Haem precursor δ -aminolaevulinic acid induces activation of the cytosolic iron regulatory protein 1

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Control of cellular iron homeostasis is performed by iron regulatory protein 1 (IRP1) through post-transcriptional modifications. This protein is sensitive to intracellular iron availability, being activated at low iron levels and inactivated at high iron levels, conditions that signal the increased expression of the transferrin receptor or of ferritin respectively. IRP1 is known to be activated by some oxidants such as H_2O_2 and NO. δ -Aminolaevulinic acid (ALA), previously found to produce reactive oxygen species and a carbon-centred radical, to release iron from ferritin, and to increase rat liver and brain non-haem iron and ferritin, was investigated for its effects on IRP1 activity

in cultured hamster pulmonary fibroblasts. We have found that 1–2 mM ALA produced a 2–3-fold activation of IRP. On incubation with 1–4 mM succinylacetone methyl ester, a potent ALA dehydratase inhibitor, a 3–4-fold activation of the protein was observed, accompanied by a 40% increase in the intracellular ALA concentration. When cells were incubated in the presence of ALA or succinylacetone methyl ester, *N*-acetylcysteine inhibited IRP1 activation, suggesting that the observed effect is mediated by an oxidative process. We surmise that ALA-induced IRP1 activation might act as a co-sensor of iron homeostasis.

INTRODUCTION

Studies on iron uptake and storage have shown a tightly regulated system for preventing toxicity from excess iron, because reduced iron species and oxygen can interact to form reactive oxygen species, including superoxide, H_2O_2 and the highly reactive hydroxyl radical. Studies from several laboratories have led to insights into the intracellular regulation of iron metabolism [1–3]. When intracellular iron supplies are adequate, the mRNA for transferrin receptor (TfR) is degraded rapidly, resulting in a decrease in its levels. When intracellular iron levels are low, TfR biosynthesis increases, whereas the rate of mRNA translation of both ferritin and erythrocyte δ -aminolaevulinic acid synthase (eALAS), the rate-limiting enzyme in haem biosynthesis, decreases. These regulatory changes are mediated by a post-transcriptional regulatory system that involves binding of iron regulatory protein 1 (IRP1) to stem-loop structures in the mRNA known as iron-responsive elements (IREs). Functional IREs are found in the 5' untranslated region of the genes that encode ferritin and eALAS and in the 3' untranslated region of the gene encoding TfR [1,4]. IRP1 activation and inactivation influence its capacity to bind TfR 3'-IRE mRNA and ferritin or eALAS 5'-IRE mRNA respectively. eALAS transcription is known to be regulated by IRP1 [5], but not the so-called housekeeping ALAS, present in all mammalian tissues other than erythrocyte cells. eALAS and ALAS genes are located on chromosomes X and 3 respectively [6].

IRP1 displays two functions, either enzymic aconitase activity or RNA-binding activity, depending on the iron load [4]. Changes in IRP1 activity occur without significant changes in the amounts of immunologically detectable protein [7,8]. Indeed, IRP1 function is due to the disassembly and reassembly of its [4Fe–4S] cluster [9]. The apoprotein, with complete disintegration of the cluster, is the IRE-binding form. Another related protein involved in the control of iron homeostasis has recently been described [10,11]. This protein, now known as IRP2, also functions as a

translational repressor by binding to IRE-containing RNA species. However, unlike IRP1, IRP2 does not have aconitase activity and its cytoplasmic levels are modulated by iron [12–14].

The central role of the cluster in IRP1 function has been supported by studies showing that mutations of cysteine residues involved in cluster ligation produce the apoprotein, which binds RNA constitutively regardless of the iron status of the cell. The actual mechanism of cluster disintegration is not yet known. Assembly of the cluster *in vitro* occurs spontaneously if the required ingredients, Fe, sulphide ions and a thiol reductant, are present. In turn, disassembly is accomplished *in vitro* by oxidation of the cluster with O_2 or ferricyanide [15,16].

More recently it was shown that IRP1 is rapidly activated by exposure of cells to H_2O_2 [17,18] and NO [19,20]. Activation by H_2O_2 was not seen *in vitro*. It depends on cell integrity, showing that there is no direct interaction between H_2O_2 and the cluster.

On the basis of the above findings, Rouault and Klausner [9] suggested that IRP1 simultaneously senses levels of oxidants and iron. They infer that the holoprotein can function as a sensor of oxidants, whereas the apoprotein can function as a sensor of intracellular iron levels. Thus iron levels might have little impact on the process of cluster disassembly, determining instead whether the cluster can be reassembled and thereby controlling the transition from apoprotein to holoprotein.

In the past few years it has been demonstrated that δ -aminolaevulinic acid (ALA), formed in the rate-limiting step of the haem biosynthetic pathway, can act as a pro-oxidant both *in vitro* and *in vivo* [21–24]. ALA was shown to undergo enolization and subsequent oxidation catalysed by iron complexes at pH 7–8, with formation of reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 and $\cdot OH$) and ALA enolyl radical (structure II in Scheme 1). *In vitro*, both ALA-generated $O_2^{\cdot-}$ and the carbon-centred ALA enolyl radical promoted iron release from ferritin [24]. *In vivo*, ALA treatment significantly increased the total non-haem iron concentration in rat liver [24] and cerebral cortex, and ferritin levels in brain [23]. These results suggest that ALA by itself might be

Abbreviations used: ALA, δ -aminolaevulinic acid; ALAd, ALA dehydratase; eALAS, erythrocyte ALA synthase; IRE, iron-responsive element; IRP1, iron regulatory protein 1; NAC, *N*-acetylcysteine; SAME, succinylacetone methyl ester; SOD, superoxide dismutase; TfR, transferrin receptor.

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Scheme 1 Oxidation of ALA

Iron-catalysed aerobic oxidation of the enolic form (**I**) of ALA to 4,5-dioxovaleric acid (DOVA) with generation of reactive oxygen species and a carbon-centred ALA enolyl radical (**II**).

directly involved in the regulation of iron uptake and storage. In this respect, Minotti [25] raised the possibility that ALA-generated $\text{O}_2^{\bullet -}$ or the ALA enolyl radical might be important in the mobilization of iron embedded in the endoplasmic reticulum for iron-requiring cell functions.

In the present work we study the effect of ALA on IRP1 activation. The haem biosynthetic pathway is considered to be the major consumer of intracellular iron in mammalian cells, with ALA biosynthesis being the rate-limiting step. We here hypothesize that ALA oxidative behaviour, at least when ALA is overproduced inside the cells, could have an important role as an intracellular regulator of iron metabolism by means of IRP1 activation.

EXPERIMENTAL

Materials

N-Acetylcysteine (NAC), ALA, δ -aminolaevulinic acid dehydratase (ALAd), Bromophenol Blue, catalase, dithiothreitol, EDTA, Hepes, heparin, 2-mercaptoethanol, Nonidet P40, succinylacetone, sucrose, superoxide dismutase (SOD) and Tris were purchased from Sigma. Acrylamide and bisacrylamide were from Bio-Rad. Dulbecco's modified Eagle's cell culture medium was from Gibco. Acetonitrile and *o*-phthalaldehyde were from Merck. Fetal calf serum was from Cultilab. *N*-Methyl-*N*-nitroso-4'-toluene sulphonamide (Diazald®) was from Aldrich. [α - ^{32}P]UTP was from Amersham. All other reagents were of analytical grade and the water was deionized by a Labconco Water system.

Cells

The experiments were performed on M8 cells, an established line of V79 Chinese hamster pulmonary fibroblasts. Human erythroleukaemic K562, rat hepatocyte BRL and glioma C6 cell lines

were also used. Cells were routinely grown in Dulbecco's modified Eagle's medium, pH 7.0, supplemented with 10 % (v/v) fetal calf serum, 472 i.u./ml penicillin and 94 mg/ml streptomycin. Cells were incubated in a humidified air/ CO_2 (19:1) atmosphere at 37 °C. Cell treatment was performed by addition of the various reagents to the cell medium at the indicated concentrations and times shown in the legends to the figures.

Cell extract

Cells (10^6) were washed twice with PBS buffer, pH 7.2, then detached from the flask by addition of 1 ml of 1 mM EDTA in PBS and collected in a 1.5 ml Eppendorf tube. The cell pellet, obtained by centrifugation at 12000 *g* for 5 min, was resuspended with 20 μl of Munro's buffer [MB; 10 mM Hepes/3 mM MgCl_2 /40 mM KCl/5 % (v/v) glycerol/1 mM dithiothreitol (pH 7.6)] [26] containing 0.2 % (v/v) Nonidet P40 and homogenized by resuspension with a micropipette. After a second addition of 40 μl of MB, the mixture was centrifuged at 12000 *g* for 15 min. The supernatant was used for the assays immediately or within 1 week of storage at -20°C and its protein content was determined by the method of Bradford [27].

RNA probe and binding to IRP1

The plasmid pSPT-fer [28], kindly provided by Dr. Lukas Kühn (Lausanne, Switzerland), contains the IRE sequence for human ferritin H-chain mRNA and the T7 and SP6 promoters. The ^{32}P -labelled probe was synthesized with a kit from Promega and [^{32}P]UTP. The binding reaction was performed as described [29]. The following ingredients were added to an Eppendorf tube: cell extract (2 μg of protein diluted in 4 μl of MB), 4 μl of MB, with or without 4 % (v/v) 2-mercaptoethanol (the addition of 2-mercaptoethanol was to produce IRP1 activation *in vitro*), 0.1 ng of the [^{32}P]RNA probe (15000 c.p.m.) in 4 μl of MB, 1 unit of

RNase T1 in 4 μ l of MB (to destroy unprotected RNA not bound to IRP1), 4 μ l of MB containing 50 mg/ml of heparin and 4 μ l of sample buffer [30 mM Tris/HCl (pH 7.5)/40% (w/v) sucrose/0.2% Bromophenol Blue]. Each addition was followed by brief centrifugation to mix the reagents. The samples were then applied to the wells of polyacrylamide gel for electrophoresis.

Band-shift electrophoresis

The method followed was essentially that described by Konarska and Sharp [30]. The gel mixture contained 6% (w/v) polyacrylamide/bisacrylamide (29:1) in 0.3 $\times$ Tris/borate/EDTA buffer [89 mM Tris/borate/10 mM EDTA (pH 8.0)]. The gel plate was 12 cm $\times$ 12 cm, 1 mm thick, contained 12–15 wells of 6 mm width and was run vertically for 1 h at 200 V. At the end of the run, the gel was transferred to a piece of 3MM paper, covered by a PVC film and dried in a Bio-Rad gel drier (Model 583) at 80 $^{\circ}$ C for 45 min. The gel was exposed for 48 h to an autoradiographic X-OMAT-K Kodak film, before development. The band intensities on the autoradiographic film were determined by a Shimadzu CS9000 densitometer.

ALA determination

ALA assays were performed by HPLC with electrochemical detection. Sample (standard ALA solution or biological sample) (40 μ l) was placed in a small conical vessel, to which 5.0 μ l of *o*-phthalaldehyde reagent (36 mM) was added. This solution was vortex-mixed and a 20.0 μ l aliquot injected into an HPLC system after incubation for 1 min.

ALA level determinations were performed in the same cell extracts used for the band-shift assay, diluted to 0.5 mg of protein per ml, or in the extracellular medium. Samples were frozen at -20° C for no more than 2–3 days.

Chromatographic conditions

Isocratic liquid determinations with ALA-*o*-phthalaldehyde derivatives were performed with a chromatograph equipped with an LC10AD pump coupled to an LECD 6A electrochemical detector from Shimadzu Corp. (Kyoto, Japan). The detection working electrode was maintained at 0.6 V with respect to Ag/AgCl and its signal delivered to a 386 ASA computer with data collection and handling provided by Scientific Software (San Ramon, CA, U.S.A.). For the ALA assay a reverse-phase C₁₈ Novapak column (15.0 cm $\times$ 3.9 mm internal diam.) from Waters Corp. (Milford, MA, U.S.A.) and 0.05 M phosphate buffer, pH 7.0, with 10% (v/v) acetonitrile and 2.4 mM EDTA as the mobile phase were used. The sample was introduced with a 7125-055 injector equipped with a 20 μ l external loop from Rheodyne (Cotati, CA, U.S.A.), and eluted with the mobile phase circulated at 1.5 ml/min. The chromatographic ALA peak was confirmed by the incubation of standard solutions or biological samples in the presence of ALAd (10 units/ml) in PBS buffer, pH 6.5, for 2 h at 37 $^{\circ}$ C. Afterwards, samples were injected into the HPLC system to confirm the disappearance of the ALA peak. Another control was made by spiking samples with authentic ALA at known concentrations.

Succinylacetone methylation

Succinylacetone methyl ester (SAME) was prepared by direct reaction of succinylacetone with diazomethane in ethyl ether as solvent. After evaporation of the ethyl ether at room temperature, the SAME residue was dissolved in deionized water to be used in the cell treatments. Diazomethane was prepared from Diazald[®] as described previously [31].

Haem determination

Haem was determined in whole cells by the fluorimetric method described previously [32]. Pelleted cells (10⁶) were treated with 2 M oxalic acid (100 μ l) at 100 $^{\circ}$ C for 30 min. After incubation, samples were resuspended in 0.9 ml of PBS and centrifuged at 14000 *g* for 15 min. Fluorescence emission was determined in the supernatant in a spectrofluorimeter (Fluorolog Spex, model 1681), with excitation and emission wavelengths of 405 and 600 nm respectively. A standard curve of protoporphyrin IX was run in parallel.

RESULTS

To determine whether IRP1 is activated by ALA, M8 cells were treated with authentic ALA or SAME, a potent ALAd inhibitor, to hinder ALA utilization and ensure its accumulation inside

Figure 1 Effect of ALA on IRP1 activity

The band-shift assay was performed with extracts from M8 cells incubated in culture medium supplemented with fetal calf serum. (A) Cells were exposed to ALA at the indicated concentrations for 2 h. (B) Cells were exposed to 1 mM ALA at the indicated times. The top panels show the IRP1/IRE complex from cells exposed to the indicated treatments; the bottom panels show the same extracts treated with 2% (v/v) 2-mercaptoethanol (EtSH). The tabulation shows IRP1 activity in the extracts as a percentage of full activation produced by the 2% 2-mercaptoethanol treatment. Values are means $\pm$ S.D. for four independent experiments.

Figure 2 Effect of SAME on IRP1 activity

The experimental conditions were the same as described in the legend to Figure 1. **(A)** Cells were exposed to SAME at the indicated concentrations for 2 h. **(B)** Cells were exposed to 4 mM SAME at the indicated times. The top panels show the IRP1/IRE complex from cells exposed to the indicated treatments; the bottom panels show the same extracts treated with 2% 2-mercaptoethanol (EtSH). The tabulation shows IRP1 activity in the extracts as a percentage of full activation produced by the 2% 2-mercaptoethanol treatment. Values are means ± S.D. for three independent experiments.

cells. Afterwards, cell extracts were assayed for the activation of IRP1 by band-shift electrophoresis. The corresponding bands of the autoradiogram depicted in Figure 1(A) show IRP1 activation of cells incubated for 2 h in culture medium supplemented with fetal calf serum and exposed to ALA at increasing concentrations. Increased activity of IRP1 was observed at all concentrations tested (0.5–8 mM). Interestingly, activation reached a maximum at 2 mM and decreased with higher ALA concentrations. As depicted in Figure 1(B), IRP1 activation by 1 mM ALA increased with time for 1 h after incubation. Activation remained increased until 2 h of incubation, but no activation was evident after 15 h (results not shown). Presumably, IRP1 activity would be influenced by enhanced haem levels after 15 h of incubation with ALA. This could also explain the decrease in IRP1 activity with ALA concentrations higher than 2 mM. Published results show that haemin inhibits IRP1 activity [33], in agreement with the

Figure 3 Intracellular ALA levels

The experimental conditions were the same as described in the legend to Figure 1. Determinations were performed in the M8 cellular extracts utilized for the band-shift assay. Intracellular ALA levels were determined after incubation at each indicated time in the presence of 4 mM SAME and 1 mM ALA. The inset represents intracellular ALA levels determined after 2 h of incubation at the indicated SAME concentrations. Results are those for three independent experiments where values agreed within 10%.

induction of ferritin synthesis during increased haem synthesis or haem oxygenase expression [34,35] and with lowered TfR number and expression [36]. Augmented ferritin synthesis and decreased TfR synthesis are considered to be related to IRP1 deactivation. Figure 2(A) shows IRP1 activity in M8 cells after incubation at increasing SAME concentrations (0.5–8 mM). Under these conditions IRP1 activity increased greatly. The highest activity was obtained at 4 mM SAME; increasing SAME concentrations did not lead to enhanced IRP1 activity. The time course of IRP1 activation by 4 mM SAME showed increased activation until 2 h of incubation (Figure 2B) and, unlike ALA, activation by 4 mM SAME was observed for 15 h incubation (results not shown). This result was thought to be related to the accumulation of ALA and not to alterations in haem level because, after 2 h incubation in the presence of 4 mM SAME, intracellular ALA was found to be elevated without alteration of intracellular haem levels.

When cells were incubated in the presence of 0.5 mM ALA plus 1 mM SAME, activation was significantly higher than with 1 mM ALA alone and similar to that with 4 mM SAME (results not shown). Activation by both ALA and SAME could be seen after cell incubation either in culture medium supplemented or not with fetal calf serum, or in PBS containing 0.8 mM CaCl₂. The level of activation by 1 mM ALA or 4 mM SAME in all cell lines tested (human erythroleukaemic K562, rat hepatocyte BRL and glioma C6 cells) was similar to that in fibroblasts.

In parallel, intracellular ALA concentrations were measured after ALA and SAME treatments; the ALA levels were measured in the extracellular medium after incubation in the presence of SAME. Intracellular ALA concentrations after incubation with 1 mM ALA and 4 mM SAME are shown in Figure 3. As depicted, when cells were incubated in the presence of 1 mM ALA, its uptake was observed up to 30 min, after which ALA levels remained approximately steady. Intracellular haem increased 2-fold, from 3.9 nmol per 10⁶ cells in control cells to 8.0 nmol per 10⁶ cells after 2 h of incubation in the presence of 1 mM ALA.

ALA levels increased greatly after 15 min of incubation in the presence of 4 mM SAME (92% higher than in control samples), subsequently decreasing and reaching a plateau at 2 h of in-

For comparative purposes, M8 cells were incubated in the presence of 10 μ M menadione to promote H_2O_2 production inside cells. Under this condition, almost total inhibition of menadione-stimulated IRP1 was observed when NAC was added to the medium (Figure 4). H_2O_2 production during ALA oxidation was determined after addition of 1 mM ALA to the cell culture medium (10^6 cells). At increasing times, catalase was added to the system and the O_2 released was followed with an oxygen monitor (Yellow Spring model YSI 153 with a standard ISI 5331 electrode). Under this condition, extracellular H_2O_2 generated by 1 mM ALA during 2 h of incubation was 24 μ M. Martins et al. [17] reported the activation of IRP1 by H_2O_2 at extracellular concentrations of at least 100 μ M in M8 fibroblast cells, and Pantopoulos and Hentze [18] by 50 μ M in murine B6 fibroblast cells. Thus the amount of extracellular H_2O_2 produced during oxidation of 1 mM ALA would be insufficient to induce IRP1 activity. The induction of IRP1 by ALA might be an intracellular event where ALA oxidation can occur at a specific site facilitating H_2O_2 , or another oxidizing species, to induce IRP1 activity.

We conclude that the effect of ALA on IRP1 activity is driven by intracellular ALA oxidation and not oxidative species generated outside cells. Activation of IRP1 promoted by ALA or SAME could be partly due to H_2O_2 generated inside the cell. Other oxidative species produced during ALA oxidation, such as the ALA enolyl radical (structure II in Scheme 1), 4,5-dioxo-valeric acid (Scheme 1) or $\text{O}_2^{\cdot-}$, could also be involved in the observed activation. One possibility would be the direct interaction of the ALA enolyl radical with the IRP1 iron-sulphur cluster, promoting iron release and disassembly of the cluster, followed by the induction of IRP1 mRNA-IRE-binding activity. This possibility must be explored.

Intracellular ALA might function as a co-sensor in the regulation of iron metabolism. Published results support the suggestion that IRP1 activity is regulated by different metabolic states, such as cellular signalling (for example protein phosphorylation) [38], intracellular iron availability [1,4], oxidative species such as NO [19,20], and oxidative stress [17,18].

Because the haem biosynthetic pathway is considered to be the greatest consumer of iron in mammalian cells, one might consider that some intermediate of haem synthesis could have an important regulatory role in iron uptake and storage by cells. The fact that eALAS mRNA has a functional IRE motif [5,6] also supports this idea. It is possible that the activation of IRP1 by excess ALA, and the consequent inhibition of eALAS mRNA translation, constitutes a mechanism for regulating ALA levels and makes them adequate for iron availability. It must be emphasized that only eALAS is regulated by IRP1, because mRNA of housekeeping ALAS does not have an IRE motif. It is interesting that experiments performed with the erythro-leukaemic cell line K562 gave similar results to those shown here with M8 fibroblasts.

It must be confirmed whether ALA has the physiological role in iron metabolism regulation by means of IRP1 modulation that is hypothesized here. Nevertheless one might suppose that at least under conditions of ALA overload, such as those found in inherited porphyrias, saturnism and tyrosinosis [39], ALA might induce metabolic changes in intracellular iron.

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, the Conselho de Desenvolvimento Científico e Tecnológico, CNPq and the Programa de Apoio ao Desenvolvimento Científico e Tecnológico, PADCT. E.J.H.B. is the recipient of a fellowship from the J. S. Guggenheim Memorial Foundation.

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