic nitrite increased NO generation, but this was not significantly different compared with LPS alone (Panel A). Simultaneous incubation with L-NAME abolished the NO generation in LPS activated macrophages, but did not significantly change NO formation in activated macrophages treated with nitrite (Panel B). Data are means±SEM (n=15). *, P<0.05 vs indicated group. LPS=lipopolysaccharide (10 ng/ml), NO_2=sodium nitrite (10 µM), L-NAME=non-selective NOS inhibitor (1 mM).

Figure 4. ***The nitrite-mediated reduction of superoxide generation in LPS-activated macrophages is dependent on xanthine oxidase but not on aldehyde oxidase***.

NOX activity was measured as lucigenin-dependent chemiluminescence of superoxide (similar to that described for Fig. 1). Incubation with Raloxifene did not change superoxide formation in control or in LPS-activated macrophages, nor did it influence the effect of nitrite during activation with LPS (Panel A). Incubation with Febuxostat increased superoxide generation in non-activated macrophages, but did not significantly influence superoxide formation in LPS-activated macrophages. Simultaneous treatment with Febuxostat abolished the effect of nitrite during activation with LPS. Data are means±SEM (n=10-12). *, P<0.05. LPS=lipopolysaccharide (10 ng/ml), NO_2=sodium nitrite (10 µM), Raloxifene=aldehyde oxidase inhibitor (5 nM), Febuxostat=xanthine oxidase inhibitor (30 nM).

Figure 5. ***Nitrite attenuates the LPS induced iNOS expression and peroxynitrite production without affecting the expression of Nox2***.

Co-incubation with inorganic nitrite alone did not influence Nox2 expression. Activation of macrophages with LPS significantly increased Nox2 expression, but this was not altered by simultaneous incubation with inorganic nitrite (Panel A). Co-incubation with inorganic nitrite alone did not influence iNOS expression, although it was slightly reduced. Activation of macrophages with LPS significantly increased iNOS mRNA expression, and this was attenuated by simultaneous incubation with inorganic nitrite (Panel B). Additionally, macrophages stimulated with LPS showed significant higher peroxynitrite generation compared to control. The presence of nitrite significantly reduced the peroxynitrite production in LPS-activated macrophages and these levels were not different from that observed in nitrite-treated non-activated macrophages (Panel C). Data are means±SEM (n=10-12). *, $P<0.05$. The peroxynitrite levels are expressed as relative fluorescence units (RFU). LPS=lipopolysaccharide (10 ng/ml), NO_2=sodium nitrite (10 µM).

Figure 6. ***Nitrite reduces superoxide generation in LPS-activated human monocytes.***

NOX activity was measured as lucigenin-dependent chemiluminescence of superoxide (similar to that described for macrophages in Fig. 1 & 2). Similar to that observed in macrophages, LPS-activated monocytes showed significantly higher superoxide generation compared with control. Co-incubation with inorganic nitrite reduced superoxide production to baseline. Data are means±SEM (n=10-12). *, $P<0.05$; LPS=lipopolysaccharide (10 ng/ml), NO_2=sodium nitrite (10 µM)

Figure 7. ***Schematic illustration of the proposed mechanisms for nitrite-mediated reduction of NOX-derived superoxide generation and iNOS in activated macrophages.***

Activation of macrophages with lipopolysaccharide (LPS) is associated with increased NOX-derived superoxide (O_2^-) generation and activation of iNOS. A simultaneous increase in NO and $O_2^{\bullet-}$ will lead to formation of peroxynitrite ($ONOO^-$). Inorganic nitrite (NO_2^-) can be reduced by xanthine oxidases (XO) in activated macrophages to form nitric oxide (NO), which may reduce NOX activity and superoxide (O_2^-) generation. Increased levels of NO, or reduced levels of $O_2^{\bullet-}$ can reduce iNOS activation, and will also result in less formation of $ONOO^-$.

Graphical Abstract (for review)

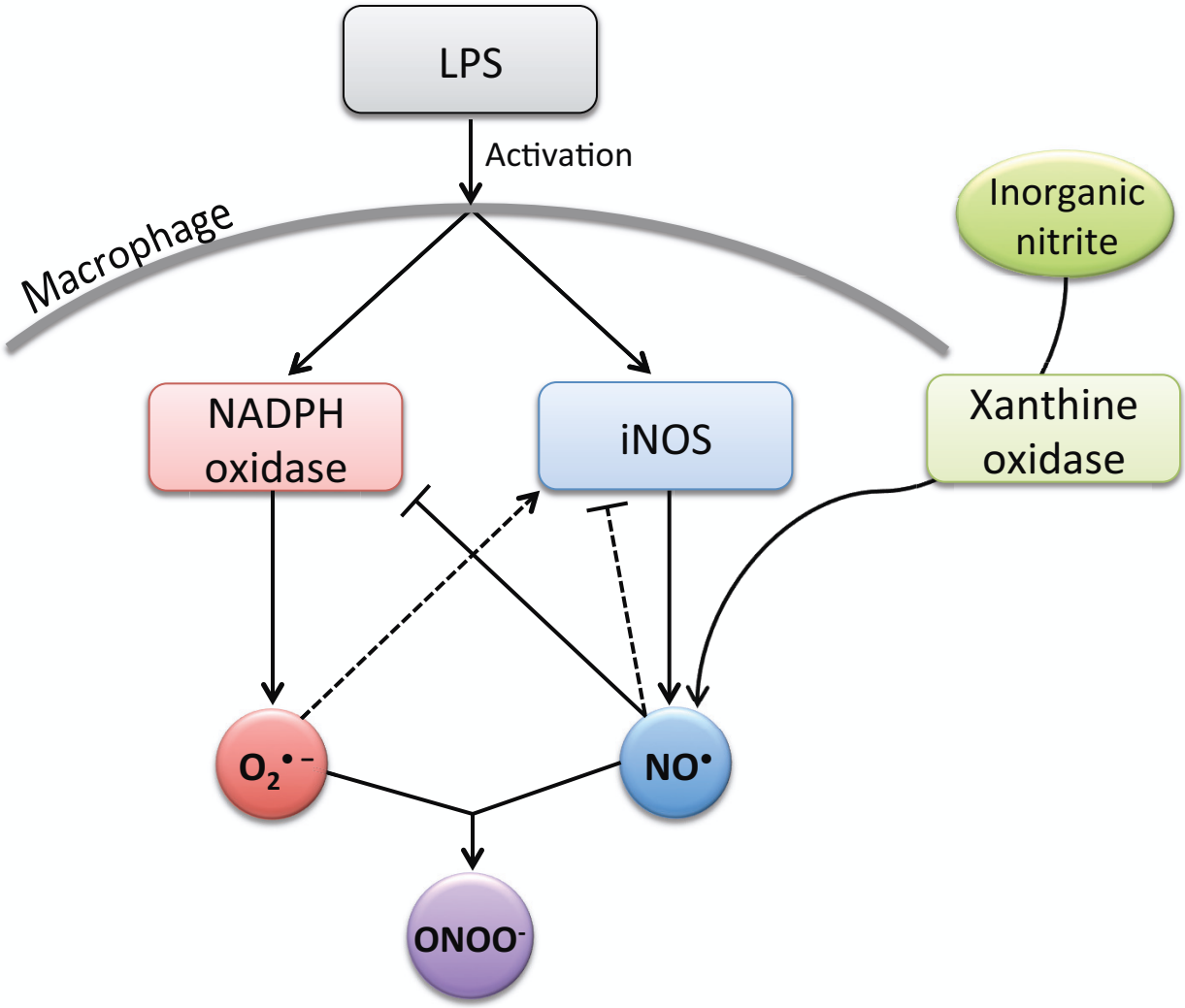


Figure 1

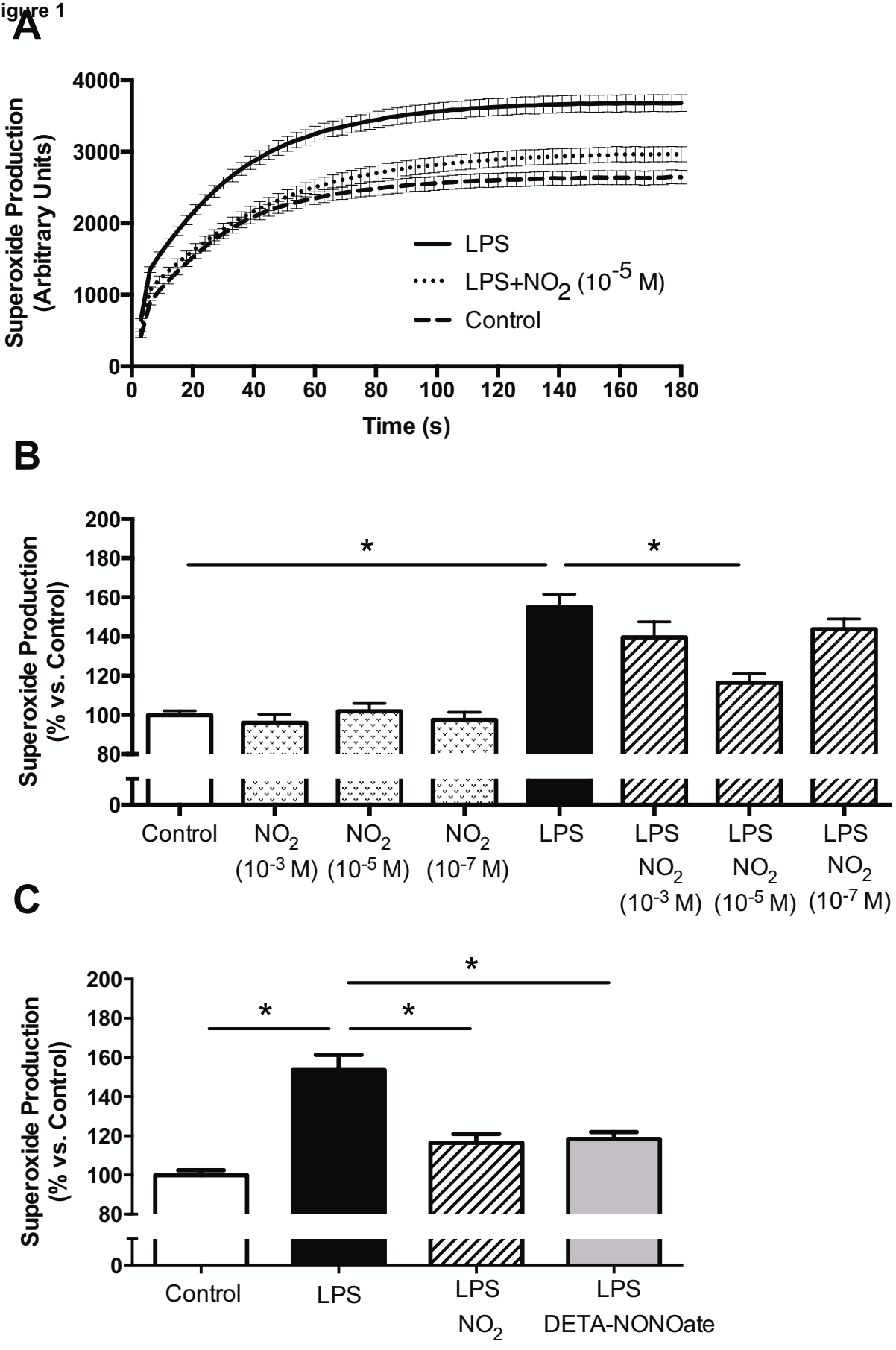


Figure 2

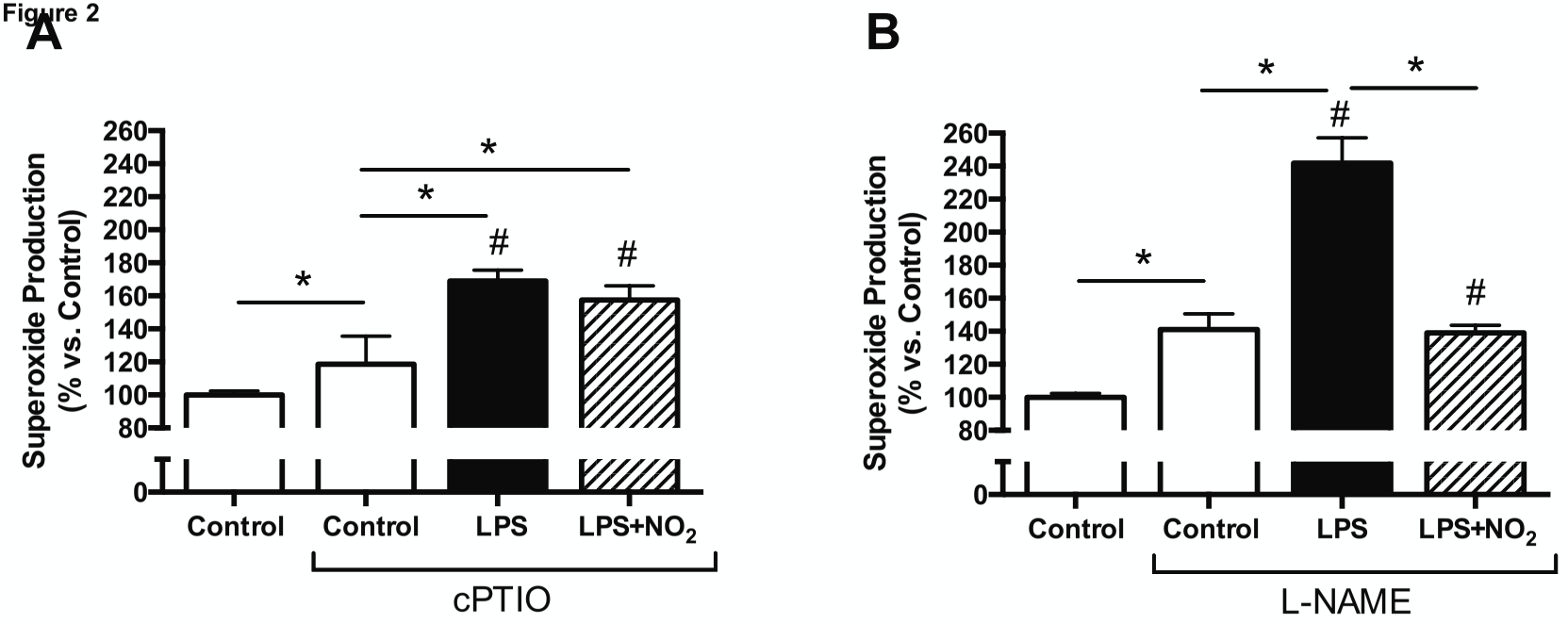


Figure 3

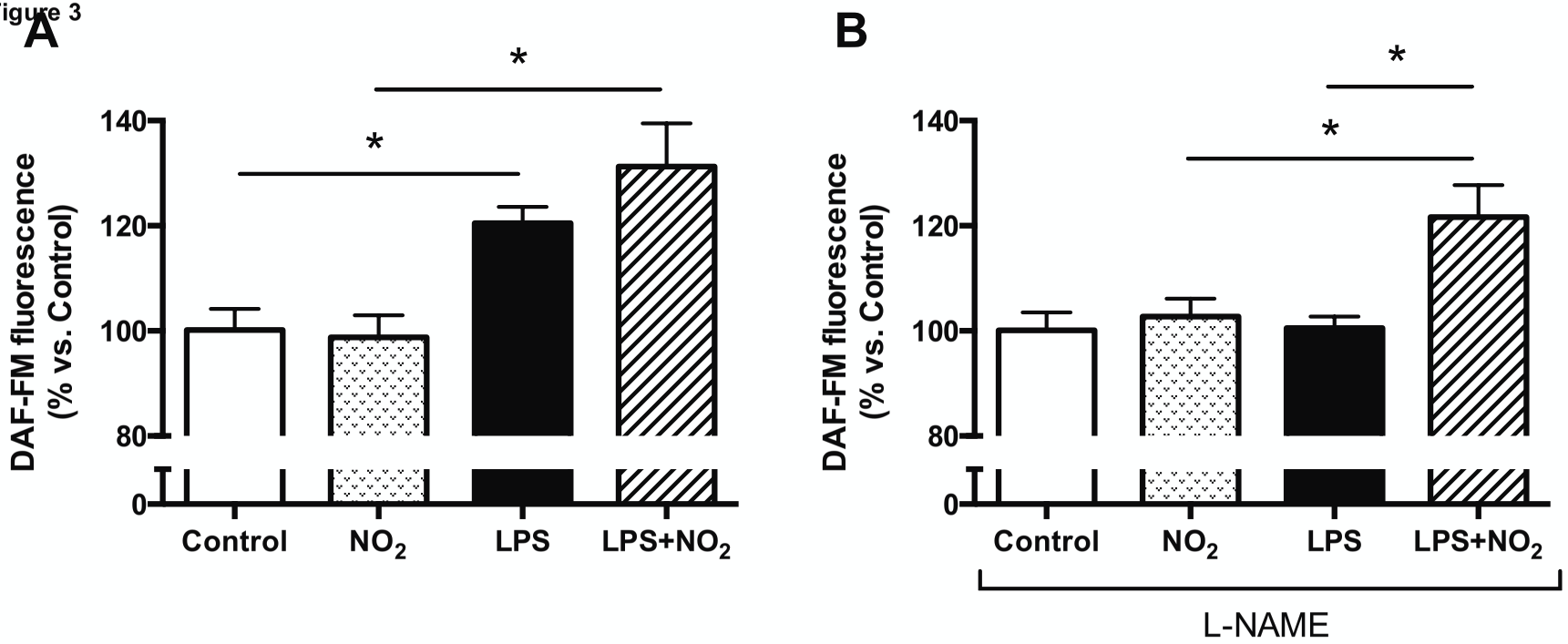


Figure 4

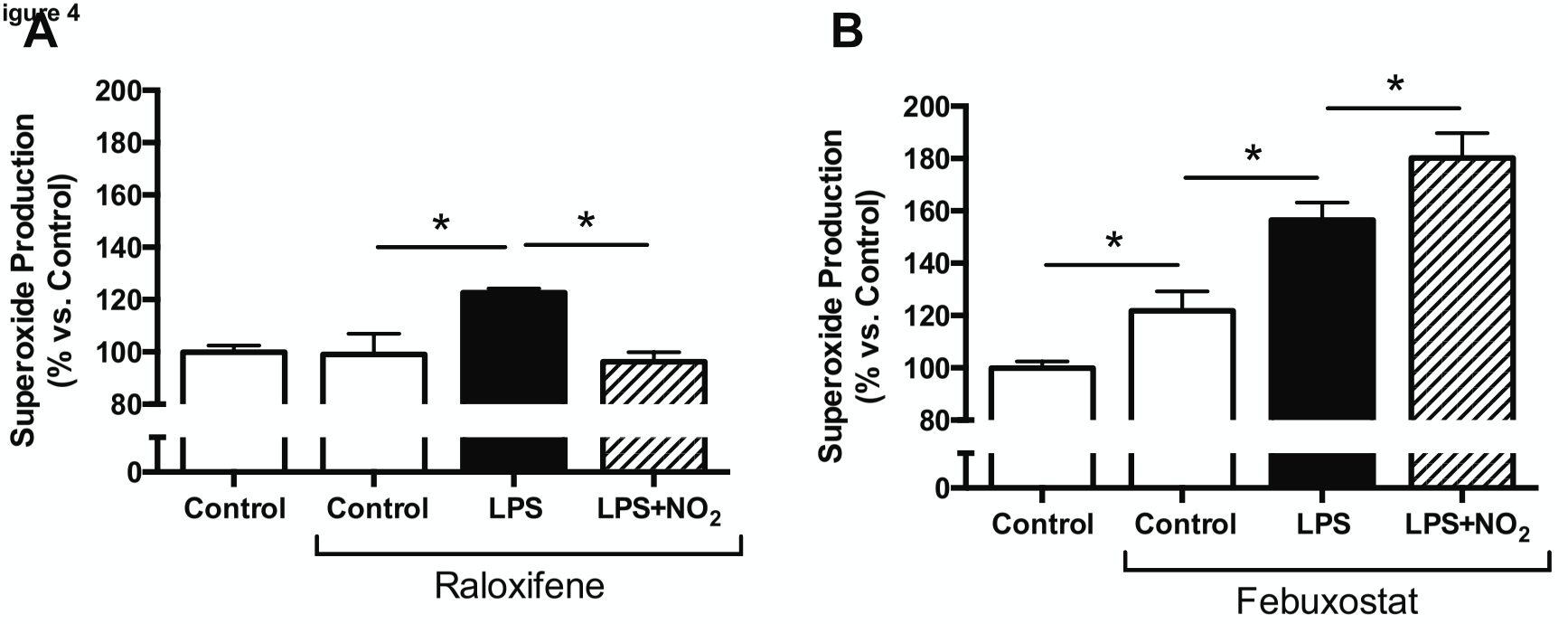
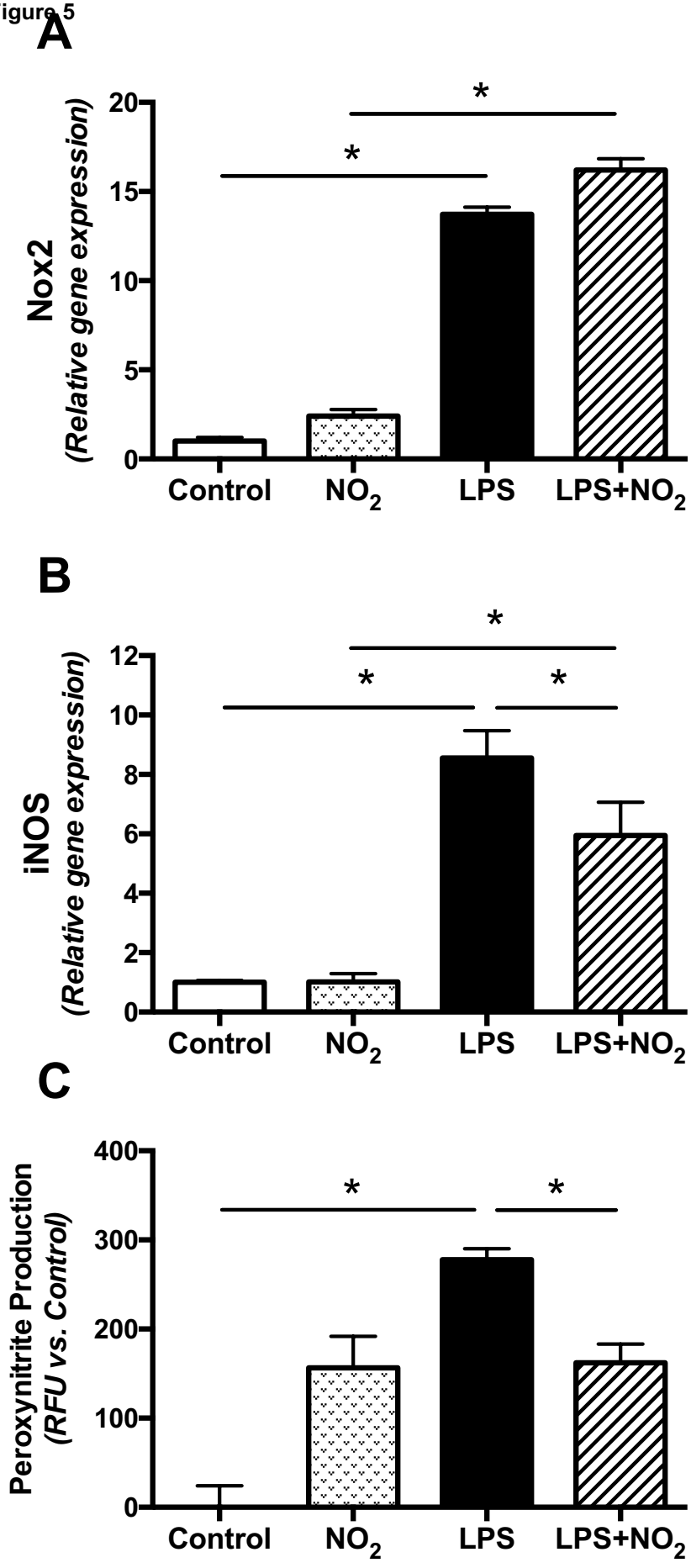


Figure 5



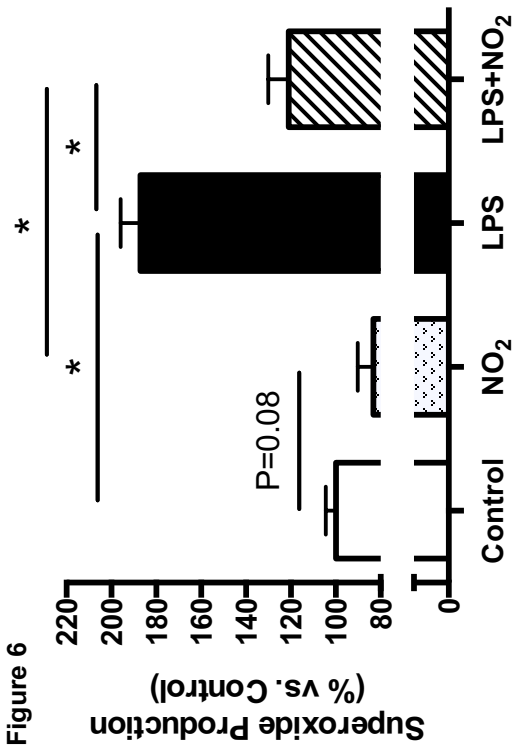
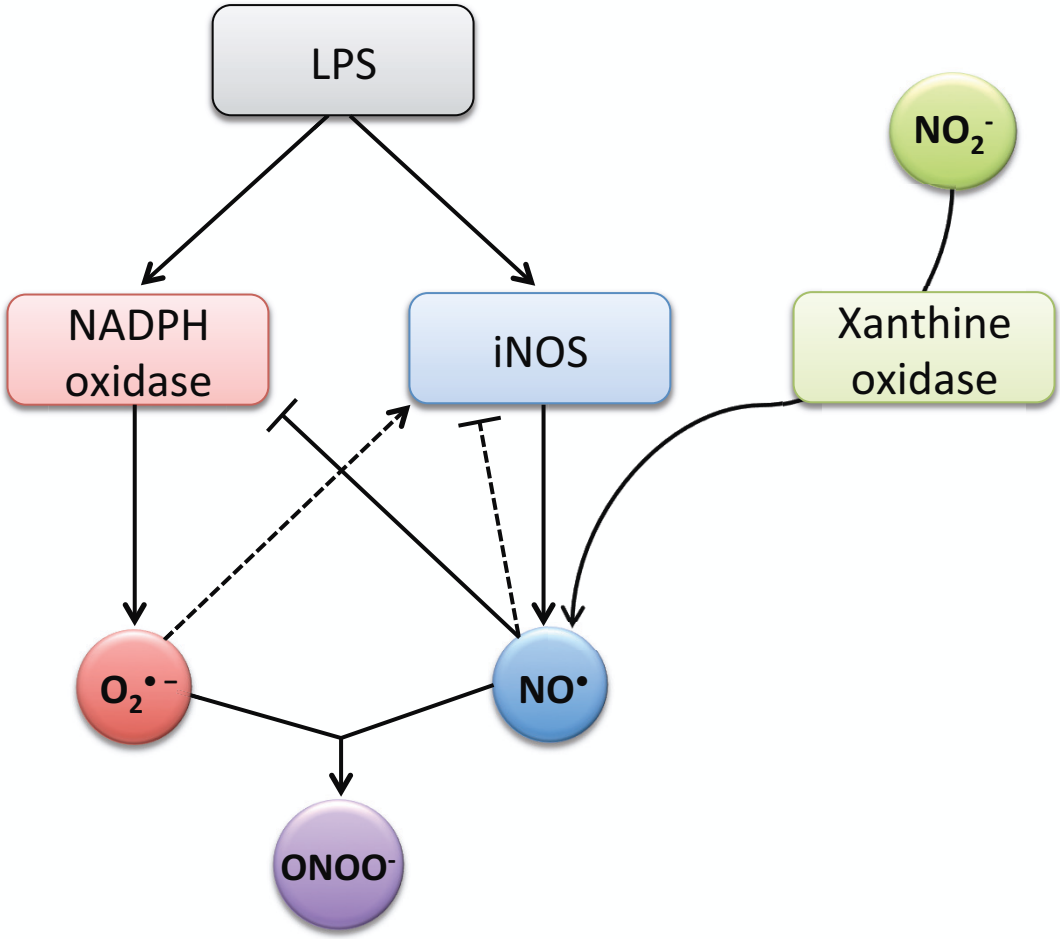


Figure 6

Figure 7


LPS
NO_2^-
NADPH oxidase
iNOS
Xanthine oxidase
$O_2^{\bullet -}$
$NO^{\bullet}$
$ONOO^-$