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Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway

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Nitric oxide (NO) produced from L-arginine by NO synthases (NOS) is a transmitter known to be involved in diverse biological processes, including immunomodulation, neurotransmission and blood vessel dilatation. We describe a novel role of NO as a signaling molecule in post-transcriptional gene regulation. We demonstrate that induction of NOS in macrophage and non-macrophage cell lines activates RNA binding by iron regulatory factor (IRFs), the central *trans* regulator of mRNAs involved in cellular iron metabolism. NO-induced binding of IRF to iron-responsive elements (IRE) specifically represses the translation of transfected IRE-containing indicator mRNAs as well as the biosynthesis of the cellular iron storage protein ferritin. These findings define a new biological function of NO and identify a regulatory connection between the NO/NOS pathway and cellular iron metabolism.

Key words: aconitase/anemia/iron sulfur proteins/post-transcriptional regulation/RNA–protein interactions

Introduction

Nitric oxide (NO) is a labile, diffusible product of mammalian cells. It serves as a short-lived messenger molecule involved in diverse biological phenomena such as the regulation of blood pressure, modulation of platelet function, neurotransmission and cytotoxicity (Ignarro, 1991; Moncada *et al.*, 1991; Lowenstein and Snyder, 1992; Nathan, 1992; Stamler *et al.*, 1992). Specific enzymes, NO synthases (NOS), generate NO from L-arginine in many different cells. NOS exist in distinct constitutive (endothelial and neuronal) or γ-interferon (γIFN)/lipopolysaccharide (LPS)-inducible (macrophage) forms. They require heme, FAD, FMN and tetrahydrobiopterin as cofactors (Kwon *et al.*, 1989; Tayeh and Marletta, 1989; Hibbs, 1991; Mayer *et al.*, 1991; McMillan *et al.*, 1992).

Many of the biological effects of NO are chemically based on direct interactions with iron-containing proteins, such as guanylyl cyclase (heme iron), ribonucleotide reductase (non-heme iron) or aconitase (iron sulfur) (Drapier and Hibbs, 1988; Lepoivre *et al.*, 1989; Palacios *et al.*, 1989; Schmidt *et al.*, 1991; Weinberg, 1992). Moreover, the cytotoxic and tumoricidal activities of NO have been considered in view of its effects on iron metabolism (Weinberg, 1992). These properties of NO prompted us to investigate whether the regulation of cellular iron metabolism and the NO/NOS pathways were interconnected.

The regulation of iron metabolism is largely exerted post-transcriptionally by specific mRNA–protein interactions between iron regulatory factor (IRF, also referred to as IRE-BP, FRP or P90) and iron-responsive elements (IREs) contained in ferritin, transferrin receptor and erythroid 5-aminolevulinate synthase (eALAS) mRNAs (Theil, 1990; Kühn and Hentze, 1992; Klausner *et al.*, 1993; Melefors and Hentze, 1993). Binding of IRF to IREs localized in the 5′ UTR of ferritin and eALAS mRNAs represses translation (Walden *et al.*, 1989; Goossen *et al.*, 1990; Goossen and Hentze, 1992; Melefors *et al.*, 1993), whereas similar interactions with IREs in the 3′UTR stabilize transferrin receptor mRNA against degradation (Casey *et al.*, 1988; Müllner and Kühn, 1988; Casey *et al.*, 1989; Müllner *et al.*, 1989). Iron regulation is achieved by post-translational modulation of the IRE-binding activity of IRF. Mounting evidence indicates that IRE binding by IRF is regulated in response to the status of an iron sulfur cluster located near the center of the protein (Hentze and Argos, 1991; Rouault *et al.*, 1991; Constable *et al.*, 1992; Haile *et al.*, 1992a,b; Emery-Goodman *et al.*, 1993; N.K.Gray, S.Quick, B.Goossen, A.Constable, H.Hirling, L.C.Kühn and M.W.Hentze, submitted). In iron-replete cells, IRF contains a cubane 4Fe-4S cluster which prevents IRE binding. In this state, IRF displays aconitase activity. In iron-starved cells, such an Fe-S cluster does not exist and IRF functions as an IRE-binding protein.

In this report, we identify a regulatory pathway which involves NO as an effector molecule that controls the IRE-binding activity of IRF *in vivo*. Induction of NO synthesis in J774 macrophage and K562 erythroleukemia cell lines is shown to augment the IRE-binding activity of IRF. We demonstrate that this increase in IRE-binding activity results in translational repression of IRE-containing indicator mRNA as well as endogenous ferritin mRNA. The possible mechanism of NO regulation of IRF, as well as potential biological and pathophysiological implications, are discussed.

Results

Nitric oxide synthesis stimulates IRE binding of IRF in activated J774 cells

To explore the possibility of a regulatory connection between the NO/NOS pathway and cellular iron metabolism, we first investigated the mouse macrophage cell line J774, where induction of NOS by γIFN/LPS is well documented (Stuehr and Marletta, 1987). Figure 1 shows a gel retardation assay assessing IRE binding by IRF in extracts prepared from

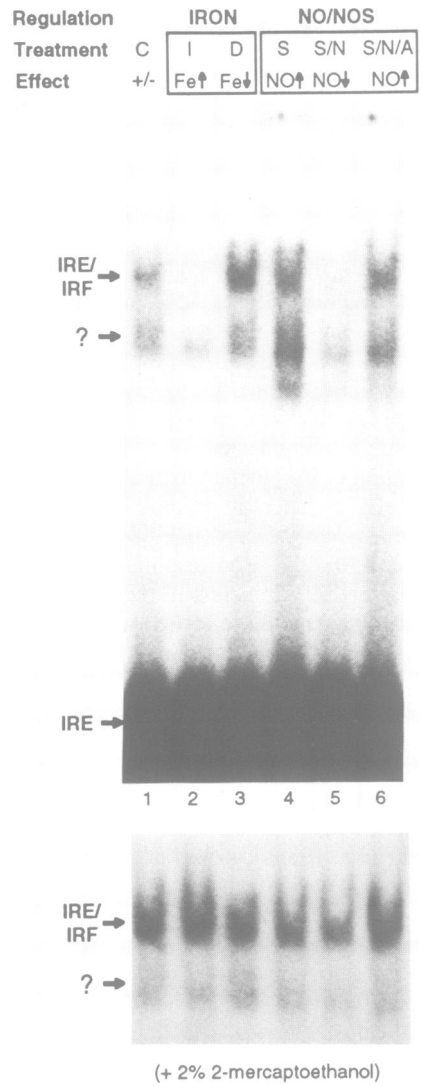

Fig. 1. Induction of nitric oxide synthase (NOS) in J774 cells regulates IRE binding by iron regulatory factor (IRF). J774 cells were treated for 18 h with 50 μM $Fe(NO_3)_3$ (I), 100 μM desferrioxamine (D), 50 U/ml γIFN and 10 μg/ml LPS (S), 250 μM N^G-methyl-L-arginine (N) and 10 mM L-arginine (A) as indicated or remained as untreated controls (C). Equal aliquots (20 μg) of detergent extracts were analyzed for IRE-binding activity in the presence (**lower panel**) and absence (**upper panel**) of 2% 2-mercaptoethanol by gel retardation assay with excess ^{32}P-labeled IRE probe as described previously (Leibold and Munro, 1988; Rouault *et al.*, 1988; Hentze *et al.*, 1989). The positions of the IRE/IRF complex and of excess free IRE probe are indicated by arrows. The IRE-binding partner in the complex marked '?' has not yet been unambiguously identified, the occurrence of this complex is characteristic for extracts from rodent cells (Leibold and Munro, 1988; Rouault *et al.*, 1988; Leibold *et al.*, 1990; Dandekar *et al.*, 1991). Only RNA–protein complexes are depicted in the lower panel. A representative example of >7 independent experiments is shown.

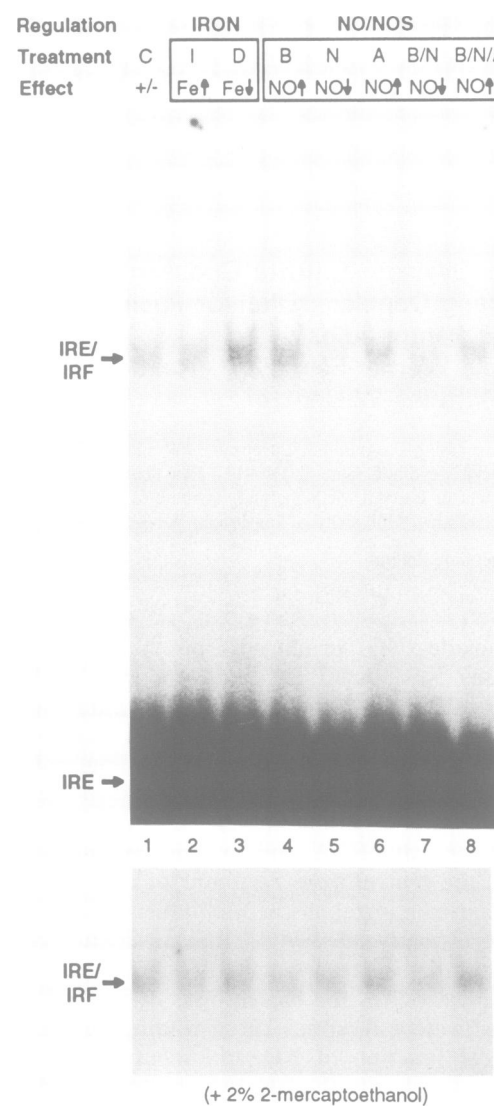

Fig. 2. Nitric oxide synthase activity in K562 cells affects IRE binding of iron regulatory factor (IRF). K562 cells were treated for 18 h with 50 μM $Fe(NO_3)_3$ (I), 100 μM desferrioxamine (D), 50 μM tetrahydrobiopterin (B), 250 μM N^G-methyl-L-arginine (N) and 10 mM L-arginine (A) as indicated, or remained as untreated controls (C). Equal aliquots (20 μg) of detergent extracts were analyzed for IRE-binding activity in the presence (**lower panel**) and absence (**upper panel**) of 2% 2-mercaptoethanol by gel retardation assay with excess ^{32}P-labeled IRE probe as described previously (Leibold and Munro, 1988; Rouault *et al.*, 1988; Hentze *et al.*, 1989). The positions of the IRE/IRF complex and of excess free IRE probe are indicated by arrows. Only RNA–protein complexes are depicted in the lower panel. A representative example of >12 independent experiments is shown.

untreated cells (C, lane 1), iron-perturbed cells (lanes 2 and 3) and cells in which NOS was induced by γIFN/LPS treatment (S, lanes 4–6). Two RNA–protein complexes were resolved in extracts from untreated cells. The upper complex represents murine IRF bound to the radiolabeled IRE probe (Dandekar *et al.*, 1991), the composition of the faster migrating complex (labeled '?' in the figure) and its relationship to an IRE/IRF interaction have not been completely characterized (Leibold *et al.*, 1990; Dandekar *et al.*, 1991). As predictable from previous work (Leibold and Munro, 1988; Rouault *et al.*, 1988; Hentze *et al.*, 1989), cells treated with $Fe(NO_3)_3$ (lane 2) displayed reduced IRE-binding activity, whereas treatment with the iron chelator desferrioxamine strongly induced IRE binding (lane 3). These responses to iron perturbation were used as a reference for the evaluation of NO/NOS-mediated effects (lanes 4–6). Induction of NO synthesis by γIFN/LPS induced IRE binding to levels equal to or exceeding those of desferrioxamine treatment (compare lanes 4 and 3). Pharmacological blockage of NO synthesis using N^G-methyl-L-arginine (N), a substrate analog of L-arginine (Olken *et al.*, 1991),

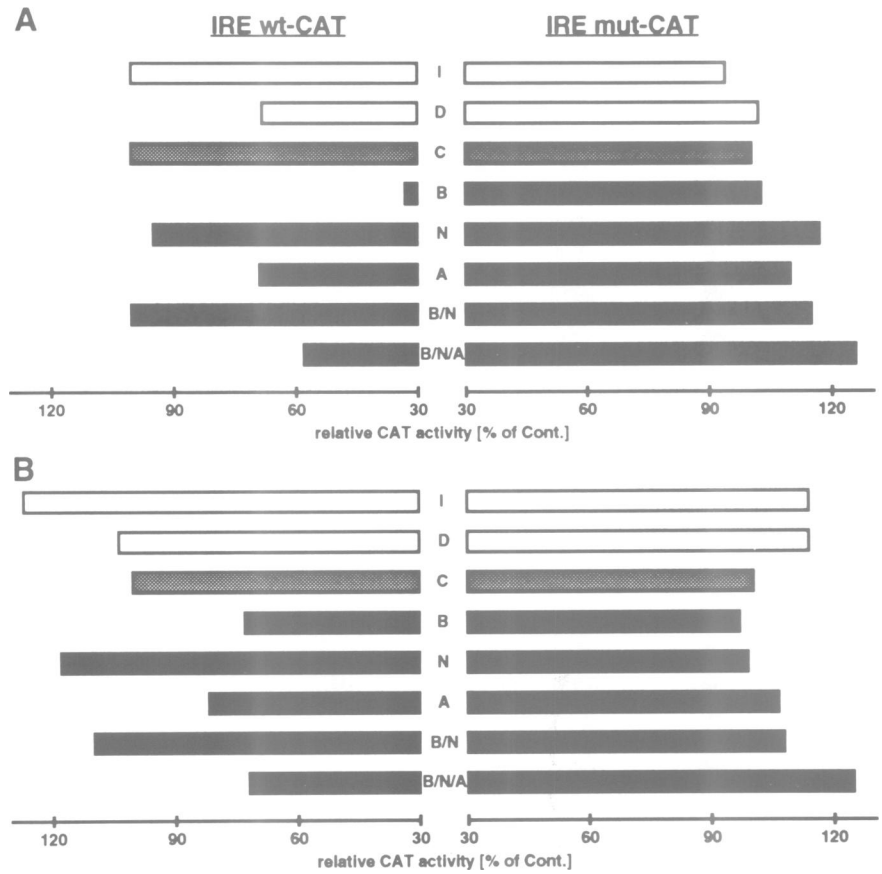

Fig. 3. Regulation of CAT indicator mRNAs by NO/NOS via the IRE. K562 cells were transiently transfected with 5 μg of plasmids encoding CAT mRNAs which bear a functional IRE (left side) or a point-mutated non-functional IRE (right side) in their 5′ UTRs, and which were previously characterized for iron regulation (Hentze *et al.*, 1988). Twenty-four hours after transfection, cells were treated for 18 h exactly as described in the legend to Figure 2 prior to cell lysis and CAT assay as described previously (Hentze *et al.*, 1988). CAT activities/μg cellular protein from iron-perturbed (white bars) or NO/NOS-perturbed (black bars) cells are expressed relative to the values of untreated controls (Cont, stippled bars). **Panels A** and **B** show representative examples of experiments (n = 3 in panel A, n = 4 in panel B) that were grouped according to their responses to iron chelation (D) or iron administration (I), as explained in the text.

repressed IRE binding to levels as low as those of iron-treated cells (compare lanes 5 and 2). The blockage of NOS by N^G-methyl-L-arginine could be overcome by incubation with excess L-arginine, resulting in restored NO synthesis and high IRE-binding activity (lane 6). Thus, pharmacological perturbation of the NO/NOS pathway elicits regulation of IRF resembling that of iron regulation. Similar to iron regulation (Hentze *et al.*, 1989), the NO/NOS-mediated responses of IRF appear to occur post-translationally because IRE-binding activity can be recovered *in vitro* from all extracts by treatment with 2% 2-mercaptoethanol (Figure 1, lower panel).

NO regulation of IRF in K562 erythroleukemia cells

Since iron regulation via IRF is a ubiquitous cellular function, we tested whether NO/NOS regulation was restricted to macrophages or could also be observed in cells expressing a constitutive form of NOS. K562 human erythroleukemia cells were chosen because iron metabolism in these cells is well characterized (Klausner *et al.*, 1983; Mattia *et al.*, 1986) and NOS activity has been demonstrated (Werner-Felmayer *et al.*, unpublished data).

In K562 cells, the NOS cofactor 5,6,7,8-tetrahydrobiopterin (B) was used to stimulate NO synthesis (Werner-Felmayer *et al.*, 1993a,b, 1990). Following an experimental rationale identical to that described above, we noticed that iron regulation of IRF was less pronounced in K562 than in J774 cells (Figure 2, lanes 2 and 3). Similar to the inducible NOS in macrophages, augmentation of NOS activity increased IRE binding, as was seen in extracts from iron-deprived cells (compare lanes 4, 6 and 8 with lane 3), whereas the NOS inhibitor N^G-methyl-L-arginine (N) elicited a reduction in IRE binding reminiscent of that seen in iron-treated cells (compare lanes 5 and 7 with lane 2). Incubation of cellular extracts with 2-mercaptoethanol restored IRE binding to similar levels (Figure 2, bottom panel), confirming the results obtained with J774 cells. Moreover, when K562 or J774 cells were treated for only 4–6 instead of 18 h (as was done in the experiments shown in Figures 1 and 2), qualitatively similar results were obtained (data not shown). Taken together, all experimental data support the notion that NO regulates the IRE-binding activity of IRF by a reversible, post-translational mechanism.

CAT indicator mRNAs are regulated by NO via an intact IRE in transfected K562 cells

Having demonstrated regulation of the IRE-binding activity of IRF by the NO/NOS pathway, we next assessed whether this regulation changed the translation of IRE-containing mRNAs *in vivo*. While attempts to transfect J774 cells were unsuccessful, K562 cells could be transfected with chloramphenicol acetyltransferase (CAT) indicator plasmids

bearing a wild-type (wt) or a point-mutated (mut) IRE within the 5′ UTR of the encoded CAT mRNA (Hentze *et al.*, 1988). The results obtained with these constructs from multiple transfection experiments could be classified into two groups, based on the responses of IRE wt-CAT to iron perturbations. A representative experiment of each group is shown in Figure 3A and B (left panels, white bars). The first group displayed reduced CAT activity compared with untreated control cells (C, stippled bar) after treatment with the iron chelator desferrioxamine, but showed little stimulation of CAT expression by iron (Figure 3A). The second group was characterized by elevated CAT expression after iron treatment, but exhibited little response to iron chelation (Figure 3B). This phenomenon has consistently been observed before (Caughman *et al.*, 1988; Dandekar *et al.*, 1991; Goossen and Hentze, 1992) and has been attributed to differences in the iron content of culture media, as well as to relative differences in cellular iron status related to the growth rate and density of cells. Comparison of iron regulation of CAT activity with the NO/NOS-mediated responses (black bars) revealed that conditions which stimulate NO production and cause increased IRE binding (Figure 2) repress CAT expression (B, A, B/N/A in Figure 3A and B), in most cases even exceeding the effect of iron starvation. Inhibition of NOS, correlated with low IRE binding (Figure 2), permitted CAT expression resembling that of iron-treated cells (Figure 3A and B, compare N and B/N with I). No such regulation of CAT activity was found in cells transfected with IRE mut-CAT (Figure 3, right panels), confirming that regulation required the presence of an intact IRE. We conclude from these experiments that NO/NOS-induced changes in IRE binding control the translation of IRE wt-CAT mRNA via the IRE. However, as previously discussed in the case of iron perturbations (Caughman *et al.*, 1988; Dandekar *et al.*, 1991; Goossen and Hentze, 1992), the CAT activity determinations underestimate the range of translational regulation and only allow semiquantitative conclusions to be drawn, because the enzymatic activity measured is affected by CAT accumulation before the regulatory stimulus and protein stability in addition to the translation rate of the CAT mRNA.

Translational regulation of ferritin expression by the NO/NOS pathway

To obtain direct evidence for NO/NOS-mediated regulation of an endogenous, IRE-controlled mRNA, we finally investigated the regulation of ferritin expression by NO. J774 cells were treated under conditions similar to those employed for the gel retardation experiment in Figure 1, followed by pulse labeling with [^{35}S]methionine and immunoprecipitation of ferritin polypeptides. Since this technique monitors the translation rate of an mRNA more directly than an enzymatic (CAT) assay, the data obtained by this approach are also quantitatively reliable. Iron regulation of ferritin biosynthesis displayed a pattern similar to that seen in Figure 3B, i.e. increased expression after iron treatment and little effect of iron chelation (Figure 4, lanes 1–3). Perturbation of the NO/NOS pathway caused drastic effects on ferritin biosynthesis (Figure 4, lanes 4–6). The range of NO/NOS-mediated ferritin regulation between lanes 4 (or 6) and 5 was estimated by phosphoimaging of the SDS–polyacrylamide gel to be ~9-fold. Both iron regulation

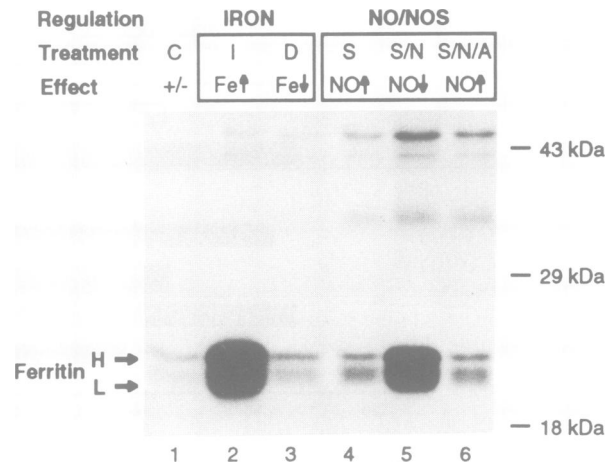

Fig. 4. Regulation of ferritin biosynthesis by NO/NOS. J774 cells were treated for 4 h, exactly as described in the legend to Figure 1, prior to metabolic pulse-labeling with [^{35}S]methionine for 2 h, cell lysis, immunoprecipitation of equal quantities of trichloroacetic acid-insoluble radioactivity with polyclonal ferritin antibodies and SDS–PAGE as described previously (Melefors *et al.*, 1993). The positions of specifically precipitated ferritin H- and L-chain polypeptides and molecular size standards are indicated.

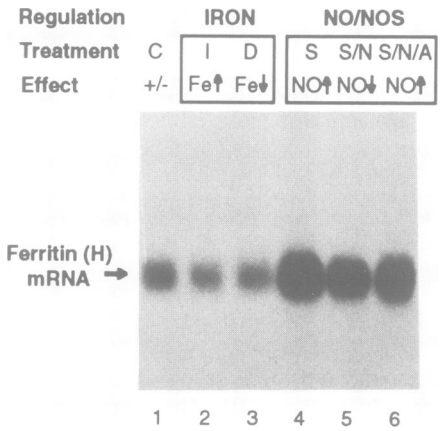

Fig. 5. Analysis of ferritin mRNA levels by Northern blotting. J774 cells were treated in parallel with the cells described in Figure 4. Total RNA was extracted and subjected to Northern blotting (10 μg) with a human ferritin H-chain cDNA probe (Melefors *et al.*, 1993). The position of murine ferritin H-chain mRNA is indicated. Small differences between lane 4 and lanes 5/6 are also apparent for ribosomal RNAs on the ethidium bromide-stained gel and represent technical variation.

(Figure 5, lanes 1–3) and NO/NOS regulation (Figure 5, lanes 4–6) of ferritin biosynthesis were confirmed by Northern blotting to occur without corresponding changes in ferritin mRNA levels. In addition, treatment of J774 cells with γIFN/LPS increased ferritin H-chain mRNA levels when compared with non-treated cells (compare lanes 4–6 with lanes 1–3). Note that the increase in ferritin mRNA levels only depends on the treatment of J774 cells by γIFN/LPS, and is independent of whether NO synthesis occurs (lanes 4 and 6) or is blocked (lane 5). Interestingly, the increase in ferritin mRNA levels is not fully reflected by a similar rise of ferritin biosynthesis (compare Figures 5 and 6). It is possible that the NO-induced augmentation of the IRE-binding activity of IRF (Figure 1, compare lanes 4 and 6 with lane 1) represses ferritin mRNA translation more

efficiently. We conclude from these data that NO controls ferritin biosynthesis by changing the rate of translation of its mRNA.

Discussion

We have established that NO synthesized by macrophage and non-macrophage NOS regulates IRF, the central post-transcriptional regulatory molecule of mRNAs involved in iron metabolism. This response, which is probably mediated by a post-translational mechanism (see below), controls the translation of IRE-CAT indicator mRNAs and endogenous ferritin mRNA. We have not yet studied NO/NOS regulation of other IRE-containing mRNAs, such as the transferrin receptor or the erythroid 5-aminolevulinate synthase transcripts.

Mechanism of the NO-induced increase in the IRE-binding activity of IRF

Since NO displays chemical reactivity towards iron, one can envision two alternative scenarios for how NO regulates IRF: it may either directly interact with IRF (Figure 6A) or it may alter the availability of iron from the so-called 'regulatory iron pool' (Figure 6B), which is thought to determine the activity of IRF in response to cellular iron loading or starvation (Rouault *et al.*, 1985; Theil, 1990; Kühn and Hentze, 1992; Klausner *et al.*, 1993). While the possibility of NO-mediated regulation of IRF via the regulatory iron pool must be considered, recent advances in understanding of structure/function relationships of IRF allow a plausible mechanistic model for the direct control of IRF by NO to be proposed (Figure 6A). IRF has been found to be highly similar to the mitochondrial iron sulfur protein aconitase (Hentze and Argos, 1991; Rouault *et al.*, 1991) and was demonstrated to function as an iron-dependent cytoplasmic aconitase and IRE-binding protein (Constable *et al.*, 1992; Haile *et al.*, 1992a,b; Kennedy *et al.*, 1992; Emery-Goodman *et al.*, 1993; Gray *et al.*, submitted). An intact 4Fe-4S cluster was required for aconitase activity, whereas its (partial) inactivation/disassembly was suggested to be required for IRE binding. We propose that NO reacts with the 4Fe-4S cluster of IRF (a cytoplasmic aconitase) in a fashion akin to its well-studied direct interaction with the 4Fe-4S cluster of mitochondrial aconitase, where iron–nitrosyl complexes are formed (Drapier and Hibbs, 1986; Hibbs *et al.*, 1988; Lancaster and Hibbs, 1990; Pellat *et al.*, 1990). Formation of an iron–nitrosyl complex could trigger an allosteric change of the protein allowing for IRE binding (Figure 6A, upward arrow), similar to the activation of soluble guanylyl cyclase by NO (Stamler *et al.*, 1992). Alternatively, the iron–nitrosyl complex could cause the release or be recognized as a substrate for subsequent (enzymatic) disassembly of the iron sulfur cluster (Figure 6A, downward arrows).

Possible pathophysiological implications of the NO-mediated regulation of IRF

Our findings may also warrant consideration in view of the pathophysiology of the commonly observed anemias of chronic disease. This group of anemias is characterized by increased serum levels of γIFN and pteridine derivatives

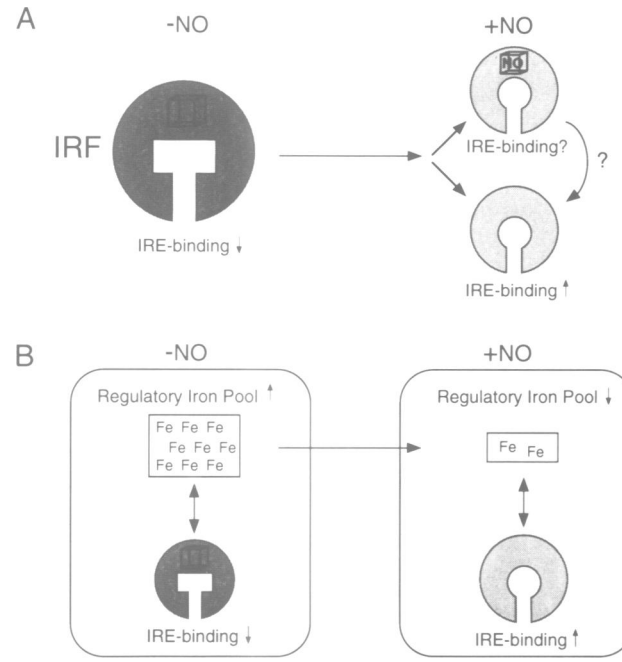

Fig. 6. Models for the mechanism of the NO-induced increase in the IRE-binding activity of IRF. IRF is depicted as a circular object which exists in two different conformations. Dark gray symbolizes the form with low IRE-binding activity, whereas the light gray object represents the IRE-binding form of IRF. In **panel A**, NO is suggested to exert a direct effect on the cubane 4Fe-4S cluster of IRF. In **panel B**, the primary effect of NO is considered to be a reduction in the size of the regulatory iron pool, leading to removal of the 4Fe-4S cluster and active IRE binding. For a further discussion, see the text.

such as neopterin and tetrahydrobiopterin, as well as by multiple abnormalities in iron metabolism (Fillet *et al.*, 1989; Cazzola *et al.*, 1990; Fuchs *et al.*, 1991). A blockage of iron utilization within the maturing red blood cell has been suggested to play a crucial role in the pathogenesis of this disease (Weinberg, 1984; Means and Krantz, 1992). Interestingly, the expression of eALAS, a rate-limiting enzyme in erythroid heme synthesis, is translationally controlled by IRF (Melefors *et al.*, 1993). Could an NO-mediated increase in erythroid IRF activity leading to reduced eALAS expression constitute the molecular basis of the 'iron utilization block' in (some of) the anemias of chronic disease?

A novel biological role of NO in the regulation of gene expression and cellular iron metabolism

Irrespective of such a possibility, our findings reveal a novel aspect in the biology of the NO/NOS system: direct control of gene expression at the post-transcriptional level. In addition, they demonstrate a regulatory connection between the control of cellular iron metabolism and NO synthesis, which itself acts by forming iron–nitrosyl adducts with some of its target enzymes. In contrast to the NO/NOS system, for which the currently reported functions are all cell type specific, IRE/IRF regulation serves as a 'housekeeping' regulatory circuit of most mammalian cells. Whether modulation of IRF activity represents a novel 'housekeeping' function of the NO/NOS system which might account for its widespread occurrence in many different cell types, will require further studies.

Materials and methods

Generation of ^{32}P-labeled RNA probes

^{32}P-labeled RNA probes (sp. act. ~3.2 × 10^6 c.p.m./μg) were generated by the method of Milligan *et al.* (1987) using cloned T7 RNA polymerase; the sequence of the DNA template for the transcription of the IRE probe was 5′ GGGATCCGTC CAAGCACTGT TGAAGCAGGA TCCCTAT-AGT GAGTCGTATT A 3′. Synthetic DNA oligonucleotides and the labeled *in vitro* transcription products were purified by gel electrophoresis [15% polyacrylamide:bis-acrylamide (20:1), 8 M urea] and eluted according to standard procedures (Sambrook *et al.*, 1989). The RNA was finally resuspended in H_2O.

Gel retardation assays

J774.A1 cells (1 × 10^7) grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 ng/ml streptomycin were treated for 18 h with 50 μM $Fe(NO_3)_3$, 100 μM desferrioxamine, 50 U/ml murine γ-interferon plus 10 μg/ml LPS from *Escherichia coli*, 250 μM N^G-monomethyl-L-arginine or 10 mM L-arginine. K562 cells (1 × 10^7) maintained in RPMI 1640 plus additives were treated for 18 h with 50 μM 5,6,7,8-tetrahydrobiopterin (Schircks Lab., Jena, Switzerland) or as indicated above. Detergent extracts were prepared from J774 and K562 cells as previously described (Hentze *et al.*, 1989). Equal aliquots (20 μg) of cellular extract were incubated with 12 000 c.p.m. of IRE probe at 22°C; where indicated, 2% (v/v) 2-mercaptoethanol was added to the extract 2 min prior to the addition of probe. After 30 min, 3 mg/ml heparin were added for an additional 10 min. Analysis of RNA–protein complexes by non-denaturing gel electrophoresis and autoradiography was performed as previously described (Leibold and Munro, 1988).

Cell culture and transfections

K562 cells (1 × 10^6) were transfected by lipofection (Boehringer Mannheim, Germany) with 5 μg of plasmids L5(+26mer)-CAT or Δ-168 (Hentze *et al.*, 1988) as described previously (Stamatatos *et al.*, 1988). After transfection, cells were treated for 18 h as indicated above, lysed, and assayed for CAT activity (Hentze *et al.*, 1987, 1988).

Metabolic labeling and immunoprecipitation of ^{35}S-labeled proteins

J774.A1 cells (1 × 10^7) were grown for 4 h in culture medium supplemented as described above, washed twice with methionine-free medium and labeled with [^{35}S]methionine (50 μCi/ml) for an additional 2 h at 37°C. Quantitative immunoprecipitation from equal amounts of trichloroacetic acid-insoluble radioactivity with polyclonal ferritin antibodies (Boehringer Mannheim, Indianapolis, USA), analysis by SDS–PAGE and autoradiography were performed as described previously (Caughman *et al.*, 1988; Melefors *et al.*, 1993).

Northern blotting

Total cellular RNA was prepared from J774 cells cultured in parallel with the cells used for metabolic labeling. Cells were solubilized in guanidinium isothiocyanate and RNA was recovered after ultracentrifugation through a CsCl cushion. Equal aliquots (10 μg) of RNA were subjected to electrophoresis through 0.7% agarose gels in the presence of formaldehyde and to electrotransfer onto nylon membranes. Equal loading of the different lanes was assessed by inspection of ribosomal RNAs of the ethidium bromide-stained gels. The nylon membranes were analyzed by hybridization with a ^{32}P-labeled human ferritin H-chain cDNA probe.

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