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Iron Loading Exaggerates the Inflammatory Response to the Toll-like Receptor 4 Ligand Lipopolysaccharide by Altering Mitochondrial Homeostasis

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Abstract

Background—Perioperative and critically ill patients are often exposed to iron (in the form of parenteral iron administration or blood transfusion) and inflammatory stimuli, but the effects of iron loading on the inflammatory response are unclear. Recent data suggest that mitochondrial reactive oxygen species have an important role in the innate immune response, and that increased mitochondrial reactive oxygen species production is a result of dysfunctional mitochondria. We tested the hypothesis that increased intracellular iron potentiates Lipopolysaccharide-induced (LPS) inflammation by increasing mitochondrial reactive oxygen species levels.

Methods—Murine macrophage cells were incubated with iron then stimulated with LPS. C57BL/6 WT mice were intraperitoneally injected with iron and then with LPS. Markers of inflammation and mitochondrial superoxide production were examined. Mitochondrial homeostasis (the balance between mitochondrial biogenesis and destruction) was assessed, as were mitochondrial mass and the proportion of non-functional to total mitochondria.

Results—Iron loading of mice and cells potentiated the inflammatory response to LPS. Iron loading increased mitochondrial superoxide production. Treatment with MitoTEMPO, a mitochondria-specific antioxidant, blunted the pro-inflammatory effects of iron loading. Iron loading increased mitochondrial mass in cells treated with LPS and increased the proportion of non-functional mitochondria. Iron loading also altered mitochondrial homeostasis to favor increased production of mitochondria.

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Conflict of Interest:

Dr. Bagchi is a consultant for Lungpacer Medical Inc., Burnaby, British Columbia, Canada. The other authors of this manuscript have no conflicts of interest.

Conclusions—Acute iron loading potentiates the inflammatory response to LPS, at least in part by disrupting mitochondrial homeostasis and increasing the production of mitochondrial superoxide. Improved understanding of iron homeostasis in the context of acute inflammation may yield innovative therapeutic approaches in perioperative and critically ill patients.

INTRODUCTION

Iron is an essential trace element¹. Increased appreciation of the adverse effects of anemia in the perioperative period together with awareness of the risks of blood transfusion have led to the pre-operative use of intravenous iron preparations as part of patient blood management protocols^{2–4}. Critically ill patients in intensive care units are also exposed to acute iron loading (defined here as an increase in intracellular iron concentration in response to iron administration) in the form of blood transfusions as well as oral and parenteral iron treatment^{5, 6}. Although patients are often exposed to both iron loading and inflammatory stimuli such as major surgery or critical illness, the effects of iron loading on the inflammatory response are incompletely understood.

Because of the ability of iron to generate reactive oxygen species (ROS) by the Fenton reaction⁷, iron administration might be expected to potentiate the response to subsequent inflammatory stimuli. Studies supporting a pro-inflammatory role for iron include those by Zager et al, who showed that iron administration worsened *E. coli* induced sepsis⁸, and Wang et al, who reported that the iron chelator deferoxamine blunted LPS induced inflammation⁹. However, Pagani et al found that iron deficient mice had a more robust inflammatory response to LPS than iron-replete mice¹⁰, and De Domenico and colleagues demonstrated that iron administration diminished the inflammatory response to LPS¹¹. The reason for the lack of agreement between these studies is unclear, but may be related to differing routes and timing of iron administration.

Iron homeostasis is tightly controlled to minimize the risk of toxicity. Hepcidin is a key regulator of iron homeostasis^{12, 13}. Hepcidin binds to and downregulates ferroportin (FPN), the only known iron exporter in mammals. Acute inflammation has been shown to increase hepcidin levels¹⁴, inducing systemic hypoferremia with an increase in intracellular iron.

Mitochondria have an important role in the acute inflammatory response. Pathogen associated molecular patterns (PAMPs) such as LPS, a toll-like receptor 4 (TLR4) agonist, induce inflammation in part by increasing the production of mitochondrial reactive oxygen species (mtROS)¹⁵. Mitochondria are a major site of iron utilization within the cell¹⁶, and exist in a dynamic equilibrium between biogenesis and mitophagy (removal of dysfunctional mitochondria by autophagy)¹⁷. Perturbations in mitochondrial homeostasis (defined here as the balance between mitochondrial biogenesis and mitophagy) may increase the proportion of damaged or nonfunctional mitochondria, increasing mtROS production¹⁸.

In this study, we examined the effect of acute iron loading on the inflammatory response using *in vivo* and *in vitro* models of inflammation. We hypothesized that iron loading would exaggerate the pro-inflammatory effect of LPS by increasing mtROS production.

MATERIALS AND METHODS

Reagents and chemicals

Escherichia coli LPS (O55:B5), a TLR4 agonist, was purchased from List Biologicals (Campbell, CA). The TLR2 and TLR3 agonists Pam-3-Cys (P3C) and Poly I:C (PIC) (respectively) were obtained from Invivogen (San Diego, CA). Formyl peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), iron dextran, 20% dextran, ferric ammonium citrate (FAC), desferoxamine, antimycin and 3-methyladenine were purchased from Sigma Aldrich (St. Louis, MO). Calcein-AM, MitoSOX, MitoTracker Deep Red, MitoTracker Green and SYTOX Blue were purchased from Molecular Probes (Invitrogen, ThermoFisher Scientific, Waltham, MA). Antibodies, isotype controls and reagents for flow cytometry were obtained from BD Biosciences (San Jose, CA). MitoTEMPO, a mitochondria specific antioxidant,¹⁹ was purchased from Enzo Life Sciences (Farmingdale, NY).

Animals

The Institutional Animal Care and Use Committee at the Massachusetts General Hospital approved the animal studies. Male C57BL/6 mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed a standard, iron-replete diet and were injected intraperitoneally with one dose of iron-dextran (1 g/kg in a volume of 10 µL/g) or normal saline (control) for iron loading experiments. Pilot experiments were performed with a range of iron doses (0.5 – 2.0 g/kg) and serum iron levels and liver hepcidin mRNA were measured at various time points (1, 3, 4 and 7 days, data not shown). Mice injected with 1 g/kg iron –dextran demonstrated both a sustained increase in serum iron levels and a strong induction of hepcidin after 3 days, leading us to choose this 72-hour time point for further investigation. A separate group of WT mice was injected with normal saline or 7.5% dextran (10 µL/g, the same volume as iron dextran). Three days later mice were injected intraperitoneally with LPS (5 mg/kg) or an equal volume of normal saline (control). The mice were sacrificed 6 hours later and blood and organs (lungs and liver) were collected. Serum and organs were stored at –80°C until use.

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Cell Collection (Manassas, VA). RAW 264.7 (hereafter referred to as “RAW”) cells were cultured in 6 well- (at 8×10^5 cells/well) or in 96 well- tissue culture plates (at 1.28×10^5 cells/well) in DMEM with 10% fetal calf serum (FCS) with glutamine, penicillin and streptomycin. For iron supplementation experiments, the cells were incubated with ferric ammonium citrate (FAC, final concentration of elemental iron, 200 µM) overnight and then treated with LPS (150 ng/ml) or medium alone for 6 hours. The concentration of iron was chosen so as to approximate the serum iron concentrations used in pilot, *in vivo* dose-response experiments.

Human monocyte isolation

The Institutional Review Board at the Massachusetts General Hospital approved the collection of whole blood from volunteers for the purpose of monocyte isolation (IRB

protocol # 2014P001656). Mononuclear cells were isolated from human whole blood collected in EDTA using Polymorphprep density gradient (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. Mononuclear cells were incubated (at 8×10^5 cells/well) in 6 well tissue culture plates in serum-free DMEM medium for 4 hours. The cells were then washed with serum-free DMEM to remove non-adherent cells, and incubated overnight in DMEM medium with 10% fetal calf serum. The cells were incubated with iron and/or LPS as in the RAW cell experiments.

Mouse serum studies

Serum IL6 and TNF levels were measured using mouse IL-6 and TNF Quantikine ELISA kits (R&D Systems, Minneapolis, MN). Serum iron levels were measured using an Iron-SL assay (Sekisui, Osaka, Japan).

Quantitative RT-PCR

TaqMan primers for qRT-PCR were purchased from ThermoFisher Scientific. SYBR green primers were synthesized by the Massachusetts General Hospital DNA core facility. The sequences of SYBR green and TaqMan primers used in this study are listed in Supplemental Table 1. Total RNA was extracted from mouse liver and lung tissues or RAW cells using Trizol (Invitrogen, ThermoFisher Scientific). Reverse RNA transcription was accomplished using MMLV-RT (Promega, Madison, WI). Quantitative RT-PCR was performed using Applied Biosystems SYBR Green or TaqMan master mix (ThermoFisher Scientific) and an Eppendorf MasterCycler RealPlex2 (ThermoFisher Scientific). The level of target transcripts was normalized to the level of 18S rRNA using the relative CT method.

Intracellular labile iron measurement

Intracellular labile iron was measured as described previously²⁰. Briefly, cells were incubated with Calcein-AM, which was transported across the cell membrane by viable cells and de-esterified, producing intracellular, fluorescent, free calcein. Calcein binds with intracellular labile (or free) iron, a reaction that quenches calcein fluorescence. The concentration of labile iron in a cell is inversely proportional to the intensity of calcein fluorescence. SYTOX Blue was used to identify and exclude non-viable cells, which do not take up Calcein-AM, and may thereby confound results.

Flow Cytometry

Mouse whole blood was incubated in red blood cell lysis buffer for 3 minutes and washed twice in flow cytometry buffer (phosphate buffered saline (PBS) with 2% FCS). The cells were then incubated with PerCP-Cy5.5-conjugated mouse monoclonal anti-CD11b antibody, APC-conjugated mouse monoclonal anti-Ly6G antibody or isotype controls. The cells were then incubated for 30 min with Calcein-AM (0.125 μ M). RAW cells incubated with iron and/or LPS were treated with Calcein-AM (0.125 μ M), MitoSOX (2.5 μ M), MitoTracker Deep Red (50 nM) or MitoTracker Green (50 nM) for 30 minutes at 37°C in DMEM. Flow cytometry was performed using a FACS Aria III machine (BD Biosciences), and results were analyzed using FlowJo software (TreeStar, Ashland, OR). In all cases, gating parameters were set to exclude doublets and non-viable cells.

Determination of the ratio of mitochondrial to nuclear DNA

Total genomic DNA was isolated from RAW cells with a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). Quantitative PCR was used to measure the amount of cytochrome c oxidase I (CO1, a mitochondrial gene), and 18S ribosomal DNA (18S, a nuclear gene), as previously described²¹. The ratio of CO1 to 18S was used as a measure of the relative proportions of mtDNA and nuclear DNA.

Statistics

For *in vitro* studies, data are expressed as means and standard deviations of individual experiments replicated thrice. Data were tested for a normal distribution by the Shapiro-Wilk test, and analyzed using Student's t-test (or Mann-Whitney U if the data were not normally distributed) or two-way ANOVA (iron X LPS). If the iron X LPS interaction was statistically significant, we applied all possible pairwise comparisons (Bonferroni's post hoc tests). If the interaction was not statistically significant we interpreted the main effects only and refrained from post hoc testing. For data that were not normally distributed we used the Kruskal-Wallis test (with Dunn's post hoc tests for all possible pairwise comparisons). For the sake of clarity, not all pairwise comparisons have been reported in the figures. Hypothesis testing was 2-tailed. Values of $p < 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.0 (La Jolla, CA). Sample sizes for *in vivo* experiments were based on our prior experience with LPS injection without *a priori* power calculations. Conditions in the *in vivo* experiments were non-sequential, and processing of samples for the *in vivo* experiments was performed by investigators who were blinded to the experimental conditions.

RESULTS

Iron-dextran administration increases serum iron and intracellular iron in circulating neutrophils and monocytes

Injection of mice with iron-dextran increased serum iron levels ten-fold (Figure 1 A). LPS injection alone reduced serum iron levels by more than 50%, consistent with previous reports²². Mice that were treated with both iron and LPS had iron levels similar to those of mice injected with iron alone. Iron loading or LPS administration each increased hepatic hepcidin gene expression, as has been described by others²³ (Figure 1 B). Intracellular labile iron levels in circulating neutrophils (Ly6G positive cells) and circulating monocytes (CD11b positive cells) were elevated as shown by decreased calcein fluorescence in iron treated mice (Figures 1 C and D). These observations demonstrate that parenteral administration of iron-dextran induces hepcidin production and increases intracellular iron levels in circulating neutrophils and monocytes.

Iron administration potentiates the inflammatory effects of LPS

Iron administration alone did not induce inflammation in mice, as determined by the absence of increase in either serum protein levels or mRNA levels (in liver and lungs) of the cytokines IL6 and TNF α (Figure 2 A–F). A non-lethal LPS challenge induced a marked increase in serum IL6 and TNF α levels as well as at the corresponding mRNA levels in

mouse liver and lungs. Iron-treated mice challenged with LPS showed more than 5-fold higher serum cytokine levels than mice challenged with LPS alone. Similarly, the mRNA levels of *Il6* and *Tnfa* in lungs and liver of mice treated with iron and LPS were between 1.5 to 2.5 fold greater than mice treated with LPS alone. Iron treatment and subsequent LPS challenge did not alter mRNA levels of the anti-inflammatory cytokine IL10 in the lung compared to LPS challenge alone (Supplemental Figure 1). In control studies, we showed that 7.5% dextran (the vehicle for iron) did not have an independent pro-inflammatory effect on a subsequent stimulation with 5 mg/kg LPS (Supplemental Figure 2 A and B). Taken together, these observations indicate that parenteral iron administration strongly augments the pro-inflammatory response to LPS in mice.

Pre-incubation with iron augments LPS-induced cytokine mRNA induction in human monocytes and RAW cells

Human monocytes were found to have a more intense response to LPS stimulation after being iron-loaded (Figure 3 A and B). *In vitro* incubation of RAW cells with iron increased intracellular labile iron (non-ferritin bound, catalytically active iron) concentration in RAW cells. Deferoxamine (DFO), an iron chelator, was used as an assay control, demonstrating the inverse relationship between cellular labile iron levels and calcein fluorescence (Figure 4 A). Iron-loaded RAW cells stimulated with LPS had significantly higher mRNA levels of *Il6* and *Tnfa* than cells treated with LPS alone (Figures 4 B and C). Iron-loaded RAW cells stimulated with LPS also produced significantly more IL6 protein than cells stimulated with LPS alone (Supplemental Figure 3). Iron-treated RAW cells challenged with LPS showed increased mRNA expression of the chemokine monocyte chemoattractant protein 1 (*Mcp1*) compared to cells treated with LPS alone (Figure 4 D). Iron pre-treatment with subsequent LPS reduced the expression of the anti-inflammatory cytokine *Il10* compared to cells treated with LPS alone (Figure 4 E). Conversely, RAW cells pre-incubated with 30 μ M DFO showed a blunted response to LPS (Figure 5 A and B). Of note, incubation of cells with 200 μ M iron had no adverse effects on cell viability, as determined by flow cytometric detection of non-viable cells (data not shown). These results demonstrate that iron loading augments the pro-inflammatory effect of LPS on RAW cells *in vitro*, and that the use of an iron chelator blunts the cytokine response to LPS. Similarly, pre-treatment of human monocytes with iron augments the pro-inflammatory effect of a subsequent exposure to LPS.

The response to iron loading *in vitro* differs depending on the type of inflammatory stimulus

We examined the effect of iron loading on the response of RAW cells to three additional pro-inflammatory mediators, P3C, PIC and fMLF. P3C (Pam-3-Cys) is a TLR2 agonist found in gram positive bacteria, PIC (Poly I:C) is a viral TLR3 agonist, and fMLF (N-Formyl-Met-Leu-Phe) is a formylated peptide found in bacteria and mitochondria, and is an example of a damage-associated molecular pattern, or DAMP. In contrast to the 2.5 fold increase in *Il6* mRNA levels induced by iron and LPS, compared to LPS alone, iron-loaded cells stimulated with PIC did not show any significant increase in *Il6* mRNA compared to cells treated with PIC alone. The combination of iron and P3C produced a 1.5 fold increase in *Il6* mRNA compared to P3C alone. Iron together with the mitochondrial DAMP fMLF produced a 2.5 fold increase in *Il6* mRNA compared to DAMP alone (Figure 5 C). These results suggest

that the effects of iron loading on enhancing the inflammatory response are pathway specific and depend on the type of pro-inflammatory stimulus.

mtROS contribute to the pro-inflammatory effect of iron loading

To determine the effect of iron loading on mtROS levels, RAW cells were incubated with iron and stained with MitoSox, a fluorescent dye that specifically detects mitochondrial superoxide²⁴. Compared to untreated RAW cells, RAW cells exposed to iron had a 40% increase in fluorescence intensity, suggesting that iron loading results in an increased level of intracellular mtROS. The combination of iron and LPS increased RAW cell mtROS levels by 50% compared to cells treated with LPS alone (Figure 6 A and B).

To consider the possibility that an anti-oxidant might blunt the pro-inflammatory effect of iron, RAW cells were incubated with MitoTEMPO (100 μ M), a mitochondria specific anti-oxidant¹⁹, prior to stimulation with LPS. Pre-incubation with MitoTEMPO significantly blunted (but did not abolish) the inflammatory response to LPS compared to similarly treated cells that were not exposed to MitoTEMPO (Figures 6 C and D showing *Il6* and *Mcp1* mRNA levels, respectively). These results demonstrate that iron induces mtROS in RAW cells, and that inhibiting mtROS production diminishes the inflammatory response in iron-loaded cells treated with LPS. However, increased mtROS alone (as caused by iron loading) is not sufficient to increase inflammatory mRNA levels, as cells treated with iron alone did not express increased cytokine mRNA levels (Figure 4 B and C).

Iron administration modifies mRNA levels of genes involved in mitochondrial homeostasis

Compared to control mice, mice treated with LPS had lower mRNA levels of genes associated with mitochondrial biogenesis (*Pgc-1 α* , and *Ampk*, Figures 7 A and B) in the liver, but higher expression of *Lc3b*, a gene involved in mitophagy (Figure 7 C). Conversely, iron-treated mice that were challenged with LPS had greater expression of *Pgc-1 α* and *Ampk* than mice treated with LPS alone (Figures 7 A–C), suggesting a shift towards either greater mitochondrial biogenesis or decreased mitophagy. We found similar changes in *Ampk* gene expression in mouse lungs and in RAW cells (Supplemental Figure 4 A and B). These results suggest that LPS alone induces a relative shift towards mitophagy, while iron pre-treatment before LPS administration tilts the balance towards mitochondrial biogenesis.

Iron loading increases the proportion of non-functional to total mitochondria in RAW cells

To examine the effect of iron treatment on the functional status of mitochondrial, we stained RAW cells that were treated with iron and/or LPS with MitoTracker Deep Red (a dye that stains functional mitochondria only) and MitoTracker Green (a dye that stains all mitochondria, functional and otherwise)¹⁸. The proportion of RAW cells stained with MitoTracker Green alone (i.e. [MT Green – MT Deep Red]/MT Green) was significantly increased in RAW cells treated with iron compared to control cells, and was also significantly higher in iron-loaded cells treated with LPS than in LPS-only cells (Figure 8 A–B). This suggests that iron loading increases the proportion of non-functional mitochondria in RAW cells.

Mitochondrial genomic DNA content, a measure of mitochondrial mass, was higher in iron-loaded RAW cells treated with LPS than in cells treated with LPS alone (Figure 8 C). The mtDNA content in iron-loaded cells that were not treated with LPS was similar to LPS treated cells that were not iron-loaded. Taken together, these data suggest that iron-loaded cells stimulated with LPS have both a higher mitochondrial mass and a greater proportion of non-functional mitochondria than cells that were treated with LPS alone.

DISCUSSION

In this study, we observed a strong pro-inflammatory effect of iron loading on a subsequent challenge with lipopolysaccharide, *in vivo* and *in vitro*. Parenteral iron loading increased intracellular labile iron in circulating neutrophils and monocytes, and strongly increased the cytokine response to a subsequent LPS challenge. Similarly, addition of iron to RAW cells increased intracellular labile iron, and further augmented the LPS-induced increase in mRNA levels of pro-inflammatory genes. As with RAW cells, iron-loaded human peripheral blood monocytes also had higher inflammatory cytokine mRNA levels after LPS stimulation. Iron loading induced mtROS, and inhibition of mtROS formation blunted the augmented response to LPS in iron-loaded RAW cells. Iron loading in conjunction with LPS stimulation also increased the expression of genes associated with mitochondrial biogenesis *in vivo* (in liver and lungs) and increased mitochondrial genomic DNA *in vitro* (in RAW cells), suggesting that iron loading alters mitochondrial homeostasis. Iron loading increased the proportion of non-functional mitochondria in RAW cells. Taken together, the data suggest that a combination of iron loading together with an inflammatory stimulus results in an increased proportion of defective mitochondria and increased mtROS production.

Anesthesiologists often provide care for patients given parenteral iron supplements prior to major surgery, and to critically ill patients in intensive care units, who may be iron-loaded from blood transfusion or iron therapy. Thus the effect of acute iron loading on the inflammatory response is of particular relevance in perioperative medicine, especially because there are few studies that have examined the effects of iron infusions on outcomes²⁵.

Wang et al used a mouse model of hemochromatosis (*Hfe* knockout mice) to show that low intracellular labile iron in *Hfe* KO macrophages caused a diminished inflammatory response to LPS²⁶. In addition, pre-treatment of macrophages from wild type mice with an iron chelator reduced the inflammatory response to LPS²⁶. Other studies showed that the iron chelator deferoxamine attenuated the inflammatory response to LPS in RAW cells and decreased inflammation in mouse models of endotoxemia⁹, and peritonitis²⁷. In contrast, Pagani et al found that iron-deficient mice (with low macrophage iron levels) had a greater inflammatory response to LPS compared to iron replete mice¹⁰. De Domenico et al found that oral iron supplementation followed by an LPS challenge blunted the response of mice to LPS¹¹. In both of the last two studies the authors attributed their findings to anti-inflammatory effects of the iron regulating hormone hepcidin. Iron supplementation increases hepcidin production, while low hepcidin production in iron-deficient mice induces an exaggerated response to LPS. However, the mechanism proposed for the presumptive anti-inflammatory effect of hepcidin (activation of the JAK-STAT pathway by hepcidin-

ferroportin binding¹¹) has since been questioned²⁸. In this study, we demonstrated a robust pro-inflammatory response to iron loading in spite of an increase in hepcidin gene expression (Figure 1 B). The mode of iron supplementation (enteral and parenteral) as well as the formulation of iron may impact the bioavailability of iron and hence intracellular iron concentrations^{7,29}, possibly accounting for differences between our study and that of De Domenico and colleagues.

We found that the response to iron loading differs depending on the type of pro-inflammatory stimulus, suggesting the presence of specific pathways that are influenced by intracellular iron. Indeed, Wang et al found that intracellular iron influences LPS signaling specifically by modifying the MyD88-independent adaptor TRIF-related adaptor molecule (TRAM) related response to LPS²⁶.

To further examine the mechanisms responsible for the pro-inflammatory effects of iron loading, we measured mtROS production. Iron loading increased mtROS production in RAW cells, and a mitochondrial-specific antioxidant (MitoTEMPO) blunted the pro-inflammatory effect of iron on RAW cells, reducing cytokine (*Il6*) and chemokine (*Mcp1*) mRNA levels to those of macrophages treated with LPS alone. These findings suggest that iron-induced mtROS may have a 'priming' effect on macrophages, augmenting the response to a subsequent LPS challenge. In spite of the lack of demonstrated increase in mtROS production with LPS, LPS stimulation appears to increase mtROS production, because treatment with MitoTEMPO blunts the inflammatory response to LPS alone. These results are consistent with findings reported by Bulua et al, who found that mouse embryonic fibroblasts have a decreased response to LPS when pretreated with a different mitochondrial superoxide inhibitor, MitoQ³⁰. Bulua and colleagues also did not find increased MitoSOX staining in the fibroblasts treated with LPS alone³⁰. We speculate that while LPS does increase mtROS production, the effects of LPS on mtROS levels may be opposed by increased mitophagy, resulting in no change in net mtROS levels.

Other studies have found that increased intracellular iron increased mtROS production, and that mtROS was associated with increased inflammation in different cell types, such as cardiomyocytes³¹ and macrophages³². The results of this study therefore add to the existing literature by showing that iron loading potentiates inflammation by augmenting mtROS production.

Although the *in vitro* data in our study were derived from monocytes and macrophages, it is possible that iron loading may impact ROS production by neutrophils, which are a major source of ROS *in vivo*³³. Sampaio et al showed that iron-dextran administration in a streptozotocin-induced model of diabetes in rats was associated with a strong increase in neutrophil ROS production³⁴. Iron loading may therefore be pro-inflammatory in both monocytes and neutrophils.

Iron-loaded cells exposed to LPS had a greater mitochondrial mass, as determined by the relative abundance of mitochondrial DNA to nuclear DNA. Of note, we did not find a significant difference in the proportion of mtDNA to nuclear DNA between iron-loaded cells and LPS treated cells. Iron loading RAW cells increased the proportion of non-functional

mitochondria relative to total mitochondria. Others have shown that damaged or non-functional mitochondria produce more mtROS^{18, 35}. Mitochondrial mass can be increased by increasing mitochondrial biogenesis, decreasing mitophagy, or both. In this study, the combination of iron and LPS increased mRNA levels of genes involved in mitochondrial biogenesis, *Pgc-1α* and *Ampk* *in vivo*. LPS treated mice had higher expression of hepatic *Lc3b*, a gene involved in mitophagy, which is consistent with prior data reporting that LPS increases mitophagy³⁶. The data therefore suggest that the combination of iron loading and LPS stimulation increases mitochondrial biogenesis *in vivo*. Of note, increased mitochondrial biogenesis is not necessarily deleterious – some studies have shown that mitochondrial biogenesis imparted a pro-survival phenotype in acute inflammatory states³⁷.

Studies using mouse models of defective mitophagy showed that accumulation of non-functional mitochondria potentiated mtROS formation and induced a more potent inflammatory response to innate immune stimulants, including LPS^{18, 21, 38}. Duvigneau et al showed that endotoxin-induced iron accumulation in cells was associated with altered mitochondrial respiration and mitochondrial dysfunction³⁹. Lowering intracellular iron levels using iron chelators induced mitophagy in a *C. elegans* model of *Pseudomonas* infection⁴⁰, while iron loading promoted mitochondrial biogenesis in osteoclasts⁴¹. These reports are consistent with our finding that intracellular iron levels modulate mitochondrial homeostasis.

This study has some limitations. We used iron dextran, rather than iron sucrose or iron gluconate, because iron dextran was previously shown to be the least likely to cause direct iron-induced toxicity⁴². Because we did not test other forms of iron *in vivo*, we cannot comment on the effects of other iron formulations. A second potential limitation is that we examined the effects of increased intracellular iron on the early inflammatory response, and thus cannot comment on the effect of iron loading on the temporal course of inflammation. In addition, while we have found that iron loading has a pro-inflammatory effect on macrophages, we did not investigate the effect of iron loading on macrophage phenotype, although others have shown that iron loading induced an M1 phenotype in macrophages⁴³. In RAW cells, we observed a decrease in *Il10* mRNA in response to iron loading, but a similar effect was not observed in murine lungs *in vivo* (Supplemental Figure 1). Finally, our data demonstrating the effects of iron loading on mitochondria function are limited to *in vitro* assays. Further work is needed to determine whether iron loading alters mitochondrial function *in vivo*.

The results of this study suggest that increased intracellular iron leads to an increased pro-inflammatory response to the TLR4 ligand LPS, raising the possibility of targeting intracellular iron as a therapeutic modality in acute inflammatory states. However, many questions need to be addressed before intracellular iron can be considered a viable biologic target. Critically ill patients are often hypoferremic, and iron chelators will likely exacerbate hypoferremia. Therapy with iron chelators^{44, 45} may increase the risk of bacterial infections by organisms that can extract iron from the iron-chelator complex. Finally, we do not currently have reliable assays for monitoring intracellular iron levels to guide therapy.

CONCLUSION

Our results suggest that iron loading alters mitochondrial homeostasis leading to the accumulation of defective mitochondria and increasing production of mtROS. The production of mtROS primes macrophages for a 'second hit' such as exposure to LPS, greatly augmenting the inflammatory response to the second stimulus. Figure 9 presents a schematic of the role increased intracellular iron plays in modulating the acute inflammatory response. The data presented here highlight the pro-inflammatory effects of iron loading in acute inflammation, and suggest that clinicians should consider the risks of treatments that result in iron loading in acutely ill patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Effects of parenteral iron overload in mice

(A) Mice treated with iron had significantly higher serum iron levels, while mice treated with LPS alone became hypoferremic (two-way ANOVA with Bonferroni's post hoc tests performed on $\log_{10}$ transformed values; p values adjusted for all possible comparisons; Int: Interaction p value). (B) Iron and LPS independently induce hepcidin (*Hamp*) mRNA levels in the liver (two-way ANOVA, interaction between iron and LPS not significant, therefore only main effects reported). $N = 7-9$ mice/group for figures 1 A and B. (C) and (D) Iron injection increases intracellular labile iron in circulating neutrophils and monocytes. Both neutrophils (Ly6G positive cells) and monocytes (CD11b positive cells) from the iron treated mice had lower calcein fluorescence intensities, consistent with higher intracellular iron levels. Representative histograms are from one vehicle treated and one iron treated mouse. The dot plots show mean fluorescent intensities of 3–4 mice/group (unpaired t test).

FIGURE 2. Effect of parenteral iron administration on inflammatory gene expression and serum cytokine levels

Serum, lung and liver samples were harvested from C57Bl/6 mice treated with iron and/or LPS, and analyzed for serum *Il6* (A), serum *Tnf α* (B) and mRNA levels of the two cytokines in the lungs (C–D) and livers (E–F). In each case, iron supplementation by itself did not induce inflammation. LPS alone induced increases in cytokine protein levels as well as mRNA levels. Serum cytokine levels in the control and iron treated groups were below the detection level of the assay in many cases. Iron administration significantly enhanced the

effect of LPS induced inflammation in the mice. N = 7–9 mice per group. (Mann Whitney U test (**2 A** and **B**), and two-way ANOVA with Bonferroni's post hoc tests (**2 C–F**, p values adjusted for all possible comparisons; Int: Interaction p value).

FIGURE 3. Effect of iron loading on human monocytes *in vitro*

Human monocytes were isolated from peripheral blood and incubated in 6 well plates. The cells were incubated with iron (200 μ M) or control overnight, and then stimulated for 6 h with LPS (150 ng/ml) or medium. LPS alone induced *IL6* mRNA (A) and *TNF α* mRNA (B). Iron loading significantly increased mRNA levels in response to LPS (two-way ANOVA with Bonferroni's post hoc tests, p values adjusted for all possible comparisons; Int: Interaction p value, n = 3 replicates/condition).

FIGURE 4. Effect of iron loading on RAW 264.7 cells *in vitro*

(A) RAW cells treated with LPS, iron or Deferoxamine (DFO, an iron chelator) were incubated with calcein and subjected to flow cytometry, and calcein fluorescence quantified. Calcein fluorescence decreased in iron-treated cells (one-way ANOVA with Bonferroni's post hoc tests, p values adjusted for all possible comparisons). A representative histogram from one of three independent experiments is shown. The dot plots show mean fluorescent intensities of 3 replicates/condition. (B–E) RAW cells were incubated with iron (200 μ M) or control overnight, and then stimulated for 6 h with LPS (150 ng/ml). LPS alone induced *I/6*

and *Tnfa* mRNA. Iron loading significantly increased mRNA levels in response to LPS. **(D)** The combination of iron and LPS increased *Mcp1* mRNA levels to a greater extent than LPS alone. **(E)** Conversely, the combination of LPS and iron resulted in less *Il10* mRNA levels than with LPS alone **(B–E: two-way ANOVA with Bonferroni's post hoc tests, p values adjusted for all possible comparisons; Int: Interaction p value, n = 3 replicates/condition).**

FIGURE 5. (A–B) Effect of an iron chelator on RAW 264.7 cells *in vitro*

RAW cells were pre-treated with 30 μ M Deferoxamine (DFO, an iron chelator) and subsequently stimulated with LPS for 6h. DFO pre-treatment decreases *I/6* mRNA induced by LPS significantly. There is a trend towards decreasing TNF mRNA in DFO pretreated cells that did not reach statistical significance (two-way ANOVA with Bonferroni's post hoc tests, p values adjusted for all possible comparisons; Int: Interaction p value, $n = 3$ replicates/condition). (C) RAW cells were incubated with iron (200 μ M) or control overnight, and then stimulated for 6 h with LPS (150 ng/ml), Pam-3-Cys (P3C; 1 μ g/ml) Poly I:C (PIC; 25 μ g/ml) or N-Formyl-L-methionyl-L-leuyl-L-phenylalanine (fMLF; 20 μ M). *I/6* gene expression was normalized to the response to LPS alone. The response to iron loading is not uniform across different pro-inflammatory agents. Iron loading augments the inflammatory response to LPS (a TLR4 ligand) and P3C (a TLR2 ligand), but not to PIC (a TLR3 ligand). Iron loading also augments the response to fMLF - a formylated peptide that is considered a DAMP, or damage associated molecular pattern (unpaired t tests, $n = 3$ replicates/condition).

FIGURE 6. The effect of iron and LPS on mitochondrial reactive oxygen species production
(A) Effect of iron supplementation on mtROS production. The shaded histogram depicts unstained cells. The histogram in red shows the effects of Antimycin (20 $\mu\text{g/ml}$), an inhibitor of mitochondrial complex III used as a positive control. Iron naïve cells treated with LPS do not show increased MitoSOX fluorescence compared to controls (middle panel). However, iron pre-treated RAW cells exposed to LPS have more mtROS production compared to cells treated with LPS alone (right panel), although the increase is similar to that caused by iron incubation alone (left panel). Data are representative of three experiments. **(B)** Summary bar graphs showing relative MitoSOX fluorescence (two-way ANOVA, interaction between iron and LPS not significant, therefore only main effects reported; $n = 3$ replicates/condition). **(C–D)** Incubating RAW cells with 100 μM of the mitochondrion specific anti-oxidant MitoTEMPO blunted the mRNA response to LPS (*Il6* and *Mcp1*) in iron-loaded macrophages (unpaired t tests, $n = 3$ replicates/condition).

FIGURE 7. Iron loading changes the balance between genes responsible for mitochondrial biogenesis and mitochondrial autophagy (mitophagy)

C57Bl/6 mice were treated with iron and/or LPS, $N = 7-9$ mice per group. Total RNA was extracted from mouse liver and RT-qPCR was performed using primers for *Pgc-1α* (a mitochondrial biogenesis associated gene, **A**), *Ampk* (a mitochondrial biogenesis associated gene, **B**) and *Lc3b* (a mitophagy associated gene, **C**). LPS alone increases *Lc3b* mRNA levels, while decreasing *Pgc-1α* and *Ampk* mRNA levels. Iron-loaded mice stimulated with LPS had the opposite profile, with an increase in the mRNA levels of the mitochondrial

biogenesis associated gene *Pgc-1 α* and *Ampk*. (two-way ANOVA, interaction between iron and LPS not significant, therefore only main effects reported).

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FIGURE 8. Iron loading increases the proportion of non-functional mitochondria in RAW 26.7 cells

RAW cells were treated with iron and/or LPS, then incubated with MitoTracker Deep Red and MitoTracker Green and subjected to flow cytometry. (A) Shows density plots for each condition, representative of 3 independent experiments. The gate shown contains the cells that are MitoTracker DeepRed negative/MitoTracker Green positive cells (non-functional mitochondria). 3-Methyladenine (3MA), an inhibitor of autophagy, was used as a positive control. (B) A summary histogram of the cytometry results (two-way ANOVA, interaction between iron and LPS not significant, therefore only main effects reported; $n = 3$ replicates/condition). (C) Iron-loaded, LPS treated RAW cells have higher mitochondrial mass defined by mitochondrial gene: nuclear gene ratio (two-way ANOVA with Bonferroni's post hoc tests, p values adjusted for all possible comparisons; Int: Interaction p value, $n = 3$ replicates/condition).

FIGURE 9.

A schematic view of the role of mitochondrial homeostasis (the balance between mitochondrial biogenesis and mitophagy) in the acute inflammatory response. (A) LPS induced inflammation induces inflammatory gene transcription. Simultaneously, maintenance of mitochondrial homeostasis allows removal of damaged mitochondria by mitophagy (mitochondrial ‘quality control’), resulting in a blunted inflammatory response, which may be why mtROS does not appear to increase in LPS stimulated cells. In the presence of excess intracellular iron (B) the balance of mitochondrial homeostasis shifts towards increased biogenesis and less effective mitophagy, resulting in an accumulation of damaged mitochondria, increased mtROS and an augmented pro-inflammatory effect.