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Nitrosative and oxidative modulation of iron regulatory proteins

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Abstract. Cytokine-driven nitric oxide (NO) synthase II provides cells with effectors for reactions at redox-sensitive site(s) of proteins. Iron regulatory proteins (IRP1 and IRP2), two post-transcriptional regulators of gene expression, are particularly sensitive to NO synthesis and to oxidative stress. IRP1 possesses a redox-active Fe-S cluster and can also exhibit aconitase activity. IRP2 has no Fe-S cluster but exhibits several redox-sensitive cysteine residues. Under proper redox conditions, both IRPs bind to iron-responsive elements in the untranslated region of mRNAs encoding proteins involved in iron metabolism and energy production. This review describes and compares the effects of NO, peroxynitrite, and reactive oxygen species on these two chemosensitive proteins.

Key words. Nitric oxide; redox modulation; iron metabolism; gene regulation.

Introduction

A redox-sensitive biochemical pathway involving nitric oxide (NO) and/or reactive oxygen species as cellular signaling molecules has been identified in various systems [1; see also the article by Broillet in this issue]. This original pathway is mainly based on the reactivity of NO and its derivatives with metal- or thiol-containing enzymes. In addition to enzymes, some transcription factors can be modulated by NO and oxidative stress. For example, OxyR, which is involved in H_2O_2 detoxification in *Escherichia coli* and *Salmonella typhimurium*, can be activated by S-nitrosylation of a cysteine residue [2]. This is also the case with SoxR which induces transcription of *soxS* in *E. coli* upon activation by NO. The *soxS* gene product is also a transcription factor that activates many defense genes against oxidative stress [3]. The presence of two 2Fe-2S clusters in SoxR permits its activation by NO. In higher eukaryotes, the best example of gene regulation by redox signaling is provided by iron regulatory proteins (IRPs), which regulate iron metabolism at a post-transcriptional level. Homeostasis of intracellular iron in mammalian cells is mostly maintained by a coordinated regulation of the expression of both transferrin receptor (Tf-R) and ferritin (Ft). Indeed, cells acquire iron from plasma transferrin after its binding to Tf-R and internalization in endosomal vesicles. After transit through an ill-defined intracellular labile pool, iron is used either for heme or non-heme iron protein or stored in Ft. By controlling the level of expression of Tf-R and Ft, cells can determine both iron uptake and sequestration. Even though Ft, for example, is also regulated at the transcriptional level [4, 5], the major players involved in intracellular iron control are the two IRPs. In this mini-review, I will therefore focus on the post-transcritional regulation of iron metabolism by the IRP/iron-responsive element (IRE) system which constituted the first example of direct involvement of NO in the regulation of gene expression.

IRP1: a bifunctional protein

IRP1, previously referred to as FRP, P90, IRF or IRE-BP, is a cytosolic protein of 98 kDa. It was the first protein in mammalian cells identified as being able to maintain homeostasis of intracellular iron through a post-transcriptional mechanism. IRP1 recognizes specific sequences called IREs on mRNA [6]. IREs are stem-loops made of 30 nucleotides with a bulge (fig. 1). The loop has a conserved sequence 5′-CAGUGN 3′, N

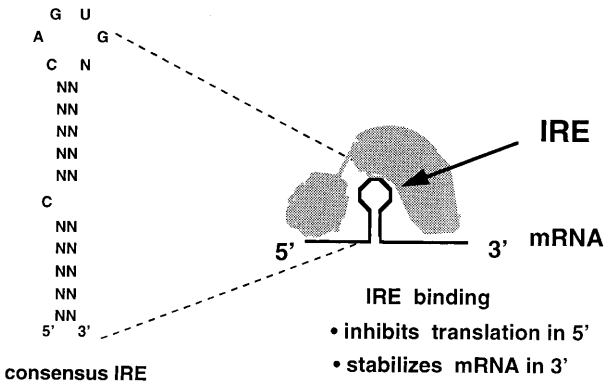

Figure 1. Consensus sequence of IRE located in the 5′- or 3′-untranslated regions of several mRNAs. The IRE is composed of approximately 30 nucleotides as a hairpin structure. The consensus IRE has a conserved terminal loop 5′-CAGUGN-3′ where N is an invariable pyrimidine. It also shares base-paired stems interrupted by a C-bulge. The high stem usually comprises five base pairs, where NN can be any complement base pair. The 'lower' stem varies in size.

usually being a pyrimidine. The first functional IRE was localized in the 5′-untranslated region of mRNA of human Ft H- and L-chains [7, 8]. Other IRE sequences have since been discovered at the 5′ end of the mRNAs of various proteins: δ-aminolevulinate synthase of erythroid cells (δALAS), a limiting enzyme of heme synthesis [9], as well as mitochondrial aconitase [10–12], and subunit b of *Drosophila melanogaster* succinate dehydrogenase [11, 13, 14] which both play a part in energy metabolism. IRP1/IRE interaction with the 5′ end inhibits translation (fig. 2). The molecular mechanism of IRP1-mediated inhibition of translation of Ft and δALAS mRNA has been partially elucidated. When close to the cap structure, the IRP1/IRE complex prevents the recruitment of the small ribosomal 43S pre-initiation complex [15], probably by steric hindrance [16]. A second type of post-transcriptional regulation of iron metabolism mediated by IRPs is illustrated by the regulation of the Tf-R (fig. 2). Tf-R receptor mRNA contains five IREs, all located at its 3′ end [17]. The interaction between IRPs and IREs increases the stability of the mRNA. Indeed, the presence of the IRP/IRE complex in the regulating region seems to mask an endonuclease cleavage site [18]. Finally, an iron transporter named 'divalent-cation transporter' (DCT1) has recently been cloned and characterized [19]. Its expression is ubiquitous but it is preferentially expressed in the duodenum. In most tissues, iron deficiency greatly increases the expression of DCT1 mRNA. In its 3′-untranslated region, DTC1 mRNA contains a sequence similar to those of the IREs present on Ft and Tf-R mRNAs. Thus, DCT1 could also be controlled by the IRE/IRP interaction.

Much information about its structure and biological function has emerged fom the purification of IRP1 and from the molecular cloning of the cDNA encoding

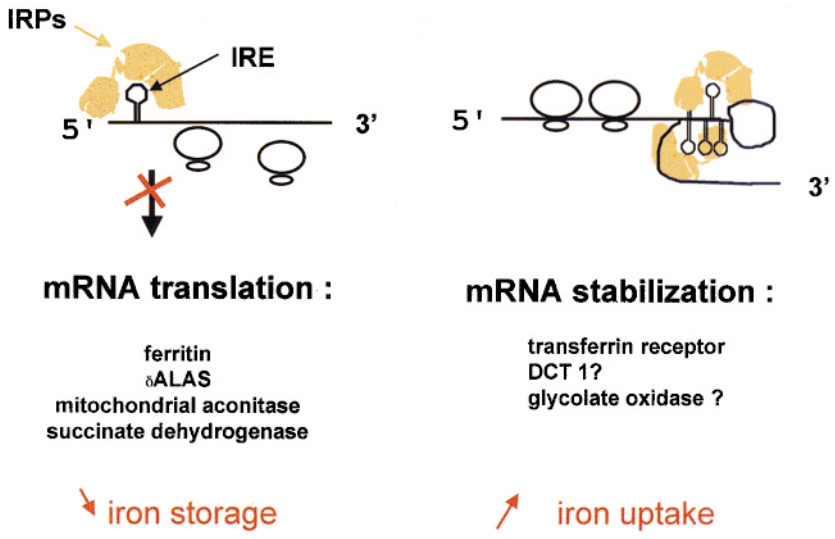

Figure 2. IRP-mediated post-transcriptional regulation of iron and energy metabolism. IRPs inhibit translation by binding IRE(s) located in the 5′-untranslated region of mRNAs. This process prevents attachment of the small 43S ribosomal subunit to the cap. In contrast, IRE/IRPs interactions at the 3′ end of mRNAs stabilize them by preventing endonucleotide cleavage.

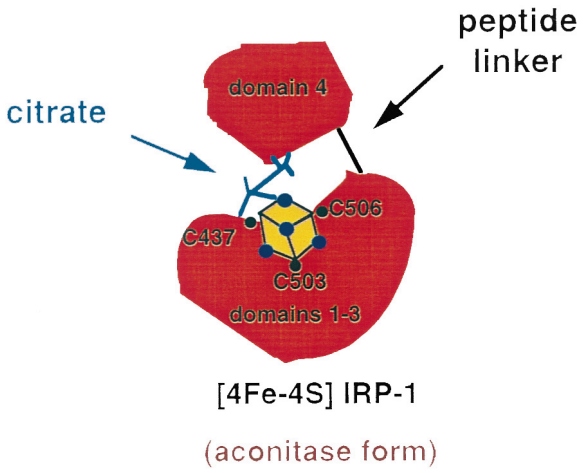

Figure 3. Schematic representation of 4Fe-4S IRP1, based on the crystal structure of mt-aconitase. IRP1 comprises three domains with N-terminal domains (1–3) connected to the fourth domain by a peptide linker. A 4Fe-4S cluster is inserted into the active site and is linked by three cysteine residues (Cys437, 503 and 506). The fourth labile iron, termed Fe_a, interacts with citrate and enables its rotation and then its conversion to isocitrate.

human IRP1. Computerized comparison of amino acid sequences revealed striking homology between human IRP1 and pig heart or *Saccharomyces cerevisiae* mitochondrial (mt)-aconitase [20, 21]. IRP1 and mt-aconitase exhibit approximately 31% identity and about 56% similarity. X-ray crystallography of mammalian mt-aconitase has provided insight into the biochemistry and structure of Fe-S enzymes and has allowed modeling of two forms of IRP1 [22] (fig. 3). Like mt-aconitase, IRP1 comprises four domains, three at the N-terminal end which form a compact core (domains 1–3), to which the fourth (domain 4) is connected by a peptide linker. The flexibility of this hinge allows the fields 1–3 and 4 to come together around a narrow cleft corresponding to the active site of the enzyme. A 4Fe-4S center is located in the cleft and is linked to domain 3 by cysteines 437, 503, and 506 (fig. 3). The fourth iron (Fe_a) is not bound to any residue and can therefore interact directly with solvent or substrates e.g., citrate. Citrate itself binds to at least four arginines (R536, R541, R699, R780) belonging to the four domains. Thus its presence at the catalytic site contributes to the stabilization of the protein structure and to the connection of domains 1–3 to domain 4 [23]. IRP1 in this closed conformation (holo-IRP1) exhibits aconitase activity in the cytosol which converts citrate into isocitrate, with cis-aconitate as an intermediate.

An aconitase in cytosol: what for?

Despite the high degree of conservation between IRP1 and bacterial aconitases especially in the active site, the role of aconitase activity in the conversion of citrate into isocitrate in the cytosol has remained enigmatic. However, some assumptions can be made on the basis of the presence of a cytosolic NAD-dependent isocitrate dehydrogenase catalyzing the conversion of isocitrate into oxalosuccinate with simultaneous production of NADH. The conversion of citrate into isocitrate catalyzed by the holo-IRP1 may indirectly maintain the rate of cytosolic NADH by providing the substrate for cytosolic isocitrate dehydrogenase. In turn, NADH may take part in the internalization of iron in the cells via reductases anchored in the plasma membrane [24]. There is also a transmembrane oxidoreductase in endocytosis vesicules [25] where NADH may act as a cofactor in reduction of Fe^{3+} into Fe^{2+}, an essential step for the entry of iron into the cytoplasmic compartment. In addition, it is worth recalling that citrate is a low-molecular-weight iron chelator. One may therefore speculate that citrate accumulation resulting from cytosolic aconitase/IRP1 inhibition could contribute to enhancement of iron transport within the cytosol.

The mutual exclusion of the two IRP1 activities

The form of IRP1 able to bind IRE sequences with high affinity is thought to be entirely devoid of its Fe-S center [26–28]. Indeed, several studies suggest that the Fe-S center prevents the access of the IRE sequence to the residues involved in the RNA-binding domain. Ultraviolet cross-linking studies, chymotryptic digestion, as well as site-directed mutagenesis have been used to identify several amino acids of the IRE-binding domain. Peptide 121–130 of human IRP1 interacts with IREs and includes two active site amino acids: aspartate 125 and histidine 126 [29]. Residues 480–623 also participate at the RNA binding site [30]. Importantly, this sequence, at least in rabbit IRP1, includes cysteines 503 and 506, two of the three residues which hold the Fe-S center [31]. Finally, three arginines (Arg541, Arg780, and Arg536) localized at the active site of IRP1 also seem to participate in the binding to mRNA [32]. Overall, these data suggest a partial overlap between the active site and the RNA-binding domain, thus providing a clue to the mutual exclusion of the two IRP1 activities. The crystal structure of mt-aconitase provides an outline of the mechanism by which the Fe-S center of IRP1 would prevent binding to IRE [33]. The presence of both the 4Fe-4S center and citrate would prevent RNA binding by steric hindrance. Citrate would play a part in keeping the conformation of IRP1 'closed' through its many

interactions with the four domains of IRP1. Alternatively, in the absence of the Fe-S center, the fourth domain of the apo-IRP1 could move away from packed domains 1–3 due to the flexible hinge, to allow an 'open' configuration making the binding site accessible to IREs. Mutation of cysteines 437, 503, and 506 definitively proved the crucial role of the Fe-S center in IRP1 functions. Indeed, IRP1 devoid of either cys437 or both cys503 and 506 cannot insert the Fe-S center and constitutively binds IRE sequences [34].

The 'null' 3Fe-4S IRP1 form

Purification of IRP1 enabled identification of three forms of IRP1. The 4Fe-4S center-containing protein (holo-IRP1) in the 'closed' conformation described above does not bind IRE sequences, but still has high affinity for the substrate and exhibits aconitase activity. Conversely, the apoprotein form of IRP1 with an 'open' configuration binds mRNA, does not exhibit aconitase activity, and has low affinity for citrate. An intermediate form with a 3Fe-4S center is catalytically inactive despite having a high affinity for the substrates (citrate and cis-aconitate), and does not exhibit RNA-binding activity either, because the Fe-S center, although incomplete, is still present at the active site. In turn, it could mask the RNA binding site by maintaining the 'closed' conformation [35, 36].

The 'null' oxidized apo-IRP1 form

By blocking the sulfhydryl groups, diamide inhibits the *trans*-regulating activity of the apo-IRP1 [34]. Apo-IRP1 thus requires free sulfhydryls for its interaction with IRE sequences. Mutation of cys437, 503, and 506 to the corresponding serine showed that cysteine 437 is able to form a disulfide bridge with cysteine 503 or 506, which inhibits the interactions between IREs and IRP1 [34]. A slightly reducing environment mimicked by addition of 0.02% 2-mercaptoethanol is sufficient to lift this inhibition. These experiments confirmed the existence of an oxidized apoprotein form of IRP1 which had previously been described under harsh in vitro conditions, e.g., upon exposure to a high level of ferricyanide [37].

Assembly of the 4Fe-4S center within the cell

Very few studies have addressed this crucial question. However, in vitro experiments show that iron-sulfur centers can be spontaneously reconstituted in the presence of iron, sulfide, and a reducing agent like dithiothreitol. Under physiological conditions, it is still unclear whether cluster assembly is spontaneous or requires cellular factors. A protein called NifS [38] identified in bacteria is necessary for the formation of the nitrogenase 4Fe-4S center and accelerates the in vitro formation of the 2Fe-2S center of the *E. coli* transcription factor SoxR [39]. NifS is an 87.5-kDa homodimer which allows the formation of stoichiometric amounts of sulfide and L-alanine from L-cysteine [38]. It is thus tempting to speculate that NifS-like protein(s) could also be present in mammalian cells and take part in the assembly/disassembly of the IRP1 Fe-S center. Moreover, recent studies by Ding and Demple [40] strongly suggest that dithiol proteins like thioredoxin (TRX/TR system) promote the assembly of 2Fe-2S clusters of SoxR.

IRP2, a false twin

A second protein able to bind IRE sequences was recently identified by electromobility shift assays aimed at characterizing IRP1 (fig. 2). These in vitro experiments showed two complexes formed between a ^{32}P-labeled IRE probe and cytosolic proteins from rat or mouse cells [41, 42]. This second protein, named IRPB then IRP2, was characterized in rodents [43] and aroused great interest when it was identified in humans [44]. IRP2 cDNA was isolated both in humans and rat, and there is 93% identity between the two species [33, 45]. The amino acid sequence of human IRP2 exhibits 57% identity and 75% similarity with that of human IRP1. This explains why IRP2 is able to bind consensus IRE sequences with the same affinity as IRP1 [43, 44, 46].

Although IRP2 represses the translation of Ft mRNA in vitro just like IRP1 [44, 47], some structural differences between the two IRPs have been reported. IRP2 has a molecular weight of 105 kDa due to an additional 73-amino-acid domain near the N-terminal part [45, 48]. Although IRP2 has 16 of the 18 active site amino acids present in IRP1, including cysteines 512, 578, and 581 which are equivalent to those which hold the Fe-S center of IRP1, it does not exhibit aconitase activity. This lack of enzymatic activity is neither explained by the absence of serine 669 and arginine 474, whose counterparts in IRP1 are essential for enzymatic catalysis, nor by the insertion of the 73-amino-acid sequence [49]. Moreover, attempts at in vitro reconstitution of an Fe-S center in IRP2 were unsuccessful [49]. Despite its lack of both an Fe-S cluster and aconitase activity, IRP2 was classified among the Fe-S isomerases which include three distinct protein families: aconitases, isopropylmalate isomerases, and IRPs [50]. Recent studies have focused on the intrinsic role of IRP2 in intracellular iron homeostasis of cells not expressing IRP1 [51].

Post-translational regulation of IRPs

It was long believed that activity/expression of IRPs was regulated only by intracellular iron fluxes. However, recent evidence indicates that physiological redox effectors also play an important part in IRP modulation. I shall briefly review and compare the effects of iron, oxidative stress and NO and its related species peroxynitrite (table 1).

Regulation by iron

It has long been known that intracellular iron status plays a major role in the regulation of IRP activity. In vitro, exposure of cells to iron chelators induces high-affinity binding of both IRPs to the IRE sequences. Under these conditions, synthesis of Ft, δALAS, mt-aconitase, and succinate dehydrogenase (SDH) is repressed whereas Tf-R mRNA is stabilized [52–54]. In contrast, when intracellular iron increases, both IRPs lose their capacity to bind IREs. Thus mRNA containing an IRE at the 5′ end is translated and Tf-R mRNA is quickly degraded. Iron homeostasis is therefore maintained by lowering iron uptake and by increasing iron storage in Ft. Thus, a switch from 4Fe-4S IRP1 to the Fe-S center-free apoIRP1 occurs through an ill-defined post-translational mechanism [55].

In contrast to IRP1, IRP2 is degraded in iron-replete cells [56, 57]. This fast degradation depends on the presence of an additional exon coding for the above-mentioned 73-amino-acid sequence inserted near the N-terminal part of IRP2 [58]. Interestingly, the insertion of this sequence in a similar position in IRP1 generates a chimeric protein which is then quickly degraded in the presence of a high intracellular iron concentration. Conversely, IRP2 is stabilized by deletion of this sequence. The sequence which contains five cysteines, ten prolines and two CRG motives, was termed the 'iron-dependent degradation domain' [58]. Mutation of the three cysteines of the CX5CX3C motif to serines abolishes the iron-mediated fast degradation of the protein [58]. In contrast, mutation of cysteines homologous to those which bind the 4Fe-4S center in IRP1 does not affect degradation by iron. This indicates that even if IRP2 is able to accommodate an Fe-S center via these three cysteines, it would not play a role in iron-dependent IRP2 'turnover' [52]. The data on the iron-dependent degradation of IRP2 are completed by three significant sets of results: (i) degradation was prevented by specific proteasome inhibitors, suggesting that IRP2 is degraded by the 26S complex of the proteasome [58, 59]; (ii) the 'turnover ' of IRP2 is dependent on protein translation [57, 59]; (iii) IRP2 oxidation is iron dependent. Many of these oxidized residues, most of which are represented in the iron-dependent degradation domain, are carbonylated then ubiquitinated prior to degradation by the proteasome [60].

Regulation by reactive oxygenated species

Most cells, in various pathophysiological situations, may be exposed to oxidative stress. The superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which result from these biological dysfunctions interfere with the activity of several Fe-S enzymes, including bacterial and mammalian aconitases as well as IRP1 [61–64]. Protection of the active site by citrate prevents inactivation by O_2^- [64] and ascorbate can reconstitute the full 4Fe-4S cluster [65]. The reactivity of O_2^- at the IRP1 Fe-S center does not lead to the formation of an IRP1 able to bind IRE sequences, even though enzymatic activity is lost [66]. Altogether, these data suggest that O_2^- damages and/or oxidizes the 4Fe-4S center of IRP1 resulting in the formation of the 'null' 3Fe-4S center form. It has recently been reported that triggering of intact cells with extracellular H_2O_2 can activate IRE binding by IRP1 [67–70]. Activation of IRP1 by H_2O_2 is accompanied by

Table 1. Summary of the effects of iron, oxidative stress, and NO on IRP modulations in cells.

Effector	Function and proposed mechanism of IRP1 activation	Function and proposed mechanism of IRP2 activation
Iron chelators	induction of IRE binding—mechanism unknown	induction of IRE binding m accumulation of IRP2 mRNA and protein following de novo synthesis [68]
Iron source	keeps or restores IRP1 in its aconitase form—post-translational event, NifS-like proteins?	loss of IRE binding—oxidation, carbonylationand ubiquitination of IRP2 before fast proteasome-dependent degragation [60]
Oxidative stress (O_2^-, H_2O_2)	induction of IRE binding—protein phosphatase I/IIa-dependent pathway	not modulated
	induction of IRE binding:	induction of IRE binding:de novoprotein synthesis required [68]
NO	(i) fast and direct—alteration of the Fe-S cluster [76]	no modulation [76, 80]
	(ii) slow and indirect through the mobilization of an iron pool [72]	loss of IRE binding: unknown mechanism [81]

inhibition of Ft synthesis and by stabilization of Tf-R mRNA. However, direct exposure of cell cytosols to H_2O_2 is ineffective [66, 68]. The H_2O_2-triggered signaling pathway which leads to inhibition is not fully elucidated but requires a membrane-associated component, phosphorylation steps and/or ATP [71]. IRP2 activity does not seem to be modulated in response to H_2O_2 [72].

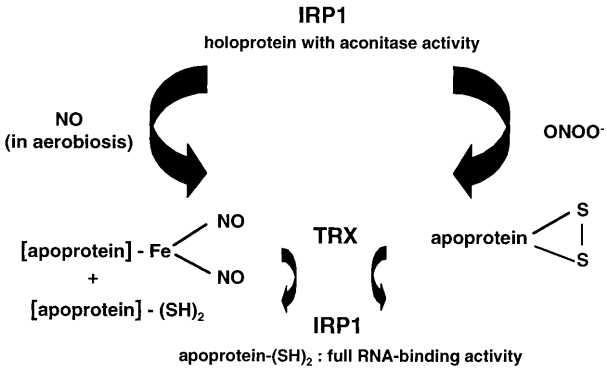

Figure 4. IRP1 activation requires cooperation between the physiological reducing system thioredoxin/thioredoxin reductase and NO or peroxynitrite ($ONOO^-$). Thioredoxin (TRX) can promote the conversion of some oxidized apoprotein form(s) of IRP1 generated by NO and $ONOO^-$ into the IRE-binding apoprotein form.

Regulation by NO

The avidity of NO for heme and non-heme iron proteins encouraged investigation into its involvement in the modulation of IRP1 activities. NO was the first biological molecule shown to be able to conversely modulate the two activities of IRP1 in activated macrophages [73, 74]. This modulation was then observed in other cell types expressing either constitutive or inducible NO synthase (NOS) [73–75]. Two hypotheses have been advanced for the mechanism by which NO reacts with IRP1. First, that modulation by NO results from reduced intracellular iron pool availability, leading to cellular iron deficiency. In this case, NO would have a delayed and indirect effect on the modulation of IRP1 [72]. Second, a direct interaction between NO and IRP1, or more precisely its Fe-S center, was proposed. This assumption was based on two sets of results: (i) the protection against NO by the substrates (which bind to the Fe-S center), and (ii) the rapidity of the effect of NO both in cell-free systems and on a reporter target cell withdrawn from the NO-generating cell monolayer [66, 76].

Furthermore, the capacity of the physiological reducing system thioredoxin/thioredoxin reductase to cooperate with NO in the activation of IRP1 was demonstrated [77]. NO in aerobiosis increases IRE-binding activity of IRP1 via the formation of a reduced apoIRP1 form. However, this activation is far from maximal, probably due to the formation of an inactive form of IRP1 possibly containing iron nitrosyl complexes [78] which could mask the IRE binding site. By reducing this inactive form, thioredoxin could largely reconstitute the *trans*-regulatory activity of IRP1 easily observed in whole cells [76, 77] (fig. 4).

Whether or not IRP2 is modulated by NO, and with what consequences, is still a matter of debate. The IRE-binding activity of IRP2 was increased in a B6 fibroblast cell line transfected to overexpress NOS II mRNA [68]. This modulation, confirmed in the Ltk – fibroblasts treated with SNAP (an S-nitrosothiol able to decompose spontaneously into NO), requires de novo protein synthesis [72]. Other studies showed an activation of IRP2 correlated with endogenous NO production in rat hepatic cells following acute inflammation [79]. On the other hand, in a rat hepatoma cell line, neither induction of NOS II by cytokines nor exposure of the cells to NO donors modulated IRP2 activity or expression [80]. Moreover, two independent studies reported that expression or *trans*-regulating activity of IRP2 was decreased in macrophages stimulated in vitro by the combination interferon (IFN)-γ/lipopolysaccharide (LPS). In one case, the authors claimed that the down-regulation observed was mediated by NO synthesis [81] whereas in the other case, the authors concluded that it was not [76].

It is important to recall that, as in many other systems, NO synthesis can not only modulate iron status in generating cells, but can also affect a target cell which does not necessarily express NOS activity. In vitro, the effector/target cell system has sometimes been used as a tool to resolve some critical issues, two examples of which follow.

1) To avoid any interference with the multipotent cytokines or endotoxin and to maintain physiological conditions, an elegant approach was followed by Hentze and co-workers [82]. Rat B6 fibroblasts stably transformed with a murine macrophage NOS plasmid (B6.NOS) were cocultured with B6 cells expressing IRE-containing human growth hormone (hGH) reporter mRNA (B6.IRE-hGH). NO production by B6.NOS resulted in repression of hGH synthesis by adjacent B6.IRE-hGH, which was abolished by inhibiting NOS activity by monomethyl-L-arginine.

2) Coculture experiments were also performed by Bouton and co-workers [76] to determine precisely the time-course of the effect of NO on IRP1. NO production by NOS requires a lag due to transcription and translation (5–6 h). This lag may create the impression

that the effect of NO is slow. To avoid this bias, adherent monolayers of RAW 264.7 macrophages were induced to produce NO, and after 16 h, were covered with non-adherent C58 pre-T cells. Target cells were withdrawn from the macrophage monolayer and analyzed for IRP1 activation. Enhancement of RNA binding by IRP1 was detectable as early as 1 h after the beginning of the coculture, pointing to a direct effect of NO on IRP1.

Because IRP binding to IREs results in repression of Ft translation and Tf-R mRNA stability [35, 82], NO synthesis, by activating IRP1, is expected to decrease Ft expression and increase Tf-R synthesis. Several lines of evidence indicate that Ft translation is repressed in response to NO-mediated activation of IRP1: Weiss et al. [74] showed that Ft H- and L-chains were drastically down-regulated in the J774 macrophage cell line stimulated for NO synthesis, whereas the Ft mRNA level was unchanged. These data pointed to post-transcriptional regulation of Ft by NO synthesis. Furthermore, the same group confirmed the regulation of Ft translation by NO using a fibroblast cell line stably transformed to constitutively express NOS II [83]. However, not all data agree with an NO-mediated reduction in Ft expression. Indeed, despite IRP1 activation, exposure of K562 erythroleukemia cells to the NO generator SNAP had no effect on the intracellular Ft level [84]. Moreover, Recalcati et al. [81] reported that stimulation of J774 cells by cytokines and LPS induced an increase in Ft synthesis. These conflicting results may be explained by the intrinsic, and at times predominant, role of IRP2 (see below).

The situation regarding NO modulation of the IRP/IRE system and Tf-R expression is also somewhat puzzling. In two independent sets of studies, it was shown that SNAP increases IRP1 activity and Tf-R expression [84, 85]. Moreover, one of these studies reported that sodium nitroprusside, which releases an NO^+-like molecule, had an opposite effect. Thus, depending on the redox environment, NO release may have different consequences for IRP activity. As pointed out by two groups, stimulation of cells by IFN-γ and LPS lowers Tf-R mRNA levels, whether NO is produced or not [80, 86]. As cells transformed to overexpress NOS II exhibit a several-fold increase in Tf-R mRNA mediated by the IRP/IRE interaction [68], it was proposed that down-regulation of Tf-R expression in response to IFN-γ and/or LPS outweighs the positive effect of NO. Furthermore, an increasing number of findings indicate that IRP2, even though it is generally less expressed than IRP1, plays a crucial role in iron metabolism. For example, Cairo and Pietrangelo [79] have reported that in livers of turpentine-treated rats, IRP2 and not IRP1 is responsible for Tf-R mRNA stabilization. It is worth noting that two independent studies indicate that stimulation of macrophage cell lines by IFN-γ/LPS down-regulates IRP2 [76, 81]. Accordingly, it is possible that despite activation of IRP1 (via NO), IFN-γ/LPS-mediated down-regulation of IRP2 is dominant in post-transcriptional regulation of Tf-R. A tentative explanation can be proposed: IRP2 has a greater affinity for IREs with an adenine in the middle of the six-membered loop, i.e., NNGAGN [87]. One the five IREs (IRE_A) of the 3′-untranslated region of Tf-R mRNA possesses such a loop [35]. Accordingly, if binding to IRE_A is predominant for mRNA stability, immunological stimulation, by decreasing IRP2, may plausibly down-regulate Tf-R expression and up-regulate Ft.

Regulation by peroxynitrite

Formation of peroxynitrite results from reaction of NO with O_2^-. It is assumed that this strong oxidant can be generated in living cells under pathophysiological conditions (see Ducrocq et al, in this issue). Indeed, several groups have observed simultaneous production of O_2^- and NO in various cell types upon activation [88–91]. More recently, production of peroxynitrite has been detected in macrophages and some other cell types by the presence of nitrated tyrosine, a specific footprint [92, 93, 94, 95; see also Ducrocq et al. in this issue]. It has been postulated that peroxynitrite rather than NO is responsible for some effects previously attributed to NO, including inhibition of Fe-S-containing proteins such as complexes I and II of the mitochondrial respiratory chain, and aconitases [64, 70, 96]. As it is believed that peroxynitrite attacks the Fe-S cluster, we decided to evaluate its effect on IRP1 IRE binding, confirming that peroxynitrite inhibits the enzymatic activity of IRP1 without degrading the protein. However, IRP1 does not gain RNA-binding activity [97]. To solve this puzzling question, the mechanism of peroxynitrite action on IRP1 was further studied in vitro. It was concluded that not only does peroxynitrite disrupt the Fe-S center of IRP1, but it also promotes formation of an oxidized apo-IRP1 exhibiting neither aconitase activity nor *trans*-regulatory activity. Furthermore, site-directed mutagenesis showed that peroxynitrite allows formation of a disulfide bridge involving Cys437. In such an oxidized form, IRP1 has no biological activity. It was concluded that peroxynitrite may predispose IRP1 to bind IRE sequences only if the protein is then placed in a very slightly reducing environment [97].

The effect of peroxynitrite on the RNA-binding activity of IRP2 was also studied. RNA-binding capacity is lost in response to peroxynitrite but protein is not degraded, as testified by recovery of full activity in the presence of 2-mercaptoethanol. Thus, IRP2 can also be modulated at a post-translational level in response to redox influence [97]. When exposed to diamide or 5′,5′-dithiobis-

(2-nitrobenzoic) acid, IRP2 activity is lost but can be recovered in the presence of 2-mercaptoethanol or dithiothreitol [49, 57]. Thus, sulfhydryl groups of IRP2, like those of apo-IRP1, are sensitive to oxido-reduction. In conclusion, both IRPs are sensitive to their redox environment. Under reducing condition, their RNA-binding activity is favored by the reduction of their sulfhydryl groups whereas it is decreased or even completely inhibited under oxidizing conditions, due to formation of disulfide bridge(s).

Two IRPs: why?

After describing the various regulations of IRP1 and IRP2, the question as to why cells require two IRPs remains open. Indeed, both IRPs bind IRE sequences with the same affinity and are equally effective as in vitro *trans*-regulators. Both IRPs can be phosphorylated, which in turn increases their binding to RNA [98]. However, although IRP1 and IRP2 bind a consensus IRE equally well, site-directed mutagenesis experiments showed that IRP1 is able to bind an IRE sequence preferentially carrying a UAGUAC loop, whereas IRP2, but not IRP1, binds IREs containing a GGGAGU loop [87]. However, none of these chimeric constructions have yet been identified on physiological mRNAs. The two IRPs could differ in distribution, and IRP1 is well expressed in kidneys and liver whereas IRP2 is better expressed in heart and muscles [45]. Moreover, both IRPs exhibit specific regulation in certain pathophysiological situations. In the well-known regulation due to iron deficiency, IRP1 undergoes a conformational change without loss of protein, whereas IRP2 becomes unstable and is degraded. Nevertheless, despite different mechanisms of regulation, the regulatory activity of both IRPs is inhibited in response to iron increase. Oxidative stress may be more selective. Indeed, it has been reported that extracellular hydrogen peroxide only activates IRP1 in Ltk − fibroblasts [72]. As activation of IRP1 decreases Ft synthesis and increases the expression of Tf-R, it has been speculated that this phenomenon could lead to an increase in intracellular iron and generation of toxic radical species by the Fenton reaction. In this context, IRP1 would have a cytotoxic function. Conversely, other in vivo studies pointed to an exclusive down-regulation of IRP2 after an oxidative stress [99]. Here, inhibition of IRP2 activity induces increased synthesis of Ft which, by collecting excess iron, could protect cells against oxidative damage. Finally, the IRPs are regulated differently in murine macrophages activated by IFN-γ and LPS. Whereas IRP1 gains the capacity to bind the IRE sequence, IRP2 seems to be degraded [76]. Despite IRP1 activation via NO, it is the loss of IRP2 which seems to control the expression of Tf-R mRNA in macrophages [83]. In brief, if IRP2 is regulated differently or even opposite to IRP1, it could help balance IRP1-mediated control of iron metabolism.

Prospects

It may be wise to change the term 'iron regulatory proteins' in the future after considering the newly revealed involvement of these proteins in energy metabolism. Indeed, the 5′-untranslated region of porcine mt-aconitase mRNA contains a conserved IRE which is recognized by the two IRPs with equivalent affinity [87]. Consequently, in vitro translation of mt-aconitase mRNA is repressed. An IRE sequence was also recently localized in the 5′-untranslated region of insect SDH mRNA as well as in the 3′ of that of mouse glycolate oxidase [100], two enzymes which are involved in cellular energy production. It has not yet been shown that NO and reactive oxygenated species can regulate the expression of these enzymes through IRP1, and in turn modify energy metabolism, but this certainly represents a promising line of investigation. It is also important to recall that two of these proteins, mt-aconitase and SDH, possess one or more iron-sulfur cluster(s) crucial for enzymatic activity. It has recently been confirmed that porcine mt-aconitase is sensitive to NO [101], and that its inactivation by NO is accompanied by the appearance of an electron paramagnetic resonance (EPR) $g = 2.02$ signal emitted by the 3Fe-4S cluster and an EPR $g = 2.04$ signal originating from a protein-bound dinitrosyl-iron-dithiol complex [78]. SDH, which also takes part in the electron transport chain as part of complex II, is inactivated by NO, or a related species, through disruption of iron-sulfur cluster(s), as testified by the appearance of an EPR-detectable nitrosyl-iron complex [102]. Thus, it is striking that NO and NO-derived species affect the yield of cellular energy through two mechanisms—direct interaction with iron-sulfur cluster(s) of SDH and mt-aconitase, and a secondary response in which the IRE/IRP1 system represses translation of these two proteins.

In conclusion, these findings and considerations point to a regulation of IRPs by NO and related species which is independent of iron and whose implications go far beyond the control of cellular iron status.

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