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Nitric oxide does not promote iron release from ferritin

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It has been previously reported that iron release from ferritin could be promoted by nitric oxide (NO) generated from sodium nitroprusside. It was thus proposed that some of the toxic effects of NO could be related to its ability to increase intracellular free iron concentrations and generate an oxidative stress. On the contrary, the iron exchange experiments reported here show that NO from *S*-nitrosothiols is unable to promote iron release from ferritin. The discrepancy may be explained by the disregarded ability of ferrozine, the ferrous trap used in the previous report, to mobilize iron both from ferritin and from sodium nitroprusside spontaneously.

Keywords: ferritin, iron release, nitric oxide, *S*-nitrosothiol

Introduction

Ferritin is the intracellular iron storage protein of all organisms and may incorporate as many as 4000 iron atoms within its protein shell, composed of 24 subunits. It prevents free low-molecular mass iron complexes from accumulating within cells and thus limits iron-dependent generation of toxic oxygen radicals. This function requires that iron uptake and mobilization mechanisms be highly controlled (Harrison & Lilley 1989).

Iron enters ferritin as Fe^{2+} and polymerizes after oxidation by molecular oxygen. The reaction is catalysed by the protein which thus displays a ferroxidase activity. On the other hand, iron is mobilized from ferritin by reduction of the mineral core. Electrons may be provided by a great variety of natural (ascorbate) or synthetic reducing agents. Photoreduction is also rather efficient (Laulhère *et al.* 1990, Aubailly *et al.* 1991, Macur *et al.* 1991). In fact, under reducing conditions, iron uptake and release occur simultaneously, but this is rarely noticed (Laulhère & Briat 1993).

In most *in vitro* investigations, Fe^{2+} traps, such as ferrozine, are used for assaying iron release from ferritin. The resulting iron complex is strongly light-absorbing in the visible region, allowing a simple and quantitative monitoring of the reaction by spectrophotometry. However, the chelator changes the driving force of the reduction of the iron core, thus facilitating iron release, and has a strong inhibitory effect on the ferroxidase activity and the iron uptake. Moreover, ferrozine may withdraw some loosely bound iron in the absence of electrons. As a consequence, the extent of iron release is generally overestimated and the simultaneous iron uptake is disregarded.

Recently, it was reported that iron release from ferritin could be promoted by nitric oxide (NO) (Reif & Simmons 1990). This observation has, later on, provided a strong support to the theory, now widely accepted, that some of the toxic effects of NO could be related to its ability to mobilize iron from ferritin, to increase intracellular free iron concentrations and thus to generate an oxidative stress.

We were puzzled by several methodological and basic aspects of this study. First, ferrozine was used as a Fe^{2+} trap for assaying iron release and, as discussed above, might have perturbed the reaction. Second, the NO donor used was sodium nitroprusside (SNP), a nitrosyl-iron complex which is photosensitive and may have been a source of iron not related to ferritin (Farrel 1989). Third, NO is a rather ineffective reducing agent and, on the basis of redox potentials, is not expected to transfer electrons to ferritin iron (Fontecave & Pierre 1994). We thus re-investigated the reaction between NO and ferritin, with special care given to the above discussed points. In this paper we demonstrate that NO, generated during decomposition of *S*-nitrosothiols, is not able to mobilize iron from ferritin significantly.

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Materials and methods

Materials

Cadmium-free ferritin (horse spleen) was obtained from Boehringer (Mannheim, Germany). SNP was purchased from Sigma (L'Isle d'Abeau, 38297 France). *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *S*-nitrosocysteamine were synthesized in the laboratory by Dr Jean Luc Decout and Loïc Le Hir de Fallois.

Iron uptake by ferritin

This was measured using $^{59}Fe^{3+}$-citrate as an iron source in the presence of ascorbate or *S*-nitrosothiols as previously described (Laulhère & Briat 1993). The exchange was performed at 25°C in the dark. The reaction was stopped by the addition of ferrozine followed immediately by agarose gel electrophoresis in the presence of EDTA.

^{59}Fe-labelled ferritin

This was obtained by reducing 100 μM ^{59}Fe-citrate with 6 mM ascorbate and allowing iron uptake by ferritin for 30 min. Stock solutions of radioiron were made by mixing 2 volumes of ^{59}Fe (Amersham IFS1, 3.7 MBq ml^{-1}) with 1 volume of 2 mM ferrous sulphate and 8 mM citrate. Horse ferritin was 50 μg ml^{-1} in 100 mM Tris–maleate buffer, pH 7.5. Adventitious radioiron was eliminated by two successive treatments of the protein with Chelex resin.

Specific radioactivity of ferritin iron

This was measured after purification of ferritins by agarose gel electrophoresis, as previously described (Laulhère & Briat 1993). Ferritin iron content and specific radioactivity can alternatively be measured as follows. The ferritin band was cut off from agarose gel and melted in weighted Eppendorf tubes containing 250 μl of 0.8 M HCl. After 10 min boiling, the solution was cooled, and 100 μl of 3 M sodium acetate (pH 5.2), 20 μl of 10 M NaOH and 50 μl of 1 M Na-ascorbate were added. Sample volumes were adjusted to 0.8 ml with water (actually to 800 mg in weight). Aliquots of 180 μl received either 20 μl water and were counted in a liquid scintillator or 20 μl of 0.01 M ferrozine. Absorbance at 562 nm, characteristic of the Fe^{2+}–ferrozine complex, was measured at least 1 h later, when a stable value was reached, using a Uniscan II spectrophotometer.

Iron release by ferritin

This was quantitated from the loss of iron or radioiron in ferritin samples. After the exchange experiment, ferritin samples were electrophoretically purified as described above. When ferrozine was used as a ferrous trap, Fe(II) release was monitored spectrophotometrically at 562 nm. Samples were not treated with Chelex resins prior to release measurements. The effect of light was studied by irradiating the solution with a 100 W electric bulb held 50 cm above the samples.

Results

NO does not promote iron uptake by ferritin

Iron uptake by ferritin could be evaluated from experiments in which 66 μM radioactive ^{59}Fe-labelled ferric citrate was incubated with ferritin and increasing concentrations of ascorbate or NO donors. Ascorbate was used as a positive control to define optimal conditions for iron uptake by ferritin. The ferritin sample was from horse spleen and contained 2080 iron atoms per molecule, giving a ferritin iron concentration in the reaction mixture of 208 μM. After

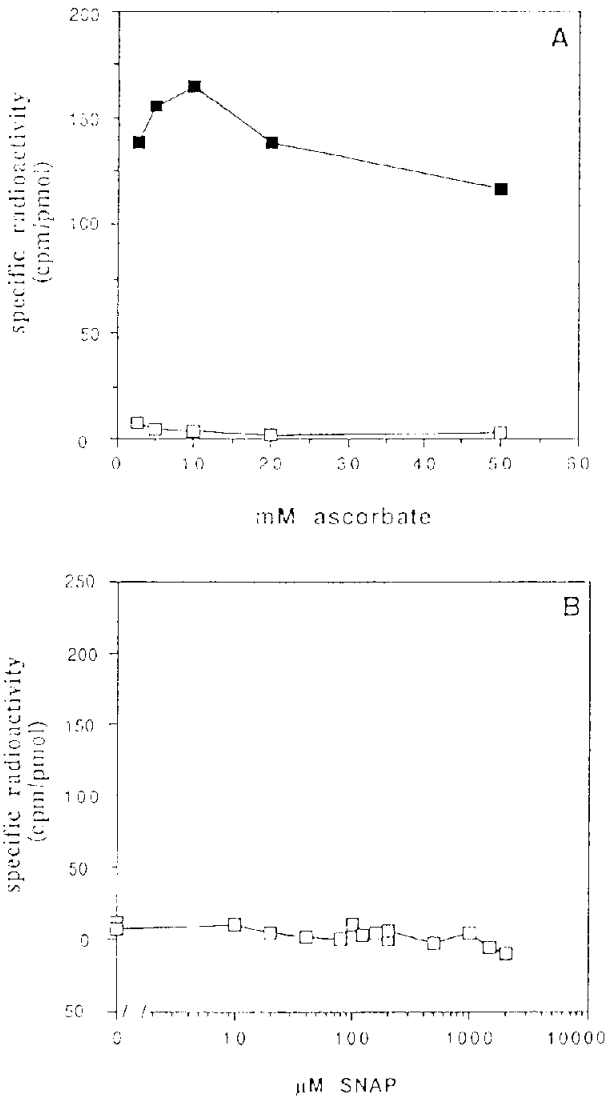

Figure 1. Iron uptake by ferritin in the presence of ascorbate or SNAP. (**A**) Specific radioactivity of ferritin iron (c.p.m. pmol^{-1}) after iron exchange during incubation of ^{59}Fe-citrate with horse spleen ferritin and increasing concentrations of ascorbate in the absence (■) or in the presence of ferrozine (□). (**B**) Specific radioactivity of ferritin iron after iron exchange during incubation of ^{59}Fe-citrate with ferritin and increasing concentrations of SNAP (□). The experiments were repeated three times at pH 7.5.

1 h of reaction, the specific radioactivity of the protein was measured, as an assay for iron incorporation. Moreover, the total iron content of ferritin was assayed.

Maximal iron incorporation was achieved for a concentration of 10 mM ascorbate, used as standard reducing agent (Figure 1A). Iron uptake was greatly inhibited by ferrozine and experiments in the presence of 0.2 mM ferrozine provided blank values which were systematically subtracted from the positive values. Very low amounts of iron were exchanged with regard to the initial ferritin iron content. Accordingly, at any ascorbate concentration, the total iron content of ferritin remained approximately constant (data not shown).

The effect of NO on iron uptake was studied with *S*-nitrosothiols as a source of NO and not with SNP. These compounds spontaneously decompose in aqueous solution yielding NO and the corresponding disulphide quantitatively (Roy *et al.* 1994). They here have the great advantage over SNP of being free of iron. Figure 1(B) shows that during incubation of ferritin with ^{59}Fe-citrate and increasing concentrations of SNAP no radioactivity was found to be incorporated into the protein.

NO does not promote iron release from ferritin

The iron release assay in this experiment was based on the loss of radioactivity from a ^{59}Fe-labelled ferritin, during incubation with increasing concentrations of *S*-nitrosothiols at pH 7.4. After 1 h of reaction, the ferritin radioactivity was determined as described in Materials and methods. As shown in Figure 2, both SNAP and *S*-nitrosocysteamine, the latter chosen for its very fast decomposition rate (Roy *et al.* 1994), failed to lower the iron content of ferritin, even at the highest concentrations (3–4 mM). Addition of ferrozine had no effect on iron release (data not shown). It should be noted that the sensitivity of the present method does not allow us to detect less than 1% iron variations, which is in the range of reported iron mobilization (below 0.8%).

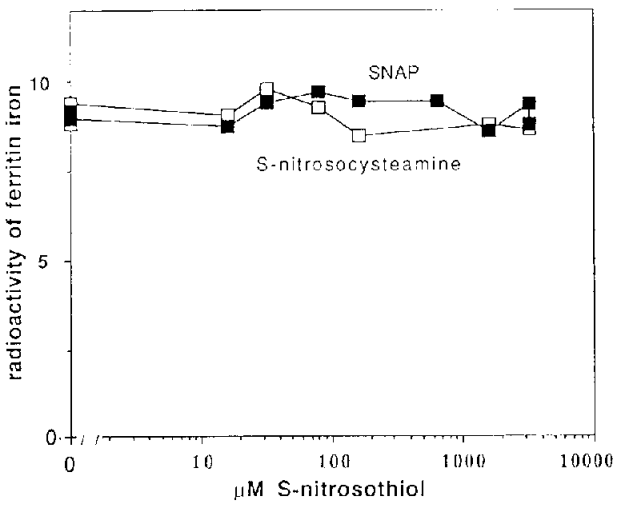

Figure 2. Iron content of ferritin after treatment of ^{59}Fe-labelled ferritin with SNAP (■) or *S*-nitrosocysteamine (□) (expressed in 1000 c.p.m. per ferritin band on a gel).

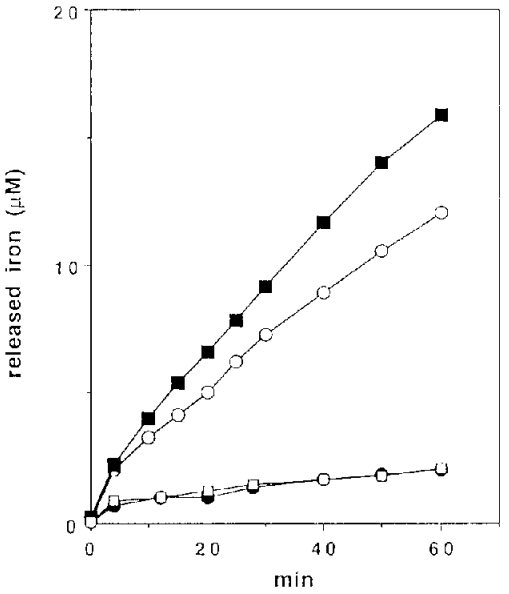

Figure 3. Iron release from ferritin promoted by ascorbate (○, 2.5 mM; ■, 10 mM) or 4 mM SNAP (□) at pH 5.5. A blank experiment was performed in the absence of ascorbate and SNAP (●). Similar experiments using SNAP and ascorbate, performed at pH 6.5 and 7.5 in 50 mM Tris–maleate–NaOH buffer, gave similar results. The values reported represent the average of three experiments.

Whether lower pH would facilitate a NO-dependent iron release has been investigated, since iron release by ascorbate is known to be greatly pH dependent. In those experiments, ferrozine was used as a trap for released iron and the reaction monitored spectrophotometrically. The assay is actually sensitive and much easier to carry out than the radioactive iron loss assay. Moreover, it is now legitimated by the absence of iron uptake reactions. Figure 3 clearly shows that 4 mM SNAP was unable to promote iron release from ferritin at pH 5.5. The same was observed when the reaction was carried out at pH 6.5 and 7.5 (data not shown). For comparison, experiments with 2.5 and 10 mM ascorbate at the same pHs have been carried out (Figure 3).

SNP does not mobilize iron from ferritin

Why should NO be active when generated from SNP, as shown by Reif & Simmons (1990), and not from SNAP, as shown here? In order to understand this discrepancy, we studied the iron release from ferritin by SNP, under the conditions used in Figure 3. The results are displayed in Figure 4. As previously reported (Reif & Simmons 1990), during incubation of ferritin with SNP iron was recovered as the iron–ferrozine complex in a time-dependent reaction. After 30 min of reaction, about 2.5 μM iron was released, a value consistent with that reported by Reif & Simmons (1990).

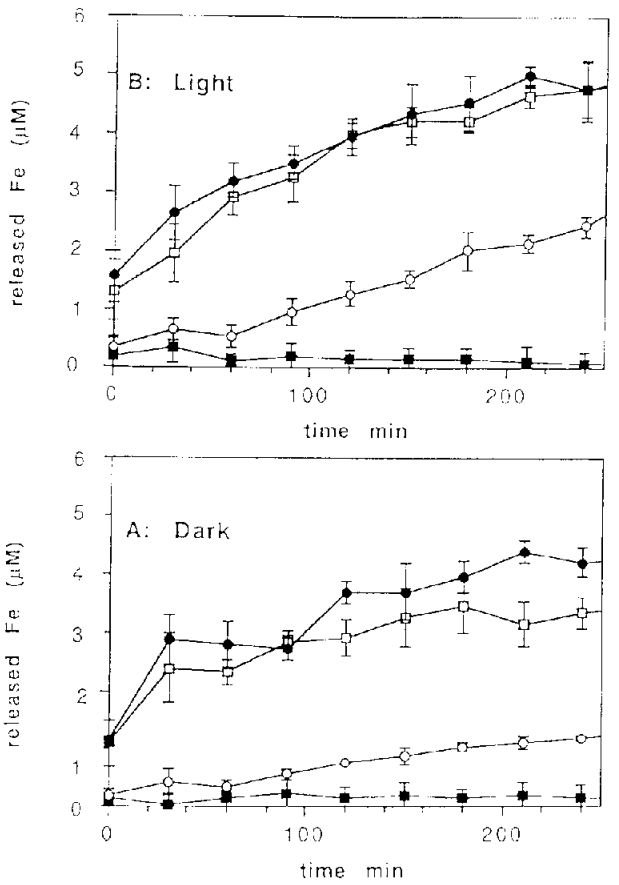

Figure 4. Iron release in the presence of ferrozine, from ferritin (□) or from SNP (○), or from their mixture (●), in HEPES buffer, pH 7.0, in the dark (**A**) and in the light (**B**). Blank experiments (■) were carried out in the absence of both ferritin and SNP. Vertical bars represent SD.

However, the control experiments, in which SNP had been omitted, gave comparable amounts of iron ferrozine complex, confirming that ferrozine was able to remove iron from ferritin directly. The reaction was less effective in the dark. Figure 4 also shows that SNP itself may slowly transfer iron to ferrozine in a reaction stimulated by light. This then shows that SNP is not able to mobilize iron from ferritin significantly.

Discussion

In cytokine-activated cells such as macrophages, NO is generated (Feldman *et al.* 1993) and has been regarded as a molecule with highly effective cytostatic and cytotoxic potential. The mechanisms by which NO exerts its action against tumour cells, bacteria or parasites are still to be elucidated. However, recent studies have identified possible molecular targets of NO. For example, enzymes containing essential redox active cysteines, such as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Molina y Vedia *et al.* 1992) or ribonucleotide reductase (Lepoivre *et al.* 1990, Roy *et al.* 1995), a key and rate-limiting enzyme in DNA synthesis, may be nitrosated and thus inactivated. Recently, it has been shown that proteins containing zinc–sulphur clusters such as zinc fingers, involved in transcriptional regulation, may lose their metal ion and their capacity to bind to DNA during reaction with NO (Kröncke *et al.* 1994). The inactivation is due to the nitrosation of the cysteine ligands. Enzyme iron centres may also be altered or mobilized by NO, because of the high affinity of iron for NO. Such reactions have been demonstrated with a variety of iron–sulphur enzymes, such as aconitase, and complex I and II of the mitochondrial electron transport chain (Granger & Lehninger 1982, Hibbs *et al.* 1984, Drapier & Hibbs 1986, Wharton *et al.* 1988). In most cases the inactivation of the protein is reversible, suggesting some regulatory role of NO. For example, NO may modulate the RNA-binding activities of the iron regulatory factor by acting at the level of its iron–sulphur centre (Drapier *et al.* 1993, Weiss *et al.* 1993). Heme proteins such as cytochrome oxidase have also been shown to be inhibited by NO (Cleeter *et al.* 1994).

During activation of macrophages, NO accumulates in target cells and reacts with intracellular iron complexes. The resulting stable paramagnetic iron-nitrosyl complexes can be easily observed by EPR spectroscopy (Drapier *et al.* 1991). Characteristic EPR signals have been detected directly in both normal and tumour whole cells. However, the exact chemical nature of the complexes and the source of iron responsible for the signals are poorly understood. It has been recently shown, on the basis of EPR spectroscopy, that NO reacts with ferritin iron yielding stable iron-nitrosyl complexes within the protein, suggesting that ferritin might serve also to store NO (Lee *et al.* 1994). The EPR spectra of ferritin iron-nitrosyl complexes are strikingly similar to those of NO-treated cells.

NO has been suggested not only to bind to ferritin iron but also to mobilize it, as an explanation for the loss of iron from tumour target cells during interaction with activated cytotoxic macrophages. This was suggested by Reif & Simmons (1990) and, later on, accepted as a possible mechanism for NO toxicity, since mobilization of iron from intracellular storage sites (ferritin) would increase the generation of reactive oxygen species and the damage to DNA, proteins or lipids.

The results of the present study now demonstrate that iron-free NO donors do not release iron from ferritin to a significant extent, even at very high and non-physiological concentrations. By avoiding strong ferrous chelators in iron-release assays, our methodology avoids ligand-promoted iron release from ferritin. Spontaneous iron release from ferritin has been proved to be significantly enhanced by light, especially in the presence of ferrozine (Laulhère *et al.* 1990, Aubailly *et al.* 1991, Macur *et al.* 1991). Reported observations on the steady presence of accessible ferrous iron on ferritin coats (Theil & Raymond 1994, Harris *et al.* 1994) could account for the observed ability of ferrozine alone to release iron from ferritin. This reaction was not accelerated by the addition of either SNP or SNAP. The incapacity of NO donors to mediate iron release from ferritin

is now consistent with the poor reducing power of NO. Considering that iron release from ferritin requires low redox potential electrons, NO is not expected to be an efficient iron mobilizer.

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