Iron attenuates nitric oxide level and iNOS expression in endotoxin-treated mice

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Abstract The effect of exogenous Fe-citrate complex (Fe doses of 120 and 240 μmol/kg) on nitric oxide (NO) production in vivo has been studied in blood and liver tissue of endotoxin-treated mice. Fe-citrate complex was administered to mice subcutaneously at the same time with intravenous injection of *Escherichia coli* lipopolysaccharide (LPS). Iron-dependent decrease in NO_2^-/NO_3^- and nitrosyl hemoglobin levels in blood of animals was detected at 6 h after LPS administration, suggesting systemic attenuation of NO generation. NO production in the liver tissue of LPS-treated mice was decreased after Fe administration judging from the amount of mononitrosyl-iron complexes formed in the tissue by diethyldithiocarbamate. The iNOS protein determination in the liver tissue of LPS-treated mice demonstrated iron-dependent inhibition of iNOS expression. We have found previously that exogenous iron does not affect systemic NO level when it is given at 6 h after LPS injection, i.e. after iNOS expression. This is a first report demonstrating iron-dependent iNOS down-regulation in endotoxin-treated mice.

Key words: Septic shock; Nitric oxide; Iron; Inducible nitric oxide synthase; Nitrosyl iron complex; Spin trapping

1. Introduction

The early studies on interaction between iron and infections yielded controversial results. Iron supports bacterial growth [1], but it can inactivate bacterial exotoxins and endotoxin [2]. It has been recently established that these bacterial products trigger harmful overproduction of nitric oxide (NO) leading to cardiovascular collapse, multiple organ failure and death [3]. Cardiovascular response to endotoxin and animal survival improved in mice lacking iNOS enzyme, which generates NO during infection [4,5]. Intracellular iron is a major target of endogenous NO, which alters the activity of iron-dependent enzymes [6]. Nitric oxide controls free iron level in cells by activating iron regulatory factor [7] and affecting enzymes involved in heme metabolism [8]. Weiss et al. [9] have proposed feedback regulation between iron metabolism and NOS pathway, based on iron control of nuclear transcription for iNOS in macrophages in vitro. However, in vivo interaction between NO pathway and iron may be more complicated since macrophages and other iNOS-expressing cells are subjected to a complex activation and suppression by cytokines, reactive oxygen species (ROS) and nitric oxide. In addition, the traditional view of the iron as a prooxidant and proinflammatory factor [10] suggests Fe-induced ROS production leads to activation of nuclear transcription factor NF-κB and subsequent iNOS expression in tissue. Holotransferrin (diferric transferrin), for example, has been reported to induce iNOS mRNA expression in rat cultured aortic smooth muscle cells [11]. In this study we have examined the effect of exogenous Fe-citrate complex on the level of nitric oxide in blood and tissue as well as iNOS expression in the liver of endotoxin-treated mice. Our results demonstrate that exogenous iron attenuates nitric oxide production in vivo in endotoxin-treated mice via down-regulation of iNOS expression.

2. Materials and methods

2.1. Materials

ICR mice (female, 30–35 g) were supplied by Harlan Sprague-Dawley (Indianapolis, IN). All animal experiments were guided by the principles for the care and use of laboratory animals as recommended by the US Department of Health and Human Services, and approved by the George Washington University Animal Care and Use Committee. Lipopolysaccharide (LPS, *Escherichia coli* O26:B6) and desferroxamine mesylate were from Sigma (St. Louis, MO). Diethyldithiocarbamate (DETC) was purchased from Aldrich (Milwaukee, WI), 3-morpholinosydnonimine (SIN-1) was from Cayman Chemical Company (Ann Arbor, MI). Polyclonal rabbit anti-iNOS antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and horseradish peroxidase (HRP) chemiluminescent substrate Supersignal was from Pierce (Rockford, IL). Other chemicals and reagents were purchased from Sigma (St. Louis, MO). NO_2^- was measured by the Griess colorimetric assay [12]. NO_3^- was first converted to NO_2^- by *E. coli* nitrate reductase [13] and measured as described above.

2.2. Animal procedure

LPS (6 mg per mouse) was given via the tail vein and at the same time iron-citrate complex (33 mg/kg $FeSO_4 \cdot 7H_2O$ plus 167 mg/kg of sodium citrate) was administered subcutaneously (iron dose 120 μmol/kg). In some experiments animals received a double dose of iron (240 μmol/kg). Control animals received LPS plus Na-citrate. At 6 h after LPS, the mice received 2.2 mmol/kg DETC, they were killed 30 min later and liver tissue obtained. Some animals treated with iron did not receive DETC, they were killed at 6 h after LPS; blood samples obtained from these animals were used for nitrosyl hemoglobin (HbNO) measurement; sera were used for NO_2^-/NO_3^- assay and liver tissue was used to determine total amount of free iron by iron-desferal EPR assay [14].

2.3. EPR measurements

EPR measurements were performed at 77 K with a Bruker ER 200 X-band spectrometer using an EPR finger Dewar (Wilmad Class,

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Abbreviations: EPR, electron paramagnetic resonance; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; MGD, *N*-methyl-D-glucamine dithiocarbamate; DETC, diethyldithiocarbamate; SIN-1, 3-morpholinosydnonimine; HbNO, nitrosyl hemoglobin; HRP, horseradish peroxidase

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Buena, NJ). $DETC_2$-Fe-NO complexes were determined by EPR in the liver tissue frozen in liquid nitrogen. Whole blood obtained from mice without anticoagulant (0.5 ml sample volume) was frozen immediately at 77 K for EPR detection of HbNO. Total amount of $DETC_2$-Fe complex formed in tissue by DETC was determined after additional incubation of tissue with SIN-1 as described earlier [15]. Total amount of free iron was determined in the liver tissue homogenate of mice untreated with DETC after additional incubation with desferal as described [14,15].

2.4. Determination of iNOS protein by Western blot

Protein samples from the liver of control mice, LPS-treated mice, and mice treated with LPS plus iron were homogenized and electrophoretically size-separated as we have previously described [16]. Equivalent amounts of total liver protein from individual mice (100 μg) were loaded into adjacent lanes and separated using a discontinuous system consisting of an 8% polyacrylamide resolving gel and a 6% stacking gel on an 8×10 cm electrophoresis cell (Bio-Rad, Hercules, CA). Following separation, the proteins were electrophoretically transferred to a nitrocellulose membrane which was blocked with 12% non-fat dried milk in Tris-buffered saline (NFM/TBS) for a minimum of 2 h. The membranes were incubated with a 1:1000 dilution of polyclonal rabbit anti-iNOS antibody in 4% NFM/TBS for 60 min at room temperature. Membranes were then incubated with HRP-labeled goat anti-rabbit IgG (1:1000) in 4% NFM/TBS for an additional 60 min at room temperature. Thorough rinsing of membranes was performed between each step using three rinses of Tris-buffered saline with 0.8% Tween-20 for a minimum of 5 min each. The bound antibody was detected with a chemiluminescent HRP substrate and exposed to X-ray film (BIOMAX MR, Eastman Kodak, Rochester, NY). The films were processed with a Kodak M35 X-OMAT autoprocessor (Eastman Kodak), and densitometry was performed using a Phosphorimager Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

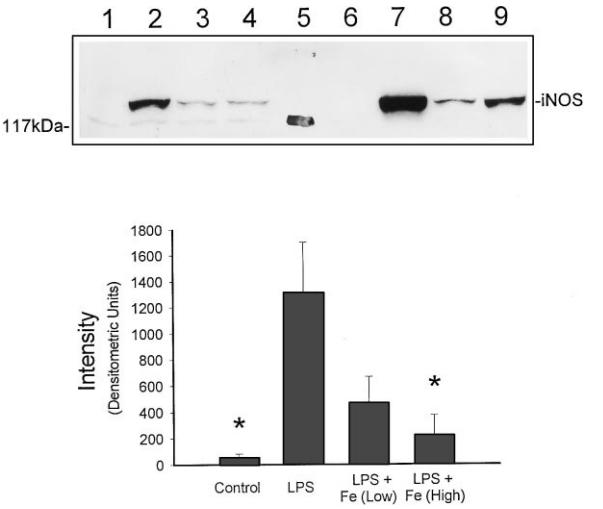

Fig. 1. Top: Representative Western blot for iNOS protein in which 100 μg of total liver protein from mice treated with vehicle control (lanes 1 and 6), LPS (lanes 2 and 7), LPS plus 120 μmol/kg iron (lanes 3 and 8) and LPS plus 240 μmol/kg iron (lanes 4 and 9) were loaded. Lane 5 contains the size markers. Bottom: Densitometric analysis of iNOS protein band in mice treated with vehicle control ($n = 6$), LPS ($n = 6$), LPS plus 120 μmol/kg iron (designated Fe Low, $n = 4$), and LPS plus 240 μmol/kg iron (designated Fe High, $n = 4$). $^*P < 0.05$ compared to LPS-treated mice.

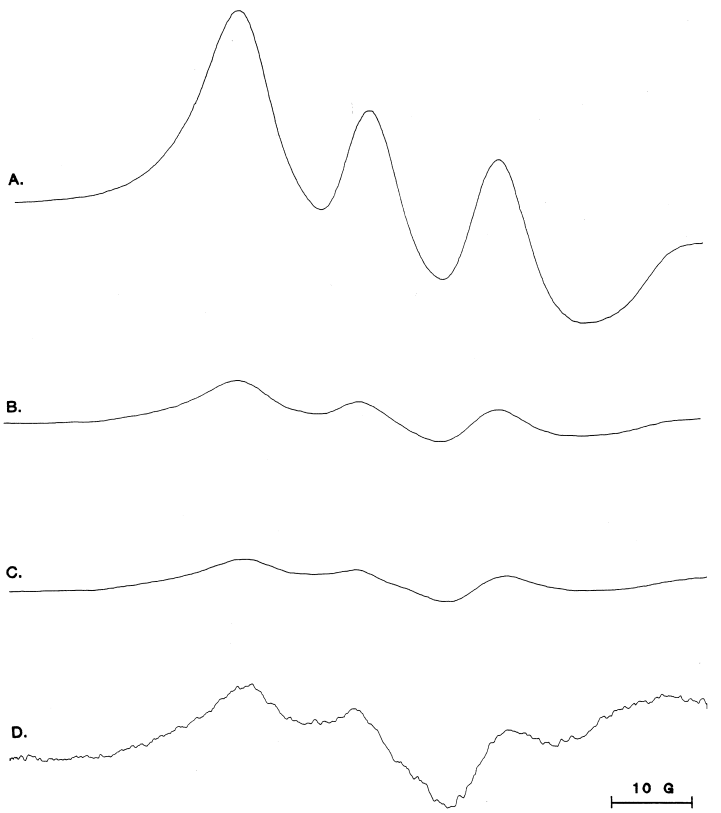

Fig. 2. EPR spectra (77 K) obtained from the liver tissue of septic shock mice, which received 6 mg LPS i.v. and simultaneously s.c. injection of Na-citrate (A) or citrate complex of iron: 120 μmol/kg (B), 240 μmol/kg (C) plus i.p. injection of DETC 2.2 mmol/kg (at 6 h after LPS administration). Normal mice (D) received only DETC injection. All mice were killed 30 min after DETC injection, then liver tissue samples were frozen at 77 K and EPR spectra recorded. The spectra show only triplet EPR signal of $DETC_2$-Fe-NO complex at $g_\perp = 2.041$. The receiver gain for spectrum (D) was 10-fold higher than for other spectra. EPR conditions were: microwave frequency 9.40 GHz, microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 3.2 G, center field 3292 G, scan rate 42 G/min, time constant 0.5 s.

Table 1
The effect of Fe-citrate administration on NO_2^-/NO_3^- level in sera of endotoxin-treated mice[a]

Experimental group	$NO_2^- + NO_3^-$ (μM)	Free Fe (nmol/g of liver wet wt.)[b]
A. LPS, citrate[c]	340 ± 20 (3)	75 ± 11 (4)
B. LPS, Fe (120 μmol/kg)	155 ± 23 (3)**	161 ± 18 (4)*
C. LPS, Fe (240 μmol/kg)	71 ± 11 (4)***	572 ± 137(4)*
D. Normal mice	23 ± 7 (5)****	110 ± 10 (4)

[a]Mice received LPS *E. coli* i.v. via the tail vein (6 mg per animal), Fe-citrate was given simultaneously with LPS as s.c. injection. Mice were killed at 6 h after LPS injection. Control mice (group A) received LPS and sodium citrate (167 mg/kg).
[b]The amount of free iron in liver tissue was determined by EPR in desferal-treated liver homogenate as described [14,15].
[c]The data are presented as mean ± standard error (number of animals); $*P < 0.02$; $**P < 0.005$; $***P < 0.001$; $****P < 0.0005$ compared to group A.

2.5. Statistical analysis

Data are expressed as the mean ± standard error. Differences between groups were evaluated with a one way analysis of variance and a Tukey test. A probability level of $P < 0.05$ was considered significant.

3. Results and discussion

Administration of endotoxin to mice induced iNOS protein expression (Fig. 1) in the liver tissue of animals (iNOS protein was not found in the normal tissue). We observed a concomitant increase in the nitric oxide level in liver tissue of LPS-treated mice after administration of DETC, yielding $DETC_2$-Fe-NO complexes with a characteristic triplet EPR signal at $g_\perp = 2.041$ (Fig. 2) in tissues of mice (only a trace amount of $DETC_2$-Fe-NO complex observed in the liver of normal animals). The augmentation of NO level had a systemic character judging from the increase in the amounts of NO_2^-/NO_3^- (Table 1) and nitrosyl hemoglobin (Fig. 3) in murine circulation. HbNO in the whole blood of endotoxin-treated mice produced characteristic triplet EPR signal of nitrosyl heme ($a^N = 17.5$ G) centered at $g = 2.012$ (Fig. 3). Previously, we have demonstrated that short-term (30 min) Fe exposure did not affect HbNO and NO_2^-/NO_3^- levels in blood of septic shock mice, when iron was given after iNOS expression (i.e. at 6 h after LPS administration) [15]. In this study simultaneous administration of endotoxin (6 mg per animal, i.v.) and iron (120 or 240 μmol/kg, s.c.) produced significantly lower iNOS protein expression in the liver (Fig. 1), decreased NO_2^-/NO_3^- level in sera (Table 1) and HbNO level in whole blood 6 h later (Fig. 3), compared to LPS-treated animals. Incidentally, the dose of iron administered to mice (120 μmol/kg) is close to the amount of iron which mice consume daily in the diet (33 mg/kg per day) [17]. The amount of free iron in the murine liver tissue homogenate has been determined by EPR as [Fe^{III}-desferal] complex, which produced a broad EPR signal at $g = 4.3$. Free Fe in the liver rose about two-fold after the iron dose of 120 μmol/kg and more than six-fold after the Fe dose of 240 μmol/kg (Table 1). We have demonstrated previously that DETC and desferal sample the same pool of free iron in liver tissue [15]. Administration of DETC in vivo in iron-treated mice produced increased amounts of DETC-Fe complex in the liver tissue, compared to untreated animals (data not shown). However, the amount of $DETC_2$-Fe-NO complex formed in liver of Fe-treated septic shock mice was only 39 ± 9% ($n = 6$, Fe dose 120 μmol/kg) and 11 ± 2% ($n = 3$, Fe dose 240 μmol/kg), compared to endotoxin-treated mice (Fig. 2). DETC has been chosen in this study because it binds free iron in tissue, while *N*-methyl-D-glucamine dithiocarbamate (MGD) requires simultaneous administration of exogenous Fe to produce MGD_2-Fe-NO complex in tissue (this additional iron injection would complicate the interpretation of the experiment).

Iron administration after iNOS expression (i.e. at 6 h after

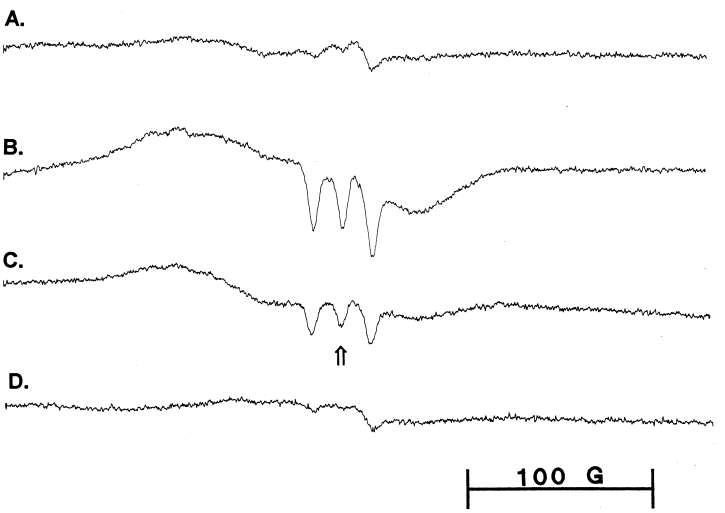

Fig. 3. EPR spectra (77 K) of whole blood (0.5 ml sample volume) obtained from normal mice (A) and mice which received 6 mg LPS i.v. and simultaneously s.c. injection of Na-citrate (167 mg/kg) in saline (B) or s.c injection of citrate complex of iron: 120 μmol/kg (C), 240 μmol/kg (D) in saline. The animals were killed at 6 h after LPS injection. EPR conditions were the same as described for Fig. 2 except: center field 3350 G, scan rate 49 G/min, modulation amplitude 5 G and the receiver gain was 8-fold higher. The triplet EPR signal (⇑) at $g = 2.012$ is that of nitrosyl heme.

LPS) did not affect systemic NO level in LPS-treated mice [15]. Therefore, in vivo iNOS activity is not likely to be affected by iron administration in this experiment. In agreement with the above described attenuation of nitric oxide level in endotoxin-treated mice, we observed iron-dependent down-regulation of iNOS protein expression in the liver tissue of mice (Fig. 1).

Direct iron regulation of nuclear transcription for cytokine-inducible NOS mRNA has been suggested in macrophages. iNOS activity decreased by half in cells activated with interferon γ plus LPS in the presence of 50 μM ferric iron [9]. However, in cultured rat aortic smooth muscle cells ferric and ferrous ions (up to 400 μM) were without effect on iNOS mRNA expression [11]. In our experiment in vivo, NO level decreased more than two-fold, when free Fe content in liver tissue rose up to 86 ± 20 nmol/g wet weight ($n = 4$) above the control level (Fe-citrate complex was given simultaneously with LPS). More distant effects of iron treatment in vivo should be considered as well. For example, in early studies, direct iron binding to LPS molecule has been suggested to modulate LPS interaction with cells in vivo, thus attenuating LPS toxicity in rabbits [2]. However, to achieve this effect, preincubation of LPS and Fe (6 h) in vitro was required before injection [2]. In our study, LPS and iron were injected separately via different routes. Iron is rapidly chelated by transferrin in the circulation, thus limiting the possibility of interaction between iron and LPS in vivo. In addition, Fe-citrate binding with endotoxin should antagonize *all* the actions of LPS. This was not the case in our study (LPS preserves its lethal effect in mice treated with 120 μmol/kg of Fe plus LPS). Moreover, iron continues to exert its NO inhibitory activity even when it is given after LPS, but before iNOS expression (data not shown).

Others have pointed out prooxidant effect of iron [18] and direct nitric oxide scavenging by iron complexes [19]. Interestingly, nitrosyl iron complexes exert much less prooxidant activity in vitro [20] and in rat hepatocyte culture [21] than 'free' iron complexes in the absence of NO. Therefore, in tissues of septic shock animals, the prooxidant effect of free iron should be significantly alleviated due to massive NO production [15,22]. Beneficial effects of iron [2] and iron complexes [23] were reported in endotoxin-treated animals. Fe-citrate can bind NO in vitro, as evidenced by weak EPR resonance similar to that in a neutral solution of $FeSO_4$ treated with NO [24]. However, NO depletion in iron-treated septic shock mice could not be explained simply as NO scavenging by iron complexes. This explanation does not take into account down-regulation of iNOS protein expression after iron administration. In addition, Fe-citrate injection does not change NO_2^-/NO_3^- and nitrosyl hemoglobin levels in blood of animals, when it is given after iNOS activation (i.e. at 6 h after LPS) [15].

In conclusion, this study demonstrates that exogenous iron, when it is given before iNOS induction, attenuates nitric oxide production in vivo in endotoxin-treated mice via down-regulation of iNOS expression.

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