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Role of nitric oxide in cellular iron metabolism

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Abstract

Iron regulatory proteins (IRP1 and IRP2) control the synthesis of transferrin receptors (TfR) and ferritin by binding to iron-responsive elements (IREs) which are located in the 3′ untranslated region (UTR) and the 5′ UTR of their respective mRNAs. Cellular iron levels affect binding of IRPs to IREs and consequently expression of TfR and ferritin. Moreover, $NO^{\bullet}$, a redox species of nitric oxide that interacts primarily with iron, can activate IRP1 RNA-binding activity resulting in an increase in TfR mRNA levels. We have shown that treatment of RAW 264.7 cells (a murine macrophage cell line) with NO^{+} (nitrosonium ion, which causes S-nitrosylation of thiol groups) resulted in a rapid decrease in RNA-binding of IRP2, followed by IRP2 degradation, and these changes were associated with a decrease in TfR mRNA levels. Moreover, we demonstrated that stimulation of RAW 264.7 cells with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) increased IRP1 binding activity, whereas RNA-binding of IRP2 decreased and was followed by a degradation of this protein. Furthermore, the decrease of IRP2 binding/protein levels was associated with a decrease in TfR mRNA levels in LPS/IFN-γ-treated cells, and these changes were prevented by inhibitors of inducible nitric oxide synthase. These results suggest that NO^{+}-mediated degradation of IRP2 plays a major role in iron metabolism during inflammation.

Introduction

Iron (Fe) is essential for life, functioning as a metal cofactor for many proteins containing either heme or non-heme Fe (Klausner *et al.* 1993; Hentze & Kuhn 1996; Richardson & Ponka 1997a). Hemoproteins have crucial biological functions, such as oxygen binding (hemoglobin), oxygen metabolism (oxidase, peroxidase, catalase, *etc*), and electron transfer (cytochromes). Many non-heme Fe-containing proteins catalyze key reactions involved in energy metabolism (e.g. mitochondrial aconitase and [Fe-S] proteins of the electron transport chain) and DNA synthesis (ribonucleotide reductase).

On the other hand, the chemical properties of Fe, which are exploited for a remarkable range of biological functions, have created problems for living organisms. In aqueous solution, Fe may exist in two oxidation states, Fe^{2+} and Fe^{3+}, which can donate and accept electrons, respectively. At physiological pH and oxygen tension, Fe^{2+} is readily oxidized to Fe^{3+}, which rapidly forms essentially insoluble $Fe(OH)_3$ polymers. Moreover, unless appropriately chelated, Fe, due to its catalytic action in one electron redox reactions, plays a key role in the formation of harmful oxygen radicals that ultimately cause oxidative damage to vital cell structures (Halliwell & Gutteridge 1990). Thus, living systems have evolved specialized molecules for the acquisition, transport and storage of Fe in a soluble non-toxic form to satisfy the requirements of the body and cells for this metal. Moreover, cells are equipped with exquisite mechanisms that maintain sufficient amounts of Fe for synthesis of physiologically active Fe-containing molecules and yet keep 'free iron' at its lowest possible level.

Physiologically, the majority of cells in the organism acquire Fe from a well-characterized plasma glycoprotein, transferrin (Tf). Fe uptake from Tf involves the binding of Tf to the Tf receptors (TfR), internalization of Tf within an endocytic vesicle by receptor-mediated endocytosis, and the release of Fe from Tf by a decrease in endosomal pH. Following Fe release

from Tf within endosomes, Fe^{2+} passes through the endosomal membrane by divalent metal transporter1 (Fleming *et al.* 1997, 1998), and then enters the poorly characterized intracellular labile pool. Intracellular Fe that exceeds the requirement for the synthesis of functional heme and nonheme Fe-containing proteins is stored within ferritin (Richardson & Ponka 1997a).

Control of intracellular Fe homeostasis

Generally, sensitive control mechanisms exist that monitor Fe levels in the intracellular labile pool to prevent its expansion, while still making the metal available for Fe-dependent proteins. In proliferating non-erythroid cells, iron dependent regulation of both TfR and ferritin occurs post-transcriptionally, and is mediated by nearly identical iron responsive elements (IREs) in the UTRs of numerous mRNAs (Hentze & Kuhn 1996; Richardson & Ponka 1997a; Aisen *et al.* 1999). The opposite situation develops when this pool is depleted of iron. Pivotal players in this regulation are cytosolic iron regulatory proteins (IRP1 and 2) which sense iron levels in the labile pool. As will be described in detail below, IRP1 and IRP2 perform their function by binding to specific nucleotide sequences, termed iron-responsive elements (IREs), which are present in the mRNAs for numerous proteins involved in iron metabolism (Figure 1).

The IRE is composed of a stem-loop structure with an upper double-stranded, 5-bp-long helix of variable sequence and a six-membered loop whose sequence is 5′-CAGUGN-3′. Below this short paired stem, there is a small asymmetrical bulge with an unpaired cytosine as the first nucleotide 5′ of the stem (Hentze *et al.* 1988). In some IREs, this bulge is a single unpaired C nucleotide whereas in others, two additional 5′ nucleotides appear to be in opposition to one free 3′ nucleotide. Beyond the bulge, there is another base paired region without any obvious sequence constraints. The IRE motifs have been identified in untranslated regions (UTR) of mRNA for ferritin (one IRE in 5′ UTR, Leibold & Munro 1987), TfR (five IREs in 3′ UTR, Casey, *et al.* 1988), the erythroid 5-aminoleuvulinic acid synthase (one IRE in 5′ UTR, Dandekar *et al.* 1991), mammalian mitochondrial aconitase (Kim *et al.* 1996) and some other proteins.

IRP1 is a 98 kDa bifunctional protein with mutually exclusive functions of RNA binding and aconitase activity (Kaptain *et al.* 1991; Haile *et al.* 1992) which shares 30% identity with mitochondrial aconitase (Kennedy *et al.* 1992), an enzyme of the Krebs cycle. Importantly, 18 active site residues of mitochondrial aconitase are conserved in IRP1, including the three cysteines (427, 503, and 506) (Rouault *et al.* 1991; Philpott *et al.* 1993) that are involved in the binding of Fe in the [4Fe-4S] cluster (Hirling *et al.* 1994). The structural analysis of mitochondrial aconitase has revealed that the [Fe-S] cluster rests at the cleft between the two regions of the protein. One region includes domains 1 to 3, and the second represents domain 4. The [Fe-S] cluster has been known to play a crucial role in regulating the enzymatic activity of mitochondrial aconitase as well as IRP1 (cytosolic aconitase). The holo-form of IRP1 contains a [4Fe-4S] cluster and has aconitase activity but cannot bind to IREs. On the other hand, when the [Fe-S] cluster is disassembled, IRP1 loses aconitase activity and acquires IRE binding activity (Hentze & Kuhn 1996). Thus, the aconitase and RNA binding activities of IRP1 are mutually exclusive (Figure 1).

A second IRE-binding protein, IRP2, was initially identified in rat hepatocytes, and subsequently cloned from a variety of mammalian tissues and cells (Henderson *et al.* 1993; Guo *et al.* 1994). IRP2 shares 62% amino acid sequence identity with IRP1 but differs in a unique way; it harbors a 73-amino acid insertion in its N-terminal region and lacks the [Fe-S] cluster. This additional region contains a cysteine-rich sequence that is known to be responsible for targeting the protein for degradation *via* the ubiquitin-proteasome pathway when cellular Fe levels are high (Iwai *et al.* 1995; Guo *et al.* 1995; Iwai *et al.* 1998). IRP2 does not have aconitase activity, probably due to the lack of the [Fe-S] cluster (Phillips *et al.* 1996) (Figure 1).

Recent research in numerous laboratories (Rouault, *et al.* 1991; Klausner *et al.* 1993; reviewed in Hentze & Kuhn 1996; Richardson & Ponka 1997a) has revealed that the interactions of IRPs with IREs regulate iron metabolism in non-erythroid cells in the following manner: When cellular iron becomes limiting, IRP2 is present in the cytosol, and IRP1 is converted into the high affinity binding state. The binding of IRPs to the IRE in the 5′ UTR of ferritin mRNA blocks the translation of ferritin, whereas the association of IRPs with IREs in the 3′ UTR of TfR mRNA stabilizes this transcript. On the other hand, when intracellular iron is abundant, IRP1 is unable to bind to IREs, and IRP2 is degraded, resulting in efficient translation of ferritin mRNA and rapid degradation of TfR mRNA (Figure 2).

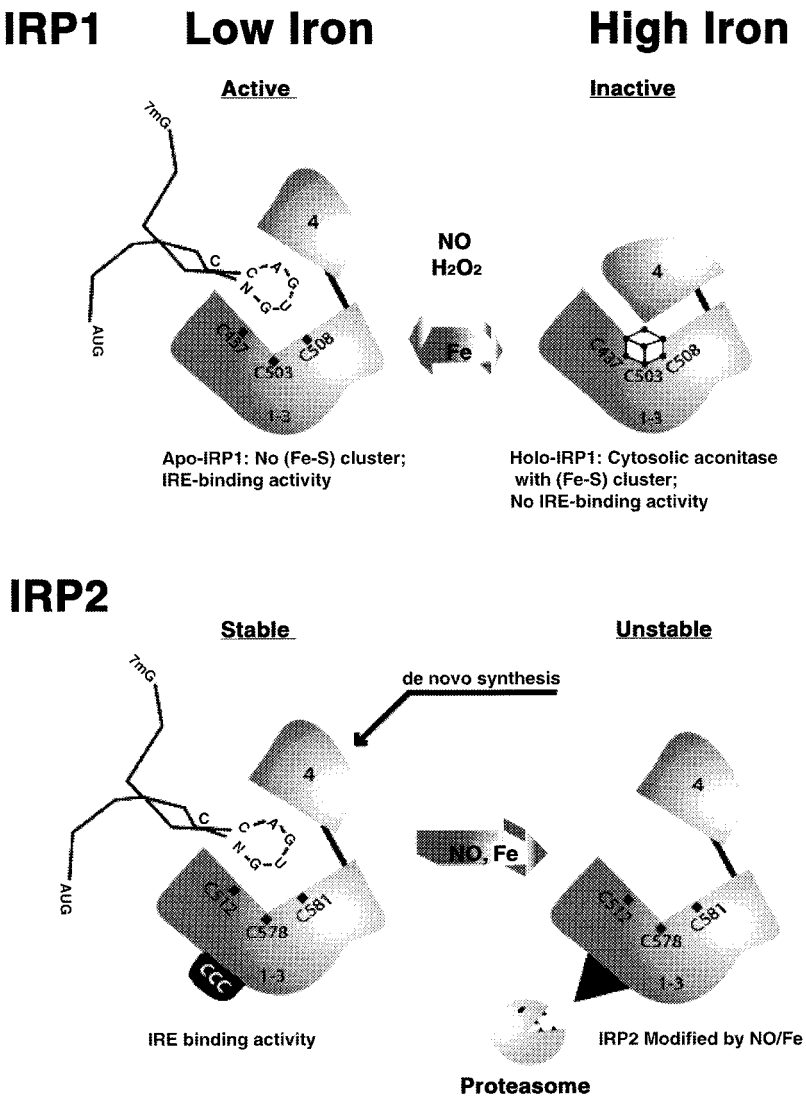

Fig. 1. The IRE/IRP system. IRPs simultaneously regulate the expression of TfR and ferritin by interacting with IREs which are located in the 5′ UTR of ferritin mRNA and 3′ UTR of TfR mRNA. When cellular iron becomes limiting, the binding of IRPs to the IRE of ferritin mRNA blocks the translation of ferritin, whereas an association of IRPs with IREs of TfR mRNA stabilizes this transcript. On the other hand, when intracellular iron is abundant, IRPs are unable to bind to IREs, resulting in an efficient translation of ferritin mRNA and rapid degradation of TfR mRNA.

It needs to be pointed out that Fe is not the only player that modulates IRP1 binding activity, IRP2 levels and, consequently, transferrin receptor and ferritin expression. As will be discussed in more detail below RNA binding activities of both IRP1 and IRP2 are modulated by nitric oxide (NO). Besides NO, hydrogen peroxide (H_2O_2) is another factor that has been shown to modulate IRP1 binding activity. When cells are exposed to H_2O_2, IRP1 binding activity is rapidly ($\sim$ 1 h) converted to its IRE-binding form (Pantopoulos & Hentze 1995), probably due to the disassembly of the [Fe-S] cluster in IRP1 (Pantopoulos 1996). Moreover, as compared to Fe chelators whose continuous presence is required for IRP1 activation, the presence of H_2O_2 is only required for 10–15 min, and then the activation of IRP1 can be completed in the absence of the effector (Pantopoulos *et al.* 1997). IRP1 activation seems to require an interaction of H_2O_2 with a membrane component, suggesting that H_2O_2 must be 'sensed' to stimulate a pathway leading to IRP1 activation (Pantopoulos & Hentze 1998). As expected, based on the IRE/IRP paradigm, exposure of

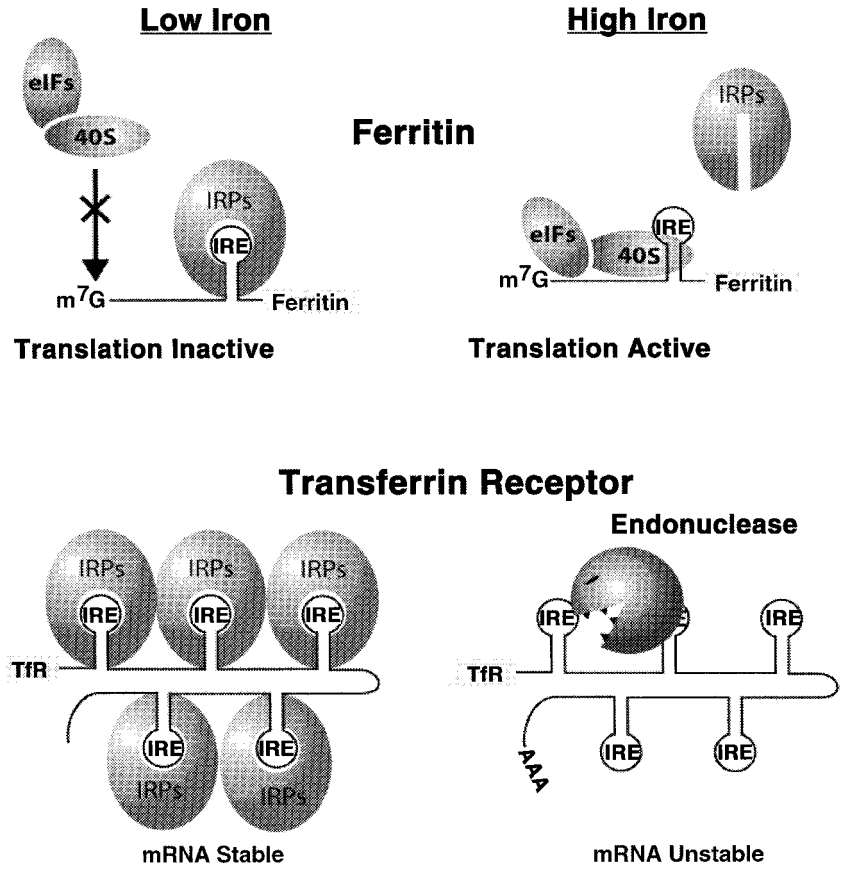

Fig. 2. Regulation of iron regulatory proteins. Both IRP1 and IRP2 have four homologous domains which bind to the IRE. In iron replete conditions, IRP1 assembles the [Fe-S] cluster with aconitase activity but is unable to bind IRE, while IRP2 does not have the cluster and is targeted for proteasomal degradation. In iron starved conditions, IRP1 loses the [Fe-S] cluster and acquires IRE binding properties, and IRP2 is stabilized.

cells to H_2O_2 stimulates TfR mRNA expression (Pantopoulos and Hentze, 1995) and, consequently, lead to an increase in iron uptake. (Caltagirone, *et al.* 2000).

Role of nitric oxide in iron metabolism

Nitric oxide (NO) is a highly reactive diatomic molecule that is produced in mammalian cells by the enzyme NO synthase, which catalyzes the stepwise conversion of L-arginine to NO and L-citrulline (Marletta 1993; Abu-Soud *et al.* 1997). In biological systems, NO exists in three redox-related species and in the context of this article, NO is used as a generic term to encompass all these species. Redox-related species of NO include $NO^{\bullet}$, which can modulate Fe-containing proteins by direct coordination to Fe centers (see below) and can have a cytotoxic effect by reacting with superoxide anion ($O_2^{\bullet -}$) to produce peroxynitrite ($ONOO^-$). The second important species of NO, the nitrosonium ion (NO^+), can nitrosylate thiol groups of proteins, a modification that may have important regulatory functions (Stamler *et al.* 1992a; Stamler *et al.* 1992b; Hess *et al.* 2001). Nitrosonium ion has an extremely short half-life ($\approx 10^{-10}$ s) in solution at physiological pH (Stamler *et al.* 1992b), and binds extremely rapidly to thiol groups. Following such binding, the resulting $-$SNO compounds maintain 'nitrosonium character' and can transfer NO^+ to other thiols (Stamler *et al.* 1992a; Lipton *et al.* 1993; Stamler 1994). Another form of NO is NO^-, nitronyl anion, which was reported to have a similar reactivity as NO^+ (Kim *et al.* 1999), leading to S-nitrosylation; however, the physiological relevance of this species is not clear.

NO has two principal functions in cells, servoregulation and cytotoxicity. For regulatory functions, NO is produced in small amounts under physiological

conditions and mediates vasorelaxation, controls the adhesion and aggregation of platelets and neutrophils, and is involved in neurotransmission (Bredt & Snyder 1994). Most of these actions are mediated through the binding of NO to iron in the heme prosthetic group of soluble guanylate cyclase, which catalyzes the conversion of GTP to cyclic GMP (Ignarro 1996). The importance of Fe in mediating the functions of NO is also apparent when examining the cytotoxic effects of the molecule. Cytotoxic functions of NO are observed when it is produced in much larger quantities by macrophages, hepatocytes and other cells following their exposure to cytokines or microbial products (Moncada *et al.* 1991). NO produced via such high-output systems inhibits proliferation of intracellular pathogens and tumor cells (Hibbs Jr. *et al.* 1984; Hibbs Jr. *et al.* 1988). These effects can be explained by the reactivity of $NO^{\bullet}$ with Fe in the [Fe-S] centers of several important macromolecules, including aconitase, and complex I and complex II of the electron transport chain (Drapier & Hibbs Jr. 1988; Drapier & Hibbs 1986; Henry *et al.* 1993). The high affinity of $NO^{\bullet}$ for iron probably results in both the removal of Fe from [Fe-S] centers and the formation of nitrosyl-Fe species within [Fe-S] proteins. In fact, cocultivation of tumor cells with activated macrophages results in the inhibition of target cell DNA synthesis and a concomitant loss of a large fraction of intracellular Fe (Hibbs Jr. *et al.* 1984; Hibbs Jr. *et al.* 1988). All these effects of NO severely impair ATP synthesis. In addition, $NO^{\bullet}$ inhibits another Fe containing enzyme, ribonucleotide reductase, the rate limiting enzyme involved in the conversion of ribonucleotides to deoxyribonucleotides which are necessary for DNA synthesis (Kwon *et al.* 1991; Guittet *et al.* 1998). Thus $NO^{\bullet}$ exerts its function through its interaction with the Fe of heme and non-heme Fe proteins.

Numerous investigators examined the effects of NO on ferritin synthesis and TfR mRNA or protein levels, but the results obtained are remarkably controversial. While some investigators observed that NO caused a marked decrease in ferritin synthesis (Weiss *et al.* 1993) (as would be predicted based on NO-mediated activation of IRP1 RNA-binding activity), others reported that NO increased ferritin synthesis and accumulation (Recalcati *et al.* 1998). These latter findings are in agreement with numerous studies showing that inflammation and inflammatory cytokines stimulate ferritin synthesis (Konijn & Hershko 1977). Similarly, according to some reports (Richardson *et al.* 1995; Pantopoulos & Hentze 1995b; Pantopoulos *et al.* 1996) a NO-mediated increase in IRE-binding activity of IRP1 is associated with increases in TfR mRNA levels. However, these observations are in apparent conflict with studies showing that treatment of macrophages with IFN-γ and LPS (known to cause iNOS induction followed by increased NO production) leads to a dramatic decrease in TfR levels (Hamilton *et al.* 1984; Byrd & Horwitz 1989; Weiss *et al.* 1997).

A possible explanation of these paradoxes could be in the fact that NO has markedly different biological effects depending upon its redox state (Stamler *et al.* 1992b; Stamler 1994). Recently, this laboratory reported that while $NO^{\bullet}$ increased IRP1 binding activity, TfR mRNA and TfR number, NO^{+} decreased IRP1 activity, TfR mRNA and Tf binding in K562 cells (Richardson *et al.*, 1995). It is likely that $NO^{\bullet}$, which binds iron, can ligate to the [Fe-S] centre and activate IRP1 by disrupting the cluster (Hentze & Kuhn 1996; Weiss *et al.* 1993), a condition known to stabilize TfR mRNA. On the other hand, we demonstrated that treatment of K562 cells with an NO^{+} donor prevented the binding of IRP1 to the IRE, leading to TfR mRNA degradation (Richardson *et al.* 1995); it was concluded that S-nitrosylation of critical thiol groups in IRP1 was responsible for a decrease in RNA binding activity of IRP1.

Since NO can regulate Fe metabolism via interacting with IRPs (Drapier *et al.* 1993; Weiss *et al.* 1993; Richardson *et al.* 1995; Hentze & Kuhn 1996; Richardson & Ponka 1997b), we examined IRE-binding activities of IRPs in RAW 264.7 cells exposed to NO donors as compared to those treated with IFN-γ/LPS which stimulate NO production in macrophages (Moncada, 1991). In IFN-γ/LPS-treated cells, RNA-binding activity of IRP1 increased while that of IRP2 decreased (Figure 3A, lane 4), and these changes were associated with a decrease in TfR mRNA levels (Figure 3B, lane 4). Treatment of RAW 264.7 cells with *S*-nitroso-*N*-acetylpenicillamine (SNAP, $NO^{\bullet}$ generator) enhanced the RNA-binding activity of IRP1 without affecting IRP2 (Figure 3A, lane 5). Treatment of RAW 264.7 cells with SNAP (10 h) was associated with an increase in TfR mRNA levels (Figure 3B, lane 5) and a decrease in ferritin synthesis (Kim & Ponka 2002), changes that can be explained by the increase in IRP1 binding activity. However, the changes in Fe metabolism induced by the $NO^{\bullet}$ donor, SNAP, are clearly different from those occurring in RAW 264.7 following their exposure to IFN-γ/LPS (Figure 3A, B,

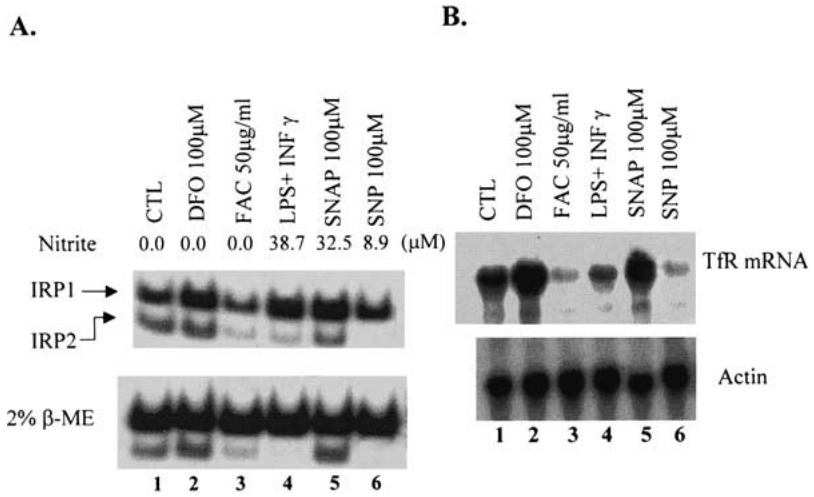

Fig. 3. Effects of NO donors on RNA-binding activities of IRPs, and TfR mRNA levels. RAW 264.7 cells were incubated (as indicated) without or with desferrioxamine (DFO, 100 μM), ferric ammonium citrate (FAC, 50 μg ml), lipopolysaccharide (5 μg ml) *plus* interferon-γ (IFN-γ, 100 U ml), SNAP (100 μM), and SNP (100 μM) for 10 h. *A*. Nitrate concentrations in media collected following 10 h incubation of RAW 264.7 cells with either cytokines or NO-donors (SNAP and SNP) were measured using Griess reagent (Kim & Ponka 1999). Gel-retardation assay was used to measure the interaction between IRPs and IREs using established techniques. Briefly, following various experimental manipulations, 4×10^6 cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in 40 μl of extraction buffer (10 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 40 mM NaCl, 5% glycerol, 1 mM DTT, 0.2% Nonidet P-40). After lysis, 10 μg aliquots were analyzed for IRP binding activities by incubating with 0.1 ng of ^{32}P labeled pSRT-fer RNA transcript (Muller *et al.* 1989; Kim & Ponka 1999). In parallel experiments, samples were treated with 2% β-mercaptoethanol before the addition of the RNA probe. *B*. RNase protection assays to measure TfR mRNA levels were performed using a kit from Pharmingen (Mississauga, ON) as described in the manufacturer's manual. ^{32}P-labeled antisense RNAs were generated using T7 polymerases. Actin mRNA was used as a control (Reprinted from Kim & Ponka 1999 with permission.)

lane 4). Importantly, a 10-h exposure of RAW 264.7 cells to the NO^+ generator, sodium nitroprusside (SNP), dramatically decreased RNA-binding activity of IRP2 (Figure 3A, lane 6) which was associated with a decrease in TfR mRNA levels (Figure 3B, lane 6) and an increase in ferritin synthesis (Kim & Ponka 2002). Hence, Fe metabolism changes in RAW 264.7 cells treated with the NO^+ donor, SNP are very similar to those seen in IFN-γ/LPS-treated RAW 264.7 cells.

In iron-replete cells IRP2 is degraded *via* the ubiquitin-proteasome degradation pathway (Iwai *et al.* 1995; Guo *et al.* 1995; Iwai *et al.* 1998). Hence, we examined whether SNP-mediated IRP2 degradation also occurs in proteasomes and to test this we exploited the protein synthesis inhibitor, cycloheximide, as well as more specific proteasome inhibitors, MG132 and lactacystin. RAW 264.7 cells were pre-treated with cycloheximide, MG132 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 (Figure 4A, B, lanes 5–7). As expected, Fe caused a slight decrease in IRP2 binding and protein level (Figure 4A, B, lane 3) that was completely blocked by proteasome inhibitors (Figure 4A, B, lanes 8–10). Both cycloheximide and MG132 prevented the loss of IRP2 binding activity as well as its degradation (Figure 4A, B, lanes 11–12). Lactacystin failed to attenuate the SNP-mediated decrease in RNA-binding activity of IRP2 but prevented the degradation of this protein. Importantly, MG132, but not lactacystin, prevented the SNP-mediated decrease in TfR mRNA levels (Figure 4C, lane 6 *vs.* lanes 9 and 10). It is of interest to mention that, after SNP treatment, the residual IRP2 protein can be seen on Western blot analysis in the absence of any detectable IRP2 RNA binding activity (Figure 4A, B, lane 4). It is possible that the ubiquitination of IRP2 prevents its RNA binding, before the protein is totally degraded. Our results suggest that in SNP-treated cells critical SH groups of IRP2 are *S*-nitrosylated 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 IRP2 is responsible for iron-mediated proteolytic degradation of this protein. Although the mechanisms involved are not fully understood, it appears that iron-dependent oxidation converts IRP2 into a substrate for ubiquitination (Iwai *et al.* 1998) and consequent targeting for proteasomal degradation. Importantly, when 3 out of 5 cysteines in the 73 amino acid 'degrada-

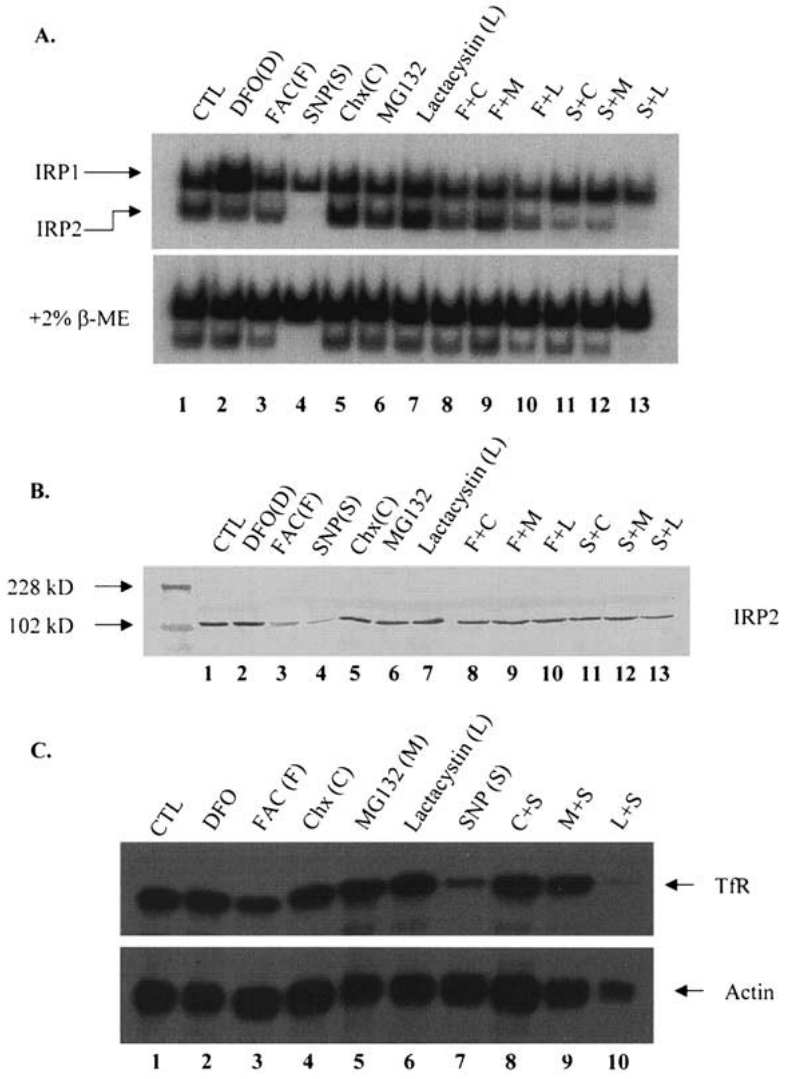

Fig. 4. Effects of proteasome inhibitors on RNA binding activities of IRPs (A), their protein levels (B) and TfR mRNA levels (C). RAW 264.7 cells were grown in control medium (lane 1, CTL), or were pre-incubated (lanes 5–13) with cycloheximide (Chx, 20 μg ml), MG132 (50 μg ml), or lactacystin (20 μM) for 30 min, following which the cells were incubated for 3 h with DFO (100 μM), FAC (50 μg ml), or SNP (S, 100 μM) as indicated in the figure. (Reprinted from Kim & Ponka 1999 with permission.)

tion domain' of IRP2 are mutated, the degradation of this protein is completely blocked (Iwai *et al.* 1995), indicating that these residues play a critical role in controlling IRP2 degradation. Based on our investigation with the NO^+ donor, SNP, we propose that *S*-nitrosylation of cysteines in the 'degradation domain' of IRP2 is a distinctive feature that predisposes this protein for ubiquitination and consequent proteasomal degradation. Stimulation of RAW 264.7 cells with IFN-γ/LPS, which induces endogenous NO production, causes a dramatic decrease in IRP2 activity/levels as well as in TfR mRNA levels (Figure 3) and an increase in ferritin synthesis (Kim & Ponka 2002), and these changes are similar to those occurring in NO^+-treated RAW 264.7 cells. We are currently investigating whether IRP2 is ubiquitinated in RAW 264.7 cells treated with either NO^+ donors or IFN-γ and LPS.

As already discussed, NO^+ caused a significant decrease in IRP2 binding to the IRE, associated with a dramatic decrease in TfR mRNA levels in RAW 264.7 cells (Figure 3). Similar changes occur in RAW 264.7 cells following their treatment with LPS/IFN-γ (Figure 3), suggesting that their ef-

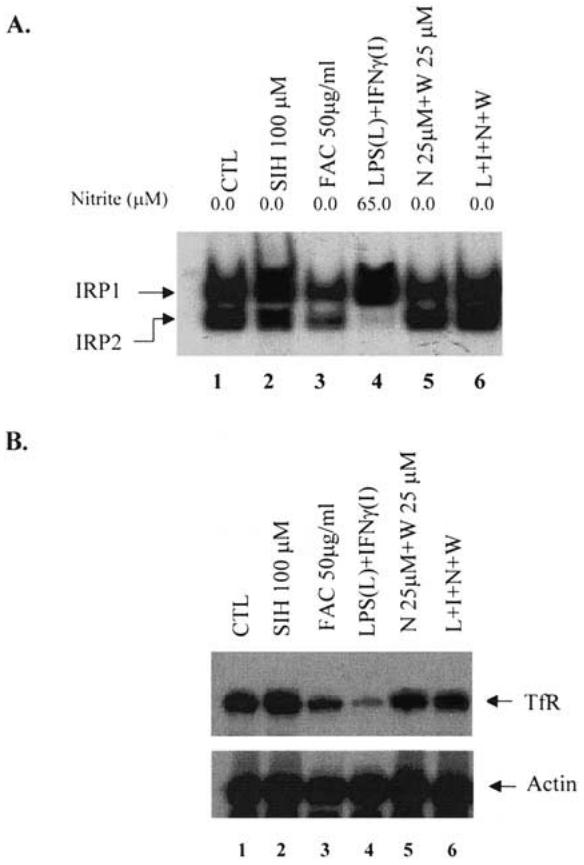

Fig. 5. Effect of iNOS inhibitors on IRP binding activities (A) and TfR mRNA levels (B) in RAW 264.7 cells. The cells were incubated (10 h) with various agents as indicated. *N*-(3-(aminomethyl)-benzyl)acetamidine (1400W, W) and L-N^6-(1-iminoethyl)-lysine (L-NIL, N) are specific inhibitors of iNOS. Protein extracts were assayed for IRE-binding activities using gel-retardation assays as described (Kim & Ponka 1999). TfR and actin mRNA levels were determined by RNase protection assay. (Reprinted from Kim & Ponka 2000 with permission.)

fects are NO-mediated. To test this, we examined the effect of specific inhibitors of inducible NOS (iNOS) on IRP2 and TfR mRNA levels in LPS/IFN-γ-treated cells. Figure 5A shows that specific iNOS inhibitors prevented the LPS/IFN-γ-induced decrease in IRP2 RNA-binding, indicating that the iNOS inhibitors prevented IRP2 degradation. This result strongly suggests that the LPS/IFN-γ-mediated decrease in IRP2 levels in RAW 264.7 cells occurs *via* an NO-related mechanism. This conclusion is in conflict with the one proposed by Bouton *et al.* (Bouton *et al.* 1998), but these investigators did not use iNOS specific inhibitors. N^G-monomethyl-L-arginine did not completely block NO production in LPS/IFN-γ-stimulated macrophages (Bouton *et al.* 1998), while iNOS-specific inhibitors, used in this study, did (Figure 5A). Moreover, we also found that the iNOS inhibitors attenuated LPS/IFN-γ-mediated decrease in TfR mRNA levels (Figure 5B), indicating that this decrease is also NO-mediated. It is well documented that cytokine-activated RAW 264.7 cells have dramatically increased levels of nitrosoglutathione (GS-NO) (Akaike *et al.*, 1997), a species that is capable of inducing S-nitrosylation of proteins in activated macrophages (Simon *et al.* 1996). It is reasonable to expect that GS-NO causes *S*-nitrosylation of IRP2 in LPS/IFN-γ-treated RAW 264.7 cells. GS-NO can be formed from $NO^{\bullet}$ by an iron-dependent reaction (Vanin *et al.* 1997) that is likely to occur in cytokine-activated macrophages. Hence, the presence of both $NO^{\bullet}$ and GS-NO (which has nitrosonium character) can explain activation of IRP1 while IRP2 is deactivated. Although our experiments strongly suggest that IRP1 is unable to maintain normal TfR mRNA levels in the absence of IRP2 (Kim & Ponka 1999), it can be argued that cytokines may cause a decrease in transcriptional activity of the TfR gene. However, INF-γ does not seem to regulate TfR gene transcription (Kim & Ponka 2000). Moreover, our experiments clearly demonstrated that inhibitors of iNOS prevented IRP2 degradation and attenuated TfR mRNA decrease in LPS/IFN-γ-stimulated RAW 264.7 cells (Figure 5). Despite the report that IRP2 has a low expression level in some tissues (Henderson *et al.* 1993; Guo *et al.* 1995; Henderson 1996), our results strongly support the idea that NO-mediated IRP2 degradation is responsible for the decrease in TfR mRNA and corroborate the previous findings from other laboratory (Schalinske *et al.* 1997). Moreover, deletion of IRP2 in mice led to a dramatic increase in the ferritin level in the brain (LaVaute *et al.* 2001). Collectively, these results support the view that IRP2 alone can play a significant role in the control of TFR mRNA levels and ferritin synthesis.

Conclusion

In conclusion, this study showed that chemically produced NO^+, which causes *S*-nitrosylation of thiol-groups of proteins, decreased RNA-binding activity of IRP2, followed by IRP2 degradation and a subsequent decrease in TfR mRNA levels and an increase in ferritin synthesis. Moreover, we have demonstrated that the decrease in TfR mRNA in IFN-γ- and LPS-treated macrophages is caused by a selective decrease

in IRP2. Furthermore, our results together with the observations of others (Recalcati *et al.* 1998) suggest that NO^+ from GS-NO may play a role in IRP2 decrease in LPS/IFN-γ-stimulated macrophages. In addition, we demonstrated that IRP2 alone plays a significant role in controlling TfR mRNA levels since IRP1, although available for RNA binding in LPS/IFN-γ-treated macrophages, was unable to prevent the decrease in TfR mRNA levels. To sum up, we have provided further evidence for a dichotomy in NO effects on cellular iron metabolism and shown that it is likely caused by the existence of two redox-related species of NO ($NO^\bullet$ and NO^+). Moreover, our experiments explained at least some of the paradoxical observations regarding iron metabolism changes in cytokine-treated macrophages.

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