Control of Transferrin Receptor Expression via Nitric Oxide-mediated Modulation of Iron-regulatory Protein 2*

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Cellular iron storage and uptake are coordinately regulated post-transcriptionally by cytoplasmic factors, iron-regulatory proteins 1 and 2 (IRP-1 and IRP-2). When iron in the intracellular transit pool is scarce, IRPs bind to iron-responsive elements (IREs) in the 5′ untranslated region of the ferritin mRNA and 3′ untranslated region of the transferrin receptor (TfR) mRNA. Such binding inhibits translation of ferritin mRNA and stabilizes the mRNA for TfR, whereas the opposite scenario develops when iron in the transit pool is plentiful. However, we (Richardson, D. R., Neumanova, V., Nagy, E., and Ponka, P. (1995) Blood 86, 3211–3219) and others reported that the binding of IRPs to IREs can also be modulated by nitric oxide (NO). In this study, we showed that a short exposure of RAW 264.7 cells (a murine macrophage cell line) to NO study, we showed that a short exposure of RAW 264.7 cells (a murine macrophage cell line) to NO, sodium nitroprusside (SNP), caused a significant decrease in IRP-2 binding to the IREs followed by IRP-2 degradation and that these changes occurred without affecting IRP-1 binding. The SNP-mediated degradation of IRP-2 in RAW 264.7 cells could be prevented by MG-132 or lactacystin, known inhibitors of proteasome-dependent protein degradation. A SNP-mediated decrease in IRP-2 binding and levels was associated with a dramatic decrease in TfR mRNA levels and an increase in ferritin synthesis. Importantly, the proteasome inhibitor MG-132 prevented the SNP-mediated decrease in TfR mRNA levels. These observations suggest that IRP-2 can play an important role in controlling transferrin receptor expression.

Iron is essential for all living organisms and is involved in cell proliferation, respiration, oxygen and electron transport, and DNA synthesis (1–5). However, “unshielded” iron is potentially toxic to cells. This toxicity of iron comes from its high reactivity with hydrogen peroxide that can lead to the production of hydroxyl radicals (via the Fenton reaction), which can damage membrane lipids and other cellular components (6). Therefore, organisms have evolved mechanisms to sequester free iron, both in the circulation (via transferrin, iron transport protein) and within the cells (via ferritin, iron storage protein).

Physiologically, the majority of cells in the organism acquire iron from a well characterized plasma glycoprotein, transferrin (Tf). Iron uptake from Tf involves the binding of Tf to the Tf receptors (TfRs), internalization of Tf within an endocytic vesicle by receptor-mediated endocytosis, and the release of iron from Tf by a decrease in endosomal pH. Following iron release from Tf within endosomes, iron passes through the endosomal membrane by ill understood mechanisms and then enters the poorly characterized intracellular labile pool. Intracellular iron that exceeds the requirement for the synthesis of functional heme and nonheme iron-containing proteins is stored within ferritin (4, 5).

Iron-regulatory proteins 1 and 2 (IRP-1 and IRP-2) are cytoplasmic proteins known to interact with specific nucleotide sequences, called iron-responsive elements (IREs), which are located in the 3′-untranslated region (UTR) of Tf mRNA (1, 2, 7) as well as the 5′-UTRs of mRNAs for ferritin (1, 2, 8), erythroid-specific 5-aminolevulinic acid synthase (9), and mitochondrial aconitase (10). When cellular iron becomes limiting, the IRP-1 is recruited into the high affinity binding state. The binding of IRPs to the IRE in the 5′-UTR of the ferritin mRNA represses the translation of ferritin, while an association of IRPs with IREs in the 3′-UTR of Tf mRNA stabilizes the transcript. On the other hand, the expansion of the labile iron pool inactivates IRP-1 and leads to degradation of IRP-2, resulting in an efficient translation of ferritin mRNA and rapid degradation of Tf mRNA (reviewed in Refs. 1–4).

IRP-1 is homologous to, and shares 30% identity with, mitochondrial aconitase (11), an enzyme of the Krebs cycle, and in iron-replete cells IRP-1 also has aconitase activity (12, 13). Importantly, 18 active site residues of mitochondrial aconitase are conserved in IRP-1, including the three cysteines (427, 503, and 506) (14, 15) that are involved in the binding of iron in the [4Fe-4S] cluster (16). The aconitase and IRE binding activities of IRP-1 are mutually exclusive. The IRP-1 form with an intact [4Fe-4S] cluster exists in iron-replete cells and has aconitase activity but lacks IRE binding activity. On the other hand, in iron-depleted cells, IRP-1 lacks the [4Fe-4S] clusters as well as aconitase activity and exhibits IRE binding activity (reviewed in Refs. 3 and 4).

A second IRE-binding protein, IRP-2, has recently been characterized by gel retardation assays and then purified and cloned from a variety of mammalian tissues and cells (17–20). IRP-2 shares 62% amino acid sequence identity with IRP-1 but differs in a unique way, having a 73-amino acid insertion in its N-terminal region. This region contains a cysteine-rich sequence that is responsible for targeting the protein for degradation via the ubiquitin-proteasome pathway when cellular...
iron levels are high (21, 22). Moreover, IRP-2 cannot assemble an [Fe-S] cluster and lacks aconitase activity.

Nitric oxide (NO) is an important endogenous regulator, many of whose functions are mediated via its binding to iron either in the heme of guanylate cyclase or in the [Fe-S] centers of important nonheme iron proteins (3, 4, 23). Iron interacts primarily with NO• (free radical), while its oxidized form, NO+ (nitrosionium ion) causes S-nitrosylation of thiol groups of proteins (24–26). NO is well known to modulate the activity of mitochondrial aconitase (reviewed in Refs. 3 and 23), which is, as discussed above, highly homologous to IRP-1. Previously, we showed that while NO• increases IRP-1 binding activity, TfR mRNA, and TfR number, NO+ decreased IRP-1 activity, TfR mRNA, and TfR binding in K562 cells (27). We have concluded (27) that treatment of K562 cells with the NO+ donor (sodium nitroprusside, SNP) resulted in S-nitrosylation of critical thiol groups, which may prevent the binding of IRP-1 to the IREs, a condition known to promote TfR mRNA degradation. On the other hand, NO• derived from S-nitroso-N-acetyl-penicillamine (SNAP) may directly react with the [Fe-S] cluster that may be followed by loss of the cluster, resulting in an increase in the binding of IRP-1 to IRE, a condition known to stabilize TfR mRNA.

In this study, we used RAW 264.7 cells and confirmed that two different redox forms of nitrogen monoxide, NO• and NO+, have very different and, in fact, opposite effects on IRP-1 RNA binding activity. Moreover, we found that IRP-2 may be a very important and rather unexpected target for NO• binding activity. Furthermore, a decrease in IRP-2 binding/levels following a short exposure to the NO• generator, SNP, dramatically decreased RNA binding activities of IRP-2 and did not increase RNA binding activities of IRP-1 (data not shown). In parallel experiments, samples were treated with 2% β-mercaptoethanol before the addition of the RNA probe.

Ribonuclease Protection Assay—RNase protection assays were performed using a kit from Pharmingen (Mississauga, Canada) as described in the manufacturer’s manual. 32P-Labeled antisense RNAs were generated using T7 polymerases. Actin mRNA was used as a control.

RESULTS

NO• Mimics the Effect of IFN-γ/LPS on IRP-2 and TfR in RAW 264.7 Cells—Murine macrophages are known to produce a large quantity of NO following their treatment with IFN-γ and LPS (29–31). Since NO can regulate iron metabolism via interacting with IRPs (3, 23, 27, 32, 33), we examined IRE-binding activities of IRPs in RAW 264.7 cells exposed to NO• donors as compared with those treated with IFN-γ and LPS. In IFN-γ/LPS-treated cells, RNA-binding activity of IRP-1 increased, while that of IRP-2 decreased (Fig. 1, A and B, lane 4), and these changes were associated with a decrease in TfR mRNA levels (Fig. 1C, lane 4) and an increase in ferritin synthesis (Fig. 1D, lane 4). Treatment of RAW 264.7 cells with SNAP (NO• generator) enhanced the RNA binding activity of IRP-1 without affecting IRP-2 (Fig. 1, A and B, lane 5). Since NO•-derived NO• can be easily oxidized to nitrite, we measured the effect of nitrite on IRP-1 RNA binding activity. We found that sodium nitrite in concentrations much higher than those generated by SNAP did not increase RNA binding activities of IRP-1 (data not shown). In parallel experiments, RAW 264.7 cells were treated with other NO• donors (NOC-9 or NOC-18), which produced responses in IRE binding activities that were similar to those following SNP (data not shown).

Treatment of RAW 264.7 cells with SNAP (10 h) was associated with an increase in TfR mRNA levels and a decrease in ferritin synthesis, changes that can be explained by the decreased IRP-1 activity. However, these changes in iron metabolism induced by the NO• donors are clearly different from those occurring in RAW 264.7 following their exposure to IFN-γ and LPS. Interestingly, a 10-h exposure of RAW 264.7 cells to the NO• generator, SNAP, dramatically decreased RNA binding activity of IRP-2 (Fig. 1, A and B, lane 6), which was associated with a decrease in TfR mRNA levels (Fig. 1C, lane 6) and an increase in ferritin synthesis (Fig. 1D, lane 6). Hence, iron metabolism changes in RAW 264.7 cells treated with the NO• donor are very similar to those seen in IFN-γ/LPS-treated RAW 264.7 cells. In the following experiments, we investigated the effects of NO• versus NO• donors on IRP-2 cells in more detail.
NO Targets IRP-1, while NO\(^{+}\) Affects Primarily IRP-2—The above studies indicate that NO\(^{+}\) and NO\(^{-}\) donors have distinct effects on IRP-1 and IRP-2. Hence, we investigated the kinetics of changes in IRPs following treatments of RAW 264.7 cells with different NO donors. Incubation of RAW 264.7 cells with SNAP (NO\(^{-}\) generator) activated IRP-1 within 1 h, and the maximum RNA-binding activity of IRP-1 was reached at 3 h (Fig. 2, A and B). Treatment of RAW 264.7 cells with SNP was associated with an increase in TIR mRNA levels (Fig. 2D), and this increase was apparent as early as 1 h, when IRP-1 activity was only slightly increased (Fig. 2, A and B). It is possible that a small increase in IRP RNA binding activity may cause a significant increase in Tir mRNA. However, SNAP/NO\(^{+}\) may also increase Tir mRNA levels by an IRP-1-independent mechanism, and this possibility is currently under investigation. As compared with appropriate controls, SNP treatment did not produce any appreciable change in IRP-2 (Fig. 2, A and B) during the whole incubation period (10 h). On the other hand, SNP-treated RAW 264.7 cells showed responses very different as compared with those following SNAP. Only a 1-h exposure of RAW 264.7 cells to SNP resulted in a significant decrease in IRP-2 binding to the IRE, and this binding activity was totally absent at 3 h (Fig. 2, A and B, lane 7). Moreover, IRP-2 protein levels decreased in SNP-treated cells (Fig. 2C), but this decrease occurred later (−3 h). Moreover, this decrease in IRP-2 protein levels following SNP was not associated with any appreciable decrease in IRP-1 protein levels (Fig. 2C), as can be predicted from the results of RNA binding of IRP in the presence of β-mercaptoethanol (Fig. 2A). Moreover, SNP treatment did not cause any significant decrease of total protein in RAW 264.7 cells (not shown). Importantly, a decrease in IRP-2 binding, following a short (3-h) exposure of RAW 264.7 cells to SNP, was associated with a dramatic decrease in Tir mRNA levels (Fig. 2D). These changes occurred without any change in the IRE binding activity of IRP-1 (Fig. 2, A and B), strongly suggesting that IRP-2 alone can play a significant role in controlling Tir expression.

The above experiments indicate that SNP decreases Tir mRNA levels by an IRE/IRP-dependent mechanism. Additional evidence supporting this conclusion was provided by an experiment in which we exploited mouse LtK cells transfected with human Tir lacking its 3′-UTR, which contains the IREs (28). Fig. 3 shows that neither changes in cellular iron levels nor SNP treatment affected the levels of truncated human Tir mRNA in transfected LtK cells. On the other hand, SNP treatment caused a dramatic decrease in endogenous mouse Tir mRNA in LtK cells (not shown), as was the case using RAW 264.7 cells (Figs. 1 and 2).
Fig. 3. Effects of ferric ammonium citrate, desferrioxamine, and SNP on human TfR mRNA devoid of the 3′-UTR containing IRE elements. Mouse Ltk cells, transfected with human (without IRE) TfR cDNA (28), were incubated (as indicated) without (CTL) or with ferric ammonium citrate (FAC), desferrioxamine (DFO), or SNP for 3 h. A. Tf mRNA and actin mRNA levels were revealed by Northern blot analysis (27); human TfR cDNA was used as a probe. B. densitometric analysis of Tf mRNA levels.

Specificity of NO⁺ in Causing IRP-2 Degradation—Since decomposition products of SNP could be responsible for the above described phenomena, we investigated the effects of K₃Fe(CN)₆ and KCN on the RNA binding activities of IRP-2. Fig. 4A (lanes 5 and 6) shows that neither ferricyanide nor potassium cyanide decreased the IRP-2 binding activities or protein levels (Fig. 4B). Although iron release from SNP is highly unlikely, we conducted a control experiment in which we examined whether SNP-derivied iron could be responsible for the observed effects on IRP-2 as shown in Fig. 2. We showed that EDTA, a membrane-impermeable iron chelator that would trap any iron released from SNP, did not prevent SNP-induced degradation of IRP-2 (Fig. 4B, lane 9). Moreover, the chelator that is commonly used to intercept intracellular iron, desferrioxamine, was also unable to attenuate SNP-induced degradation of IRP-2 (Fig. 4B, lane 9).

IRP-2 Appears to Play a Major Role in Controlling TfR mRNA Levels—In the aforementioned experiments, SNAP affected the IRP-1, causing an increase in its RNA binding activity, while SNP caused a decrease in IRP-2 binding and protein levels. Hence, we deemed it important to examine how the combination of both agents affects iron metabolism in RAW 264.7 cells. We found that when both NO donors (SNAP and SNP) were added simultaneously, the RNA binding activity of IRP-1 increased, while that of IRP-2 decreased (Fig. 5, A and B, lanes 7 and 8) as compared with untreated cells (Fig. 5, A and B, lane 1). However, a slightly higher dose of SNP was required to activate IRP-1 in the presence of SNAP than in the absence of this agent (Fig. 5, A and B, lanes 7 and 8 versus lanes 3–6). As expected, IRP-1 activation seen following SNAP treatment was associated with an increase in TfR mRNA levels in RAW 264.7 cells (Fig. 5C, lanes 3–6). However, rather unexpectedly, the addition of SNP together with SNAP caused a dramatic decrease in TfR mRNA levels (Fig. 5C, lanes 7–10), and these levels remained low even in RAW 264.7, which contained IRP-1 with high RNA binding activities (Fig. 5, A and C, lanes 7 and 8). Hence, it appears that the decrease in TfR mRNA levels in RAW 264.7 cells correlates with a selective decrease in IRP-2 binding, suggesting that this factor plays a crucial role in maintaining TfR mRNA levels.

SNP-mediated Degradation of IRP-2 Occurs via the Ubiquitin-Proteasome Pathway—In iron-replete cells, IRP-2 is degraded via the ubiquitin-proteasome degradation pathway (21, 22). Hence, we examined whether SNP-mediated IRP-2 degradation also occurs in proteasomes, and to test this we exploited the protein synthesis inhibitor, cycloheximide, as well as more specific proteasome inhibitors, MG-132 (34, 35) and lactacystin (36). RAW 264.7 cells were pretreated with cycloheximide, MG-132, or lactacystin for 30 min, following which SNP was added to the cultured cells for an additional 3 h. None of these agents affected RNA binding activity of IRPs or their protein levels (Fig. 6, A and B, lanes 5–7). As expected, iron caused a slight decrease in IRP-2 binding and protein level (Fig. 6, A and B, lane 3) that was completely blocked by proteasome inhibitors (Fig. 6, A and B, lanes 8–10). Both cycloheximide and MG-132 prevented the loss of IRP-2 binding activity as well as its degradation (Fig. 6, A and B, lanes 11 and 12). Lactacystin failed to attenuate the SNP-mediated decrease in RNA binding activity of IRP-2 but prevented the degradation of this protein. Importantly, MG-132, but not lactacystin, prevented the SNP-mediated decrease in TfR mRNA levels (Fig. 7).

We also examined whether SNP effects required the intact cell systems (37). Fig. 8 shows that SNP added to cell extracts at concentrations as high as 500 μM did not affect either IRP-2 binding to the IRE or its protein level. However, treatment of cell extracts with a high concentration of iron in the presence of a reducing agent caused a significant degradation of IRP-2 (Fig. 8, A and B, lane 5), confirming an earlier report (38).
These results strongly suggest that SNP-mediated degradation of IRP-2 requires intact cells and ubiquitination.

**SNP-mediated Degradation of IRP-2 May Require Carriers for NO**

The half-life of NO in aqueous solutions is very short (\(10^{-10}\) s), and it seems unlikely that NO, in its ionic form, can be transported inside cells. We have examined (Fig. 9) whether the incubation medium, used in the above described experiments, contains components that could serve as carriers for NO. RAW 264.7 cells were extensively washed with Hanks’ buffer, which is free of thiol-containing compounds that may serve as NO carriers. Cells were then incubated without or with SNP for various time intervals, following which IRP-2 binding was examined. The effect of SNP on IRP-2 deactivation was significantly delayed when cells were incubated in Hanks’ buffer (i.e., without thiols) as compared with cells incubated in the regular medium (Fig. 9). Hence, it appears that thiol-containing compounds (e.g., cysteine or components of fetal calf serum) are involved as carriers and that NO is delivered to the cells via transnitrosylations.

**DISCUSSION**

Iron is uniquely suited to carry out biochemical redox reactions without which life would be impossible, but if not appropriately shielded it may become very toxic because of its catalytic action in one-electron redox reactions (1, 4, 6). Hence, organisms were compelled to solve one of the many paradoxes of life, i.e., to keep “free iron” at the lowest possible level and yet in concentrations allowing adequate supply of the essential element for the synthesis of hemoproteins and other iron-containing molecules. The real chemical nature of iron in the intracellular labile iron pool (LIP) is ill understood, but it is known that iron in this metabolically and kinetically active pool can be intercepted by strong chelators. Moreover, organisms are equipped with remarkable regulatory mechanisms that coordinately regulate cellular iron uptake and storage and maintain iron in the LIP at appropriate levels. The level of iron in the LIP is “sensed” by IRPs, which are responsible for coordinate regulation of ferritin and TfR expression. When iron in the LIP is scarce, IRP binding to the IREs represses ferritin mRNA translation and increases TfR mRNA stability, and the opposite scenario develops when iron in the transit pool is plentiful (1–5).

However, iron is not the only player that can modulate IRPs, and IRP activities/levels can be affected by various forms of “oxidative stress” and NO (3). IRP-1 is homologous to mitochon-
1 h, and maximum activation is reached in 3 h (Fig. 2). Hence, our experiments suggest that NO\(^*\) activates RNA binding activity of IRP-1 by directly interacting with its [Fe-S] cluster, and this conclusion is also supported by recent findings of Kennedy et al. (39).

IRP-2 (105 kDa) is larger than IRP-1 (90 kDa), and this difference in size is caused by a 73-amino acid insertion containing a cysteine-rich element in the N terminus of IRP-2 (17–19). The three cysteine residues that coordinate the [Fe-S] cluster in IRP-1 are conserved in IRP-2 (20). IRP-2 is degraded in iron-replete cells (18, 19, 21, 22, 38), and the specific N-terminal amino acid insertion is necessary and sufficient to render IRP-2 susceptible to proteolytic degradation in proteasomes (21). In this study, we found that a 1-h exposure of RAW 264.7 cells to the NO\(^*\) generator, SNP, resulted in a significant decrease in IRP-2 binding activity to the IRE, and this binding activity was totally absent at 3 h (Fig. 2, A and B). We also demonstrated that IRP-2 protein levels decreased in SNP-treated RAW 264.7 cells, but this decrease occurred later than the inhibition of IRP-2 binding (Fig. 2C). Interestingly, the NO\(^*\)-mediated degradation of IRP-2 is sensitive to the protein synthesis inhibitor cycloheximide (Fig. 6), indicating that ongoing protein synthesis is required for the inactivation of IRP-2. More importantly, the SNP-mediated deactivation (in terms of IRE binding) and degradation of IRP-2 in SNP-treated RAW 264.7 cells could be prevented by MG-132 or lactacystin (Fig. 6), known proteasome inhibitors (34–36). It is of interest to mention that after SNP treatment the residual IRP-2 protein can be seen on Western blot analysis in the absence of any detectable IRP-2 RNA binding activity (Figs. 2, 4, and 6). It is possible that the ubiquitination of IRP-2 prevents its RNA binding before the protein is totally degraded.

Since until now the only described mechanism of IRP-2 degradation has been the one involving iron, we considered the possibility that this metal may somehow be involved in IRP-2 degradation in SNP-treated RAW 264.7 cells. Because SNP is an iron complex, it might be possible that treatment of cells with SNP could lead to the donation of iron and result in IRP-2 degradation. However, ferricyanide (structurally very similar to SNP) did not decrease IRP-2 protein levels under conditions when SNP did (Fig. 4). Hence iron donation to the cell via SNP could not explain the observed decrease in IRP-2 levels. Moreover, the addition of the iron chelators, either EDTA or desferrioxamine, to SNP did not prevent the decrease in IRP-2 protein levels (Fig. 4), further suggesting that the effect of SNP was not due to iron donation to the cell. We have also scrutinized the possibility that SNP-derived NO\(^*\) could increase iron levels in the LIP but found several arguments against this explanation. First, at early time intervals, NO\(^*\) did not decrease IRP-1 activity (Fig. 2), as would have been expected if iron levels in the LIP increased. In this connection, it is important to stress that iron-mediated deactivation of IRP-1 and IRP-2 occurs with similar kinetics (40). Second, we found that while NO\(^*\) donors can effectively mobilize \(^{59}\)Fe from \(^{59}\)Fe-prelabeled cells, SNP (the NO\(^*\) donor) is unable to do so,\(^a\) suggesting that NO\(^*\) is unlikely to affect intracellular iron levels and/or cause iron redistribution within the cells.

Our results together with the above considerations suggest that in SNP-treated cells critical SH groups of IRP-2 are S-nitrosoylated and that this modification targets this protein for degradation via the ubiquitin-proteasome pathway. It is well established that the 73-amino acid sequence unique to IRP-2 is responsible for iron-mediated proteolytic degradation of this protein. Although the mechanisms involved are not fully un-

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\(^a\) S. Kim and P. Ponka, unpublished observations.
It is now well established that cellular iron uptake and storage are coordinately regulated through a feedback control mechanism mediated at the post-transcriptional level by IRP-1 and IRP-2. It seems reasonable to conclude that the primary function of IRPs involves "sensing" iron levels in the LIP and maintaining the size of this pool via controlling TFR and ferritin expressions. However, cellular iron homeostasis appears to be regulated not only by iron levels per se but also by other factors that manifest themselves, e.g., during inflammation, a condition associated with increased NO production. We (27) and others (Refs. 32 and 33; reviewed in Ref. 3) previously demonstrated that NO can affect cellular iron metabolism via its interaction with IRP-1. This report extends our previous observations by showing that one of the redox forms of NO, nitrosonium ion (NO⁺), is an important regulator of IRP-2.

We found that treatment of RAW 264.7 cells with NO⁺ results in a rapid decrease in RNA-binding of IRP-2, followed by IRP-2 degradation, probably in proteasomes. The decrease in IRP-2 is associated with the decrease in TFR mRNA levels, and similar changes develop in RAW 264.7 cells following their treatment with IFN-γ and LPS. It is tempting to speculate that the decrease in TFR in activated macrophages may be beneficial in preventing iron uptake and, consequently, diminishing "oxidative stress" within the cells.

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