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Sulfide increases labile iron pool in RD4 cells

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Abstract A linkage between sulfur and iron metabolism has been suggested since sulfide has the ability to release iron from ferritin in the presence of iron acceptors in vitro. Nevertheless, this linkage is still lacking evidence in vivo as well as in cellular models. In this study we have treated human RD4 skeletal muscle cells with sodium sulfide and measured the level of the labile iron pool (LIP) as well as the intracellular sulfide concentration. We have also detected the amounts of L-ferritin protein as well as the iron regulatory protein 2 (IRP2). The sulfide treatment resulted in a 100% increase in the amount of LIP after 1 and 2 h. We also found that the raise of the LIP levels was coupled to an elevation of the amounts of intracellular sulfide that increased by 60%. The bioavailability of the released iron was confirmed by a 100% increase in L-ferritin protein as well as a 60% decrease of the IRP2 protein levels. These results suggest that there is a linkage between sulfur metabolism and intracellular iron regulation in mammalian cells.

Keywords Sulfide · Ferritin · Labile iron pool · Iron regulation

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Abbreviations

IRE	Iron responsive element
IRP	Iron regulatory protein
LIP	Labile iron pool
TfR	Transferrin receptor

Introduction

Iron is essential for all living organisms from bacteria to man for a broad variety of physiological functions, ranging from oxygen transport to DNA synthesis. However, iron can also be toxic due to its ability to catalyze the formation of highly reactive hydroxyl radicals through the Fenton reaction. The hydroxyl radicals can cause severe damage to vital biological compounds such as lipids, proteins and DNA. Therefore, it is of the greatest importance that the iron homeostasis is kept under a tight regulation (For recent reviews, see (Crickton et al.2002; Hentze et al. 2004; Andrews 2005)). In biological systems, the appropriate iron balance is kept by regulatory mechanisms enabling the cell to meet the iron demand, but to prevent an iron overload. The cytosolic iron regulatory proteins 1 and 2 (IRP1 and IRP2, respectively) regulate the levels of several key proteins involved in the cellular iron homeostasis through binding to iron responsive elements (IRE's) in the untranslated regions of their respective mRNAs. Proteins regulated by IRP's include the iron uptake

protein transferrin receptor (TfR) and the iron storage protein ferritin. The activities of IRP1 and levels of IRP2 are primarily dependent on the levels of the cytosolic iron referred to as the labile iron pool (LIP).

The ferritins are highly conserved and essential elements in the iron homeostasis in most organisms ranging from animals to plants. Ferritin has the capacity of storing substantial amounts of iron (4500 atoms/molecule) (Harrison and Arosio 1996). It is a multimeric protein, consisting of 24 polypeptides of both heavy ferritin (H-ferritin) and light ferritin (L-ferritin) chains that share the sequence homology but are derived from two different genes (Jain et al. 1985; Chou et al. 1986), forming a large cavity in which to store the iron atoms in a bioavailable non-toxic manner. Conversely, it is not fully understood how the sequestered iron is released from the ferritin core.

Sulfide is a strong reducing agent, produced by a variety of enzymes within the cell (Ubuka 2002). Cassanelli and Moulis have shown that sulfide has the ability to release iron from ferritin in the presence of iron acceptors in vitro (Cassanelli and Moulis 2001). Consequently, we intended to further study the role of sulfide in the mammalian iron homeostasis, treating human RD4 skeletal muscle cells with sodium sulfide in order to investigate whether it was possible to release iron from ferritin and make it bioavailable in a cellular model. We demonstrate that sulfide treatment of RD4 cells results in markedly elevated levels of LIP and intracellular sulfide, increased ferritin protein levels as well as decreased levels of IRP2 protein.

Materials and methods

RD4 cells were maintained at 37°C under an atmosphere of 5% CO_2 in Dulbeccos' modified Eagle's medium (DMEM) with Glutamax II and 4.5 g/l D-glucose, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. 80% confluent cells were treated for 1, 2, and 3 h respectively with freshly prepared 5 mM sodium sulfide (Sigma) in 100 mM NaOH.

The measurements of the cytosolic labile iron pool (LIP) were carried out as described (Epszteijn et al. 1997). In brief; 4×10^6 cells (in PBS) were loaded with 100 nM calcein AM (Molecular probes) followed by 15 min incubation at 37°C. After washing with PBS (to remove non-internalized calcein), the cells were transferred to a stirred cuvette and the basal calcein fluorescence measured (ex 488 nm, em 517 nm). Then, the fluorescence of the calcein was de-quenched by the addition of salicylaldehyde isonicotionyl hydrazone (SIH), and the increased fluorescence monitored until a steady signal was obtained (300 s).

The intracellular sulfide levels were measured as described (Siegel 1965). In brief; 500 µl of a cysteine solution (0.5 mM of cysteine in 20 mM Tris pH 8.0, 2 mM DTT) were mixed with 500 µg of cell lysate in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 µg/ml leupeptin) and then incubated for 2.5 h at 30°C in vials sealed with rubber caps followed by the addition of 100 µl of DPD-reagent (0.02 M *N*, *N*-dimetyl-*p*-phenylenediamine sulfate (DPD) in 7.2 N HCl) and 100 µl of $FeCl_3$-solution (0.03 M $FeCl_3$ in 1.2 N HCl) were added by Hamilton syringe followed by 20 min incubation at room temperature. The rubber-caps were removed and the samples were centrifuged at 10000 rpm for 10 min. Finally, absorbance measured at 650 nm.

For Western blotting analysis 20 µg of total extracts were prepared in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 µg/ml leupeptin) were resolved by SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and probed with antibodies corresponding to L-ferritin, IRP2 and tubulin at 1:1000 dilution. Tubulin was used as an internal standard. Densitometric analysis was performed using the Image Gauge software (Fuji).

Results

First, we intended to investigate whether the sulfide-mediated iron release that Cassanelli and Moulis have shown in vitro (Cassanelli and Moulis 2001) would be achievable in the human skeletal muscle cell line RD4. The cells were treated with 5 mM of sodium sulfide (in 100 mM of NaOH) for 1, 2 and 3 h respectively (Fig. 1A). After 1 h of sulfide treatment,

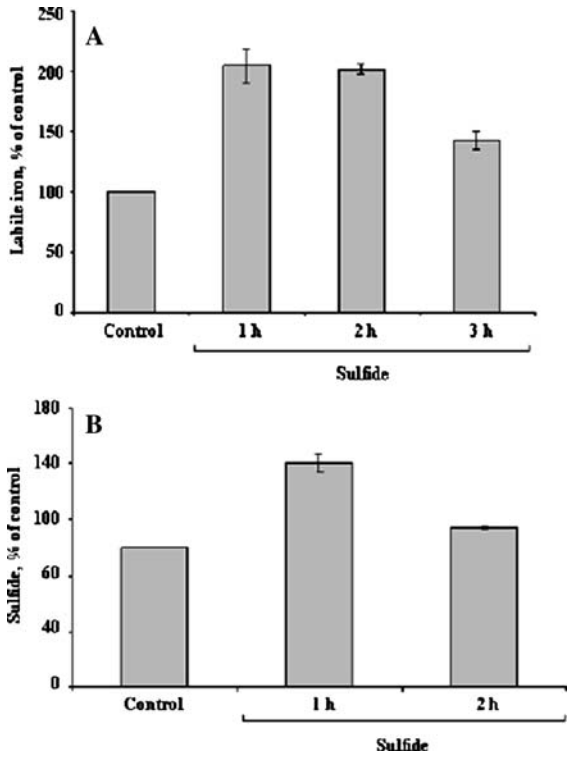

Fig. 1 Amount of LIP in RD4 cells treated with 5 mM of sodium sulfide for 1, 2, 3 h, respectively as percentage of control (**A**). Amount of intracellular sulfide in RD4 cells treated with 5 mM of sodium sulfide for 1 and 2 h, respectively, as percentage of control (**B**). The results are means of three independent experiments ± SD

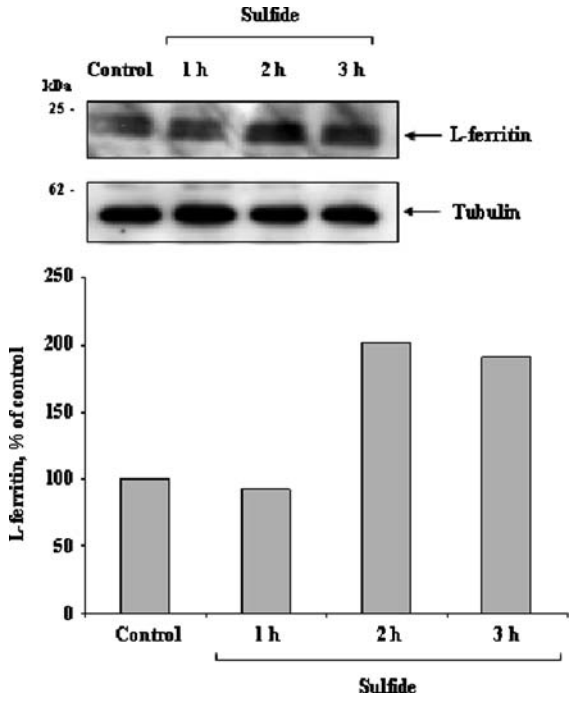

Fig. 2 Amount of L-ferritin protein in RD4 cells treated with 5 mM of sodium sulfide for 1, 2 and 3 h respectively as percentage of control. The results are representatives of those obtained in four independent experiments

we detected a 100% increase in the LIP levels as compared to control cells. After 2 h of sulfide treatment, we still could monitor a 100% raise of the LIP levels, but after 3 h we observed that the increase was lower than 50% as compared to control cells. To verify that the increase in LIP levels is coupled to the increased sulfide levels within the cell, we measured the intracellular sulfide levels (Fig. 1B). After 1 h of sulfide treatment, we detected a 60% increase in the amount of intracellular sulfide as compared to control cells. After 2 h the increase in intracellular sulfide was 20% as compared to control cells.

To investigate the bioavailability as well as the functional consequences of the elevated level of LIP, we studied the expression of L-ferritin, a protein regulated by intracellular iron (Fig. 2). After 1 h of sulfide treatment we did not notice any changes in the L-ferritin protein levels. After 2 h we measured a 100% increase in L-ferritin protein expression that was continuous over 3 h of treatment according to Western blotting.

To further examine the bioavailability of the increased amount of LIP, we intended to investigate if the raised LIP levels had an impact on the protein levels of IRP2. By using Western blotting analysis, we observed that after 1 h of sulfide treatment, no significant changes in the IRP2 expression were found (Fig. 3). After 2 h of sulfide treatment we detected a slight decrease in the IRP2 levels followed by a 50% decrease after 3 h (Fig. 3).

Discussion

Systematic iron homeostasis is essentially a closed system, regulated by the activities of IRP1 and IRP2 through their binding to IRE's in the untranslated regions of the ferritin and TfR mRNA's. IRP's function as sensors of intracellular iron by regulating the expression of TfR and ferritin according to iron levels (review: Hentze et al. 2004). When there is a lack of iron within the cell, the expression of the

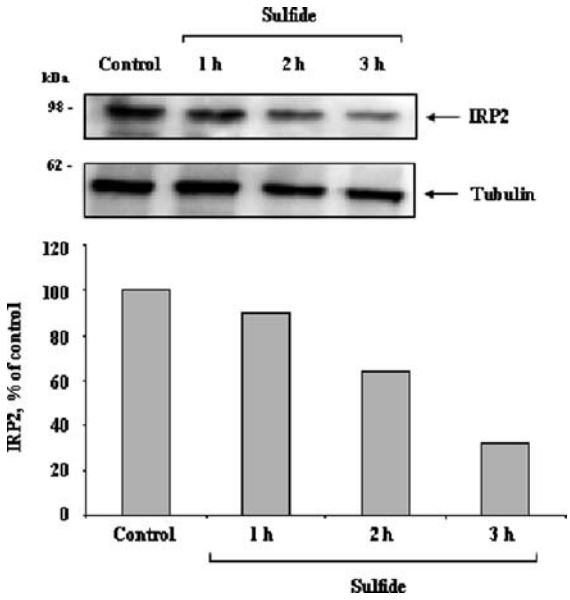

Fig. 3 Amount of IRP2 protein in RD4 cells treated with 5 mM of sodium sulfide for 1, 2 and 3 h, respectively as percentage of control. These results are representatives of those obtained in four independent experiments

ferritin protein decreases and the expression of the TfR increases. On the other hand, when there is an overload of iron within the cell the expression of the ferritin protein increases and the expression of the TfR receptor decreases.

Cassanelli and Moulis have shown the possibility to induce a substantial iron release from ferritin in vitro and our first approach was to study if such a sulfide-mediated iron release could be an option in a cellular model system (Cassanelli and Moulis 2001). We have used the human skeletal muscle cell line RD4 that expresses all proteins essential for the intracellular iron homeostasis at adequate levels. Considering our results, we assume that sulfide releases iron in a straightforward manner from ferritin in RD4 cells, demonstrated by the rapidly elevated levels of LIP that follow the increase in intracellular sulfide levels. It has been shown that sulfide can reach the ferritin core at low, high or neutral pH (St Pierre et al. 1993; Douglas et al. 1995; Cassanelli and Moulis 2001), making it likely to occur at physiological conditions in the cell. It has also been shown that sulfide, if it reaches the ferritin core, has the ability of reducing ferric ions and solubilize ferrous salts (St. Pierre et al. 1993; Douglas et al. 1995), which indicates that a sulfide induced iron release would be possible inside the cytosol.

There are several physiological sources of intracellular sulfide. First, sulfide itself has a role as a second messenger (Ubuka 2002). For instance, H_2S is present in the brain at relatively high concentrations (50–160 μM), produced by cytathione β-synthase and cytathione γ-synthase (Abe and Kimura 1996). Another player in the context of sulfide and iron metabolism is the cysteine-desulfurase IscS, a proposed donor of inorganic sulfur for the [4Fe–4S]-cluster biosynthesis (Zheng et al. 1993). However, if IscS is a link between sulfide and the iron metabolism there has to be a reduction step involved, reducing the inorganic sulfur donated by IscS to sulfide that can later eventually induce an iron-release from ferritin. We have preliminary data showing that the total amount of IscS protein in RD4 cells increases over time during sodium sulfide treatment (J.H. & T.L., unpublished data). In spite of this, the involvement of IscS can be a consequence of the iron overload, since elevated levels of intracellular iron would inactivate the IRP's and therefore raise the demand for [4Fe–4S]-cluster biosynthesis that requires more inorganic sulfur provided by IscS.

Several studies have shown that sulfide on its own, lacking an iron acceptor, does not function as an iron releasing agent from ferritin (S.t Pierre et al. 1993; Douglas et al. 1995; Cassanelli and Moulis 2001). In an adequate cell system, like in the RD4 cells used in this study, free iron levels are kept low by various chelators, and thus, the iron acceptors are present in cells. Another candidate for being such an intracellular iron acceptor might be the iron–sulfur cluster scaffold protein IscU that is involved in iron acquisition during iron–sulfur cluster assembly (Agar et al. 2000). We further demonstrate that the released iron is bioavailable according to the elevated levels of L-ferritin protein as well as the decreased levels of IRP2 protein.

We cannot neglect that we have exposed the cells to high concentrations of sodium sulfide that might have non-specific effects in cells. On the other hand, the physiological intracellular sulfide concentrations are 50–160 μM (Abe and Kimura 1996) and in our measurements the intracellular sulfide concentration was elevated by a modest 60% during the treatment. This, taken together with results by other authors

makes it likely that sulfide has a role of its own in the mammalian iron regulation.

To conclude, we have demonstrated that: (i) Treatment of RD4 cells with sulfide results in a rapid and substantial increase in LIP (ii) The increase in LIP is accompanied by an increase in the intracellular sulfide levels (iii) The elevated LIP and sulfide levels are accompanied by a decrease in IRP2 protein levels, showing that the released iron is bioavailable (iv) The protein levels of L-ferritin are increased in RD4 cells treated with sulfide.

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