Regulation of phagosomal iron release from murine macrophages by nitric oxide

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The role of NO in macrophage iron turnover was studied in macrophages from inducible nitric oxide synthase (iNOS)-deficient mice. Interferon γ/lipopolysaccharide (IFNγ/LPS)-activated bone marrow-derived macrophages from iNOS-deficient mice, following phagocytosis of ^{59}Fe-labelled transferrin–anti-transferrin immune complexes, showed reduced iron release compared with cells from wild-type iNOS littermates. Uptake of the complexes by macrophages was similar in iNOS-deficient and wild-type mice. Ferritin was up-regulated by IFNγ/LPS treatment, but NO exercised a modest opposing down-regulatory effect. No effect of iNOS deficiency was seen when iron was taken up from iron citrate, which enters via a non-phagocytic route. These results suggest that NO plays a key role in regulating iron turnover in macrophages acquiring iron by phagocytosis of erythrocytes or cell debris, and thus the supply to peripheral tissues, such as to the bone marrow for erythropoiesis.

Key words: ferritin, interferon, lipopolysaccharide, phagocytosis, transferrin.

INTRODUCTION

Macrophages play a critical role in iron metabolism. They are responsible for the processing of haemoglobin iron from senescent erythrocytes and subsequent iron supply to the bone marrow for erythropoiesis [1]. In addition, iron is essential in macrophage-mediated cytotoxicity by contributing to the production of highly toxic hydroxyl radicals via the Fenton reaction [2] and by controlling the production of NO after activation by immunological stimuli [3].

Intracellular iron homoeostasis is usually controlled by cytoplasmic iron regulatory proteins (IRP1 and IRP2), which regulate expression of several proteins by binding to iron-responsive elements (IREs) on their mRNA [4,5]. IRP binding to the IREs in the 5′-untranslated regions of ferritin (Ft) and 5-aminolaevulinate synthase mRNAs represses their translation, whereas binding of IRPs to multiple IREs in the 3′-untranslated region of transferrin (Tf) receptor (TfR) mRNA confers stability against targeted endonucleolytic degradation.

IRP binding activity is normally regulated by cellular iron levels [6,7], but can also be modulated by NO [5,8,9] and H_2O_2 [5,8,10], both of which are produced by activated macrophages. NO was shown originally to activate IRE-binding activity of both IRP1 and IRP2 [8], but we [11] and others [12] have shown more recently that activation of macrophages with interferon γ (IFNγ) causes a down-regulation of IRP2 activity, despite production of NO. In addition, we have reported that IFNγ/lipopolysaccharide (LPS) stimulation of murine J774 macrophages decreases TfR expression and Fe uptake from Tf [11]. Therefore, the regulation of iron uptake from Tf in activated macrophages seems to be very complex and does not depend solely upon NO-mediated activation of IRPs. Furthermore, NO can cause release of iron from Ft [13] and may thus be able to mobilize intracellular stores.

Although most previous studies of iron metabolism in macrophages have used Tf as the carrier to load the cells with iron, the most important function of macrophages in iron metabolism is to process effete erythrocytes and return the iron to the plasma for subsequent re-utilization [1]. In this case, iron enters the macrophage not via the TfR, but by phagocytosis, and the main intracellular compartment involved in iron processing is the phagolysosome, rather than the early endosome. Impaired iron release by macrophages probably contributes to the hypoferraemia of inflammation, and we have shown previously that tumour necrosis factor α can reduce release of iron taken up previously by phagocytosis [14]. However, the way in which the cells handle iron acquired by this route has not been studied in detail and the role of NO, which is also produced by macrophages during immune and inflammatory responses, has not been investigated. The aim of this work was therefore to clarify the role of NO in the handling of iron taken up by activated macrophages using a phagocytic route. To this end, we examined cellular iron uptake, release and distribution, and Ft expression in activated macrophages from inducible nitric oxide synthase (iNOS)-deficient mice using a model based on phagocytosis of ^{59}Fe-labelled Tf–anti-Tf immune complexes [15].

EXPERIMENTAL

Reagents

RPMI 1640 culture medium, antibiotics, PBS and fetal calf serum (FCS) were purchased from Gibco (Paisley, Scotland, U.K.). Human apotransferrin was obtained from Sigma (Poole, Dorset, U.K.) and, when required, was saturated with ^{59}Fe using Fe-nitrilotriacetate (NTA; Fe/NTA molar ratio, 1:4) [16] prepared from Na-NTA and ^{59}FeCl$_3$ (NEN, Boston, MA, U.S.A.; specific radioactivity 3–10 mCi/mg). Tf-free human serum albumin, N^G-monomethyl-L-arginine (L-NMMA), LPS, leupeptin,

PMSF, benzamidine and pepstatin A were all obtained from Sigma. Desferrioxamine was obtained from Novartis (Horsham, W. Sussex, U.K.). Nitrocellulose membranes and enhanced chemiluminescence (ECL) reagents were obtained from Amersham Bioscience (Little Chalfont, Bucks., U.K.). Rabbit anti-human Tf antibody was obtained from Dako (Glostrup, Denmark), and rabbit anti-mouse Ft antibody (αFt) was produced in our laboratory [16]. Mouse recombinant IFNγ was provided kindly by Dr G. Adolf (Boehringer Ingelheim Austria GmbH, Vienna, Austria).

Preparation of immune complexes

^{59}Fe-NTA was added in a quantity sufficient to saturate the iron-binding capacity of human apotransferrin. Then optimal proportions of Tf and antiserum to Tf (normally 1 mg of Tf/2.5 ml of antiserum) were mixed and incubated at 50 °C for 1 h [15]. The insoluble immune complexes (^{59}Fe-Tf–anti-Tf) were centrifuged at 500 g for 5 min, washed twice and resuspended in PBS.

Macrophage cultures and treatments

Bone marrow-derived macrophages (BMMϕ) were obtained from wild-type (129sv × 129sv strain) and iNOS-deficient mice, constructed as described previously [17]. It is recognized that the iNOS-deficient mice retain some residual iNOS activity which can be enhanced by treating cells with IFNγ/LPS [18], but NO production is always substantially lower than in wild-type iNOS littermates, and appropriate controls containing the NOS inhibitor L-NMMA (500 μM) were always included. Cells from mouse femurs were harvested, washed twice, grown for 5–7 days in Petri dishes in the presence of 10–20 % (v/v) L929 cell-conditioned medium at 37 °C in 5 % CO$_2$ until confluent, plated as required and incubated overnight.

Macrophages were then stimulated overnight with 50 units/ml IFNγ and 10 ng/ml LPS in RPMI 1640 medium supplemented with 10 % heat-inactivated FCS, 100 units/ml penicillin and 100 μg/ml streptomycin, pulsed for 2 h with 10 μg/ml ^{59}Fe-Tf–anti-Tf immune complexes, washed twice with PBS to remove uningested complexes, then incubated for 48 h in the presence of the same stimulants and subjected to further procedures as indicated below. In some experiments FCS was replaced by BSA (0.2 %) and the cells instead pulsed for 2 h with ^{59}Fe-citrate (1 μM Fe). Cells were fed with fresh medium containing the appropriate stimulants every 24 h and viability checked by Trypan Blue exclusion. Confocal microscopy showed that the immune complexes co-localized with Lamp-1, indicating that they had entered the late endosome and/or phagosome [19].

Iron-uptake and -release assays

Release of iron into the medium was monitored by determining ^{59}Fe activity in the culture medium at 24 and 48 h intervals using a LKB Compugamma counter. At the end of the experiments, macrophages were lysed in 2 % SDS and the remaining cell-associated radioactivity determined.

Intracellular distribution of iron

Cells were lysed in 200 μl of cytoplasmic lysis buffer (1 % Triton X-100, 40 mM KCl, 25 mM Tris/HCl, pH 7.4, 50 μg/ml leupeptin, 200 μg/ml PMSF, 1 mM benzamidine and 50 μg/ml pepstatin A) and the intracellular iron distribution determined as described previously [11]. Briefly, this consisted of centrifugation at 10000 g for 5 min to sediment the iron in insoluble material, followed by immunoprecipitation of Ft with a rabbit anti-Ft antibody. This allows separation of intracellular iron into three

compartments, containing insoluble material (consisting mainly of un-degraded immune complexes and iron bound to intracellular organelles), Ft-bound iron and soluble non-Ft iron.

Estimation of Ft

Macrophages stimulated as described above and pulsed if required with unlabelled Tf–anti-Tf immune complexes were lysed with 50–100 μl of boiling lysis solution (1 % SDS/10 mM Tris/HCl, pH 7.4). Aliquots containing 10 μg of protein were analysed by Western blotting using a 15 % acrylamide gel and overnight transfer at 150 mA to nitrocellulose membranes. The blots were developed using rabbit anti-mouse Ft antibody and ECL (Amersham Bioscience) according to the manufacturer's protocol. Membranes were then stained with a 0.1 % Ponceau Red solution (Sigma) to confirm similar protein loading in all lanes.

Measurement of nitrite and protein determination

Nitrite in the culture medium was determined by the Griess reagent [20]. The protein concentration of cell lysates was estimated by the BCA protein assay reagent (Pierce, Chester, U.K.) using BSA as a standard.

Statistical analysis

Data were analysed by ANOVA and unpaired Student's t test to determine the difference between each group. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Uptake of iron from ^{59}Fe-Tf–anti-Tf immune complexes by BMMϕ

To determine the effect of NO produced by activated macrophages on cellular iron handling, BMMϕ from wild-type and iNOS-deficient mice were stimulated with IFNγ and LPS, and then pulsed with ^{59}Fe-Tf–anti-Tf immune complexes. Stimulation of BMMϕ with IFNγ and LPS enhanced uptake
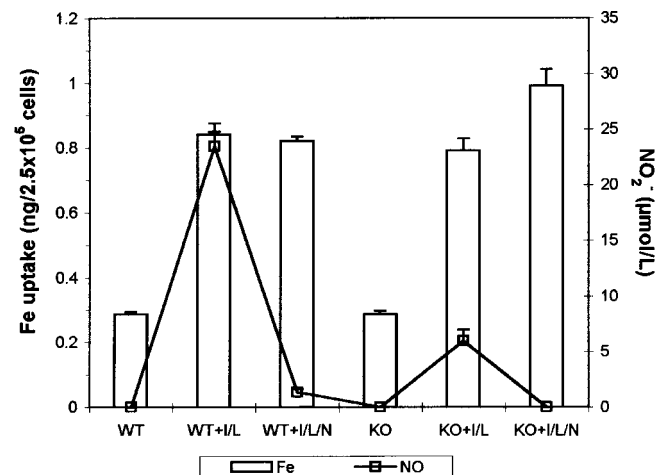

Figure 1   Effect of NO on uptake of ^{59}Fe-Tf–anti-Tf immune complexes by BMMϕ

BMMϕ from iNOS-deficient mice (KO) or wild-type littermates (WT) were stimulated with IFNγ/LPS (I/L), with or without L-NMMA (N), or left unstimulated. They were then pulsed with ^{59}Fe-Tf–anti-Tf immune complexes for 2 h and uptake determined after removal of uningested complexes. NO in the supernatant was measured 24 h after pulsing with the immune complexes.
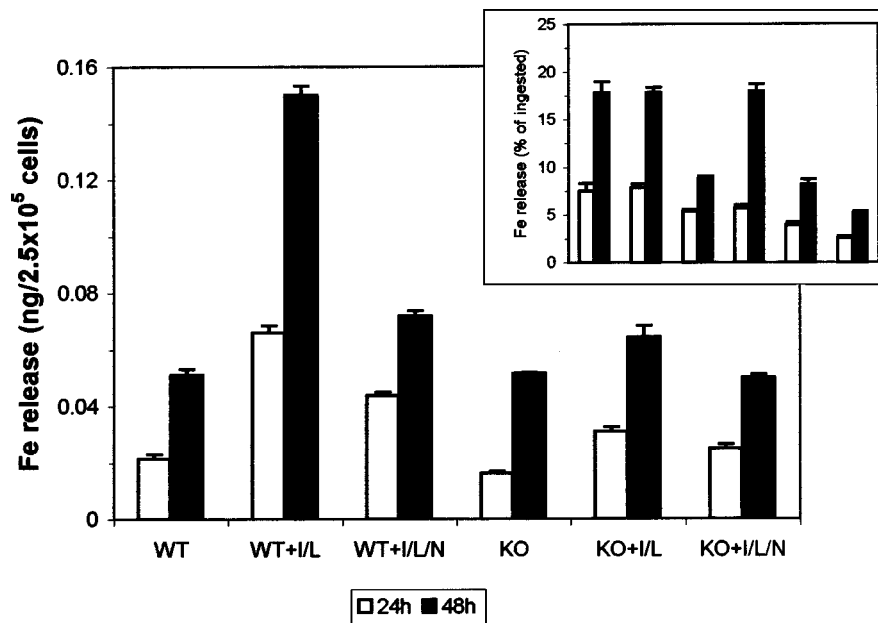
Figure 2   Release of iron by BMMϕ following uptake of ^{59}Fe-Tf–anti-Tf immune complexes

BMMϕ from iNOS-deficient mice (KO) or wild-type littermates (WT) were stimulated with IFNγ/LPS (I/L), with or without L-NMMA (N), or left unstimulated, pulsed with ^{59}Fe-Tf–anti-Tf immune complexes for 2 h and release of ^{59}Fe determined 24 or 48 h later. The inset shows the release of iron at 48 h, expressed as a percentage of ingested iron (the bars are the same as in the main figure).

of immune complexes ($P < 0.001$) in both wild-type and iNOS-deficient macrophages (Figure 1), indicating that uptake was independent of NO production. Furthermore, addition of the iNOS inhibitor L-NMMA did not affect uptake of ^{59}Fe by activated BMMϕ.

Release of iron by BMMϕ

The IFNγ/LPS-activated wild-type macrophages showed a 2–3-fold increase ($P < 0.0001$) in cellular iron release after 24 or 48 h of incubation when compared with non-activated wild-type macrophages (Figure 2). However, unlike the increased uptake of ^{59}Fe-Tf–anti-Tf immune complexes, this effect was blocked by L-NMMA after 24 and 48 h of incubation ($P < 0.01$ and $P < 0.0001$ respectively). Furthermore, activation of iNOS-deficient macrophages caused a much smaller increase ($P < 0.001$) in Fe release when compared with wild-type macrophages. This effect was again reversed by L-NMMA addition ($P < 0.05$), suggesting that residual iNOS activity was responsible for this small increase of iron release by iNOS-deficient macrophages after activation. If iron release is expressed as the percentage of ingested iron rather than the absolute amount (Figure 2, inset), the results show that in the absence of NO activated macrophages retain a greater proportion of the ingested iron than non-activated macrophages, but that in the presence of NO the proportion of iron released is restored to the level in non-activated macrophages. Similar results were obtained with peritoneal exudate macrophages (results not shown).

Intracellular distribution of ^{59}Fe in BMMϕ following ingestion of ^{59}Fe-Tf–anti-Tf immune complexes

We next investigated the intracellular distribution of the retained iron in non-activated and IFNγ/LPS-activated BMMϕ from wild-type and iNOS-deficient mice, stimulated and pulsed as
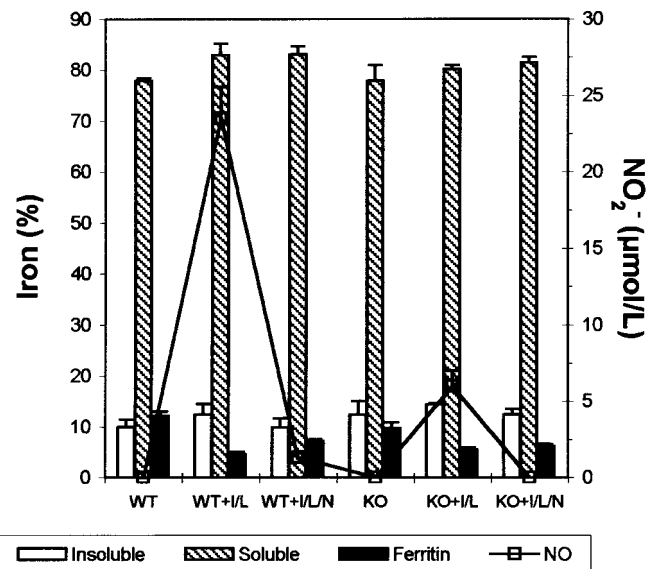

Figure 3   Intracellular distribution of iron in BMMϕ following uptake of ^{59}Fe-Tf–anti-Tf immune complexes

BMMϕ from iNOS-deficient mice (KO) or wild-type littermates (WT) were stimulated with IFNγ/LPS (I/L), with or without L-NMMA (N), or left unstimulated. They were then pulsed with ^{59}Fe-Tf–anti-Tf immune complexes for 2 h, and the uningested complexes removed. After 24 h the cells were lysed and the intracellular distribution of iron determined as described in the Experimental section. NO in the supernatant was measured 48 h after pulsing with the immune complexes.

above. After 24 h of incubation post-pulse, the majority (75–85%) of iron was in all cases present in the soluble non-Ft fraction (Figure 3). Likewise, the proportion of insoluble iron
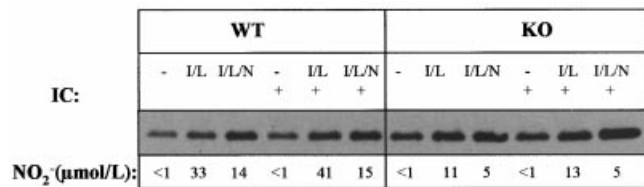
Figure 4   Ferritin synthesis by BMMφ

BMMφ from iNOS-deficient mice (KO) or wild-type littermates (WT) were stimulated as in Figure 1, then pulsed, if required, with unlabelled Fe-Tf–anti-Tf immune complexes (IC) for 2 h. After a further 24 h NO release was measured in the supernatants, the cells were lysed and Ft content determined by Western blotting. A similar protein loading in all lanes was confirmed by staining the membranes with 0.1 % Ponceau Red solution. Data are representative of two independent experiments.

did not vary greatly, constituting about 10–13 % of the total intracellular iron. However, the proportion of iron in Ft, while only a minor fraction in all cases, varied more noticeably ($P < 0.001$ by ANOVA), constituting about 10 % of total intracellular iron in non-activated macrophages but only about 4 % in activated macrophages from wild-type mice. This reduction of Ft-bound iron in activated macrophages was not totally due to NO, as it was not reversed completely by L-NMMA and was also observed in activated macrophages from iNOS-deficient mice. This suggests a diversion of iron away from the storage compartment in the more highly metabolic activated macrophages. Therefore, treatment of macrophages with IFNγ/LPS not only affected uptake and release of iron acquired by phagocytosis, but also altered iron incorporation into Ft.

Ferritin expression

Since treatment of macrophages with IFNγ and LPS altered the proportion of iron bound to Ft (Figure 3), their effect on Ft expression was investigated. Stimulation of both wild-type and iNOS-deficient macrophages with IFNγ and LPS up-regulated Ft expression (Figure 4). In the case of the wild-type macrophages, L-NMMA increased Ft expression further, whereas it had less effect on the iNOS-deficient cells. This suggests that stimulation of macrophages with IFNγ and LPS has two opposing effects on Ft expression: a NO-independent stimulatory effect that is probably transcriptional [21] and an opposing down-regulation by NO when the latter is produced in sufficient quantity, which we found to be associated with up-regulation of IRP1 activity (results not shown). When iron was added as Fe-Tf–anti-Tf immune complexes there was a further modest increase in Ft synthesis by wild-type macrophages. However, overall, activation was more effective than uptake of Fe-Tf–anti-Tf immune complexes at up-regulating Ft.

Iron uptake and release of iron from ^{59}Fe citrate by BMMφ

The ability of NO to promote Fe release by macrophages following uptake of labelled immune complexes contrasts with earlier findings with iron taken up from Tf via the TfR, where NO did not increase iron release [11]. This suggests that the effect of NO on macrophage iron is influenced by the way in which iron is acquired by the cell. It is known that macrophages can also acquire iron bound to low-molecular-mass chelators such as NTA and citrate [22]. Since such complexes enter the cells by a mechanism that probably involves direct transmembrane transport, rather than by phagocytosis or via the TfR, it was of
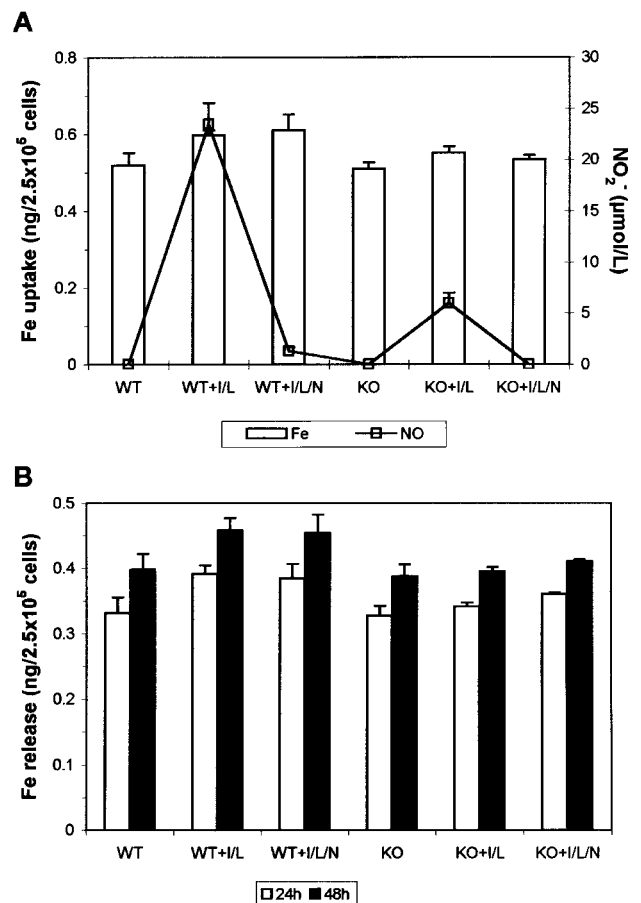

Figure 5   Effect of NO on uptake and release of ^{59}Fe-citrate by BMMφ

(**A**) BMMφ from iNOS-deficient mice (KO) or wild-type littermates (WT) were stimulated as in Figure 1, then pulsed with ^{59}Fe-citrate for 2 h in serum-free medium and uptake determined. (**B**) Subsequent release of iron was then measured after a further 24 or 48 h of incubation. NO in the supernatant was measured 48 h after incubation with ^{59}Fe-citrate.

interest to determine whether NO could influence the handling of iron taken up from Fe-citrate. IFNγ/LPS-stimulated BMMφ from wild-type and iNOS-deficient mice were pulsed with ^{59}Fe-citrate in FCS-free medium (to avoid binding of ^{59}Fe to Tf present in FCS). As can be seen in Figure 5, iron flux (uptake and release) was unaffected by NO, nor directly by IFNγ/LPS stimulation. Therefore, NO seems to affect macrophage iron release only when iron is taken up by a phagocytic mechanism.

DISCUSSION

It is well known that mononuclear phagocytes of the liver and spleen acquire iron as a result of erythrophagocytosis during the normal process of removal of effete red cells. However, the mechanisms involved in the liberation of iron by these cells in order for it to be returned to the circulation remain unclear [1]. Furthermore, during inflammatory disease there is a tendency for macrophages to retain more iron, which can eventually lead to development of anaemia, i.e. the anaemia of chronic disease. The reasons for this increased iron retention are not fully understood, although increased Ft synthesis is probably important [23–25], and it has also been suggested that NO contributes to this condition through activation of IRP1 and a subsequent increase in TfR expression [26]. However, it is

uncertain whether iron uptake from Tf is relevant to iron acquisition by macrophages *in vivo*, where phagocytosis of effete erythrocytes is likely to be the main source of iron. In an attempt to better understand how macrophages handle scavenged iron, we have investigated the role of NO in this process, using a system based on phagocytosis of ^{59}Fe-Tf–anti-Tf immune complexes as a model for erythrophagocytosis. This model has the advantage of permitting uptake of radioactive iron of high specific activity by a phagocytic route, analogous to erythrophagocytosis, but without the need for haem oxygenase to release iron from its carrier. The phagosomal/secondary lysosomal routing of the immune complexes was confirmed by their co-localization with the Lamp-1 marker [19].

To study the role of NO in macrophage iron metabolism, we used BMMϕ from mice defective in the expression of the iNOS [17]. It was found that cells from normal mice showed an increase in iron release following activation with IFNγ and LPS and a pulse of ^{59}Fe-Tf–anti-Tf immune complexes, which could be reversed by the NOS inhibitor L-NMMA. In contrast, cells from the iNOS-deficient mice showed a much smaller increase in iron release following activation, this residual effect probably reflecting the fact that the cells from the iNOS-deficient mice can still produce small amounts of NO. In contrast, when the cells were loaded with ^{59}Fe-citrate instead of immune complexes, NO had no effect on iron release. Thus NO appears to act only when iron is delivered by a phagocytic route, as we have shown previously that release of iron acquired from Tf by the J774 macrophage cell line is not increased by NO [11]. These results would suggest that NO, instead of contributing to the hypoferraemia of inflammation, may actually have a counterbalancing effect by promoting iron release from macrophages. Moreover, the ability of NO to increase uptake of iron from Tf, suggested as an alternative mechanism by which NO might contribute to the hyperferraemia of inflammation [26], may not be tenable as there is now evidence that TfR expression is regulated mainly by IRP2 rather than IRP1 [27], and that IRP2 activity is decreased rather than increased when macrophages are stimulated with IFNγ and LPS [9,11,12].

Although the mechanisms involved in cellular iron release from macrophages are not fully understood, it has generally been assumed that iron released by macrophages must first enter the cytoplasm from the phagosome for subsequent export across the cytoplasmic membrane. Iron-containing material phagocytosed by macrophages is very likely routed to the lysosomes, where a combination of proteolytic activity and low pH would release iron from carrier molecules. NO may assist in this process by promoting formation of low-molecular-mass iron nitrosyl complexes [28]; it is known that nitrite/nitrate can permeate into lysosomes [29] where the low pH will reconvert the anion to NO. In addition, haem oxygenase can remove iron from haem, which will be present after phagocytosis of erythrocytes. The interaction of NO with iron-containing phagocytosed material will be enhanced in IFNγ/LPS-activated macrophages due to slowed membrane trafficking [30]. Iron-nitroso compounds may subsequently decompose [31] or perhaps could themselves serve as a vehicle for iron release from the cell. Otherwise, subsequent events probably involve exit of iron from the phagolysosome to the cytoplasm, an event that may be mediated by the phagosomal metal transporter Nramp1, although the direction of cation transport by Nramp1 is controversial [32–34]. Once in the cytoplasm iron may be released across the cytoplasmic membrane, a process now thought to be mediated by the ferroportin membrane iron transporter [35]. However, this model would not explain why NO did not enhance release of iron acquired from Fe-citrate (Figure 5B) or Fe$_2$Tf [19].

NO can cause release of iron from Ft [13] and this might also be a mechanism whereby macrophage iron release is increased following activation with IFNγ/LPS. Although the proportion of iron bound to Ft was reduced in wild-type macrophages following IFNγ and LPS stimulation, this effect was not fully reversed by L-NMMA and a similar decrease occurred in the iNOS-deficient macrophages, suggesting that other mechanisms may operate. Moreover, the proportion of Fe incorporated into Ft was low and activation of the cells increased rather than decreased Ft levels, especially in the iNOS-deficient cells and in the wild-type cells stimulated in the presence of L-NMMA. It therefore seems rather unlikely that increased mobilization of Ft-bound iron is the mechanism of enhanced Fe release by NO.

An alternative possibility is that iron is released from activated macrophages directly via a lysosomal secretory pathway involving Nramp1, as we [19] and others [36] have suggested previously. Given the potentially damaging influence of high cytoplasmic iron on mRNA stability [37], this may prove the safest route for recycling of iron from effete red cells. NO is known to enhance secretory and/or excretory mechanisms in other cells [38], which could account for its ability to enhance iron release here. Direct extracellular secretion of iron from phagosomal contents would also imply that most if not all iron entering a cell by phagocytosis never enters the cytoplasm. Loading the cells with iron via Tf–anti-Tf immune complexes had little effect on Ft expression (Figure 4), compatible with this proposal. Although some radioactive iron was found in Ft, suggesting that some ingested iron can reach the cytoplasm, the amounts are relatively small compared with those seen in J774 cells loaded with Tf [11].

In conclusion, NO appears to participate in aiding the release of iron taken up by macrophages through phagocytosis, and may be involved in the maintenance of iron homoeostasis. Since NO synthesis in macrophages is induced by IFN and/or LPS, it may therefore be of particular importance during immune and inflammatory responses, when iron tends to accumulate in macrophages. Its role may be to counterbalance the effect of other cytokines, such as tumour necrosis factor and interleukin-1, which promote iron retention through increased Ft synthesis.

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