REVIEW ARTICLE
Iron regulatory proteins in pathobiology

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The capacity of readily exchanging electrons makes iron not only essential for fundamental cell functions, but also a potential catalyst for chemical reactions involving free-radical formation and subsequent oxidative stress and cell damage. Cellular iron levels are therefore carefully regulated in order to maintain an adequate substrate while also minimizing the pool of potentially toxic 'free iron'. Iron homoeostasis is controlled through several genes, an increasing number of which have been found to contain non-coding sequences [i.e. the iron-responsive elements (IREs)] which are recognized at the mRNA level by two cytoplasmic iron-regulatory proteins (IRP-1 and IRP-2). The IRPs belong to the aconitase superfamily. By means of an Fe-S-cluster-dependent switch, IRP-1 can function as an mRNA-binding protein or as an enzyme that converts citrate into isocitrate. Although structurally and functionally similar to IRP-1, IRP-2 does not seem to assemble a cluster nor to possess aconitase activity; moreover, it has a distinct pattern of tissue expression and is modulated by means of proteasome-mediated degradation. In response to fluctuations in the level of the 'labile iron pool', IRPs act as key regulators of cellular iron homoeostasis as a result of the translational control of the expression of a number of iron metabolism-related genes. Conversely, various agents and conditions may affect IRP activity, thereby modulating iron and oxygen radical levels in different pathobiological settings. As the number of mRNAs regulated through IRE–IRP interactions keeps growing, the definition of IRPs as iron-regulatory proteins may in the near future become limiting as their role expands to other essential metabolic pathways.

Key words: aconitase, Fe-S cluster, mRNA, post-transcriptional control.

INTRODUCTION

In 1976, Hamish Munro et al. reported a study [1] that still represents a cornerstone in the field of cell iron metabolism, as well as in the broader field of post-transcriptional control of gene expression, and, with remarkable foresight, they proposed a model of the translational control of ferritin (Ft) synthesis that still holds. The additional insights provided by a further 25 years of investigation have made the regulation of intracellular iron metabolism the best characterized system of the post-transcriptional regulation of gene expression. The iron regulatory proteins (IRPs) that are the subject of the present review (IRP-1 and IRP-2) are the key players in this complex interaction insofar as they represent the sensors of cytoplasmic iron and the controllers of Ft and the transferrin receptor (TfR). These proteins are used by cells to adjust intracellular iron concentration to levels that are adequate for their metabolic needs, but below the toxicity threshold. As iron is essential for fundamental vital activities, iron deprivation threatens cell survival, thus making iron deficiency in humans a public health problem throughout the world. On the other hand, a number of disease states are pathogenetically linked to excess body iron stores (such as genetic or acquired iron overload) or to the delocalization of intracellular iron (e.g. inflammation, atherosclerosis etc.). We shall here mainly concentrate on the role of IRPs in cell pathobiology. More detailed aspects of IRP structure and function have been recently covered by other excellent reviews [2–6].

MAINTENANCE OF INTRACELLULAR IRON HOMOEOSTASIS

Mammalian cells react to iron deficiency by presenting a higher number of TfRs at the cell surface in order to internalize iron-laden transferrin (Tf). At the same time, the synthesis of the iron-storage protein Ft is halted to enhance metal availability. The opposite is true when iron overload occurs: TfR is down-regulated in order to stop iron uptake, and Ft synthesis is increased to sequester excess iron in newly formed Ft shells. Although iron-controlled transcriptional regulation of the Ft H and L subunits [7–12] and the TfR [13,14] gene has been described, intracellular iron homoeostasis is mainly controlled at the post-transcriptional level. As described in detail below, when iron is scarce in the so-called 'labile iron pool' (LIP), Ft and TfR mRNAs are specifically recognized and bound by the active form of IRPs, cytoplasmic proteins that modulate Ft and TfR mRNA translation and stability respectively. By contrast, when iron is abundant, IRPs are devoid of mRNA-binding activity and target transcripts are freely accessible to translation complexes or nucleases. The close inverse relationship between cellular iron levels and changes in IRP activity has been recently confirmed by studies directly measuring variations in the level of the LIP [15,16]. IRPs therefore control cell iron status by means of divergent, but co-ordinated, regulation of Ft and TfR levels.

A still open question is the role of IRPs in the control of systemic iron homoeostasis, e.g. in intestinal iron absorption. Analysis of the activity of IRPs has provided information on the

level of the LIP in iron-absorbing gut cells in a rat experimental model [17], in knock-out mice defective for the *HFE* gene [18], in a human cell line [19] and in patients with disorders of iron metabolism, including haemochromatosis [12]. These results offered important insights into the relationship between body iron deposits and intestinal iron levels. In particular, the observation that IRP-binding activity is inappropriately elevated in the enterocytes of haemochromatotic patients [12] indicates that these cells, together with reticuloendothelial cells [20], are deprived of iron despite the existence of a total body iron overload and this may help us to understand why intestinal absorption in these patients is not tuned to body iron deposits or needs.

IRP-1 AND IRP-2

The molecular cloning of IRP-1 offered insights into the structural/functional correlation that allows it to undergo changes in activity and function with no appreciable variations in protein content. In fact, this evolutionarily conserved [21–24] 98 kDa protein coded in (human) chromosome 9 [25] is highly homologous with mitochondrial (m-) aconitase, which converts citrate into isocitrate in the tricarboxylic acid cycle, with 100 % identity of the amino acids involved in the formation of the catalytic core [26]. The abundance of information concerning the biochemistry and structure of m-aconitase has allowed the construction of an IRP-1 model that implies a post-translational switch between an apoprotein form capable of binding iron-responsive-element (IRE) motifs and an enzymically active holoprotein endowed with a 4Fe-4S cluster [2–6]. In the holoprotein, the four domains are in a closed conformation and permit the assembly of a 4Fe-4S cubane cluster co-ordinated by cysteine residues. By contrast, as a result of cluster disassembly, the apoprotein can accommodate the RNA in a cleft between domains 1–3 and 4. The switch between these two mutually exclusive functions of IRP-1 is regulated by intracellular iron levels, because a high degree of aconitase activity is present under conditions of iron overload and full IRE-binding capacity exists in iron-depleted cells. IRP-1 forms devoid of both functions probably exist, such as the 3Fe-4S or oxidized apo-IRP-1 forms. The mechanisms underlying the insertion and removal of the cluster, and hence the conversion between the two functions of IRP-1, remain poorly defined. Experiments with iron-loaded and iron-depleted cells first indicated that the shift from the RNA-binding to the aconitase conformation was a relatively fast process, whereas the induction of binding activity occurred much more slowly, probably reflecting a need for the new synthesis and accumulation of IRP-1 apoprotein. Although the fourth labile iron atom can be removed from the cluster *in vivo*, cluster disassembly beyond the 3Fe-4S state has only been obtained under rather unphysiological conditions, with the possible exception of cluster attack by nitrogen and oxygen radicals or xenobiotics (see below). Regardless of the iron status, cluster stability also depends on the phosphorylation state of a serine residue required for RNA binding [27]. Conversely, it is thought that cluster reconstitution occurs spontaneously under physiological conditions provided that iron and reducing agents are available, although this idea has recently been put in doubt by the cloning of the human homologue of a bacterial enzyme required for Fe-S cluster assembly [28]. Why an aconitase should be placed in the cytoplasm remains to be understood. An interesting hypothesis has been put forward in recent studies in yeast [29]. The conversion of IRP-1 into aconitase in response to iron may increase NADPH levels, thereby providing the cell with additional reducing equivalents. The latter may help the cell to maintain the redox balance and hence face iron-promoted oxidative stress (see below).

Although two IRE-binding proteins were initially detected by RNA bandshift assays in rat cells [30], IRP-2 was identified and characterized only several years after IRP-1, probably because it is usually less abundant and can be electrophoretically distinguished from IRP-1 only in murine cell extracts. IRP-2 is highly homologous with IRP-1, but has two major differences: the presence of a 73-amino-acid insertion in the N-terminus and a lack of aconitase activity (probably due to an inability to assemble a 4Fe-4S cluster) ([2,31] and references cited therein). The IRP-2 specific sequence mediates the characteristic way by which this protein is regulated: in the presence of high iron levels, IRP-2 is rapidly targeted to proteasome-mediated degradation [32], and it has also been claimed that haem, rather than free iron, is involved in IRP-2 degradation [33].

Although the two IRPs bind consensus IRE sequences with similar affinity and specificity ([2,31] and references cited therein), it has been shown that IRP-2 can recognize an exclusive IRE subset [34,35] (Table 1).

Table 1   IRE structure/IRP specificity

The Table shows the binding of IRP to IRE with respect to changes from the consensus sequences and the differences of affinity of IRP-1 and IRP-2 for various IREs. For sequences generated by SELEX procedures or present in patients with hereditary hyperferritinaemia–cataract syndrome (HFCS), letters in **bold** show deviations from consensus. Examples have been selected from [34,35,40,43] to illustrate the importance of the various regions of the IRE motifs. Affinity for IRP-1 seems more affected by mutations in the loop, whereas IRP-2 is more sensitive to variations in the bulge. Abbreviation: eALAS, ε-aminolaevulinate synthase.

Sequence	Position in IRE	Binding by IRP-1	Binding by IRP-2	Presence in mRNA
CAGUGX	Loop	Good	Good	Ft, TfR, etc.
UAGU**A**C	Loop	Poor	Null	None
GGGA**G**U	Loop	Null	Good	None
GAGA**G**U	Loop	Poor	Null	None
CCGA**G**C	Loop	Null	Poor	None
CA**C**UGU	Loop	Null	Poor	Mutated L Ft (HFCS)
UU**A**AAC	Upper stem	Poor	Poor	Mutated L Ft (HFCS)
UU**U**CUU	Bulge	Poor	Null	Mutated L Ft (HFCS)
GU**GGGG**	Lower stem	Poor	Poor	Mutated L Ft (HFCS)
UUGCUU	Bulge	Good	Good	Ft
UAUCGG	Bulge	Good	Poor	TfR
GUUCGU	Bulge	Good	Poor	eALAS
CAUCUU	Bulge	Good	Poor	m-aconitase

Table 2   RNA binding activity of IRP-1 and IRP-2 in different tissues (a) and cell lines (b)

The values have been derived from different reports and are therefore given in arbitrary units. The relative activities of the two IRP are not to be compared between tissues and cell lines.

(a)

Tissues ...	RNA-binding activity						
	Liver	Lung	Spleen	Kidney	Heart	Duodenum	Brain
IRP-1	+ + +	+ +	+ + +	+ + +	+ + +	+ +	+ +
IRP-2	+	+	+	+ +	+	+ +	+ +

(b)

Cell lines ...	RNA-binding activity						
	Hepatomas	Fibroblasts	Macrophages	Melanomas	Erythroleukaemia	T-lymphocytes	Microglia
IRP-1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
IRP-2	+ +	+	+ +	+ +	+	+ +	+

Furthermore, growing evidence suggests that IRP-2 plays a specific role, thus increasing the complexity of the post-transcriptional regulation based on IRE–IRP interaction: in addition to a specific modulation in response to stimuli and agents other than iron (see below), IRP-2 has a characteristic pattern of tissue expression ([2,31] and references cited therein) (Table 2). Furthermore, a preferential increase of IRP-2 expression is present in most cell lines (Table 2), perhaps because of the higher sensitivity to changes in iron levels [36]. Translational inhibition through both IRP-1- and IRP-2- specific IREs has provided evidence of the regulatory role of IRP-2 in cells [37], and it has been demonstrated that, when abundantly [38] or uniquely [39] expressed, IRP-2 can act as the major or sole modulator of intracellular iron metabolism.

A recent report has demonstrated that aconitases A and B undergoing a Fe-S cluster-dependent switch, and thus showing enzymic or RNA binding activity, also exist in *Escherichia coli*. Remarkably, the apoforms of aconitases A and B bind and stabilize their own mRNA, thus enhancing aconitase synthesis [24].

BIOLOGICAL TARGETS OF THE IRPs

IRPs recognize a conserved sequence of nucleotides in the untranslated region (UTR) of mRNA, which is known as the IRE and can be predicted to form stem–loop structures. Although the sequence of the hairpin loop is highly conserved (CAGUGU/C), a number of stem types, with different predicted secondary structures, are present in the various transcripts bound by IRP. These variations are mirrored by quantitative differences in iron-dependent regulation by the two IRPs that can provide a more finely tuned range of responses [6,40] (Table 1). Both the Ft H and L subunit mRNAs have one IRE in their 5′-UTR close to the AUG. The IREs in the Ft mRNAs have an internal loop/bulge, which means that they are very efficiently bound by IRP and are thus subjected to strong regulation in response to iron (Table 1) [6]. IRP binding keeps the Ft mRNAs in messenger ribonucleoproteins [41] by preventing the interaction between the cap-binding complex eIF4F (eukaryotic initiation factor 4F) and the small ribosomal subunit [42] and hence the formation of an efficient translation complex. *In-vitro*-mutagenesis experiments demonstrating the importance of (or need for) single nucleotides in the stem–loop structure have recently been supported by reports of spontaneous mutations in patients with hereditary hyperferritinaemia–cataract syndrome ([43] and references cited therein) (Table 1) in whom mutations in the IRE of Ft L subunit mRNA lead to high serum Ft levels in the absence of an iron overload and to cataract. A relationship between the effect of the mutation on IRP recognition, circulating Ft levels and the clinical severity of the disease has been established [43,44], although the molecular link between high serum Ft levels and cataract formation is unknown.

m-Aconitase also has an IRE in its 5′-UTR and is regulated by changes in intracellular iron status, but its modulation is more limited than that of Ft [45], possibly because of a different IRE structure [40] (Table 1). Since citrate can bind iron, aconitase-mediated changes in citrate concentration may modulate the availability of the metal in the LIP and thus affect iron homoeostasis. Furthermore, IRP-mediated changes in the abundance of m-aconitase may represent a means of coupling iron homoeostasis and cellular energy production, as has also been suggested by recent findings in prokaryotes [24]. The existence of a link between the tricarboxylic acid cycle and iron metabolism has been also shown by the finding that the transcript of succinate dehydrogenase b in *Drosophila* also contains an IRE in its 5′ end and is regulated by cellular iron levels by means of an IRP [46,47] and by the recent demonstration that the tricarboxylic acid oxalomalate interacts with an IRP [48].

Translational control mediated by IRE–IRP interactions also ensures that the synthesis of erythroid aminolaevulinate synthase, the key enzyme in haem biosynthesis, is coupled to iron availability [49,50], although for this enzyme also the level of translational control is moderate [51].

A conserved sequence, predicted to form a secondary structure typical of an IRE, is present in the 5′-UTR of fish, mouse and human cDNAs of ferroportin1, the recently discovered iron transporter presumably involved in iron egress from enterocytes and macrophages [52], which has been also named IREG1 [53] or MTP1 [54]. Yet it remains to be seen whether IRE/IRP-mediated regulation is actually involved in the regulation of this newly identified iron exporter. In particular, the presence of an IRE in the 5′-UTR, which usually mediates translational repression, is difficult to reconcile with the increased IREG mRNA levels found under conditions of iron deficiency [53].

Although all of the above mRNAs have an IRE in their 5′-UTR, the mRNA for TfR, which mediates iron uptake by internalizing iron-laden Tf, has five IRE motifs in its long 3′-UTR ([2] and references cited therein). The interaction of IRP with a still undefined number of IREs does not affect mRNA

Table 3   Biological targets of IRPs and possible pathobiological implications

The Table lists mRNAs bearing an IRE. The function of the encoded proteins, the modulating agents, and the clinical conditions possibly connected, are also indicated. Abbreviation: eALAS, ϵ-aminolaevulinate synthase.

mRNA	Function	Stimulus	Pathobiological setting
H and L ferritin	Iron storage	Iron, NO, O_2^-	ACD
TfR	Iron uptake	Oxygen, erythropoietin	Haemochromatosis Doxorubicin-toxicity anaemia
m-Aconitase	Tricarboxylic acid cycle	Iron	?
eALAS	Haem formation	Iron	Porphyria anaemia?
DMT-1	Iron uptake by enterocytes and iron transport	Iron, NO	Iron-deficiency haemochromatosis Microcytic anaemia
Ferroportin1/IREG1/MTP1	Iron egress from enterocytes and macrophages	Iron?	Iron-deficiency haemochromatosis

translation, but enhances TfR mRNA half-life by protecting it from the action of hitherto-uncharacterized RNase(s). In turn, increased transcript stability leads to higher TfR expression on the cell membrane and greater iron uptake. On the other hand, no typical IREs have been found in the UTR of transcripts for TfR2, a protein whose structure and function are very similar to those of TfR [55–57]. This may account for the lack of iron modulation of this gene [56,57].

A potential IRE is also present in the 3′-UTR of one of the two alternatively spliced transcripts of DMT-1, the main transporter of iron at the luminal site of mammalian enterocytes [58]. However, this IRE is probably a less efficient competitor for IRP than Ft IRE [59]. Indeed, the protein coded by the IRE-bearing isoform has been shown to be modulated by dietary iron in the duodenum [60], but no effect was found in the levels of IRE-containing DMT-1 mRNA in a fibroblast cell line [59]. In brief, there is still a lack of direct experimental evidence that this IRE confers iron-mediated post-transcriptional control of DMT-1.

The use of an *in vitro* SELEX (selective enrichment of ligands by exponential amplification) procedure has allowed the identification of IREs with alternative loop sequences that bind to IRPs and confer translational control to reporter constructs [34,35,61]; some of these also show a preferential interaction with IRP-1 or IRP-2 [37] (Table 1). However, naturally occurring transcripts bearing these alternative IREs have not yet been found. Furthermore, as recently shown for glycolate oxidase [62], the presence of a conserved IRE motif is not always sufficient for regulation by IRP.

## IRP REGULATION BY STIMULI OTHER THAN IRON

The 'simple' IRP model of bifunctional proteins with distinct metabolic functions that depend only on iron availability to assemble the cluster proved to be correct for the iron-dependent modulation of IRP-1, but was subsequently challenged by the finding that IRP-2, which has no cluster, may play important biological roles and by the results of experiments showing the existence of molecular species of IRP-1 incapable of binding IRE or functioning as aconitase, such as inactive forms with a 3Fe-4S cluster or oxidized cysteine residues. The latter findings have been mainly obtained by studies of the effects of various agents and pathobiological conditions on IRP activity (Table 3).

OXIDATIVE STRESS

Reactive oxygen species (ROS), which are unavoidably and constantly generated in every cell during its normal life under aerobic conditions, can cause considerable damage to a number of target molecules essential for cell homoeostasis. However, in controlled amounts, they also play a role in physiological pathways, such as signal transduction, cell signalling and redox regulation of cell proliferation and apoptosis. The modulation of iron availability is the main means by which cells keep ROS levels under strict control, because the appropriate sequestration of iron may allow the physiological roles of the relatively safe O_2^- (superoxide anion) and H_2O_2 to take place without the production of the highly reactive OH by Fenton chemistry. It is therefore no surprise that the activity of IRP, as the master regulator of cellular iron metabolism, is altered under conditions of oxidative stress. Given the role of Ft and TfR in iron chelation and uptake, one would expect their respective up- and down-regulation under conditions of oxidative stress in order to limit iron availability. Indeed, it has been demonstrated that various cell types react to a variety of stressful conditions by increasing Ft synthesis [63–66], and the oxidative stress response has been found to be reduced by Ft H subunit overexpression [67]. However, this effect is not consistent with the enhanced activity of IRP in H_2O_2-treated cells [68,69], because this would expand the LIP, and thus exacerbate ROS toxicity. A thorough analysis of the mechanisms and signals underlying IRP activation under these conditions has made it evident that this prompt effect occurs more as a response to phosphorylation-dependent signalling pathways, possibly related to the growth-promoting effects of H_2O_2 [70], than to oxidative stress itself. In fact, neither raising intracellular H_2O_2 levels [71] nor treating cytosolic extracts with H_2O_2 [68,69,72,73] led to any stimulatory effect and the response of IRP, which requires ATP, GTP and phosphorylation events [74], was restored by the addition of membranes [74]. Furthermore, it has been shown that the simultaneous generation of H_2O_2 plus O_2^-, which is more likely to occur under oxidative stress conditions than the production of H_2O_2 alone, inhibits IRP activity [72]. IRP inactivation has also been found following the treatment of cells with menadione, a redox cycling quinone that yields H_2O_2 plus O_2^- [75], and it has been shown that *in vivo* ROS production obtained by treating with dioxin [76], glutathione-depleting drugs [77], or by means of post-ischaemic rat liver reperfusion [78] down-regulates IRP activity.

All of these considerations mainly refer to the 4Fe-4S-endowed IRP-1, whereas there are fewer data concerning the effect of ROS on IRP-2. The mechanism of IRP-2 regulation, i.e. ubiquitin-dependent proteasome degradation after direct oxidative modification of residues in the degradation domain [32], suggests that the molecule is highly susceptible to changes in the redox status
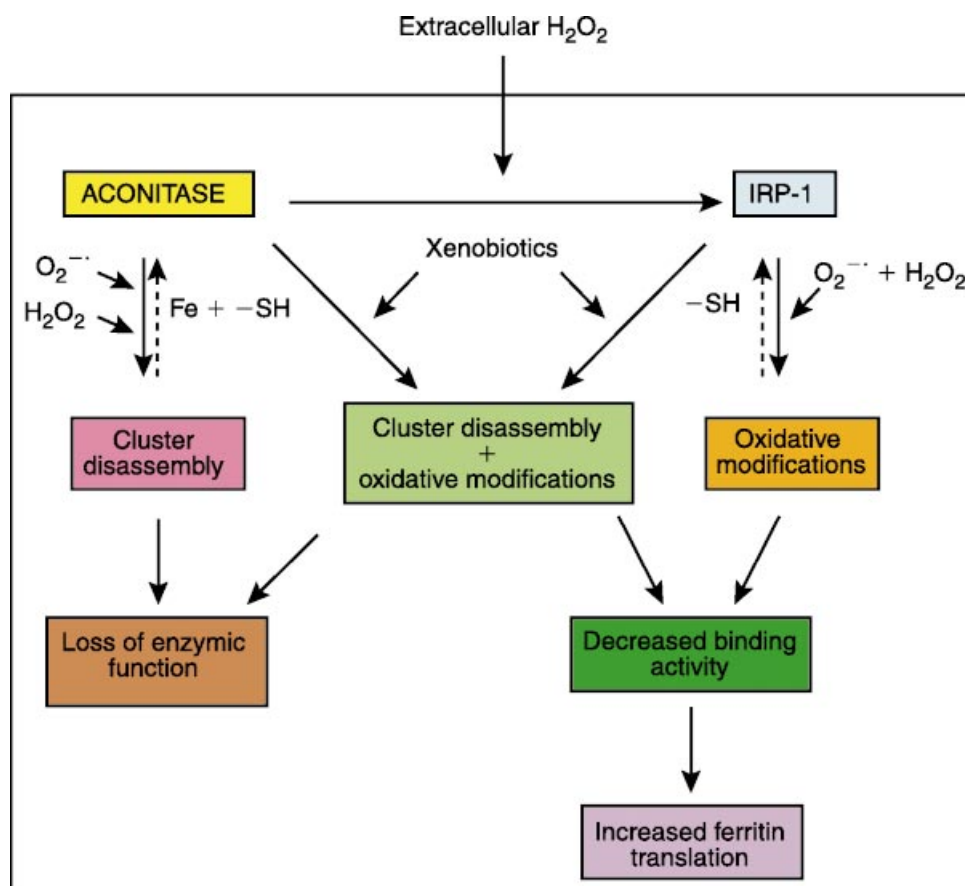
Figure 1   Redox regulation of IRP-1

The scheme summarizes the mechanistically complex interaction of ROS with IRP-1/aconitase. When produced inside the cell separately, O_2^- or H_2O_2 cause reversible loss of aconitase without acquisition of IRP-1 activity. When present together, these ROS reversibly down-regulate IRP-1 activity, but do not affect aconitase. On the other hand, extracellular administration of H_2O_2 triggers a signalling cascade that activates IRP-1 binding activity with a concomitant decrease of enzymic function. Xenobiotics which alter the redox state of the cell (e.g. doxorubicin, menadione, dioxin) cause irreversible loss of both activities. In most pathophysiological situations, decreased IRP-1 binding activity, by allowing efficient Ft translation, may favour iron sequestration and increase resistance to oxidative stress.

of the cell. Indeed, IRP-2, which is not activated by extracellular H_2O_2 [37,79], is down-regulated in rat liver after exposure to oxidative stress [77,78] and in cell cultures treated with menadione [75].

In brief, recent results have offered new perspectives concerning the role of IRPs in oxidative stress that have allowed the reconciliation of its function in a coherent framework aimed at limiting the availability of catalytically active Fe (Figure 1).

NITRIC OXIDE (NO)

The fact that Fe-S clusters are preferential targets of NO and that m-aconitase inhibition is involved in the cytotoxic effect of NO (reviewed in [80]) has prompted a number of investigators to address whether this molecule, which is known to be involved in a variety of physiological and pathological processes, also plays a role in the IRP-mediated regulation of iron metabolism. A close link between iron homoeostasis and NO has been clearly established, also in consideration of the effect of intracellular iron levels on the transcription of the inducible nitric oxide synthase gene [81]. However, depending on the cells investigated, the applied stimulus and the redox-related NO species involved etc., it has been reported that NO has a number of effects on both

IRP-1 and IRP-2. As the NO–IRP interaction has been recently reviewed [5,82], we shall here concentrate on the main findings obtained in macrophages, because the NO modulation of IRP in these cells is probably more relevant to pathophysiological conditions such as inflammation and anaemia of chronic disease (ACD).

In agreement with the first reports describing the NO-mediated activation of IRP-binding activity [83,84], increased IRP-1 activity has been repeatedly found after treatment of murine macrophagic cell lines with cytokines – leading to NO production [73] – or with NO donors [38,85,86]. It is still unclear whether the activating effect of NO depends on a direct interaction with the cluster, which would imply disassembly to beyond the 3Fe-4S stage and acquisition of the RNA-binding apoform, or a slow effect of NO on the LIP. However, recent results obtained *in vitro* by exposing cell lysates [73,87,88] or purified [89] and recombinant [87,88] IRP-1 to NO-generating chemicals support the iron-independent hypothesis, although NO-dependent mobilization of iron from cells resulting in iron depletion cannot be excluded [90]. Cluster disruption is probably insufficient, because NO-derived species would cause the formation of oxidized, and hence inactive, forms of IRP and so reduction by thioredoxin [88] or other cell reducing agents is needed for the full acquisition of binding activity.
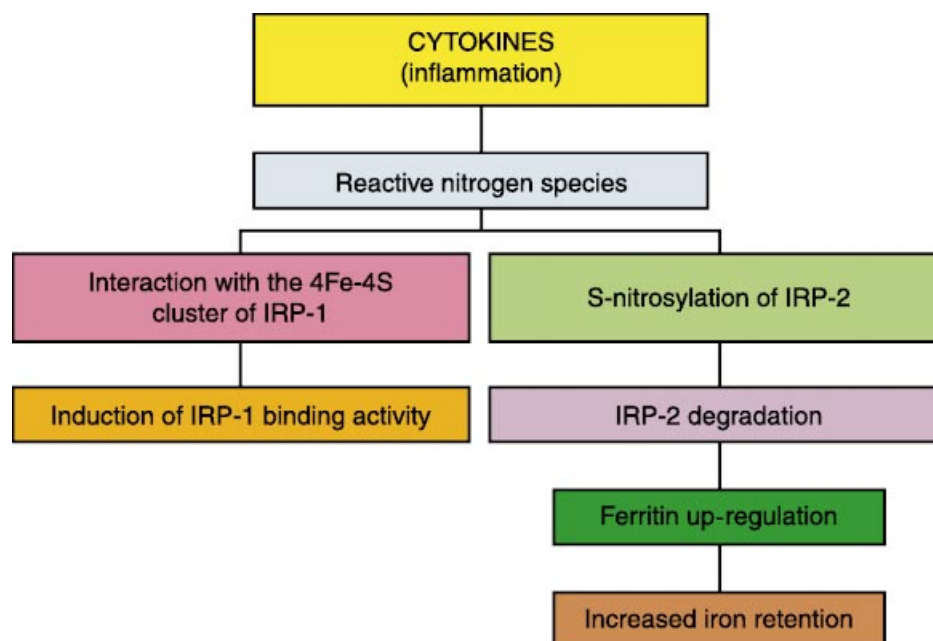
Figure 2   Post-transcriptional modifications of macrophage iron metabolism in inflammation

Cytokine-induced activation of the NO pathway differentially modulates IRP-1 and IRP-2 activities. The effect of IRP-2 down-regulation prevails and leads to stimulation of Ft synthesis which, despite the concomitant decrease of TfR expression, results in enhanced iron accumulation in the reticuloendothelial system. During chronic inflammatory states, iron sequestration in reticuloendothelial cells prevents erythroid precursors from obtaining iron for proliferation and haemoglobin synthesis, thus concurring to cause anaemia.

Conversely, the observed NO-dependent activation of IRP-2 in murine macrophages treated with lipopolysaccharides (LPS)/ interferon γ (IFNγ) [84] has not been confirmed in other studies, which have independently reported a specific down-regulation of IRP-2 [38,91] and a translationally controlled induction of Ft synthesis [38] by LPS/IFNγ. The inactivation of IRP-2 has since been confirmed by other studies, which also described a preferential effect on IRP-2 of the oxidized form of NO (NO$^+$) [86] and a dose-dependent effect of IFNγ [85]. The mechanism underlying NO-induced IRP-2 down-regulation in macrophages is probably degradation [91,92].

A number of uncertainties remain concerning the effect of NO on IRP, partly because the intrinsic chemistry of the molecule means that its reactivity also depends on the redox state of the environment in which it is generated. This is exemplified by the fact that IRP-1 treated with peroxynitrite, a not uncommon product of the reaction of NO with constantly generated O_2^-, loses its aconitase activity without gaining RNA-binding activity [87]. Furthermore, most of the information relating to the NO–IRP interaction has been obtained by treating cell extracts with NO donors. Although this is a convenient means of exploring the molecular mechanisms underlying the effect of NO on IRP, it hardly recreates *in vivo* conditions. In fact, full *in vitro* response to NO requires the addition of a cellular component, i.e. thioredoxin [88]. However, to summarize the physiological implications of the stimulation of the NO pathway on IRP, it does seem possible to conclude that, in spite of IRP-1 activation, the loss of IRP-2, which is highly expressed in macrophages, may be pathophysiologically more relevant because the cytokine-mediated activation of macrophages is accompanied by enhanced Ft synthesis [38,86] and a decreased TfR mRNA content [85,86,93] (Figure 2). The greater affinity of IRP-2 for target IREs [6] may at least help to explain this dominant effect, although down-regulation of IRP-1 protein levels by NO may also play a role [94].

These findings provide novel insights into the molecular mechanisms underlying the enhanced iron retention in macrophages that is characteristic of inflammation. A human pathological correlate is ACD, in which the supply of iron for haemoglobin synthesis to erythroid precursors is restricted as a result of its sequestration in the reticuloendothelial system. As in the case of IRP-2 down-regulation in mouse macrophage cell lines, IRP (IRP-1 plus IRP-2) activity in human monocytes/ macrophages is transiently high shortly after treatment with cytokines or NO donors, but then markedly repressed and accompanied by enhanced Ft content [95]. Moreover, the profound impairment of IRP activity in monocytes of patients with inflammation [95] indicates that *in vitro* treatment with cytokines is sufficiently representative of an *in vivo* inflammatory state. Interestingly, cytokine treatment was not found to decrease IRP activity in the monocytes of haemochromatosis patients [95]. The lack of down-regulation under inflammatory conditions supports previous results [20] indicating a defective control of iron metabolism in the reticuloendothelial cells of these subjects.

XENOBIOTICS

The IRP–IRE machinery is also a possible target of the xenobiotics that undergo redox cycling. Doxorubicin (Dox) is an anthracycline whose clinical use as an anticancer drug is limited by its severe cardiotoxicity. Reconstitution of the alcohol metabolite of Dox with human myocardial cytoplasmic fractions has been shown to impair both the enzymic and IRE-binding activities of IRP-1 [96] in a way that is independent of the presence of free radicals. The effect of Dox has highlighted the fact that exceptions to the model of mutually exclusive IRP functions can exist insofar as Doxol (a secondary alcohol metabolite of Dox) mobilizes iron from the cluster, leading to a consequent loss of aconitase activity, but cluster disassembly is

not accompanied by the acquisition of IRE-binding activity (Figure 1). This damage is mediated by Dox–Fe(II) complexes and reflects oxidative modifications of the –SH residues that are essential for both cluster formation and mRNA binding. Interestingly, the irreversible damage of the homoeostatic mechanism of aconitase/IRP-1 may disrupt cardiac iron homoeostasis and play a significant role in Dox cardiotoxicity [97]. A similar loss of both IRP functions has been reported in cells exposed to a quinone [75] and in the liver of mice treated with dioxin [76] (Figure 1).

GROWTH

De novo synthesis of the iron-containing proteins required to sustain cell proliferation (e.g. ribonucleotide reductase) may limit the availability of iron in the LIP and hence activate IRPs. A high level of IRP binding activity has been detected in mitogenically stimulated cells [98–100] and *in vivo* after partial hepatectomy [101]. It is also noteworthy that high IRP-2 binding activity during liver regeneration was accompanied by increased TfR mRNA levels, but not by reduced Ft expression. It has been shown that increased Ft synthesis under these conditions is due not only to the growth-dependent activation of Ft gene transcription, but also to the impaired efficiency of IRP as a translational repressor, whereas the stabilization of TfR mRNA remained unaffected [102]. Given the preferential binding of IRP-2 to Ft IRE [40], the only possible explanation for these findings is the greater availability of the eIF4F initiation complex, which competes with IRPs for IREs [103], during liver regeneration [104]. This contradiction of the paradigm of the co-ordinated and opposite control of Ft and TfR expression once again demonstrates that the IRE/IRP machinery is part of a complex regulatory system and interacts with other cell regulatory components. This view has recently been supported by the results of a study demonstrating that the oncoproteins regulating the switch between growth and erythroid differentiation affect the IRP-mediated expression of Ft (and ϵ-aminolaevulinate synthase) mRNAs [51].

Other recent findings have shown that IRP-2 induction in growing cells is not only the result of decreased iron levels: specific transcriptional IRP-2 activation has been found in cells overexpressing the c-*myc* oncogene [105], and this was associated with the transcriptional repression of Ft H transcription [105]. This result seems to reinforce the idea that efficient cell multiplication requires an expanded intracellular iron pool obtained by suppressing Ft H synthesis at both the transcriptional and post-transcriptional levels. IRP-2 could therefore be a target of oncogenes as part of a specific design aimed at complying with the special metabolic requirements of cancer cells and thus giving them a selective advantage [106].

The demonstration that the RNA binding activity of both IRP-1 and IRP-2 is induced by protein kinase C phosphorylation [107] suggests that growth stimuli and extracellular stress signals (see above for the effect of phosphorylation pathways on H_2O_2-mediated IRP activation) stimulate binding activity by means of a signal-transduction pathway.

HYPOXIA

The finding that Tf [108] and TfR [14,109,110] are two of the hypoxia-inducible genes has reinforced the hypothesis of a close link between iron metabolism and oxygen homoeostasis. IRPs are themselves subject to regulation by exposure to low oxygen tension, but conflicting results concerning the effects of hypoxia

on IRP activity have been reported: two studies found decreased IRP activity during hypoxia [110,111], whereas another [109] reported a marked increase. Interestingly, the fact that rodent IRPs are easily separated by means of bandshift analysis, allowed Leibold et al. to demonstrate that hypoxia differentially regulates the IRP binding activity in murine cells, leading to a decrease in IRP-1, accompanied by the acquisition of aconitase activity, and an increase in IRP-2 binding [112]. The latter derives from a post-translational mechanism involving protein stability and has an intriguing mechanistic parallel with the hypoxia-induced stabilization of hypoxia-inducible factor 1 (HIF-1) [113].

The functional consequences of the different behaviour of the two IRPs are still obscure, but, if the opposite modulation observed in mouse cells is maintained in human cells, the increased TfR expression and reduced Ft accumulation found in hypoxic cells [109] suggest that IRP-2 up-regulation plays a predominant role. On the other hand, other studies have indicated that TfR up-regulation in hypoxia is transcriptionally determined by HIF-1 [14,110], despite IRP down-regulation [110]. Furthermore, hypoxia-induced IRP inactivation may well account for the increased Ft synthesis found in hypoxic oligodendrocytes [114]. The real meaning of this differential modulation remains to be established, but it may be connected not only with the regulation of the known proteins of iron metabolism, but also with that of other specific IRE-bearing mRNAs necessary for hypoxic adaptation. Nevertheless, this example adds to the list of conditions and agents that specifically modulate the two IRPs in opposite directions.

IMPACT OF THE 'IRP SYSTEM'

Given the fundamental importance of iron homoeostasis for a variety of physiological and pathological processes, it is perhaps not surprising that it is not regulated via a simple linear pathway. IRPs play a pivotal role in iron homoeostasis by post-transcriptionally controlling the proteins involved in the acquisition, storage and transport of this essential metal, and thus add an efficient and rapid regulatory step to the (transcriptionally regulated?) pattern of expression of the individual iron proteins. IRPs may exert this regulatory role at both the cellular and body levels. With regard to intracellular iron homoeostasis, IRPs have presumably evolved in such a way that they simultaneously 'sense' and 'control' cell iron levels, with the latter function being aimed at keeping the LIP at the 'critical' level in order to ensure an adequate iron supply for important cell functions while avoiding the toxic events, such as oxidative stress, associated with an expanded LIP. This critical balance requires biological flexibility and the complexity of the IRP system offers cells an efficient means of tuning such important metabolic pathways (not only iron homoeostasis, but also energy production, growth, etc.). This complexity involves the synthesis of two distinct proteins that are regulated by entirely different mechanisms, have different affinities for a multiplicity of biological targets (IREs are differently recognized by IRPs), and different sensitivities to iron and non-iron stimuli (Table 3). In fact, IRPs may be specific preferential targets of a number of factors other than iron (see above) which, by modulating IRP activity, may modify iron metabolism. This regulation may replace or supplement iron-dependent control in order to fine tune iron homoeostasis in such a way as to allow cells to respond better or adapt to stressful events. In general, the regulation of IRP activity by environmental stimuli takes place at the post-translational level by modifying or degrading the two proteins and is superimposed over a transcriptional regulation that sets the total amount and the IRP-1/IRP-2 ratio of any particular cell.

These observations have clear clinical ramifications (Table 3). In relation to the control of body iron homoeostasis, IRPs may play a pivotal role in determining the amount of iron taken up, stored and transferred by enterocytes, and thus control the key steps of iron absorption (see above). Although the functional role of the IRE in the mRNAs of the recently discovered DMT-1 and ferroportin 1 has not been clearly established, the potential control exerted by IRPs over the main proteins responsible for intestinal iron absorption is an intriguing question that deserves future research. Inappropriate activation of IRPs in enterocytes of patients with haemochromatosis [12] may cause enhanced iron absorption through increased luminal iron uptake (due to enhanced DMT-1 expression) [115] and possibly basolateral transfer (enhanced ferroportin expression?).

Apart from iron deficiency and overload, IRPs are clearly involved in other pathobiological settings (Table 3). In our view, particular attention should be paid to inflammation and malignant cell growth. In this regard, the elucidation of iron metabolism in activated macrophages and further information about the interaction between IRPs and the NO pathway may provide unexpected explanations for clinical phenomena in patients with inflammatory states and the ensuing ACD. Moreover, while the necessity of iron for cell replication is well recognized, the specific mechanisms by which iron-binding proteins modulate cell growth have not been elucidated. Recent evidence indicated that activated oncogenes might favour unremitting growth by co-ordinately altering a gene expression programme controlling iron homoeostasis [105]. Thus further clarification of the interconnection between modulation of IRP activity and cell proliferation may offer insights into the alterations of iron metabolism that accompany cancer development and progression.

In conclusion, the recent insights into the multifaceted regulation and far-reaching controlling effects of IRPs raise more questions than they answer, but will undoubtedly help to pave the way towards a better understanding of the mechanisms underlying the physiology and pathophysiology of iron metabolism. This will also contribute significantly towards the molecular characterization of the different human disorders in which iron is involved.

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