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Nitric oxide-mediated inactivation of mammalian ferrochelatase *in vivo* and *in vitro*: possible involvement of the iron–sulphur cluster of the enzyme

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To investigate the role of the iron–sulphur cluster in mammalian ferrochelatases, the terminal enzyme of the haem biosynthetic pathway, we examined the interaction of nitric oxide (NO) and ferrochelatase. When macrophage cell line RAW 264.7 cells were treated with interferon-γ and lipopolysaccharide NO synthesis in the cells was stimulated, and a decrease in ferrochelatase activity was observed, with no change in the amount of ferrochelatase. The addition of N^G-monomethyl-L-arginine, a selective inhibitor of NO synthesis, reduced the effect of interferon-γ and lipopolysaccharide, while the effect of N^G-monomethyl-L-arginine was suppressed by the addition of L-arginine, a substrate of NO synthase. When purified recombinant human ferrochelatase was treated with 3-morpholinosydnonimine, a NO-generating compound, ferrochelatase activity decreased with disappearance of characteristic absorbance spectra of the iron–sulphur cluster. *S*-Nitroso-*N*-acetylpenicillamine also reduced the activity, in a dose-dependent manner. These results indicate that ferrochelatase activity can be modulated by NO synthesis probably through disruption of the iron–sulphur cluster. We propose that inactivation of ferrochelatase mediated by NO (or NO-derived species) may play a role in the regulation of haem metabolism.

INTRODUCTION

Ferrochelatase (EC 4.99.1.1), at the last step of the haem biosynthetic pathway, catalyses the insertion of ferrous iron into protoporphyrin to form protohaem. Eukaryotic ferrochelatase is nuclear encoded, cytoplasmically synthesized, and translocated into the inner membrane of mitochondria, where the active site faces the matrix side of the membrane [1–4]. The understanding of the enzyme at a molecular level is a prerequisite to elucidate the regulation of iron and haem metabolism. Genes and cDNAs for yeast [5], mouse [6,7], human [8], *Escherichia coli* [9] and *Bradyrhizobium japonicum* [10] ferrochelatase have been isolated. Structural diversity among species has been characterized: the C-terminus of the eukaryotic ferrochelatase is about 30 amino acids longer, when compared with that of prokaryotic ferrochelatase [11].

Nitric oxide (NO) is a highly diffusible and reactive free radical with effects on various proteins which possess haem, iron–sulphur cluster, free radical, and thiol groups [12,13]. NO has been implicated in the activation of iron responsive element-binding protein (IRE-BP), which also has aconitase activity [14,15]. IRE-BP has the [4Fe–4S] cluster; the binding of NO to the cluster and subsequent disruption of the cluster may lead to the loss of aconitase activity and the protein would have iron responsive element (IRE) binding activity. IRE-BP regulates cellular iron metabolism through binding to IRE on the 3′-untranslated region of transferrin receptor mRNA, and the 5′-untranslated regions of ferritin and erythroid-type δ-aminolaevulinate synthase mRNAs [16,17]. Accordingly, NO may be involved in the regulation of iron and haem metabolisms [14,15].

Recently, it was reported that mammalian ferrochelatase also has an iron–sulphur cluster [18,19]. The cluster is the [2Fe–2S] type and located at the C-terminal region of the protein. The C-terminal-truncated human ferrochelatase was reported to be inactive [11], thereby underscoring the importance of the iron–sulphur cluster. However, the role of the cluster in mammalian ferrochelatase is not well understood. Iron–sulphur clusters as active centres of enzymes are usually involved in oxidation–reduction, except for a group of (de)hydratases [20]. The reaction of ferrochelatase does not require oxidation or reduction, and the cluster does not seem to be required for catalytic activity; bacterial and yeast ferrochelatases do not possess iron–sulphur clusters [5,9,10]. The importance of the iron–sulphur cluster for regulation has remained unclear [18]. We report here that the formation of NO results in a decrease in ferrochelatase activity, in macrophage cell lines. The importance of the iron–sulphur cluster as the possible target of NO-mediated inactivation is given focus.

MATERIALS AND METHODS

Materials

Recombinant mouse interferon-γ (IFN-γ) (2×10^5 units/ml) was purchased from PharMingen (CA, U.S.A.). *Escherichia coli* lipopolysaccharide (LPS) was obtained from Sigma. N^G-Monomethyl-L-arginine (MMA), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine (SIN-1) were from RBI (MA, U.S.A.) and from BIOMOL (PA, U.S.A.) respectively. Mesoporphyrin IX was a product of Porphyrin Products. Blue-Sepharose CL-6B was purchased from Pharmacia Co. All other chemicals used were of analytical grade.

Cell culture and preparation of cell lysates

Macrophage cell lines RAW 264.7 and J774 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal-calf serum, 20 mM glucose, 25 mM Hepes (pH 7.2), penicillin (50 units/ml) and streptomycin (50 μg/ml). For use in experiments, the cells were detached by vigorous pipetting, centrifuged, and plated in fresh medium (2×10^6 cells/ml) containing the indicated reagents and cultured for the indicated

Abbreviations used: IRE-BP, iron responsive element-binding protein; IRE, iron responsive element; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MMA, N^G-monomethyl-L-arginine; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; DTT, dithiothreitol; $ONOO^-$, peroxynitrite.

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time. Cell viability was assessed by Trypan Blue exclusion. The cell viability was > 90% for RAW 264.7 cells treated with 10 ng/ml LPS and 100 units/ml IFN-γ, and > 95% for others. The cells were collected, washed twice with phosphate-buffered saline and disrupted by sonication in 10 mM Tris/HCl buffer, pH 8.0, containing 20% glycerol and 1 mM dithiothreitol (DTT). The supernatant obtained after centrifugation at 600 ***g*** for 10 min was used to measure ferrochelatase activity and for immunoblotting.

Preparation of recombinant human and *E. coli* ferrochelatase

The cDNA encoding the human mature ferrochelatase was placed under the transcriptional control of T7 RNA polymerase, and the protein was overexpressed in *E. coli*, as described previously [21]. The cells were collected, washed with 10 mM Tris/HCl buffer, pH 8.0, suspended in 10 mM Tris/HCl buffer, pH 8.0, containing 20% glycerol, 1 mM DTT, 2 mM EDTA, and disrupted by sonication. After centrifugation at 10000 ***g*** for 10 min the supernatant was collected. The recombinant human ferrochelatase was purified using Blue-Sepharose CL-6B, as described elsewhere [21], with slight modification. During enzyme purification, solutions containing 1 mM DTT, 20% glycerol and 2 mM EDTA were used to stabilize ferrochelatase. The obtained ferrochelatase was apparently homogeneous by SDS/PAGE. *E. coli* strain DH5α, overexpressing *E. coli* ferrochelatase, was kindly provided by Dr. K. Miyamoto of Kyoto University. The cells were grown overnight in LB broth at 32 °C, then at 42 °C for 3 h [22]. The cells were collected and lysates were prepared as described above.

Treatment of recombinant ferrochelatase with NO donors *in vitro*

Purified ferrochelatase (400 μg/ml) was incubated with SIN-1 (0–1 mM) in 50 mM Tris/HCl, pH 8.1, containing 20% glycerol, 1 mM DTT and 2 mM EDTA for 30 min at 37 °C prior to measurement of ferrochelatase activity and the electronic absorption spectrum. The cell lysates of *E. coli* overexpressing recombinant human and *E. coli* ferrochelatase were incubated at 37 °C for 30 min with 0–1 mM SIN-1 or SNAP.

Measurement of nitrite

Nitrite concentration in the culture medium and reaction mixture was determined using the Griess reagent containing 0.5% sulphanilamide, 0.05% *N*-1-naphthylethylenediamine dihydrochloride and 2.5% phosphate [23].

Assay of ferrochelatase activity

The reaction mixture containing 25 μM mesoporphyrin, 50 μM zinc acetate, 40 μg/ml palmitic acid, 0.1% Tween-20, 100 mM Tris/HCl buffer, pH 8.0, and cell lysate or purified ferrochelatase was incubated for 30 min at 37 °C. The reaction was stopped by adding dimethyl sulphoxide/methanol (3:7, v/v) containing 10 mM EDTA. Zinc-mesoporphyrin was separated from mesoporphyrin by HPLC with a Cosmosil 5C18-AR column (0.46 cm × 15 cm, Nacalai Tesque) [21]. The elution was performed with 1 M ammonium acetate (pH 5.16)/methanol (12:88, v/v) at a flow rate of 1.5 ml/min. Zinc-mesoporphyrin was determined spectrofluorometrically by measuring the emission fluorescent intensity at 580 nm, with excitation at 410 nm [24].

Immunoblot analysis

Antibodies against bovine ferrochelatase were prepared as described previously [25]. These antibodies showed cross-reactivity to mouse ferrochelatase. The lysates of RAW 264.7 cells were subjected to SDS/PAGE and electroblotted on to a poly(vinylidene difluoride) membrane. Immunostaining was done using an ECL blotting detection agent (Amersham Co., Amersham, Bucks., U.K.) and the anti-ferrochelatase antibody as the primary one.

Other methods

Protein concentration was determined according to the method of Lowry et al. [26]. The assay of δ-aminolaevulinate dehydratase activity was carried out by the method of Tsukamoto et al. [27]. Electronic absorption spectrum of ferrochelatase was measured on a Beckman DU-64 spectrophotometer.

RESULTS

Induction of NO synthesis by IFN-γ and LPS reduces ferrochelatase activity in RAW 264.7 cells

To explore the possibility that stimulation of NO synthesis alters ferrochelatase activity, we first examined this activity in the mouse macrophage cell line RAW 264.7, in which the induction of NO synthase by IFN-γ and LPS is well documented [14,28,29]. As shown in Table 1, the concentration of nitrite, one of the end products of the NO pathway, in culture medium increased after addition of IFN-γ and LPS in combination, whereas IFN-γ or LPS alone had little effect. Nitrite concentration in the medium was 27.9 ± 2.1 μM in the presence of 10 ng/ml LPS and 10 units/ml IFN-γ, and 34.5 ± 1.2 μM with 10 ng/ml LPS and 100 units/ml IFN-γ after 24 h incubation respectively. Ferrochelatase activity in the cell lysate was 14.0 ± 0.41 nmol of zinc-mesoporphyrin formed/mg of protein per h in controls, and decreased to 54.7% of control when the cells were treated with 10 ng/ml LPS plus 10 units/ml IFN-γ and to 44.1% with 10 ng/ml LPS plus 100 units/ml IFN-γ. A slight decrease in ferrochelatase activity was observed when the cells were treated with IFN-γ or LPS alone although nitrite concentration in the medium was not increased significantly. As a control experiment, the activity of δ-aminolaevulinate dehydratase, the second enzyme in the haem biosynthetic pathway, was also measured with the same samples from IFN-γ- and/or LPS-treated cells. The activity was 2.1, 2.2, 2.1 and 2.2 nmol of porphobilinogen formed/mg of protein per h in control, 10 units/ml IFN-γ-treated, 10 ng/ml LPS-treated and 10 units/ml IFN-γ plus 10 ng/ml LPS- treated cells respectively; thus, there were no significant changes in the activity. When J774 cells, another macrophage cell line, were treated with IFN-γ and LPS, a

Table 1 Alterations of ferrochelatase activity and nitrite production in RAW 264.7 macrophages treated with IFN-γ and LPS

RAW 264.7 macrophages were cultured for 24 h in the presence or absence of IFN-γ and/or LPS. The concentration of nitrite in culture medium was determined, and cell extracts were analysed for ferrochelatase activity, as described in the Materials and methods section. Data represent the mean ± S.D. of four experiments.

Treatment	Ferrochelatase activity (nmol of zinc-mesoporphyrin formed/mg of protein per h)	Nitrite (μM)
None	14.0 ± 0.4	0.7 ± 0.5
LPS (10 ng/ml)	11.5 ± 0.4	1.5 ± 1.1
INF-γ (10 units/ml)	12.1 ± 0.9	0.8 ± 0.7
LPS (10 ng/ml) + INF-γ (10 units/ml)	7.6 ± 0.7	27.9 ± 2.1
LPS (10 ng/ml) + INF-γ (100 units/ml)	6.1 ± 0.9	34.5 ± 1.2

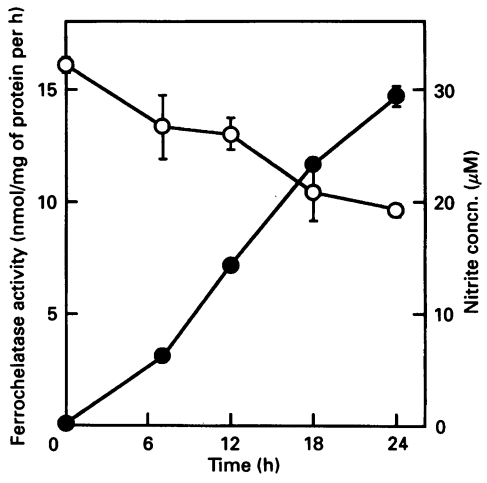

Figure 1 Time course of nitrite production and ferrochelatase activity in IFN-γ plus LPS-treated RAW 264.7 macrophages

Cells were treated with 10 units/ml IFN-γ plus 10 ng/ml LPS. At the indicated time, the concentration of nitrite was determined in the culture medium (●), and ferrochelatase activity in the cells was measured (○). Data are the means and errors of duplicate experiments.

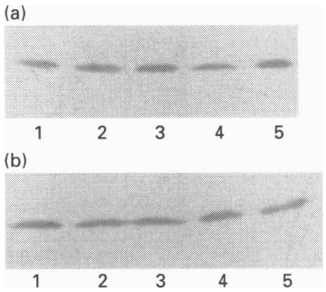

Figure 2 Immunoblot analysis of ferrochelatase in immunostimulated RAW 264.7 macrophages

Cell lysate samples (100 μg of protein) were from the same preparation used for the measurement of ferrochelatase activity, and were analysed by SDS/PAGE. Immunoblotting was performed using anti-ferrochelatase as the primary antibody. (**a**) The cells were untreated (lane 1), or treated for 24 h with 10 units/ml IFN-γ (lane 2), 10 ng/ml LPS (lane 3), 10 ng/ml LPS and 10 units/ml IFN-γ (lane 4), and 10 ng/ml LPS and 100 units/ml IFN-γ (lane 5). (**b**) The cells were untreated (lane 1), or treated for 24 h with 10 ng/ml LPS and 10 units/ml IFN-γ (lane 2), 10 ng/ml LPS, 10 units/ml IFN-γ, and 50 μM MMA (lane 3), 10 ng/ml LPS, 10 units/ml IFN-γ, and 500 μM MMA (lane 4), 10 ng/ml LPS, 10 units/ml IFN-γ, 500 μM MMA and 50 mM L-arginine (lane 5).

Table 2 Effect of NO synthesis inhibitor on ferrochelatase activity and nitrite production in RAW 264.7 macrophages

RAW 264.7 cells were treated with the reagents for 24 h. The concentration of nitrite and ferrochelatase activity was determined as described in the Materials and methods section. Data represent the mean ± S.D. of four experiments.

Treatment	Ferrochelatase activity (nmol of zinc-mesoporphyrin formed/mg of protein per h)	Nitrite (μM)
None	16.1 ± 1.2	0.9 ± 0.3
LPS (10 ng/ml) + INF-γ (10 units/ml)	9.0 ± 0.3	33.4 ± 2.1
LPS (10 ng/ml) + INF-γ (10 units/ml) + MMA (50 μM)	10.4 ± 0.9	22.7 ± 0.6
LPS (10 ng/ml) + INF-γ (10 units/ml) + MMA (500 μM)	11.6 ± 0.2	14.2 ± 0.3
LPS (10 ng/ml) + INF-γ (10 units/ml) + MMA (500 μM) + Arg (50 mM)	8.9 ± 0.8	37.8 ± 1.1

decrease in ferrochelatase activity with cell lysates and an increase in nitrite concentration in culture medium were also observed (data not shown). The time course of changes in ferrochelatase activity with the cell lysates and nitrite concentration in the culture medium is shown in Figure 1. The ferrochelatase activity decreased with time and was accompanied by an increase in nitrite concentration.

As shown in Table 2, by the addition of 50 μM MMA, a selective and stereospecific inhibitor of NO synthesis, to the culture medium the nitrite concentration decreased by 33% of that in IFN-γ plus LPS-treated cells and the ferrochelatase activity was restored by 15%. When the MMA concentration was elevated to 500 μM, nitrite concentration in the medium decreased by 57% and the ferrochelatase activity was restored by 30%. When 50 mM L-arginine was included in the culture medium with MMA, IFN-γ and LPS, the nitrite concentration increased to 37.8 ± 1.1 μM, a value similar to that in the case of IFN-γ plus LPS-treated cells, and ferrochelatase activity decreased to a level similar to that observed in IFN-γ plus LPS-treated cells.

To examine the variation of ferrochelatase in RAW 264.7 cells treated with IFN-γ plus LPS, immunoblots were prepared. As shown in Figure 2(a), there was no change in the amount of ferrochelatase in the treated cells. IFN-γ or LPS alone was also without effect. Simultaneously, neither inhibition of NO synthesis by MMA nor restoration of NO synthesis by L-arginine affected the amount of the protein (Figure 2b).

These results are taken to mean that the induction of NO synthesis leads to a decrease in ferrochelatase activity, without changes in the amount of enzyme in the cells.

Effect of SIN-1 on recombinant human ferrochelatase *in vitro*

Figure 3 shows absorption spectra of the purified human ferrochelatase. The spectrum of purified recombinant human ferrochelatase showed absorbance bands centred at 330 nm and 415 nm with pronounced shoulders at 460 nm and 550 nm, and this spectrum was similar to that reported by Dailey et al. [19]. The band at 415 nm probably arose from haem and the other from the [2Fe–2S] cluster at the C-terminal region [19]. To examine the effect of NO on ferrochelatase activity, purified recombinant human ferrochelatase was incubated with SIN-1, which spontaneously liberates NO [14]. By incubation with increasing concentrations of SIN-1, these characteristic absorbance bands gradually diminished. Concentrations of nitrite in the reaction mixture after 30 min incubation with 0.1, 0.2, 0.5 and 1 mM SIN-1 were 10.9, 31.5, 67.5 and 127.0 μM respectively. As the absorbance at 330 nm was most prominent but was overlapped by the absorbance of SIN-1, we used the absorbance at 460 nm to quantify the iron–sulphur cluster. The relationship between the absorbance at 460 nm and ferrochelatase activity is shown in the inset of Figure 3. The loss of absorbance at 460 nm depended on the loss of ferrochelatase activity, which suggests that disruption of the iron–sulphur cluster by NO (or NO-derived species) resulted in the inactivation of ferrochelatase.

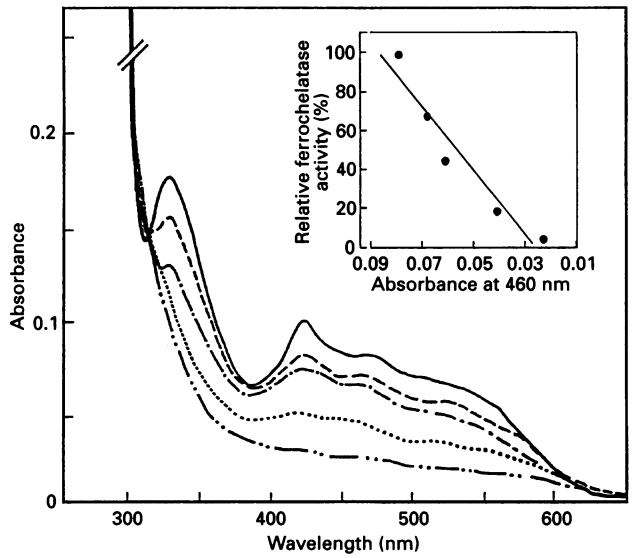

Figure 3 Effect of SIN-1 on the absorption spectrum and the activity of purified recombinant human ferrochelatase

The electronic absorption spectra of 400 μg/ml of purified recombinant human ferrochelatase incubated without (——), or with 0.1 mM (- - -), 0.2 mM (-··-), 0.5 mM (····) and 1.0 mM SIN-1 (-···-) for 30 min at 37 °C are shown. Inset: Relationship between ferrochelatase activity and the absorbance at 460 nm. Aliquots were withdrawn from the samples before measuring the absorption spectra, properly diluted and analysed for ferrochelatase activity. Relative ferrochelatase activity (percentage of untreated control) in the samples is plotted against the absorbance at 460 nm.

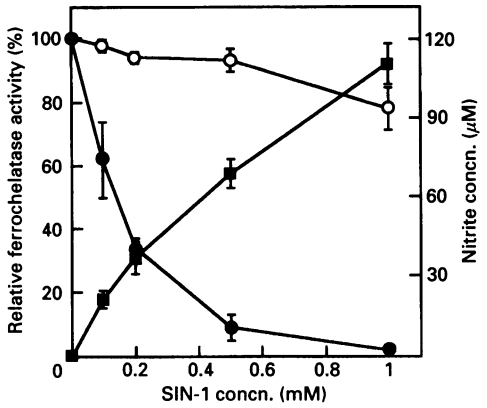

Figure 4 Effect of SIN-1 on the activities of recombinant human and *E. coli* ferrochelatase

Cell lysates from bacteria overexpressing recombinant human (●) or *E. coli* (○) ferrochelatase were incubated with the indicated concentration of SIN-1 at 37 °C for 30 min. Nitrite concentration in the reaction mixture was determined (■) and aliquots were withdrawn, properly diluted and measured for ferrochelatase activity, as described. Data represent the mean ± S.D. of four experiments.

Comparison of the effects of NO donors between human and *E. coli* ferrochelatase

E. coli ferrochelatase, in contrast to mammalian ferrochelatase, does not possess iron–sulphur clusters [9,19]. The effect of NO donors on human and *E. coli* ferrochelatase was compared to confirm the necessity for the iron–sulphur cluster in NO-mediated ferrochelatase inactivation. As shown in Figure 4, when the extract of cells overexpressing recombinant human ferrochelatase was incubated with increasing concentrations of SIN-1, the activity decreased concomitant with an increase in nitrite concentration. The activity decreased to 61.5 ± 11.1 % of the untreated control following incubation with 0.1 mM SIN-1 at 37 °C for 30 min and was almost undetectable with 1 mM SIN-1; only 22 % loss of enzyme activity was observed in the case of *E. coli* ferrochelatase, even with 1 mM SIN-1.

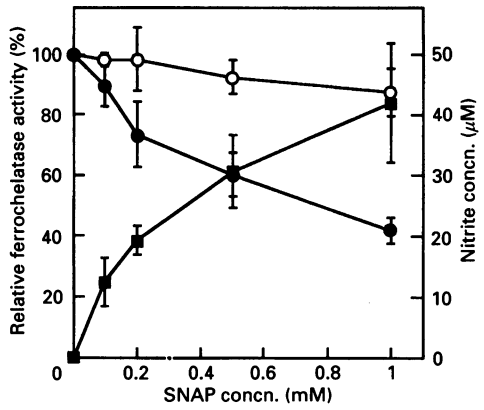

Figure 5 Effect of SNAP on activities of recombinant human and *E. coli* ferrochelatase

Lysates from the cells overexpressing recombinant human (●) or *E. coli* (○) ferrochelatase were incubated with the indicated concentrations of SNAP at 37 °C for 30 min. Nitrite concentration (■) and ferrochelatase activity were measured as described in the legend for Figure 4. Data represent the mean ± S.D. of four experiments.

The effect of another type of NO donor, SNAP, was also examined. As shown in Figure 5, the activity of human ferrochelatase decreased with incubation with SNAP but not to the same extent seen with SIN-1. The activity of human ferrochelatase was 41.9 ± 4.2 % of untreated control values after incubation with 1 mM SNAP. The activity of *E. coli* ferrochelatase was 87.5 ± 8.0 % of the control after treatment with 1 mM SNAP. Thus, the decrease in activity was more extensive in human ferrochelatase than that in *E. coli* ferrochelatase with both NO donors, SIN-1 and SNAP.

DISCUSSION

Ferrochelatase activity in RAW 264.7 cells decreased when these macrophage cells were treated with IFN-γ and LPS, where NO synthesis was induced. This means that ferrochelatase activity is under the control of physiological immunomodulators and correlates with NO synthesis in macrophage cells. When ferrochelatase was purified from bovine liver mitochondria, the enzyme showed an absorption spectrum similar to that obtained with recombinant ferrochelatase, indicating that ferrochelatase in mammalian cells contains the iron–sulphur cluster (S. Taketani, unpublished work). Under conditions of NO synthase stimulation, an increase in IRE-binding activity and decrease in aconitase activity of cytosolic aconitase/IRE-BP was noted [14,15]. We found that ferrochelatase activity was partially suppressed by the stimulation of NO synthesis, while a complete loss of aconitase activity was reported by Drapier et al. [14] in RAW 264.7 cells treated with IFN-γ and LPS. The difference in sensitivities to NO may be related to differences in the structures of their iron–sulphur clusters, since the cluster of IRE-BP is cubic type [4Fe–4S] in which the fourth Fe is thought to be labile [12]. Although changes in ferrochelatase activity were more moderate

than those seen in the aconitase activity of IRE-BP [14], addition of MMA, which inhibits NO synthase [14], restored ferrochelatase activity, and the effect of MMA was overcome by excess L-arginine (Table 2), thereby confirming the relationship between NO synthesis and ferrochelatase activity. However, the slight but significant decrease in ferrochelatase activity caused by IFN-γ or LPS alone, without increase in nitrite concentration in the culture medium, suggests participation of other regulatory mechanism(s).

The decrease in ferrochelatase activity in the absence of changes in the protein level in cells treated with IFN-γ plus LPS (Figure 2) indicates a direct effect of NO (or NO-derived species) on ferrochelatase. Incubation of purified recombinant human ferrochelatase with SIN-1, which spontaneously liberates NO [14], resulted in a loss of ferrochelatase activity. The absorbance bands of the protein characteristic for iron–sulphur proteins disappeared (Figure 3), representing disruption of the cluster, with the loss of ferrochelatase activity. This observation strongly suggests that the iron–sulphur cluster could be the target, and disruption of the cluster could lead to the loss of ferrochelatase activity. Dailey et al. [11] reported that the C-terminus-truncated human ferrochelatase which lacks the [2Fe–2S] cluster did not show the activity; hence, the iron–sulphur cluster would be essential for the activity. Human ferrochelatase was more susceptible to the effect of NO donors, both SIN-1 and SNAP, than was *E. coli* ferrochelatase which lacks the iron–sulphur cluster, further evidence that the cluster is the site of NO-mediated inactivation. However, inactivation of *E. coli* enzyme, though much less compared with that found on human ferrochelatase, suggests the possibility of other target(s) of NO-mediated inactivation on the protein.

The concentration of SIN-1 needed for inactivation of ferrochelatase (Figures 3 and 4) was much higher than that reported for inactivation of cytosolic aconitase (IRE-BP) [14], findings which agreed well with observations with RAW 264.7 cells. Although the different sensitivity to NO may be attributable to the structure of their iron–sulphur clusters, i.e. ferrochelatase has the [2Fe–2S] cluster and IRE-BP has the labile [4Fe–4S] cluster, the other factor is considered to be the buffer containing 1 mM DTT used in the present study. When high concentrations of DTT (> 1 mM) were used, the inhibitory effect of SIN-1 on human ferrochelatase activity was reduced, and with a low concentration of DTT (< 1 mM) the effect of SIN-1 was enhanced (T. Furukawa, H. Kohno, and S. Taketani, unpublished work). In the case of human ferrochelatase, the presence of thiols in the reaction buffer seems to protect against the effect of NO. Since DTT is a prerequisite to stabilize ferrochelatase [30], we could not carry out the experiments in the absence of DTT, where the sensitivity of ferrochelatase to NO could be higher than that observed in the present study.

NO reacts in biological systems with oxygen, superoxide, transition metals and thiol. NO_x, peroxynitrite ($ONOO^-$), metal–NO adducts and RS–NO are produced by those reactions and these NO-derived species, together with NO, react with haem, iron–sulphur cluster, free radical and thiol groups on proteins [12,13]. Diverse reactivity of NO and NO-derived species and multi-existence of targets on proteins complicate mechanisms involved in NO-related alterations of structure and/or functions of protein. Each NO-donor has a different character. For example, SIN-1 generates NO and superoxide stoichiometrically, an event which facilitates the formation of $ONOO^-$, and SNAP has an RS–NO structure. Inactivation or modulation at its active-site thiol of glyceraldehyde-3-phosphate dehydrogenase by SIN-1 and sodium nitroprusside, another NO-generating compound, has been reported to be efficient in the presence of DTT [31,32]. Under these conditions, it is proposed that RS–NO is formed by the reaction of NO and DTT, which supports the transfer of the NO moiety to the thiol group on the active site of the protein [31]. This inactivation mechanism through RS–NO does not seem to be applicable for human ferrochelatase, at least not so predominantly, as SIN-1 was more effective in lower concentrations of DTT. Recently it was proposed that aconitases, both mitochondrial and cytosolic (IRE-BP) types, are inactivated by $ONOO^-$, but not by NO [33,34]. The greater inhibitory effects of SIN-1 than SNAP on human ferrochelatase can be explained if inactivation of ferrochelatase relies on $ONOO^-$ formation. More detailed studies are required to determine what kind of molecule, i.e. NO, $ONOO^-$ or other NO-derived species, is responsible for the NO-mediated inactivation of ferrochelatase.

NO may be responsible for the loss of iron from NO-producing macrophages [35–37]. Drapier et al. [14] suggested that the activation of IRE-BP may compensate for the NO-induced iron depletion. The synthesis of several proteins involved in iron metabolism is apparently regulated in a similar manner by both iron and haem: ferritin synthesis is up-regulated by iron and haem while transferrin receptor synthesis is down-regulated by the same treatment [38]. Inhibition of haem synthesis down-regulates the syntheses of ferritin [39], transferrin receptor [39], and ferrochelatase [40]. Therefore, the NO-mediated increase in IRE-BP activity and NO-mediated decrease in ferrochelatase activity are likely to be closely related to regulation of intracellular levels of iron and haem, and function in the depletion of haem in cells. We propose that inactivation of ferrochelatase mediated by NO has a role in regulating iron and haem metabolisms.

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