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Induction of Ferritin Synthesis by Oxidative Stress

TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION BY EXPANSION OF THE "FREE" IRON POOL*

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Gaetano Cairo‡§, Lorenza Tacchini‡, Giovanna Pogliaghi‡, Elena Anzon‡, Aldo Tomasi¶, and Aldo Bernelli-Zazzera‡

From the ‡Centro di Studio sulla Patologia Cellulare, Consiglio Nazionale delle Ricerche and Istituto di Patologia Generale, Università di Milano via Mangiagalli 31, 20133 Milano, Italy and the ¶Dipartimento di Scienze Biomediche, Università di Modena, 41100 Modena, Italy

Ferritin, by regulating the "free" intracellular iron pool, controls iron-catalyzed generation of reactive oxygen species, but its role in oxidative damage is still unclear. We show that ferritin synthesis is significantly stimulated in the liver of rats subjected to oxidative stress by treatment with phorone, a glutathione-depleting drug. RNA-bandshift assays document reduced activity of iron regulatory factor, in particular of IRF_B, the cytoplasmic protein that post-transcriptionally controls ferritin mRNA translation. Furthermore, Northern blot analysis shows increased accumulation of H and L subunit mRNAs, and nuclear run-on experiments provide evidence of transcriptional activation. Direct measurements of intracellular free iron levels by EPR indicate that the increased ferritin synthesis can be mediated by an expansion of the free iron pool. An early drop of ferritin content after phorone treatment indicates that part of the iron that fuels the free pool might derive from ferritin degradation. Present data seem to suggest that, under conditions of oxidative stress, liver ferritin can represent either a pro- or an anti-oxidant in a time-dependent manner. In fact, its early degradation contributes to expand the intracellular free iron pool that, later on, activates multiple molecular mechanisms to reconstitute ferritin content, thus limiting the pro-oxidant challenge of iron.

Ferritin is a multimeric protein composed of 24 subunits of two types (H and L), which surround a cavity in which iron can be stored in a readily available but non-toxic form (1, 2). Changes in iron availability regulate ferritin gene expression at several levels, with the translational control being quantitatively more relevant (3–5). Ferritin mRNA translation is controlled by a cytosolic protein, iron regulatory factor (IRF),[1] whose regulated binding to an iron-responsive element (IRE) in the 5′-untranslated region of the mRNA finely tunes ferritin mRNA translation to intracellular iron levels (see Refs. 6–9 for review). A number of factors in addition to iron stimulate ferritin synthesis (10), which, in iron-independent pathophysiological situations, is predominantly regulated at pretranslational level (11).

Reactive oxygen species (ROS) are generated in small amounts in the normal metabolism of the cells and in increased amounts under many conditions of altered cell physiology; they are responsible for many kinds of cell injuries (12) and have been recently shown to induce a significant reprogramming of gene expression (13). Since intracellular iron catalyzes the generation of ROS (14, 15), ferritin, with its iron-segregating capacity, plays an important role in modulating cellular sensitivity to oxidant insults. While earlier studies pointed to ferritin as a source of catalytically active iron (16), more recent work showed that ferritin synthesis increases in different types of cultured cells subjected to oxidative stress conferring resistance to a subsequent insult (17–21). However, the intracellular triggers of this activation have not been as yet clearly identified. In fact, although the involvement of an increased free iron pool was hypothesized, direct measurements have not been provided. Moreover, it has not been defined whether other sources in addition to heme degradation by heme oxygenase (20, 21) contribute to the increased metal availability. Furthermore, the molecular mechanisms leading to enhanced ferritin synthesis under conditions of oxidative stress have not been studied in detail.

We studied the regulation of ferritin synthesis in an *in vivo* model in which a condition of oxidative stress is established by administration of phorone, a glutathione-depleting drug (22), which, by altering the balance between pro-oxidant and anti-oxidant molecules, amplifies the effects of ROS produced by cellular metabolic activity (23). We describe here that after phorone treatment, an early increase in the free iron pool, caused both by heme destruction and by ferritin degradation, stimulates ferritin synthesis transcriptionally and post-transcriptionally.

MATERIALS AND METHODS

Animals—Male Wistar rats (250–300 g) were housed, fed, and handled in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals. To induce oxidative stress, rats were injected intraperitoneally with phorone (diisopropylidene acetone) (30 mg/100 g, body weight, in sunflower oil). This drug reduces glutathione concentration, as we previously reported (22), and increases the activity of heme oxygenase (24).

Protein Synthesis by Liver Slices—Livers were sliced as in Ref. 25, and the slices were incubated for 1 h at 37 °C in the presence of 100 μCi of [^{35}S]methionine and homogenized as previously described (26). Equal amounts of labeled proteins were immunoprecipitated using anti-rat liver ferritin polyclonal antibody as previously described (27). Immunoprecipitation products were run on 15% SDS-polyacrylamide gels, and radioactivity in ferritin was revealed by fluorography and quantificated by densitometric scanning.

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§ To whom correspondence should be addressed: Centro di Studio sulla Patologia Cellulare, CNR, c/o Istituto Patologia Generale, Università di Milano, via Mangiagalli 31, 20133 Milano, Italy. Tel.: 39-2-70630821; Fax: 39-2-26681092.

[1] The abbreviations used are: IRF, iron regulatory factor; IRE, iron-responsive element; ROS, reactive oxygen species.

RNA-Protein Gel Retardation Assay—Liver samples were homogenized in the buffer described by Leibold and Munro (28); after centrifugation at 10,000 × *g* for 5 min, the supernatant was stored at −80 °C. 2 μg of protein samples of cytoplasmic extracts (determined using the Bio-Rad protein assay kit) were incubated with a molar excess of IRE probe transcribed *in vitro* with T7 RNA polymerase in the presence of 100 μCi of [α-^{32}P]UTP (800 Ci/mmol) (Amersham Corp.) from the pSPT-fer plasmid containing the IRE of human ferritin H chain (kindly provided by L. Kuhn) (29). Incubation in the presence or in the absence of 2% β-mercaptoethanol, digestion with RNase T1, and treatment with heparin were performed as previously described (30). After separation on 6% non-denaturing polyacrylamide gels, RNA-protein complexes were visualized by autoradiography and quantificated by liquid scintillation counting of bands excised from dried gels.

RNA Isolation and Northern Blot Analysis—Total cellular RNA was subjected to Northern blot analysis as described (31). Nylon filters were sequentially hybridized with the following ^{32}P-labeled DNA probes: the rat ferritin L subunit pRLFL3 cDNA (32), the rat ferritin H subunit H 1110 cDNA (33), the rat pRHO1 heme oxygenase cDNA (34), and a rat β-actin cDNA. For quantitative determinations, autoradiographic bands in the linear range were scanned with a densitometer, and the values were calculated after normalization to the signals of β-actin.

Nuclear Transcription Assay—Liver nuclei were purified and incubated for *in vitro* transcription as described (35). ^{32}P-Labeled nuclear RNA elongated *in vitro* was purified, and equal amounts of trichloroacetic acid-precipitable radioactivity for each sample were hybridized to DNA probes fixed on nitrocellulose filters following previously published procedures (36). Hybridization signals were evaluated by densitometric scanning and normalized to the values of β-actin.

EPR Determination of Free Iron Content—Redox active iron, also denominated free iron, was measured by electron paramagnetic resonance spectroscopy (EPR) as described in Ref. 37 using desferrioxamine as chelating agent. Briefly, 0.05 ml of 10 mM desferrioxamine (Desferal, Ciba-Geigy) was added to 0.5 ml of liver homogenate (50% w/v in saline), which, after incubation at 20 °C for 10 min, was placed in a cylindrical Teflon tube, frozen, and stored in liquid N_2. EPR spectra were recorded on a Brucker 200 spectrometer at liquid N_2 temperature under the following conditions: Klystron frequency, 9.12 GHz; power, 20 mW; modulation amplitude, 2.0 millitesla. The intensity of the EPR signal at g = 4.3 was measured to estimate desferrioxamine-iron concentration.

Careful calibration procedures were carried out; free iron concentration was calculated on a calibration plot obtained by adding to a control homogenate incremental volumes of a stock solution of $FeSO_4$, whose Fe^{2+} concentration was measured using the *o*-phenanthroline assay. The homogenate was then treated as described above, and the calibrating curve between amplitude of EPR signal and concentration of added iron ions was plotted.

Determination of Liver Ferritin Content—The cytoplasmic extracts used for bandshift assays were enriched in ferritin by heating at 70 °C as described (30) and run in 5-μg aliquots on 7.5% polyacrylamide non-denaturing gels. Gels were then silver stained, and ferritin bands were evaluated by densitometry. The density values of known amounts of recombinant human ferritin run on the same gel were used to plot a standard curve that was used to assess linearity of densitometric values.

RESULTS

Ferritin Synthesis by Liver Slices—The rate of ferritin synthesis was studied in liver slices pulse labeled *in vitro*, in which expression of liver-specific genes is representative of the situation *in vivo* (38). SDS-polyacrylamide gel electrophoresis analysis of immunoprecipitation products (Fig. 1) showed that ferritin synthesis is enhanced 3 h after treatment with phorone and increases further at 6 h with preferential induction of the H chain. Quantitative determination is reported in Table I.

RNA Bandshift Analysis of IRF Activity—Ferritin gene expression is regulated primarily at the post-transcriptional level through the action of IRF (6–9); therefore, we performed RNA bandshift analysis of IRF activity. Previous studies documented the existence of two specific IRE-binding proteins in rodent cells (30, 39, 40); a typical RNA gel retardation assay illustrated in Fig. 2 shows that treatment with phorone significantly reduces binding of the fast migrating complex IRF_B both at 3 and 6 h. Quantitative evaluation showed that RNA

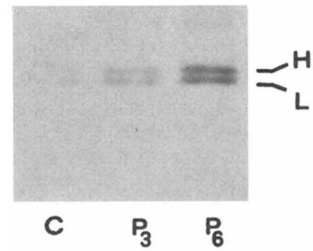

FIG. 1. **Ferritin synthesis by liver slices.** Equal amounts of labeled proteins synthesized by rat liver slices incubated in the presence of [^{35}S]methionine were immunoprecipitated with a specific anti-rat liver ferritin antibody and separated on a 15% SDS-polyacrylamide gel. Ferritin H and L subunits were visualized by fluorography. The autoradiogram shown is representative of three separate experiments. *C*, control; P_3 and P_6, 3 and 6 h after phorone treatment.

binding capacity of IRF_B in treated animals was 3-/4-fold lower than in control rats (Table I).

Northern Blot Analysis of Ferritin mRNA Levels—Ferritin mRNA levels are modulated by iron and by several other effectors (11); therefore, we analyzed the effect of oxidative stress on the amount of H and L ferritin subunit mRNAs. The Northern blot shown in Fig. 3 demonstrates that the steady state levels of both transcripts are unchanged 3 h after phorone administration but are remarkably increased 6 h after treatment, with preferential accumulation of H subunit mRNA (Table I). Phorone administration induces also a dramatic increase of the mRNA for heme oxygenase, a typical marker of oxidative stress (20, 24), while the amount of β-actin mRNA is unchanged.

Run-on Transcription Analysis—To establish whether the enhanced accumulation of ferritin mRNAs depends on an increased transcriptional rate, we assessed ferritin gene transcription by run-on analysis (Fig. 4). The transcription rate of both H and L ferritin genes increases over control levels 3 h after phorone treatment and remains sustained at 6 h. Induction seems more pronounced for the H subunit gene (Table I), which attains a response similar to that of heme oxygenase, an oxidative stress-responsive gene regulated at the transcriptional level (41). On the other hand, transcription of β-actin does not seem to be influenced by phorone treatment.

Determination of Free Iron Content—Next, we tried to identify the possible intracellular signal leading to enhanced ferritin gene expression. Since both transcription and translation of ferritin mRNAs are strictly dependent on intracellular iron availability (42), we determined the free intracellular iron content by means of EPR. Phorone significantly increases the intracellular pool of free iron in the liver both at 3 and 6 h (Table I).

Determination of Ferritin Content—To identify the origin of the iron that fuels the free pool, we measured cellular ferritin content; in fact, under conditions of oxidative stress, ferritin represents a possible iron-rich source of iron inside the cell (43). Taking advantage of its resistance to thermal denaturation, we measured the amount of ferritin in the same cytoplasmic extracts used for the IRF assay. Fig. 5 and Table I show that phorone treatment causes an early drop in ferritin content; at 6 h, the decrease is less evident, but the amount of ferritin remains below that of control animals.

DISCUSSION

The susceptibility of the cells to oxidative stress is dramatically influenced by the availability of free intracellular iron (44). The iron storage protein ferritin, which can rapidly take up and release iron, has been alternatively seen as a potentially harmful iron donor (16, 43) or as an effective anti-oxidant defense (17–21). The data of the present paper seem to suggest that, under conditions of *in vivo* oxidative stress, ferritin can

TABLE I

Effect of phorone treatment on liver ferritin gene expression and free iron content

Ferritin synthesis was calculated by scanning densitometry of fluorograms. Spontaneous IRF activity was determined by liquid scintillation counting of gel slices corresponding to the fast migrating protein-RNA complex. The values of H and L ferritin subunit mRNA steady state levels were estimated as described under "Material and Methods" after normalization for the amount of β-actin mRNA. Transcription rate of H and L genes was evaluated by densitometric scanning of autoradiograms. Ferritin content was evaluated by densitometric scanning of silver stained gels. Free iron content was estimated by EPR. All values represent mean ± S.D. of at least three separate experiments. Ferritin synthesis, IRF activity, H and L mRNAs accumulation and transcription, and ferritin content are given as a percentage of the control. Significance of difference for free iron content was tested by Student's *t* test.

	Ferritin synthesis	IRF activity	H subunit mRNA	L subunit mRNA	H subunit transcription	L subunit transcription	Liver ferritin	Free iron/g fresh liver
	%	%	%	%	%	%	%	*nmol*
Control	100	100	100	100	100	100	100	3.5 ± 0.8
Phorone (3 h)	211 ± 11	42 ± 6	97 ± 7	105 ± 12	461 ± 39	316 ± 46	52 ± 5	5.2 ± 0.9[a]
Phorone (6 h)	623 ± 47	23 ± 4	481 ± 33	306 ± 28	290 ± 15	263 ± 51	72 ± 8	5.4 ± 1.7[a]

[a] $p < 0.05$.

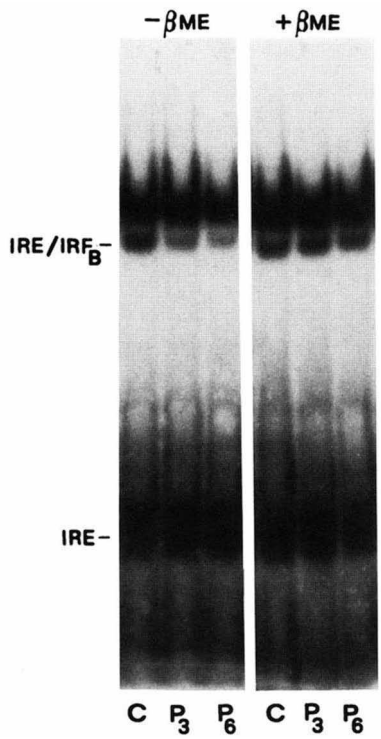

FIG. 2. **Bandshift assay of IRF activity.** Cytosolic extracts were incubated with an excess of ^{32}P-labeled IRE probe in the absence or presence of 2% β-mercaptoethanol, which is known to activate IRF binding activity. RNA-protein complexes were separated on non-denaturing 6% polyacrylamide gels and revealed by autoradiography. The exposure time of autoradiograms was twice longer for the samples without β-mercaptoethanol. The results shown are representative of four separate experiments. *C*, control; P_3 and P_6, 3 and 6 h after phorone treatment.

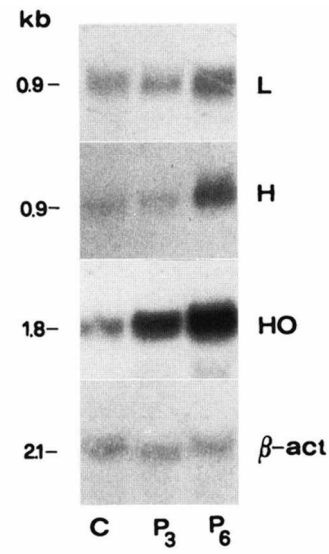

FIG. 3. **Northern blot analysis of ferritin mRNAs levels.** A filter with equal amounts of total liver RNA was hybridized with probes for H and L ferritin subunits, heme oxygenase (*HO*), and β-actin as indicated under "Materials and Methods." The autoradiograms shown are representative of three independent experiments. *C*, control; P_3 and P_6, 3 and 6 h after phorone treatment.

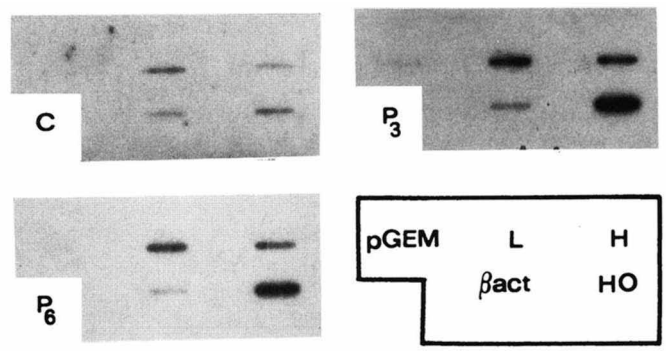

FIG. 4. **Run-on transcription assay.** Equal amounts of ^{32}P-labeled nuclear RNA synthesized *in vitro* by isolated liver nuclei were hybridized to panels of the indicated DNA probes immobilized on nitrocellulose filters. The autoradiogram shown is typical of three separate experiments. *C*, control; P_3 and P_6, 3 and 6 h after phorone treatment; *HO*, heme oxygenase.

perform both functions in a time-dependent manner. In fact, early after phorone treatment, we found higher levels of heme oxygenase mRNA and a decrease of ferritin content. The increase of heme oxygenase mRNA suggests that heme oxygenase-mediated heme degradation is a source of iron, as demonstrated to occur in UVA-irradiated fibroblasts (20, 21). However, iron originating from ferritin breakdown might also contribute to increase the pool of loosely bound redox-active iron. Since the estimated half-life of ferritin shells in rat liver is approximately 24 h (43, 45), the finding of decreased ferritin accumulation as early as 3 h after phorone treatment suggests that oxidative stress specifically induces ferritin degradation. The non-lysosomal calcium-dependent proteases activated in hepatocytes exposed to oxidative injury (46) might be implicated in this process. These findings are in agreement with a previous report indicating autophagic degradation of ferritin as a likely source of iron involved in oxidative injury of cultured rat hepatocytes (43). As a consequence of these events, an expansion of the free iron pool is likely to occur. Indeed, by EPR analysis, we directly show that phorone treatment increases free iron levels. Present data obtained *in vivo* extend therefore previous observations of ferritin induction by oxidative stress in cell cultures (20), in which an increase in the free iron pool

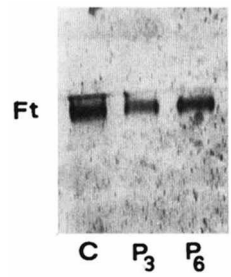

FIG. 5. **Liver ferritin content.** Cytoplasmic extracts were enriched in ferritin by heating as described under "Materials and Methods," and equal amounts of proteins were electrophoresed on non-denaturing 7.5% polyacrylamide gels. Ferritin was revealed by silver staining. The gel shown is representative of four independent experiments. *C*, control; P_3 and P_6, 3 and 6 h after phorone treatment.

was postulated, but no direct measurements were provided.

At the same time, the increased availability of iron stimulates ferritin synthesis. The preferential induction of the H subunit, where the ferroxidase activity of ferritin shells is located (47), will result in the accumulation of more acidic isoferritins better suitable for rapid uptake and sequestering of iron; this is consistent with the idea that ferritin has an antioxidant role.

The increase of ferritin synthesis seems to be regulated at both transcriptional and post-transcriptional levels. Indeed, run-on analysis directly shows a long-lasting increase of ferritin gene transcription that enhances the steady state levels of ferritin mRNAs; both H and L genes are activated, but transcription of the H gene seems to be preferentially stimulated. The increase in transcription might well be a consequence of the increased iron pool only; however, the marked stimulation of H subunit gene transcription, which is not affected by iron administration (4), suggests that ROS could be directly involved in the transcriptional activation of this gene. Oxidative stress further increases ferritin synthesis post-transcriptionally through inhibition of IRF binding activity. As recently shown to occur during liver regeneration (30), regulation is specific for the IRF_B complex. Down-regulation of IRF_B might be a consequence of increased free iron levels, but preliminary results showing inhibition of IRF activity by oxidative stress in a cell-free system suggest that ROS could directly affect IRF activity also *in vivo*.

Taken together, our data suggest that oxidative stress causes in rat liver the following cascade of events. At first, both heme cleavage by heme oxygenase and ferritin degradation cooperate to increase intracellular free iron levels. This expansion of the iron pool induces ferritin gene transcription and, by decreasing IRF binding activity, allows translation of pre-existing ferritin mRNA molecules to proceed; as a result, ferritin synthesis doubles. IRF activity remains low for a few hours and permits efficient translation of the higher amount of mRNAs that results from the previous activation of ferritin gene transcription. This causes a 6-fold increase of ferritin synthetic rate that allows reconstitution of ferritin content in the attempt to limit iron bioavailability.

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