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The role of reactive oxygen and nitrogen species in cellular iron metabolism

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Abstract
The catalytic role of iron in the Haber–Weiss chemistry, which results in propagation of damaging reactive oxygen species (ROS), is well established. In this review, we attempt to summarize the recent evidence showing the reverse: That reactive oxygen and nitrogen species can significantly affect iron metabolism. Their interaction with iron-regulatory proteins (IRPs) seems to be one of the essential mechanisms of influencing iron homeostasis. Iron depletion is known to provoke normal iron uptake via IRPs, superoxide and hydrogen peroxide are supposed to cause unnecessary iron uptake by similar mechanism. Furthermore, ROS are able to release iron from iron-containing molecules. On the contrary, nitric oxide (NO) appears to be involved in cellular defense against the iron-mediated ROS generation probably mainly by inducing iron removal from cells. In addition, NO may attenuate the effect of superoxide by mutual reaction, although the reaction product—peroxynitrite—is capable to produce highly reactive hydroxyl radicals.

Keywords: *Reactive oxygen species, nitric oxide, reactive nitrogen species, iron, iron-regulatory proteins, peroxynitrite*

Abbreviations: *c-acon, cytosolic aconitase, NOS, NO-synthase, IFNγ, interferon γ, iNOS, cytokine-inducible NOS, IRE, iron responsible element, IRP1, IRP2, iron regulatory proteins 1 and 2, LPS, lipopolysacharide, m-acon, mitochondrial aconitase, Ser, serin, SNAP, S-nitroso-N-acetyl-*D,L*-penicilamine, SOD, superoxide dismutase, ROS, reactive oxygen species, Tf–Fe*$_2$*, iron loaded transferrin, TfR1, transferrin receptor 1, TNF-α, tumor necrosis factor α*

Introduction

Iron is the most abundant transition metal in the living organisms and virtually all living cells need it for crucial metabolic pathways. Indeed, oxygen transport, ATP production or DNA-synthesis—all these basic processes require enzymes with iron as a cofactor. On the other hand, free or loosely bound iron is well known to generate free radicals that are responsible for various damages [1]. Therefore, iron has to be firmly incorporated in proteins and its homeostasis must be meticulously controlled.

Cellular iron homeostasis is managed mainly by expression of transferrin receptor 1 (TfR1) and ferritin. The first is responsible for uptake of iron into the cell, while the latter for intracellular iron sequestration and cellular storage [2–4]. Both proteins are regulated by iron regulatory proteins (IRPs).

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Iron regulatory proteins

Expression of many proteins related to iron kinetics and energy metabolism is regulated post-transcriptionally by cytoplasmic proteins called IRPs. There are two IRPs (IRP1 and IRP2) and both are able to bind to the specific sequences in the untranslated regions of mRNA known as iron responsive elements (IREs) [2–4].

Iron entering the cell seems to become firstly a part of poorly defined intracellular labile iron pool. Such pool appears to sense cellular iron stores. Under conditions of iron excess (high iron pool), IRPs do not possess affinity to IREs, on the contrary, when iron is scarce, IRPs bind to IREs. If IRE is localized at the 5′end of mRNA, e.g. in H- and L-ferritin chains, the binding of an IRP to an IRE stops protein synthesis [5]. Conversely, association of an IRP with IREs at the 3′end of TfR1-mRNA protects mRNA against degradation and synthesis of TfR1 is enhanced [6]. As a result, under the condition of iron lack (low iron pool), synthesis of TfR1 is augmented and that of ferritin stopped. When iron is abundant, the synthesis of ferritin and some other proteins involved in energy metabolism is increased, while TfR1 abated.

IRP1 is a bifunctional protein (Figure 1) which can act as an IRP (described above) or as a cytosolic aconitase (c-acon). In the state of iron repletion, this protein contains one specific $[4Fe–4S]^{2+}$ cluster with only three irons ligated directly to cysteines while the fourth (marked as Fe_a) is attached to an inorganic sulfur of the cluster [7,8]. This fourth iron is necessary for enzymatic activity. Such protein is c-acon and cannot bind IREs. When iron is scarce, this fourth iron atom is released, probably provoking the cluster decomposition, and such cluster-free protein obtains the IRE-binding activity and acts as IRP1 [9,10].

IRP2 does not contain the iron–sulfur cluster and lacks the aconitase activity. Like IRP1, IRP2 also binds to IREs in the state of iron deficiency [11]. When iron is in excess, IRP2 undergoes enzymatic degradation [12]. Mitochondrial aconitase (m-acon), an enzyme similar to c-acon, contains the Fe–S cluster as well, but it does not act as an IRP.

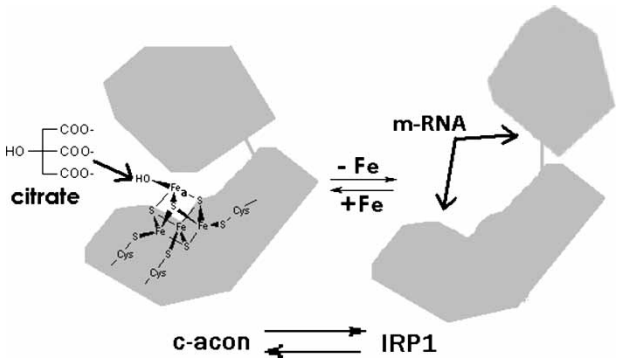

Figure 1. The dual function of cytosolic aconitase/IRP 1. The structure of the cluster and protein according to Beinert and Kennedy [7] and Klausner and Rouault [8], respectively. In iron-repleted status, the protein acts as c-acon, in iron depleted status, iron is released from the cluster, the cluster further decomposes, loses other three atoms of iron and such cluster-free protein acts as IRP1.

Free radicals

As mentioned above, under certain conditions, iron may facilitate formation of free radicals dangerous for the cells. The most potent oxidizing agent in biological systems is hydroxyl radical ($OH^{\cdot}$), which is generated by Haber–Weiss chemistry [1,13]: Superoxide ($O_2^{\cdot-}$) converts ferric ions to ferrous ions and these react with hydrogen peroxide to produce hydroxyl radicals:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

The latter reaction is known as Fenton reaction after the Fenton reagent containing hydrogen peroxide and ferrous salt. The whole process can be summarized in so-called Haber–Weiss reaction. Superoxide reacts with hydrogen peroxide in the presence of iron to produce molecular oxygen, hydroxyl radical and hydroxide anion:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe} O_2 + OH^{\cdot} + OH^{-}$$

For the hydroxyl radical production two conditions have to be fulfilled: The presence of free iron and some reactive oxygen species (ROS). NADPH-oxidase can generate superoxide, its production is generally associated with inflammation caused by neutrophils and macrophages. Many tissues contain xanthine dehydrogenase, which can be easily converted to xanthine oxidase, an enzyme also known to generate superoxide and hydrogen peroxide [13,14]. It should be noted that superoxide is produced also in the respiratory chain of mitochondria, although compartmentization seems to confine superoxide to this organelle [15,16] even though superoxide crossing through the outer mitochondrial membrane by use of a voltage-dependent channel was recently proposed [17].

Superoxide is unstable and it is decomposed either spontaneously or much faster by superoxide dismutases (SODs) into hydrogen peroxide and molecular oxygen:

$$2O_2^{\cdot-} + 2H^{+} \rightarrow O_2 + H_2O_2$$

There are two SODs in the cell: SOD-1, known as Cu, Zn-SOD, it is localized in the cytosol and SOD-2, marked as Mn-SOD, protecting the mitochondrial department.

Hydrogen peroxide is then converted by catalase or glutathione peroxidase into water and molecular oxygen:

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

It is thought that superoxide and hydrogen peroxide are present in the organism physiologically, which is supported by fact that SODs are ubiquitously and abundantly expressed [18]. Therefore, iron—the second member of the Haber–Weiss chemistry—must be meticulously regulated in order to avoid cellular damages. In man, free iron is scarce under physiological conditions. Nearly all iron is sequestered by proteins, in plasma is it bound to transferrin, in various cells it is locked in the structure of ferritin, in the red blood cells iron is firmly incorporated to hemoglobin, in muscles to myoglobin. "Free" iron means iron with at least one iron coordination site open or occupied by a readily dissociable ligand. All formerly mentioned transport and storage proteins tightly complex all six coordination sites of iron and, therefore, such iron cannot produce hydroxyl radical. In contrast, iron bound to ADP, ATP or citrate remains "free", because these molecules are not able to ligand all of its six coordination sites [19].

An imbalance in a cellular redox state, where the ROS production overwhelms anti-oxidant capacity, results in the state termed oxidative stress and recent evidence suggests that oxidative stress is a common denominator in many pathologies [1]. The prevention of cellular damages caused by elevated ROS production can be efficiently achieved with iron-chelating agents and this was demonstrated in numerous papers, including those of our group [20–23].

Superoxide and hydrogen peroxide

In the last decade, it has become obvious that superoxide and hydrogen peroxide may be involved in iron metabolism disturbances. Extracellular hydrogen peroxide stimulates within 60 min IRP1 binding to IREs together with the decrease in c-acon activity, while the withdrawal of stimulus after 15 min does not change the induction of IRP [24–27]. On the contrary, IRP2 is not significantly affected by hydrogen peroxide [27]. Interestingly, the increased IRE–IRP1 binding is not observed with hydrogen peroxide and cytosolic fractions [25,28,29] or intracellulary produced hydrogen peroxide [26], even though the c-acon activity was inhibited in all cases. Blockade of the respiratory chain evokes production of superoxide and interestingly within 2 h it activates the IRP1-binding. The latter effect appears to be mediated via hydrogen peroxide formed from superoxide, as the activation of IRP1 corresponds with emergence of intracellular H_2O_2 [26].

Hydrogen peroxide possibly reacts directly with the [4Fe–4S] cluster of c-acon, releases one iron atom (Fe_a) and subsequently inhibits the c-acon activity but it does not convert c-acon into IRP1 [28,30]. In fact, one Fe atom release from c-acon was observed in yeast with extracellulary added hydrogen peroxide, which is a diffusible molecule and, therefore, easily penetrates the membranes [9]. The hydrogen peroxide diffusion can be observed in mammals as well, but activation of IRP1 with extracellular hydrogen peroxide occurs also when no detectable increase in intracellular hydrogen peroxide was measured [26]. The effect of extracellular hydrogen peroxide in mammal is, therefore, thought not to be related to direct interaction with c-acon/IRP1, but rather via some non-soluble, probably membrane-associated protein with further conduction of the signal inside the cell. The IRP1-activation was proposed to be based on phosphorylation, as its induction by hydrogen peroxide could be inhibited by okadaic acid, which acts as an inhibitor of type I/IIa protein phosphatases [27].

The rapid activation of IRP1 by a short stimulus of extracellular hydrogen peroxide can clarify some ROS-induced damages, especially in the ischemia/reperfusion injury, when ROS, formed by xanthine oxidase, can activate iron uptake inside the cells and contribute to the Haber–Weiss chemistry with its deleterious consequences [13].

The first study examining the results obtained with cell culture experiments in a more complex system was performed by Mueller et al. [31], and indeed, the authors, using the H_2O_2-generating system in perfused rat liver, were able to show the expected increase in IRP1–IRE binding.

Escherichia coli missing cytosolic SOD reveals 8-fold increased levels of free iron as compared to the control bacteria, which clearly demonstrates the role of superoxide radical in iron release. Released iron was shown to be mainly in ferrous state. Surprisingly, most iron did not originate from ferritin but from four cytosolic bacterial enzymes containing Fe–S cluster [32]. Indeed, Fe–S cluster containing aconitase of the same bacteria is reversibly inactivated by superoxide [33]. Cu, Zn-SOD deficient mice manifest reduced enzymatic activity of c-acon, reduced IRE–IRP1 binding—probably due to a decrease in IRP1 synthesis—but no change in IRP2 expression in the liver and noteworthy normal iron metabolism as demonstrated by unchanged levels of ferritin and TfR1 [34]. Experiments in *Drosophila* with silencing and genetic mutation of the cytosolic SOD also showed abated activity of c-acon, but, in contrast, the IRP1–IRE binding was strongly activated [16]. In *Drosophila*, some additional type of regulation can be expected, which is supported by the fact, that *Drosophila* does not possess the vertebrate highly conserved site (Ser 138) of IRP1 for phosphorylation [35]. Similarly, defect in SOD-2 results in the decrease

of m-acon function [16]. Superoxide produced within the mitochondria was further shown to slightly increase the IRP1 binding and to decrease the c-acon activity [15,24]. Extracellulary produced superoxide has no effect on iron and energy metabolism [15,24] as should be expected because superoxide is not a diffusible molecule. Increased IRP1 binding and decreased c-acon activity, caused by mitochondrial superoxide, therefore, most likely reflects its conversion into hydrogen peroxide.

Treatment of rat liver lysates with xanthine oxidase, which produces superoxide a hydrogen peroxide [14,36], manifested decreased IRP–IRE binding but surprisingly it did not affect c-acon [37]. Macrophage cytosolic extracts with added xanthine oxidase showed highly reduced aconitase activity extract but did not exhibit significant effect on IRP1–IRE binding [28].

Similarly to hydrogen peroxide, superoxide also directly reacts with Fe–S clusters of various enzymes, releases iron and impairs their enzymatic activity. But in contrast to hydrogen peroxide, the cluster decomposition of c-acon seems to be more profound as documented by Flint et al. [38], who showed that bacterial enzymes containg the [4Fe–4S] cluster released at least three iron atoms when treated with superoxide. Interestingly, such cluster disintegration appears not to stimulate the IRP1–IRE binding. The degradation of Fe–S cluster is, therefore, probably not sufficient for conversion of c-acon into IRP1, or it is also possible that superoxide can oxidize some free sulfhydryl groups [18] and prevent the IRP–IRE binding. Finally, it should be emphasized, that mammals are probably better equipped with defense mechanisms against ROS, as they do not seem to develop free iron overload. In bacteria, iron, released from Fe–S clusters by superoxide, accelerates DNA damages caused by superoxide or by other ROS [32]. Whether the impairment of iron clusters caused by superoxide (and further plausible consequences, like DNA damages seen in bacteria) is minor in mammal cells, requires further examination. There are certain discrepancies among the various studies, which can be often explained by different methodical approach and this issue is further discussed in the chapter concerning the Nitric oxide (NO).

Additionally, ROS also appear to affect other iron containing molecules. Richardson and Ponka [39] examined cellular iron uptake from transferrin after exposure of cell cultures to ferric ammonium citrate. They found elevated uptake of iron which was not mediated by TfR1. Further investigation documented involvement of superoxide and/or hydrogen peroxide and possibly also hydroxyl radical in release of iron from transferrin and increased transport of iron into the cell [39]. In fact, it is well known that superoxide can release iron from ferritin [40–42]. Withdrawal of iron from iron store and transport molecules can represent an important step in ROS propagation.

Nitric oxide

NO is a free radical with very complex biological function. It is synthesized from L-arginine by three different NO-synthases (NOS). NO has high affinity to metals and many biological effects of NO can be attributed to its chemical interaction with iron: For example activation of guanylyl cyclase appears to be mediated by nitrosylation of heme iron [43] and Fe–S clusters are decomposed after interaction with NO [44].

Many authors have shown that NO reduces c-acon activity and consequently it increases the IRP1–IRE binding [27,28,45–49]. This IRP1–IRE binding is activated slowly and needs hours (3–12 h—variably in different studies, which probably depends on means of NO production and its concentration used) for full effect [24,27,46,50]. The activation seems to be analogous to induction caused by iron depletion and requires the presence of NO during the whole activation period [24,27]. The modulatory effect of NO on IRP1 seems to be stronger than that of iron repletion, as NO has been shown to activate the IRP1–IRE binding also after hemin treatment, which typically diminishes the IRP–IRE binding [48]. NO is a diffusible molecule which easily crosses biological membranes. Published data suggest that NO attacks directly the Fe–S cluster of c-acon. An iron-nitrosyl complex is formed, where NO firstly coordinates the crucial Fe_a atom of the cluster, as indicated by rapid inactivation of c-acon activity [44]. This triggers further allosteric changes of the protein and finally slowly leads to the total decomposition of the cluster and transformation of protein into IRP1 [24,27,44,46,48,51].

Unfortunately, at this point the relative harmony between different studies concerning the interaction of NO and iron metabolism ends and the findings are becoming discrepant (Table I). In our opinion, the likely explanation of such diverse results could involve many aspects of experimental procedures. Following aspects appear to play a role:

- The type of the cell culture used. First, the liver iron metabolism differs in some aspects from other tissues (for details see review [4]). Furthermore, there are some cell cultures (e.g. leukemia L1210 cells and F6 fibroblasts) that do not produce NO and these are suggested to have altered management of iron metabolism as well [46,48].
- The ratio of IRP2/IRP1. IRP2 binds with higher affinity to ferritin H-chain IRE in comparison to other IREs [12] and it seems to have, therefore, principal role in ferritin levels management. The high IRP2 content is present in macrophages and brain, while in most of other tissues it is lower [4,27,45,52].

Table I. Papers concerning more aspects of NO interaction with iron metabolism and/or IRP2.

Authors	Cell culture	NO product	Time [h]	IRP2	TfR1 mRNA	TfR1	Fe uptake	FT mRNAs	FT or FT–H	FT–Fe
Bouton et al. 1998 [60]	RAW 264.7	LPS + IFNγ	1–24	↓	–	–	–	–	–	–
Cairo et al. 2002 [45]	J774A.1*	SIN-1 + SOD	1	↓	–	–	–	–	–	–
Kim and Ponka, 1999 [50]	RAW 264.7	LPS + IFNγ	10	↓	↓	–	–	–	↑	–
		SNAP	10	0	↑	–	–	–	↓	–
Kim and Ponka, 2000 [61]	RAW 264.7	LPS + IFNγ	10	↓	↓	–	↓	0	↑	–
Mulero and Brock, 1999 [64]	J774	LPS + IFNγ	16/24	–	–	↓	↓	–	–	↓
Oria et al. 1995 [54]	K562	SNAP	24	–	↑	↑	–	↑	0	↑
Pantopoulos and Hentze, 1995 [48]	B6.NOS	NOS		↑	↑	–	–	↑	↓ × ↑	–
	RAW 264.7, J774.A1	LPS + IFNγ	12	–	↓	–	–	–	–	–
Phillips et al. 1996 [53]	FT02B	LPS + IFNγ†	4–24	↑	↓	–	–	0	↓	–
		SNAP	4–24	0	↑	–	–	–	–	–
Recalcati et al. 1998 [52]	J774A.1	LPS + IFNγ	4/24	↓	–	–	–	–	↑	–
		SNAP	24	↓	–	–	–	–	↑	–
Richardson et al.1995 [57]	K562	SNAP	18	–	↑	↑	–	–	–	–
Wang et al. 2005 [59]	B6,H1299	SNAP	8	↑	–	–	–	–	–	–

Notes. IRP2, activation of IRP2; FT, ferritin; FT–H, ferritin H-chain; FT–Fe, iron ferritin content; J774A.1, J774, RAW 264.7 are mouse macrophage cell lines; K562 is an erythroleukemic cell line; B6 is a mouse fibroblast cell line; B6.NOS: mouse B6 fibroblasts transfected with NOS; FT02B is rat hepatoma cell line; H1299 is human lung cancer cell line. *Precisely lysate of J774A.1. †In combination with tumor necrosis factor α (TNF-α); SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; IFNγ, interferon γ; NOS, NO-synthase; LPS, lipopolysacharide; SNAP, S-nitroso-*N*-acetyl-D,L-penicilamine; ↓, a decrease; ↑, an increase; 0, no change; ↓ × ↑, cannot be clearly determined.

- Experimental method of NO production and its concentration. To date, numerous different experimental approaches of NO production have been used. The most common is a stimulation of cytokine-inducible NOS (iNOS) by cytokines and lipopolysacharide. However, both agents are known to trigger multiple cellular responses and the results obtained by such treatment may be blunted by not easily eliminable confounders. The combination of LPS/IFNγ/TNF-α is documented to decrease total protein synthesis and may elicit some discrepancies among studies [53]. Other means are the iNOS gene transfection, NO gas, or the use of NO-releasing agents—e.g. the most frequently used SNAP (S-nitroso-*N*-acetyl-D, L-penicilamine) and others (Table I). It should be mentioned that there is some controversy with SNAP as penicilamine may chelate iron, even though it was manifested that SNAP has only low iron chelating properties [54].
- Duration of NO exposure. As mentioned above, IRP1 activation by NO is a slow process.
- The amount of NO produced. The most commonly used method for evaluation of NO levels is the measurement of nitrite. But it seems that nitrite levels may not be a reliable indication of biological active NO as peroxynitrite may significantly augment the nitrite levels [55]
- Redox state of the cell (i.e. the "intracellular redox background"). Under physiological conditions, NO can be interconverted to its redox form—nitrosonium (NO^+) [56]. Nitrosonium is conjectured to nitrate proteins and its effects are implied to be similar to peroxynitrite (see the next section). Nitrosonium does not activate or can even abate the IRP1–IRE binding, but it dramatically decreases the IRP2 binding activity [27,50]. Furthermore, nitrosonium has been shown to decrease the TfR mRNA levels and consequently the iron uptake by cells [55,57].
- Ambient oxygen conditions. Hypoxia is known to regulate IRP1 and IRP2 binding in opposite manner. IRP1–IRE binding appears to be diminished after hypoxia, while that of IRP2 elevated [58].

The first unresolved question is the interaction of NO with the ability of IRP2 to bind IRE. There are papers showing both an increase [27,48,53,59], a decrease [45,50,52,60,61] as well as unchanged binding [50,53]. Some light into this discrepancy gave the papers showing that production of NO by a combination of LPS and IFNγ decreased the binding activity, while SNAP did not [50], and that IRP2 decrease is associated with LPS and IFNγ independently of NO [60]. Beyond the IRP–IRE interaction, NO induces the release of iron differently from various cells [50,62,63]. The iron efflux requires glutathione, and it is probably carried out by an energy-dependent membrane transport mechanism [62]. Stimulated iron release from cells diminishes the intracellular iron pool, but such condition of iron depletion also stimulates the IRPs–IREs binding. This fact can also explain the increased IRP2–IRE binding by NO-treatment in the study by Pantopoulos and Hentze [48], where the cells were transfected with iNOS gene. Such cells produce continually NO and they could have been chronically iron depleted, which resulted in IRP2 stabilization and accumulation. A conclusion cannot be made at this moment, but it appears that NO alone does not change directly the IRP2 affinity for IRE.

It can be even speculated that activation of the IRP–IRE binding in state of iron starvation may be mediated via NO, but such mechanism is very unlikely, even though a regulatory loop between iron metabolism and the NO is known (Figure 2 and the last paragraph of this section).

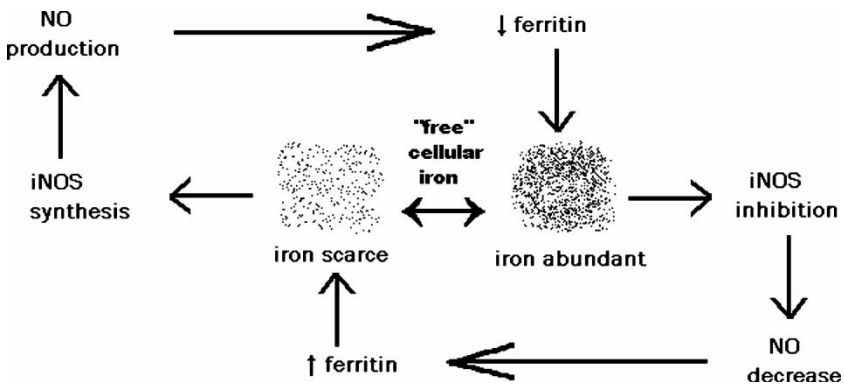

Figure 2. Probable feedback regulation between iron and NO according to Weiss et al. [67] based on their own research. When the level of intracellular iron (probably reflected by labile iron pool) needed for metabolic processes is low, the iNOS induction is provoked. This is followed by an augmented NO production, decrease in ferritin synthesis and an increase in free iron pool. In the reversed situation, when intracellular iron is abundant, iNOS is not stimulated and the resulting low NO production results to an increase in ferritin synthesis and ultimately to abated intracellular iron.

The increased IRPs–IRE binding stabilizes TfR1 mRNA and should, therefore, lead to augmented iron uptake. Studies concerning levels of TfR1 again appear to be divergent, but when the experiments using stimulation by LPS + IFNγ [48,50,53,61] are separated from other means of NO production [48,50,53,54,57], again a possible explanation arises: LPS with IFNγ may overcome the effect of NO and thus decrease the levels of TfR1 by a NO-independent manner. In agreement with this proposition, the increase in TfR1 density has been documented after NO treatment [54,57]. The only study reporting a decrease in TfR1 density [64] proved that this decrease was NO-independent and may be linked with LPS + IFNγ. Nevertheless, an expected raise in iron uptake from iron loaded transferrin ($Tf–Fe_2$) has not been observed and surprisingly the contrary process has been discovered in various cell cultures [55,61,63,64]. The explanation of this unexpected process is hard to make at the moment. Watts and Richardson [63] proposed that reduced Fe uptake results, at least partly, from a decrease in levels of ATP, which is required for $Tf–Fe_2$–TfR1-mediated endocytosis. The reduced ATP levels may be caused by inhibition of some enzymes (mainly m-acon) of respiratory chain by NO. NO did not decrease the Tf binding to TfR1 [63], but some other NO interaction with iron uptake process cannot be precluded.

NO appears to elevate ferritin mRNA levels probably by some unknown pretranslational mechanism [48,54], although in LPS + IFNγ treated cells no change in ferritin m-RNAs was reported [53,61]. Furthermore, both an increase [48,50,52,61] and a decrease in ferritin (total or H-chain) synthesis [48,49,50,53] have been described. But again, when the experiments using LPS + IFNγ are separated, NO seems to elevate ferritin m-RNA levels, but on the other hand (in the agreement with IRP–IRE theory) to decrease ferritin synthesis. It seems that LPS + IFNγ abate ferritin m-RNAs levels but paradoxically increase ferritin levels in non-hepatic cells. This statement is in harmony with known findings that ferritin synthesis is augmented in inflammation [65]. In the hepatic cells, however, an alternative iron metabolism control is expected.

In various cell cultures, the incorporation of iron from $Tf–Fe_2$ into ferritin was reduced differently after the stimulation with NO [62–64], although Oria et al. [54] reported the opposite effect in K562 cells.

For many years it was believed that NO induces iron release from ferritin [41]. Recently, however, Watts and Richardson [62] did not observe such phenomenon in cell lysates. Today, this discrepancy can be explained, as the former group used an agent afterwards shown to release NO^+ [41], while the latter a NO-releasing agent [62]. Today, NO seems to intercept iron before it reaches ferritin [62] and it can be suggested that NO does not directly interact with iron stores within ferritin but it can by some indirect mechanism mobilize iron from ferritin. Whether such mechanism simply involves an adaptation to low cellular iron levels after increased iron efflux deserves further investigation.

NO may be a biological messenger used by cells to prevent intracellular damages caused by ROS. As tumours have been shown to contain more TfR1 receptors and generally they have more pronounced iron demands [66], by elevating iron release from the cell and inhibition of respiratory chain, NO can inhibit ROS-damages and suppress cell proliferation. This is proposed to represent the defense mechanism of activated macrophages against tumour cells and pathogens.

It was demonstrated that mRNA levels of iNOS are profoundly increased in iron deficiency and reduced in the opposite condition [67]. In agreement, ferrous ions were shown to abate the iNOS-stimulated synthesis of NO and iron chelator *o*-phenantroline augmented NO-synthesis in cell cultures. Interestingly, the same group also found that iNOS-mediated NO-production is not influenced by iron in rat astrocytoma C6 cell line, supporting the assumption that NO-signaling may be controlled differently among various cells [68].

Peroxynitrite

A new interesting question arises with the possible involvement of peroxynitrite in iron metabolism. Peroxynitrite ($ONOO^-$) is the reaction product of superoxide and NO [69] and it is considered to be a strong oxidant and a major cytotoxic agent produced during inflammation, sepsis and ischemia-reperfusion. Peroxynitrite was documented to nitrate tyrosine residues of proteins [70] and in fact nitrosylation of c-acon/IRP1 was demonstrated *in vitro* [44,47]. In addition, peroxynitrite decreases c-acon activity *in vitro* more rapidly than NO and it has been shown to slightly enhance the IRP1-binding [28,44,47] or not change [27] or slightly decrease it [57]. When slight stimulation of IRP1–IRE was observed, this activation with NO and superoxide was far less potent, when compared to a situation when macrophages were stimulated to produce NO only [47]. When peroxynitrite was produced extracellulary, c-acon was slightly inhibited and some IRP1 activation was present. NO, together with superoxide (produced within mitochondria), evoked pronounced inhibition of both aconitases and, paradoxically, it significantly stimulated the IRP1–IRE binding [24].

It is suggested that peroxynitrite is a hardly diffusible molecule and the likely explanation of some increase in IRP1–IRE binding is due to the readily diffusible NO. Such proposal is supported by the fact that peroxynitrite did not stimulate IRP1

binding in macrophage cell extract, but if SOD was added, stimulation of IRP1 binding occurred [28]. The paper of Cairo et al.[45] seemingly contradicts those findings. In this study, a lower concentration of peroxynitrite-producing agent was used and in this case a significant IRP1 activation was seen. But when a higher concentration was employed, no increase in IRP1 activity was observed in concordance to previous data [45]. Similar results were reported with recombinant IRP1 [46]. Nitration and/or oxidation seem to take place in a situation when peroxynitrite is produced in sufficient quantity. It may be possible that low concentration of peroxynitrite cannot modify IRP1 more than NO alone, i.e. it removes only Fe–S cluster of c-acon and transforms it into IRP1. The decrease in c-acon activity but no significant increase in IRP1 binding caused by higher concentrations of peroxynitrite can be explained by spheric hindrance of nitro group, oxidation of free sulfhydryl groups and/or by incomplete decomposition of the Fe–S cluster of c-acon/IRP1. Chemical modification evoked by higher concentration of peroxinitrite on c-acon/IRP1 can be confirmed by the lack of recovery of c-acon activity after treatment with ferrous sulfate and cysteine, which have been proven to be able to reconstruct the c-acon cluster and its enzymatic function [45]. Activation of IRP1 binding with mitochondrial production of peroxynitrite may depend not only on NO, the potential role of superoxide and/or hydrogen peroxide cannot be omitted. In addition, peroxinitrite is known to decrease IRP-2 binding activity even in presence of an iron chelator [45].

Like superoxide and NO^+, peroxynitrite was also demonstrated to mobilize iron from ferritin *in vitro*, but surprisingly, its effect was less pronounced than that of either superoxide or NO^+[41]. In addition, peroxynitrite can also interact with iron uptake from transferrin; a decrease in TfR mRNA and in Fe-uptake was observed in various cell cultures [55,57].

It may be implied that, in macrophages during inflammatory processes (when superoxide is generated), the nitration of c-acon can on the one hand protect cells against the well-known consequences of iron excess [47] but on the other hand it cannot be omitted that peroxynitrite decomposes ($t_{1/2} = 0.5$ s) into hydroxyl and $NO_2^{\cdot}$ radicals [51]. These radicals may reduce cell viability and some effects of peroxynitrite can, therefore, be ascribed to their toxicity [57].

Conclusion

Correct iron metabolism is essential for maintenance of cellular homeostasis and both iron deficiency and iron overload are responsible for a number of even life-threatening pathologies. Reactive oxygen and nitrogen species are also abundant in cells under both physiological and pathological conditions and better understanding of the role of RONS in cellular iron trafficking is, therefore, of crucial importance.

This review aimed to show that ROS cause damage not only via the Haber–Weiss chemistry, but they can themselves affect the control of iron metabolism, provoke iron loading into the cells, mobilize iron from proteins, probably most easily from Fe–S clusters and, therefore, further aggravate the vicious circle of Fe/ROS-induced cellular damage.

On the contrary, NO appears to be involved in cellular protection against ROS. NO apparently protects cells by inhibition of exceeding iron uptake into the cells and by removing of iron from the cells.

Peroxynitrite also affects the cellular iron metabolism, but its impact seems to be weaker than that of other reactive species. On the one hand, it can be suggested that NO can protect cells against superoxide by reaction with it, but on the other hand, it has to be underlined that the reaction product—peroxynitrite—can be decomposed into highly toxic hydroxyl radical.

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