Effect of Nitric Oxide on Expression of Transferrin Receptor and Ferritin and on Cellular Iron Metabolism in K562 Human Erythroleukemia Cells

By Rosa Oria, Lourdes Sánchez, Tracey Houston, Matthias W. Hentze, Foo Y. Liew, and Jeremy H. Brock

Nitric oxide (NO) is known to increase the affinity of the intracellular iron-regulatory protein (IRP) for iron-response elements (IREs) in transferrin receptor and ferritin mRNAs, suggesting that it may act as a regulator of cellular iron metabolism. In this study, exogenous NO produced by adding the NO-generator S-nitroso-N-acetyl penicillamine gave a dose-dependent upregulation of transferrin receptor expression by K562 erythroleukemia cells and increased levels of transferrin receptor mRNA. NO did not affect the affinity of transferrin binding by the transferrin receptor. NO alone did not alter intracellular ferritin levels, but it did abrogate the inhibitory effect of the iron chelator desferrioxamine and potentiated the stimulatory effect of additional iron. NO also caused some increase in ferritin mRNA levels, which might mask any IRP/IRE-mediated inhibitory effect of NO on ferritin translation. Although NO did not affect net iron uptake, it increased release of iron from K562 cells pulsed previously with 59Fe, and subcellular fractionation showed that it also increased the proportion of intracellular iron bound to ferritin. These findings provide direct evidence that NO can affect cellular iron metabolism and suggest that NO produced in vivo by activated bone marrow macrophages might affect erythropoiesis.

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Materials and Methods

Materials. Apotransferrin was obtained from Behringwerke (Hounslow, UK) and was saturated to the required level with iron using Fe-nitrilotriacetate (FeNTA).11 Transferrin was labeled with 59Fe using 59Fe-citrate and with 111In by a modified chloramine-T procedure, as described in earlier studies.12 FeNTA (Fe:NTA ratio, 1:4) was prepared from Na-NTA and freshly prepared FeCl3 solutions13 and was added to cell cultures at a final concentration of Fe of 50 μmol/L. Human serum albumin (HSA; transferrin-free) was obtained from Schwarz-Pharma (Monheim, Germany). Rabbit antihuman ferritin, ferritins from CIBA (Horsham, UK), penicillamine from Sigma (Poole, UK), and SNAP from Schwarz-Pharma (Mönheim, Germany). Rabbit antihuman ferritin was obtained from Serotec (Oxford, UK), and the Ig fraction was prepared by ammonium sulphate precipitation and coupled to CNBr-activated Sepharose 4B (Pharmacia, Milton Keynes, UK) accoring to the manufacturer’s instructions. Chelex 100 was obtained from Bio-Rad (Gloucester, UK).

Cell cultures. K562 cells were routinely cultured in RPMI 1640 medium (Flow, Rickmansworth, UK) supplemented with antibiotics and 10% fetal calf serum. To avoid problems of variability in levels of iron and transferrin caused by the presence of serum, cells were
centrifuged and incubated for 1 hour in serum-free RPMI 1640 containing 1 mg/mL HSA and 50 μg/mL human transferrin (30% iron-saturated) to allow ectosynthesis of serum components and were then centrifuged, resuspended, and incubated for a further 24 hours in similar medium. This procedure yielded log-phase cultures in serum-free medium, which were used for all experiments.

**Transferrin binding.** Log-phase cells, obtained as described above, were incubated for a further 24 hours at 10^6/mL in serum-free medium containing transferrin and HSA. SNAP (10 μmol/L except where otherwise stated), DFO (1 mmol/L), or FeNTA (50 μmol/L Fe) were added as appropriate. The cells were then spun down, washed twice in ice-cold phosphate-buffered saline (PBS), and resuspended in fresh medium containing 50 μg/mL of unlabeled transferrin (30% iron-saturated) and HSA plus appropriate additions of test compounds. The cells were incubated for 15 hours, then centrifuged and washed twice in cold Hanks' buffered salts solution. Supernatants and washes were retained for assaying 59Fe activity. The cells were subjected to lysis and determination of intracellular iron distribution as described by Alvarez-Hernández et al. Briefly, this consisted of lysis in PBS containing 1% Triton X-100 and 1 mmol/L DFO, centrifugation at 10,000g for 5 minutes, affinity chromatography on Sepharose antiferritin, and ultrafiltration through a 10,000 molecular weight cut-off filter. This allows separation of intracellular iron into four compartments containing predominantly insoluble material (probably consisting mainly of hemosiderin and iron tightly bound to intracellular organelles), ferritin-bound iron, low molecular weight chelatable iron, and high molecular weight nonferritin iron, respectively. The 59Fe activity in each fraction was determined. Although these fractions do not necessarily contain exclusively the forms of iron stated, the method nevertheless provides a useful means of making comparative analyses of intracellular iron distribution.

**Iron uptake, release, and intracellular distribution.** Log-phase cells were centrifuged and resuspended at 3 × 10^6/mL in fresh medium containing HSA and 10 μg/mL of transferrin that had been saturated to 30% with 59Fe-citrate. The cells were incubated for 6 hours to allow incorporation of 59Fe, then centrifuged, washed 2 times in warm PBS-A, and cell-associated activity was determined. In some experiments, cells were incubated with varying amounts of 59Fe-transferrin, and the number and affinity of transferrin binding sites was determined by the Scatchard method as described previously.

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Table 1. Transferrin Binding and Ferritin Levels in K562 Cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>Transferrin Binding (pmol/10^6 cells)*</th>
<th>Intracellular Ferritin (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>38.6 ± 4.5</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>DFO (1 mmol/L)</td>
<td>64.1 ± 6.6†</td>
<td>24 ± 7†</td>
</tr>
<tr>
<td>Fe (50 μmol/L)</td>
<td>28.0 ± 4.8‡</td>
<td>68 ± 18‡</td>
</tr>
<tr>
<td>SNAP (10 μmol/L)</td>
<td>61.2 ± 4.9##</td>
<td>40 ± 13†</td>
</tr>
<tr>
<td>SNAP + DFO</td>
<td>68.2 ± 8.4†</td>
<td>40 ± 11†</td>
</tr>
<tr>
<td>SNAP + Fe</td>
<td>27.5 ± 3.1†</td>
<td>104 ± 26†</td>
</tr>
</tbody>
</table>

* Mean ± SD, n = 9.
† Mean ± SD, n = 6.
‡ P < .01 compared with control (no addition).
§ P < .05 compared with control (no addition).

The net amount of iron taken up from transferrin by the cells, ferritin mRNA levels were largely unaffected by DFO and release. There was also a significantly greater amount of iron bound to ferritin in SNAP-treated cells than in controls, but enhanced iron uptake did occur but was offset by a greater iron for the control and 11.5% transferrin receptor expression, SNAP did not greatly affect the differential level by IRP, thus enabling iron itself to control the homeostatic mechanisms.

However, there are some instances where alterations in cellular iron metabolism occur that are not directly related to changes in iron status. In particular, inflammatory and iron release was greater from SNAP + Fe than from controls, suggesting that the effects of SNAP on transferrin binding and cellular iron metabolism were mediated by NO rather than iron chelation.

DISCUSSION

Iron homeostasis is maintained by regulating the mechanisms by which cells take up, store, and release iron. The most important molecules involved in this process are the transferrin receptor, which permits cellular uptake of transferrin-bound iron,17 and ferritin, which detoxifies and stores iron acquired in excess of that needed for cellular metabolism.18 Both these proteins are regulated at the posttranscriptional level by IRP, thus enabling iron itself to control the homeostatic mechanisms.

Fig 2. Scatchard analysis of transferrin binding to K562 cells. The cells were grown for 24 hours in the presence (●) or absence (▲) of 10 μmol/L SNAP and then were exposed to 55Fe-labeled transferrin in the presence or absence of an excess of unlabeled protein, as described in Materials and Methods. For SNAP-treated cells, n = 3.78 x 10^4 (dissociation constant [kd] = 23 nmol/L); for control cells, n = 2.68 x 10^4 (kd = 26 nmol/L).
immunologic stimuli cause a slowing of iron release from sites of storage and absorption.\textsuperscript{9,20} The mechanisms mediating these changes are unclear but probably involve increased ferritin synthesis and iron retention caused by the action of cytokines such as interleukin-1\textsuperscript{21} or tumor necrosis factor- \textalpha ,\textsuperscript{22} which may upregulate both transcription\textsuperscript{23} and translation\textsuperscript{24} of ferritin. Further insight has been gained recently with the finding that NO can activate IRP-binding by converting IRP to the high-affinity form\textsuperscript{30} and thus regulate ferritin translation,\textsuperscript{8} indicating the existence of a link between a molecule involved in inflammatory processes and translational regulation of cellular iron metabolism.

The work reported here provides direct evidence for such a link. We have shown that SNAP, a compound that produces NO in solution, increased transferrin receptor expression by K562 cells, a finding consistent with the previously reported ability of endogenously produced NO in these cells to convert IRP to the form with high affinity for the IRE.\textsuperscript{8} However, such upregulation ought also to lead to a concomitant decrease in ferritin translation and, thus, to reduced cellular ferritin levels, as was indeed previously found to occur in a murine macrophage cell line in which endogenous NO production had been activated and in an IRE-chloramphenicol acetyl transferase (CAT) construct in K562 cells.\textsuperscript{8} In the present work, treatment of K562 cells with SNAP did not appreciably alter ferritin levels; moreover, SNAP both abrogated the downregulatory effect of DFO and potentiated the effect of iron. Metabolic labeling experiments also indicated that ferritin synthesis in SNAP-treated cells was similar to that in controls (data not shown). The reasons for this are not clear, but it is of interest that SNAP caused an increase in ferritin mRNA levels, which may offset decreased translation mediated by IRP. Previous work suggests that total IRP levels in K562 cells are relatively low compared with those in macrophage cell lines,\textsuperscript{8} and it may be that, if IRP levels are limiting, transferrin receptor is regulated more efficiently than ferritin. Alterations in cellular iron kinetics may also play a role, as discussed below.

With respect to ferritin, these findings differ from those reported earlier in the murine J774 macrophage cell line, in which NO markedly decreased ferritin biosynthesis.\textsuperscript{8} However, in those experiments, NO was produced endogenously by activating the cells with lipopolysaccharide and \gamma-interferon, which causes a marked NO-independent increase in ferritin mRNA levels.\textsuperscript{5} This could mask any effect of NO on transcription, and, indeed, in fibroblasts transfected with NO-synthase that constitutively produce NO, there is also a modest NO-mediated increase in ferritin mRNA levels (Pantopoulos and Hentze, unpublished data). Thus, the role of NO in ferritin gene expression may involve effects beyond those related to IRE-IRP interactions.

Despite the increased expression of transferrin receptor resulting from exposure of the cells to SNAP, there was no corresponding net increase in iron uptake. This appears to be because of the fact that SNAP-treated cells released significantly more iron than control cells, a finding that is in keeping with earlier reports showing that activated macrophages subjected to stimuli now known to result in NO release can provoke iron release from tumor cells.\textsuperscript{25,26} Therefore, NO may have the effect of increasing iron flux through the cell rather than of provoking a net increase in iron uptake or release.

The main effect of SNAP on the intracellular distribution of iron in K562 cells was a significant (albeit not particularly substantial) increase in the proportion of iron associated with ferritin. The most likely explanation for this is that iron mobilized by NO from cellular iron compounds is subsequently reincorporated into ferritin. NO interacts with cellular iron compounds to yield iron-nitroso complexes, but these can undergo ligand exchange and could, thus, yield their iron to ferritin.\textsuperscript{10} This may partly explain the failure of SNAP to decrease ferritin levels, despite the ability of NO to activate IRP, because the released iron may eventually overcome the translational block resulting from activation of IRP by NO. The fact that SNAP potentiated the effect of Fe on ferritin levels supports this explanation; however, it is possible in this case that nonspecific interactions of the cells with FeNTA, used to produce iron-loaded cells, might also affect ferritin synthesis, and it needs to be explained why transferrin receptor expression was nevertheless increased by NO. Furthermore, NO may also mobilize iron from ferritin\textsuperscript{27}; therefore, it is unclear what the net effect of NO might be.

### Table 2. Iron Release and Intracellular Iron Distribution in K562 Cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SNAP</th>
</tr>
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<tbody>
<tr>
<td>Release*</td>
<td>100</td>
<td>139 ± 18</td>
</tr>
<tr>
<td>Intra cellular iron†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insoluble</td>
<td>7.0 ± 0.9</td>
<td>7.3 ± 1.9</td>
</tr>
<tr>
<td>Ferritin-iron</td>
<td>19.9 ± 4.0</td>
<td>28.3 ± 2.0†</td>
</tr>
<tr>
<td>Low MW</td>
<td>12.4 ± 0.6</td>
<td>11.2 ± 1.2</td>
</tr>
<tr>
<td>High MW</td>
<td>60.9 ± 3.6</td>
<td>55.3 ± 1.7</td>
</tr>
</tbody>
</table>

Abbreviation: MW, molecular weight.

* Expressed as percentage of release compared with control, which varied from 18% to 37% of total cellular iron in different experiments (mean ± SD, n = 4).
† Expressed as percentage of total intracellular iron (mean ± SD, n = 3).
‡ P < .02.
on ferritin-iron. The ability of NO to increase ferritin mRNA levels, reported here, may be important because it could counterbalance any inhibition of ferritin translation. Evidently, the effect of NO on cellular iron homeostasis is complex and cannot be wholly explained by its direct effect on IRP. Detailed studies of the dynamics of IRP conversion by NO in relation to transferrin receptor and ferritin synthesis and to uptake and release of iron will be required to address this problem.

Although increased NO production in vivo occurs during inflammation, these changes in cellular iron metabolism induced in K562 cells by exogenously produced NO do not in themselves mirror the increased ferritin synthesis, iron retention, and inhibition of erythropoiesis observed in inflammation. However, the effects of inflammation on ferritin and transferrin receptor are mediated mainly by mechanisms that do not involve the IRE/IRP system and are, therefore, not likely to be influenced by NO. Thus, whereas inflammatory cytokines can reduce iron release from macrophages and uptake by erythroid precursors, NO may help to mediate a counterbalancing mechanism by restoring normal iron release from macrophages and, thus, delivery to the erythroid marrow. This is consistent with our earlier in vitro studies showing that, whereas inflammatory macrophages showed increased iron uptake and retention, immunologically activated macrophages showed increased uptake but also an increased release. On the other hand, the presence of an IRE in the 5'-untranslated region of eALAS mRNA suggests that NO might inhibit eALAS translation, which would depress rather than restore erythropoiesis. Therefore, although NO released by macrophages in the bone marrow might help to restore an adequate supply of iron to erythroid precursors, it might also potentiate the anemia. Future studies of how NO affects expression of eALAS may help to clarify its role in the anemia of chronic disease.

REFERENCES


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