Modulation of Cellular Iron Metabolism by Hydrogen Peroxide

EFFECTS OF H₂O₂ ON THE EXPRESSION AND FUNCTION OF IRON-RESPONSIVE ELEMENT-CONTAINING mRNAs IN B6 FIBROBLASTS

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Cellular iron uptake and storage are coordinately controlled by binding of iron-regulatory proteins (IRP), IRP1 and IRP2, to iron-responsive elements (IREs) within the mRNAs encoding transferrin receptor (TfR) and ferritin. Under conditions of iron starvation, both IRP1 and IRP2 bind with high affinity to cognate IREs, thus stabilizing TfR and inhibiting translation of ferritin mRNAs. The IRE/IRP regulatory system receives additional input by oxidative stress in the form of H₂O₂ that leads to rapid activation of IRP1. Here we show that treating murine B6 fibroblasts with a pulse of 100 μM H₂O₂ for 1 h is sufficient to alter critical parameters of iron homeostasis in a time-dependent manner. First, this stimulus inhibits ferritin synthesis for at least 8 h, leading to a significant (50%) reduction of cellular ferritin content. Second, treatment with H₂O₂ induces a 4-fold increase in TfR mRNA levels within 2–6 h, and subsequent accumulation of newly synthesized protein after 4 h. This is associated with a profound increase in the cell surface expression of TfR, enhanced binding to fluorescent-tagged transferrin, and stimulation of transferrin-mediated iron uptake into cells. Under these conditions, no significant alterations are observed in the levels of mitochondrial aconitase and the Divalent Metal Transporter DMT1, although both are encoded by two as yet lesser characterized IRE-containing mRNAs. Finally, H₂O₂-treated cells display an increased capacity to sequester ⁵⁷Fe in ferritin, despite a reduction in the ferritin pool, which results in a rearrangement of ⁹⁹Fe intracellular distribution. Our data suggest that H₂O₂ regulates cellular iron acquisition and intracellular iron distribution by both IRP1-dependent and -independent mechanisms.

To satisfy metabolic needs for iron, mammalian cells utilize transferrin (Tf), the iron carrier in plasma. Cellular iron uptake involves binding of Tf to the cell-surface Tf receptor (TfR), followed by endocytosis. Within the acidified endosome, iron is released from the Tf-TfR complex and transported, most likely by the Divalent Metal Transporter DMT1, across the endosomal membrane to the cytosol, where it becomes bioavailable for the synthesis of iron proteins. Excess iron is stored in ferritin, a multisubunit protein consisting of H- and L-chains, that serves as the major intracellular iron storage device (reviewed in Refs. 1–3). Sequestration of iron in ferritin is viewed as a detoxification step to reduce the risk of iron-mediated cell damage, which is based on the capacity of iron to catalyze the generation of toxic oxygen radicals (4). Balanced iron homeostasis is critical for health, and both iron deficiency as well as iron overload are associated with severe disorders (5).

At the cellular level, iron homeostasis is accomplished by the coordinate regulation of iron uptake and storage. The expression of TfR and ferritin is mainly controlled post-transcriptionally by iron regulatory proteins, IRP1 and IRP2. Under conditions of iron starvation, IRP1 and IRP2 are activated for high affinity binding to multiple "iron-responsive elements" (IREs) in the 3′-untranslated region (UTR) of TfR mRNA and to a single IRE in the 5′-UTR of the mRNAs encoding both H- and L-ferritin chains. This stabilizes TfR mRNA (6) and inhibits ferritin mRNA translation (7). Conversely, failure of IRPs to bind to cognate IREs in iron-replete cells leads to degradation of TfR mRNA and synthesis of ferritin (reviewed in Refs. 8–10). The identification of additional IRE-containing mRNAs suggests that the functional significance of the IRE/IRP system stretches out beyond the control of cellular iron uptake and storage. The mRNAs encoding the enzymes 5-aminolevulinate synthase-2 (involved in erythroid heme synthesis), mammalian mitochondrial aconitase (m-aconitase), and the insect 1p subunit of succinate dehydrogenase (both catalyzing reactions in the citric acid cycle) contain a “translation-type” IRE in their 3′-UTRs (11–16). The mRNAs encoding the more recently discovered iron transporters DMT1 (17, 18) and ferroportin/IREG1 (19–21) contain a single and, in terms of function, incompletely characterized IRE in their 3′- or 5′-UTR, respectively.

IRP1 and IRP2 share extensive homology and belong to the family of iron-sulfur cluster isomerases that also includes m-aconitase. However, their activities are controlled by distinct mechanisms. In iron-loaded cells, IRP1 assembles a cubane 4Fe-4S cluster that converts it to a cystolic aconitase (c-aconitase) and prevents IRE-binding, whereas IRP2 is oxidized and degraded by the proteasome. Iron starvation increases IRE-binding activity by disassembly of the 4Fe-4S cluster in IRP1 and stabilization/de novo synthesis of IRP2 (reviewed in Refs. 8–10, 22). Iron regulatory proteins are subjected to regulation
by additional iron-independent signals, including nitric oxide, hypoxia, and oxidative stress (reviewed in Refs. 23–25).

Of particular interest is the rapid induction of IRE binding activity of IRP1 in response to hydrogen peroxide (H$_2$O$_2$) (26, 27), because this "reactive oxygen intermediate" is implicated in iron toxicity. In the presence of catalytic amounts of ferrous iron, H$_2$O$_2$ yields highly aggressive hydroxyl radicals (Fenton reaction) that readily attack membranes, proteins, and nucleic acids (4). Exposure of different cell types to micromolar concentrations of H$_2$O$_2$ is sufficient to induce a rapid conversion of IRP1 from c-aconitase to the IRE-binding protein within 30–60 min (26, 27) by an incompletely characterized mechanism that involves signaling (28, 29). In contrast to this, H$_2$O$_2$ does not affect the activity of IRP2 (30). It should be noted that reactive oxygen species, including H$_2$O$_2$, are widely viewed as participants in a multitude of signaling pathways. These involve calcium signaling, mitogen-activated protein kinase cascades, tyrosine phosphorylation, regulation of phosphatases and phospholipases, or activation of transcription factors (reviewed in Refs. 31 and 32).

The effects of H$_2$O$_2$ on cellular iron metabolism have been as yet only partially studied. We have previously utilized mouse B6 fibroblasts, a cell line predominantly expressing IRP1 and negligible levels of IRP2, to characterize the mechanism of IRP1 induction by H$_2$O$_2$ (26, 28, 30, 33). We also showed that a treatment of these cells with 100 mM H$_2$O$_2$ for 1 h inhibits ferritin synthesis, whereas longer treatments (4–6 h) increase TIR mRNA levels, as a result of IRP1 activation (26). However, these responses have not been correlated with the biological activity of TIR and ferritin, in terms of iron uptake and sequestration. Here we extend the previous studies and investigate the effects of H$_2$O$_2$ in the expression and function of several IRE-containing mRNAs, as reflected in the uptake of $^{59}$Fe-transferrin and intracellular management of $^{59}$Fe.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—Desferrioxamine (DFO) was purchased from Novartis (Doral, Canada), and H$_2$O$_2$ was from Merck. Hemin, human apo- and holo-Tf, fluorescein isothiocyanate (FITC)-conjugated holo-Tf, and lactoferrin were from Sigma. B6 fibroblasts were grown and treated with H$_2$O$_2$ as described (26).

Metabolic Labeling with [35S]Methionine/Cysteine and Immunoprecipitation—Cells were metabolically labeled for 2 h with (50 μCi/ml) Trans35S-label (ICN, a mixture of 70:30 [35S]methionine/cysteine) and Triton X-100. Cytoplasmic lysates (1 mg) were subjected to quantitation from supernatants by addition of 0.5 ml of Sam68 antiserum (kindly provided by Dr. Stephane Richard). Immunoprecipitated material was washed twice in lysis buffer, and immunoprecipitated material was analyzed by SDS-PAGE-autoradiography (11).

Ferritin Assay—Cells were solubilized in RIPA lysis buffer (50 mM Tris-Cl, pH 7.4, 300 mM NaCl, and 1% Triton X-100). Cytoplasmic lysates (1 mg) were subjected to quantitative co-immunoprecipitation with 5 μl of rabbit polyclonal ferritin (Roche Molecular Biochemicals) and 2 μl of mouse monoclonal TIR (Zymed Laboratories Inc.) antibodies. Sam68 was then immunoprecipitated from supernatants by addition of 0.5 μl of Sam68 antiserum (kindly provided by Dr. Stephane Richard). Immunoprecipitated material was analyzed by SDS-PAGE-autoradiography (11).

Northern Blotting—RNA prepared with the Trizol® reagent (Life Technologies, Inc.) was analyzed by Northern blotting (26) with 32P-labeled mouse TfR, human ferritin H-chain, mouse actin (Sigma), or DMT1 (raised in rabbits against the peptide VFAE-AFFGKTNEQV, which corresponds to amino acids 260–275 in human DMT1). Dilutions for antibodies are indicated in the respective figure legends.

Fluorescence-activated Cell Sorting (FACS)—To determine cell surface expression or the TIR-binding capacity of TIR, cells were trapped and mixed with either 5 μl/ml FITC-conjugated mouse TfR antibody (PharMingen) or with 50 μg/ml FITC-conjugated human Tf (Sigma), respectively. Where indicated, a 50-fold excess human holo-Tf or lactoferrin was added prior to FITC-Tf. Excess FITC label was removed by washing twice with phosphate-buffered saline containing 0.1% bovine serum albumin. Cells were fixed with 3.7% formaldehyde and analyzed for fluorescence on a cell sorter (Beckman Coulter).

Generation of $^{59}$Fe-Tf—$^{59}$FeCl$_3$ (PerkinElmer Life Sciences) was mixed with sodium citrate (1:50 molar ratio in a total volume of 1 ml) and incubated for 1 h at room temperature. The resulting $^{59}$Fe-citrates were mixed with apo-Tf (2:1 molar ratio); the volume was brought up to 4 ml in 0.6 M NaHCO$_3$, and incubation was continued overnight. $^{59}$Fe-Tf was separated from $^{59}$Fe-citrate on a Centricon Plus-20 filter (Amicon), and its concentration was calculated spectrophotometrically at 465 nm (ε = 4620 μM−1 cm−1).

Cellular Uptake of $^{59}$Fe-Tf and Immunoprecipitation of $^{59}$Fe-Ferritin—Cells were labeled with $^{59}$Fe-Tf in minimal essential medium containing 25 mM Hepes, pH 7.4, 10 mM NaHCO$_3$, and 1% bovine serum albumin. Labeling was terminated by washing with ice-cold phosphate-buffered saline, and cells were monitored for radioactivity on a γ-counter. For immunoprecipitation of $^{59}$Fe-ferritin, cytoplasmic lysates were prepared in the same way as lysates of $^{35}$S-labeled cells (see above), and 1 mg was incubated at 4°C with 5 μl of rabbit polyclonal ferritin antibodies (Roche Molecular Biochemicals). Following addition of protein-A-coupled Sepharose CL-4B beads (Amersham Pharmacia Biotech), immunoprecipitated material was washed twice in lysis buffer, and radioactivity was monitored on a γ-counter.

RESULTS

H$_2$O$_2$ Elicits a Time-dependent Stimulation of TfR and Inhibition of Ferritin Synthesis—We have shown previously that treatment of cells with micromolar concentrations of H$_2$O$_2$ results in rapid induction of IRP1 to bind to IREs and that IRE binding activity remains elevated for at least 4 h following removal of the inducer (30, 33). This observation prompted us to study the effects of H$_2$O$_2$ on the expression of TIR and ferritin, two crucial proteins of iron metabolism under the control of the IRE/IRP system. Our analysis covers intervals of up to 8 h following exposure of cells to a bolus of 100 μM H$_2$O$_2$, allowing IRP1 activity to peak and decrease to basal levels (30). No apparent toxicity was observed by the trypan blue exclusion assay, under all experimental conditions employed in this study, in line with earlier observations that exogenous H$_2$O$_2$ is very rapidly degraded by these cells (33). Nevertheless, a single bolus of 100 μM H$_2$O$_2$ is sufficient to sustain a threshold of ~10 μM H$_2$O$_2$ for about 15 min, which is the minimum concentration required to elicit IRP1 activation (33). Thus, we established experimental conditions to activate IRP1 and study the effects of H$_2$O$_2$ on cellular iron metabolism in the absence of potential toxic side effects of H$_2$O$_2$. B6 fibroblasts were first treated with 100 μM H$_2$O$_2$ for 1 h and metabolically labeled with [35S]methionine/cysteine for 2 h either immediately or at different time points after treatment, and TIR and ferritin synthesis were assessed by immunoprecipitation (Fig. 1, top panel).

In cells previously treated with the iron chelator DFO (100 μM), TfR synthesis is stimulated 3.3-fold compared with untreated control cells, whereas synthesis of ferritin H- and L-chains is strongly inhibited (11 and 12% of control, respectively, lanes 1 and 2). Treatment with H$_2$O$_2$ initially does not affect TfR expression (lanes 2 and 3) but clearly stimulates TfR synthesis by 2- and 2.1-fold, within 4 and 6 h after its withdrawal, respectively (lanes 4 and 5). Soon afterward, TfR synthesis declines to almost control (1.1-fold) levels (lanes 6 and 7). In contrast, TIR, ferritin synthesis, and their expression were induced and sustained after H$_2$O$_2$ treatment; synthesis of ferritin H- and L-chains is reduced to 29 and 22% of control (lanes 2 and 3), in agreement with earlier observations (26). Ferritin synthesis remains at low levels even after 4 (28% for H- and 26% for L-chain) and 6 h (35% for H- and 31% for L-chain) following H$_2$O$_2$ withdrawal (lanes 4–7). After 8 h, ferritin synthesis only partially (60%)
Cytoplasmic cell extracts (1 mg) were subjected to quantitative immunoprecipitation and analyzed by SDS-PAGE on 12% gels (lanes 1–3). Immunoprecipitated materials were analyzed by SDS-PAGE on 12% gels (lanes 4 and 5), 4% (lanes 6 and 7), or 6% (lanes 8 and 9), and metabolically labeled for 2 h with [35S]methionine/cysteine (1 mg) were subjected to quantitative immunoprecipitation with 5 μl of ferritin (Roche Molecular Biochemicals) and 2 μl of TR (Zymed Laboratories Inc.) antibodies. Ferritin and TR-immunodepleted supernatants were incubated with 0.5 μl of Sam68 antisera (kindly provided by Dr. Stephane Richard). Immunoprecipitated materials were analyzed by SDS-PAGE on 12% gels (top panel, TR and ferritin; bottom panel, Sam68). Proteins were visualized by autoradiography and quantified by densitometric scanning (NIH Image software). The positions of TfR, ferritin (H- and L-chains), and Sam68 are indicated by arrows. The positions of molecular mass standards are indicated on the right. Induction for TfR and percentage inhibition of ferritin synthesis after H2O2 treatment (lanes 3, 5, 7, and 9) is calculated compared with respective untreated controls (lanes 2, 4, 6, and 8).

Effects of H2O2 on the Steady-state Levels of TfR and Ferritin—Analysis by Northern blotting (Fig. 2A) reveals that exposure of cells to 100 μM H2O2 for 1 h leads to a 3.4-, 4.0-, and 4.5-fold increase in steady-state levels of TfR mRNA 2, 4, and 6 h after the treatment, respectively (top panel, lanes 2–5). TfR mRNA levels drop after 8 h but are still 1.7 times higher than control (lane 6). As expected, iron chelation with DFO leads to a profound (5.7-fold) induction of TfR mRNA (lane 1). In contrast, TfR, ferritin (at least H-chain) mRNA levels are not affected by iron chelation or H2O2 (middle panel). The same holds true for non-iron-regulated β-actin mRNA (bottom panel). Thus, the time-dependent stimulation of TfR synthesis by H2O2 (Fig. 1) correlates with an increase in TfR mRNA levels, whereas H2O2-mediated inhibition of ferritin synthesis appears to be translational.

H2O2-mediated Reduction of Ferritin Pool and Accumulation of TfR—We employed an immunoturbidimetric assay to measure ferritin levels in cell extracts and to assess the effects of H2O2 on total cellular ferritin content (Fig. 2B). As expected, iron perturbations are strongly reflected in the ferritin pool; exposure of cells to hemin increases ferritin levels 3-fold, whereas iron chelation dramatically reduces ferritin to 6% of control levels (lanes 1–3). Treatment with 100 μM H2O2 for 1 h initially decreases the ferritin content to 69% (lanes 3 and 4). Further reductions to 55 and 42% are evident 2 and 4 h after H2O2 withdrawal, respectively (lanes 5 and 6). Ferritin concentration tends to increase very slightly to 49 and 47% after 6 and 8 h (lanes 7 and 8), in line with the partial recovery in de novo ferritin synthesis at these time points (Fig. 1). We conclude that H2O2 leads to a marked reduction in the ferritin pool for at least 8 h after the treatment.

To examine whether stimulation of TfR synthesis by H2O2 is associated with an increase in TfR concentration, we analyzed steady-state levels of TfR by Western blotting (Fig. 2C). Treatment of cells with 100 μM H2O2 for 1 h leads to gradual accumulation of TfR after 2–8 h (lanes 3–8). H2O2-mediated induction of TfR reaches a maximum 6 and 8 h after the treatment (1.9- and 1.8-fold, respectively). As expected, treatments with DFO or hemin result in 2.2-fold increase and 0.6-fold decrease of TfR (lanes 1–3). In this experiment, cells were solubilized in RIPA lysis buffer, to extract membrane-bound TfR efficiently, but similar results were obtained with cytoplasmic extracts (not shown).

H2O2 Leads to Increased Expression of Functional TfR on the Cell Surface—The data shown in Fig. 2C suggest that H2O2 stimulates TfR expression. We next designed experiments to address whether this is accompanied by increased Tf binding activity. The fraction of TfR expressed on the cell surface is crucial for Tf binding. In a previous report it was shown that H2O2 negatively affects the size of this fraction, at least in human hematopoietic K562 and HL-60 cells (35). In light of these findings, we analyzed relative changes in cell surface expression of TfR in mouse B6 fibroblasts by means of FACS, using FITC-conjugated Tf antibodies (Fig. 3A). The levels of TfR on the cell surface essentially remain unaltered within 2 h after exposure of cells to H2O2 (100 μM H2O2 for 1 h) (lanes 3–5), but increase by 1.4-, 1.5-, and 1.9-fold within 4, 6, and 8 h, respectively (lanes 6–8). A profound cell surface expression of TfR is achieved by treatment with DFO, whereas administration of hemin does not appear to cause any notable alterations (lanes 3–5).

By having established that exposure of cells to H2O2 is associated with increased expression of TfR, including its cell surface fraction, we then employed a functional assay to evaluate the effects of H2O2 on Tf binding activity. Cells were incubated with FITC-conjugated Tf under conditions allowing its binding to TfR. Changes in relative fluorescence were then monitored by FACS (Fig. 3B). Following treatment with H2O2 (100 μM H2O2 for 1 h), cells were mixed with 50 μg/ml FITC-Tf, either at 4 °C for 2 h or at 37 °C for 40 min. Incubation at 4 °C inhibits recycling of TfR and thus serves to evaluate binding of FITC-Tf on the cell surface. Conversely, incubation at 37 °C is preferable to examine both cell surface-bound and internalized (endosomal) FITC-Tf levels. To facilitate displacement of serum-derived Tf from TfR, incubation at 4 °C was prolonged to 2 h. Under both experimental settings, FITC-Tf binding to TfR gradually increased 4–8 h following exposure of cells to H2O2 (Fig. 3B, bars 3–8). The increase was slightly elevated when incubations were performed at 37 °C (compare 1.2- and 1.7-fold at 4 °C with 1.4- and 1.8-fold increase at 37 °C, 4, 6, and 8 h after treatment, respectively). Consistent with the data described above, iron chelation with DFO elicits stronger effects on FITC-Tf binding to TfR than H2O2 (up to 3.8-fold induction, Fig. 3B, bar 2). As expected, the effects of hemin are inhibitory (bar 1).

The specificity of the FITC-Tf binding assay is illustrated in Fig. 3C. Co-incubation of FITC-Tf with 50-fold excess non-
labeled Tf competitor strongly reduces fluorescence intensity to 24.4% in untreated and to 10% in DFO pretreated cells. In contrast, addition of 50-fold excess lactoferrin as a nonspecific competitor only slightly interferes with FITC-Tf binding (15% reduction). Incubations with these competitors were performed at 37 °C, and similar results were obtained at 4 °C (not shown). Taken together, our findings suggest that exposure of B6 cells to H₂O₂ leads not only to an increase in TfR steady-state levels but also stimulates its cell surface expression and the Tf-binding capacity. These conditions are predicted to favor enhanced cellular iron uptake from Tf.

**H₂O₂ Stimulates Uptake of ⁵⁹Fe-Tf and Storage of ⁵⁹Fe in Ferritin, Leading to Alterations in the Relative Intracellular Distribution of ⁵⁹Fe**—To determine directly the effects of H₂O₂ on iron uptake, we incubated B6 cells with 5 μM ⁵⁹Fe-Tf for 2 h and measured cell-associated radioactivity on a γ-counter. Preliminary experiments indicated that this concentration of ⁵⁹Fe-Tf is saturating (not shown). The results of the iron uptake experiment are depicted in Fig. 4A. Untreated control fibroblasts internalize ~10.5 pmol of ⁵⁹Fe/10⁹ cells during the time of labeling (2 h). Exposure of B6 cells to 100 μM H₂O₂ for 1 h results in a modest (~11.5%) but significant (p < 0.05 as estimated by Student’s t test) increase in ⁵⁹Fe uptake, 6–8 h after the H₂O₂ treatment. Iron starvation by overnight treatment with 100 μM DFO leads to a more pronounced (~24.9%) increase in ⁵⁹Fe uptake. Considering the profound effects of H₂O₂ and iron starvation on the expression of Tf and its Tf binding activity (Fig. 3), the differences in ⁵⁹Fe uptake in response to these stimuli are not particularly strong, suggesting that the Tf-TfR cycle may be subjected to additional controls. Nevertheless, these data show that H₂O₂-treated cells have an increased capacity to take up iron.

Under the conditions of the iron uptake experiment (e.g., 6–8 h following H₂O₂ treatment), ferritin synthesis is still partially repressed (Fig. 1), whereas cellular ferritin content has dropped to <50% of control levels (Fig. 2B). Since ferritin plays a major role in iron detoxification as an iron-storage sink, we wondered how cells respond to increased iron uptake when ferritin levels are reduced. To address this question, B6 fibroblasts were labeled with 5 μM ⁵⁹Fe-Tf (as in Fig. 4A) for 15 and 30 min and 1 and 2 h. Cytoplasmic extracts were analyzed by quantitative immunoprecipitation with ferritin antibodies, and ferritin-associated ⁵⁹Fe was plotted against the time of labeling (Fig. 4B). Ferritin immunoprecipitates from H₂O₂-treated cells display a marked increase in ⁵⁹Fe content compared with untreated control cells. After 2 h of labeling, ~18.16 pmol of ⁵⁹Fe/mg protein in extracts of control cells are associated with ferritin, whereas this value increases to ~14.85 pmol of ⁵⁹Fe/mg protein (181%) in extracts from H₂O₂-treated cells. Ferritin-associated ⁵⁹Fe in extracts of cells pretreated with DFO is very low (~0.9 pmol of ⁵⁹Fe/mg of protein after 2 h of labeling, representing 11% of control), most likely due to sequestration of iron by the chelator. When cells were prelabeled with ⁵⁹Fe-Tf for 2 h and then left untreated or treated with H₂O₂, no differences in the amount of ferritin-associated ⁵⁹Fe were observed (not shown).

These data suggest that H₂O₂-treated cells have an increased capacity to store newly internalized iron in ferritin, despite the reduction in the translation and in the intracellular pool of ferritin. They also imply that their fraction of ferritin-associated ⁵⁹Fe is significantly enriched. To calculate the distribution of ⁵⁹Fe in control, H₂O₂-, and DFO-treated cells, we also measured radioactivity in the ferritin-immunodepleted extracts and in the insoluble cell fraction (similar methodology has been employed by others (36)) and depicted the results in form of pie charts (Fig. 4C). Treatment with H₂O₂ leads to alterations in intracellular distribution of ⁵⁹Fe with a notable increase in the fraction of ferritin-associated ⁵⁹Fe from 15.8 to 26%. Considering that within 2 h, 10⁶ B6 cells take up ~10.5 pmol of ⁵⁹Fe if untreated and ~11.74 pmol of ⁵⁹Fe if treated with H₂O₂ (Fig. 4A), the former store ~1.66 and the latter ~3.05 pmol of ⁵⁹Fe in ferritin. This represents an almost 2-fold
increase under conditions where only half the amount of ferritin is available (Fig. 2B).

The Steady-state Levels of m-Aconitase and DMT1 Are Not Affected by \( \text{H}_2\text{O}_2 \)—By having established that \( \text{H}_2\text{O}_2 \) modulates the expression (and the function) of TfR and ferritin, we asked whether \( \text{H}_2\text{O}_2 \) also affects the abundance of m-aconitase and DMT1, both encoded by IRE-containing mRNAs. Western blotting analysis at different time points after treatment of B6 cells with \( \text{H}_2\text{O}_2 \) does not show any significant alterations in steady-state levels of m-aconitase (Fig. 5A, top panel, lanes 3–8). Overnight iron perturbations with DFO or hemin yield a similar outcome (lanes 1 and 2). Probing with an antibody against \( \beta \)-actin (bottom panel) suggests that the slight reduction in the intensity of the m-aconitase band on lane 7 is of no functional importance and rather reflects unequal loading.

The effects of \( \text{H}_2\text{O}_2 \) on DMT1 mRNA were assessed by Northern blotting. Probing with a mouse DMT1 cDNA reveals two hybridizing bands of 3.1 and 2.3 kilobases (Fig. 5B, top panel) that possibly correspond to the non-IRE and IRE-containing isoforms of DMT1 mRNAs, respectively (37, 38). By normalizing to the \( \beta \)-actin signal (bottom panel), no obvious differences in the intensity of both bands are observed between samples from untreated control (lane 5), \( \text{H}_2\text{O}_2 \)-treated (lanes 1–4) or iron-perturbed cells (lanes 5–7). This finding is also mirrored at the protein level; Western blotting with antibodies against DMT1 (Fig. 5C) shows a faint band with an apparent molecular mass of ~65 kDa that has the same intensity in samples from untreated control, \( \text{H}_2\text{O}_2 \)-treated, or iron-perturbed cells (lanes 1–5). Since the sizes of polypeptides encoded by the IRE- and non-IRE-DMT1 mRNAs differ by only 7 amino acids, the ~65-kDa band most likely corresponds to a mixture of both isoforms. The specificity of the interaction is demonstrated on lane 6. Probing the filter in the presence of excess DMT1 antigenic peptide does not produce the 65-kDa signal. Thus, in B6 cells, the expression of both non-IRE- and IRE-containing isoforms of DMT1 mRNAs does not appear to respond to iron or \( \text{H}_2\text{O}_2 \).

**DISCUSSION**

\( \text{H}_2\text{O}_2 \) as a Signal to the IRE/IRP Regulatory System—We show that a transient exposure of cells to \( \text{H}_2\text{O}_2 \) stimulates TfR and decreases ferritin translation in a time-dependent manner. TfR synthesis peaks 4–6 h following exposure of cells to 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Fig. 1) as a result of the accumulation of Tf mRNA (Fig. 2A). In contrast, ferritin (at least H-chain) mRNA levels remain unaltered for up to 8 h after \( \text{H}_2\text{O}_2 \) challenge (Fig. 2A), but ferritin (H- and L-chains) synthesis is strongly inhibited immediately after \( \text{H}_2\text{O}_2 \) withdrawal and slowly recovers afterwards (Fig. 1). As IRP1 is known to stabilize Tf mRNA and inhibit ferritin mRNA translation by binding to their respective IREs, these responses underlie the causal relationship of IRP1 induction by \( \text{H}_2\text{O}_2 \). In kinetic terms, the activation of IRP1 is in perfect agreement with the regulatory effects on its downstream targets. The translational inhibition of ferritin is rapid and temporally coincides with the increase in IRE binding activity (30), whereas accumulation of Tf mRNA is delayed and follows its stabilization by binding of IRP1. The decline of IRE binding activity to basal levels >4 h after \( \text{H}_2\text{O}_2 \) treatment (30) is associated with a decrease in TfR synthesis, as a result of Tf mRNA destabilization, and gradual recovery of ferritin mRNA translation (Figs. 1 and 2). We conclude that \( \text{H}_2\text{O}_2 \) modulates the expression of ferritin and TfR via activation of IRP1.

We have also studied the effects of \( \text{H}_2\text{O}_2 \) (and iron donors/chelators) on the abundance of m-aconitase and DMT1. Both
Regulation of Iron Metabolism by H$_2$O$_2$  

Fig. 4. H$_2$O$_2$-mediated changes in the uptake, storage, and intracellular distribution of $^{59}$Fe. B6 cells were left untreated or treated with 100 µM DFO overnight or with 100 µM H$_2$O$_2$ for 1 h. Following H$_2$O$_2$ treatment, cells were washed and further incubated for another 6 h. Subsequently, cells received 5 µM $^{59}$Fe-Tf (349 cpm/pmol $^{59}$Fe). A. Labeling was stopped after 2 h; cells were washed twice with ice-cold phosphate-buffered saline, and radioactivity was measured on a $\gamma$-counter. The amount of radioactive iron taken up by the cells, corresponding to triplicate samples, is expressed in pmol of $^{59}$Fe/10$^6$ cells in 2 h. B, labeling was stopped after 15, 30, 60, or 120 min, and cells were washed twice with ice-cold phosphate-buffered saline and lysed. Cytoplasmic extracts (1 mg) were subjected to quantitative immunoprecipitation with 5 µl of ferritin antibody (Roche Molecular Biochemicals). Radioactivity in immunoprecipitated material was measured on a $\gamma$-counter. The amount of ferritin-associated radioactive iron, expressed in pmol of $^{59}$Fe/mg protein in cell extract, is plotted against the time of labeling (ctrl, circles; DFO, triangles; H$_2$O$_2$, squares). C, samples of B were analyzed for radioactivity in the ferritin-immunodepleted supernatant ("soluble" fraction) and in the pellet obtained following cell lysis and centrifugation ("insoluble" fraction). The relative distribution of $^{59}$Fe in the ferritin-immunodepleted soluble fraction (gray), in the insoluble fraction (black), and in ferritin (white), analyzed in untreated control cells and in cells treated with DFO or H$_2$O$_2$, is plotted on a pie chart. The percentages represent mean values of triplicates. Linear regression and statistical analysis of triplicates was performed with GraphPad Prism (version 2.0) software. *, p < 0.05 versus control.

Proteins are encoded by IRE-containing mRNAs. However, under our experimental conditions, we did not observe any iron- or H$_2$O$_2$-dependent alterations in their steady-state levels (Fig. 5). The IRE in m-aconitase mRNA is located in the 5'-UTR and is functional as a translational regulator in vitro (13, 14). However, the range of iron-dependent regulation of m-aconitase translation in vivo lags orders of magnitude behind the respective range of ferritin regulation (14, 15, 39). A potential explanation for this is offered by the structural differences between m-aconitase and ferritin IREs. The former contains a C-bulge and the latter an internal loop/bulge that confer to them differential binding specificity toward IRP1 and IRP2 in vitro (40). The functionality of m-aconitase IRE in de novo synthesis of m-aconitase has been demonstrated by sensitive immunoprecipitation assays following iron perturbations and metabolic labeling of several cell lines with $^{59}$Fe-methionine (15). Relatively small but significant effects of iron on the steady-state levels of m-aconitase have been documented by Western blotting analysis of mouse (14) and rat (39) tissues following long term (over several weeks) modulation of dietary iron intake. In light of these data, we conclude that short term (<12 h) iron perturbations or treatments with H$_2$O$_2$ are not sufficient to lead to any detectable alterations in m-aconitase steady-state levels (Fig. 5A).

The IRE in DMT1 mRNA is located in the 3'-UTR and has as yet only partially been characterized. The levels of DMT1 mRNA (IRE-containing isoform) are increased in iron-deficient enterocytes from duodenal samples of hemochromatosis patients (41) or HFE$^{-/-}$ mice (42). In addition, a radiolabeled DMT1 IRE probe is functional in gel retardation assays with cell extracts (38). Taken together, these results would argue for a role of the IRE in controlling the stability of DMT1 mRNA. However, whereas a single IRE is sufficient to function as a translational control element, earlier experiments showed that the minimum requirement for regulating the stability of TIR mRNA is defined by a combination of more than one IRE together with additional non-IRE sequences (43). According to the findings in Ref. 43, the single IRE in the 3'-UTR of DMT1 mRNA would not qualify in its own right as a regulator of its stability via IRE/IRP interactions. This view is supported by the data presented in Fig. 5B. The Northern analysis does not reveal any significant differences in the abundance of the 2.3- and 3.1-kilobase transcripts, following treatments with H$_2$O$_2$ (over 8 h) or iron donors/chelators (overnight). The lack of iron responsiveness in the abundance of DMT1 mRNA is in agreement with recent data (38, 44). We speculate that the iron-dependent regulation in the expression of the IRE-containing isoform of DMT1 mRNA in enterocytes may involve additional factors that are not present in B6 cells. However, it should be noted that we do not have sufficient information on the relative distribution of DMT1 encoded by the IRE- or non-IRE forms of DMT1 mRNA, which may be an important factor for the overall interpretation of the data. Along these lines, it is not unexpected that under our experimental conditions iron or H$_2$O$_2$ essentially has no effect on DMT1 steady-state levels (Fig. 5C).

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$^2$ A. Caltagirone, G. Weiss, and K. Pantopoulos, unpublished data.
DMT1 was detected by a chromogenic (alkaline phosphatase) reaction and electrotransferred onto a nitrocellulose membrane. The membrane cells, treated as indicated, were resolved by SDS-PAGE on a 10% gel. DMT1 immunogenic peptide was used in the analysis of DMT1 by Western blotting. We wondered whether exposure of cells to H2O2 affects the expression of DMT1 and other iron regulatory proteins.

Previous studies in human hematopoietic K562 and HL-60 cells showed that oxidative stress (either in the form of menadione or extracellular H2O2) results in a rapid (within 30 min) redistribution of TfR in intracellular compartments without alterations in TfR levels (35, 50). These results appear to be IRP1-independent and are in contrast to the stimulatory effects of H2O2 on the synthesis, accumulation, cell surface expression, and Tf binding activity of TfR observed in B6 cells (Figs. 1–3). It is well established that the regulation of TfR expression is more complex in erythroid cells, the major iron consumers in the body, and involves transcriptional as well as post-transcriptional mechanisms (51–54). Thus, it is conceivable that additional, IRP1-independent pathways regulate TfR in various cell types in response to H2O2. Nevertheless, it would be interesting to investigate the effects of H2O2 on IRP1 activity in K562 and HL-60 cells.

In B6 fibroblasts, the H2O2-mediated increase in TfR expression correlates with a modest (~11.5%) but significant increase of 59Fe-Tf uptake (Fig. 4A), despite the fact that Tf binding activity is stimulated 1.7-fold (Fig. 3B). Similarly, iron-starved cells (treated with DFO) take up ~24.9% more 59Fe-Tf than untreated controls, despite a 3.1-fold induction in Tf binding activity (Figs. 4A and 3B). These findings imply that intracellular iron release during the Tf-TfR cycle may be controlled at additional checkpoints but are also compatible with the idea that subtle perturbations in intracellular iron balance may be sufficient to elicit significant pathophysiological responses.

The experiments with 59Fe-Tf yielded another unanticipated result. H2O2-treated cells have an increased capacity to store 59Fe in ferritin, at a time point where the ferritin pool is dramatically reduced (Figs. 4B and 2B), leading to changes in intracellular 59Fe distribution (Fig. 4C). The reason for this is not clear, but it is tempting to hypothesize that H2O2 signaling interferes with the incompletely defined mechanism of iron sequestration in ferritin. It has been proposed that ferritin subunits may be arranged in a flexible and dynamic structure allowing iron entry/release by localized unfolding. In this sense, it is conceivable that changes in iron entry in response to extracellular stimuli may be associated with post-translational modification of ferritin that could affect such localized unfolding (55). In fact, there is evidence in older literature that ferritin can be phosphorylated in vitro (56). Along these lines, it will be interesting to examine the phosphorylation status of ferritin in cells, following a treatment with H2O2. From a physiological point of view, the increased capacity of ferritin to

**H2O2 as a Modulator of Cellular Iron Metabolism Beyond the IRE/IRP Regulatory System**

Reactive oxygen species are implicated in a wide array of signaling pathways (31, 32). We thus wondered whether exposure of cells to H2O2 affects the expression and function of genes of iron metabolism at different levels, either upstream or downstream of the IRE/IRP regulatory system (for example transcriptionally or post-translationally). There is evidence that ferritin synthesis is transcriptionally activated in response to various forms of oxidative stress as part of a homeostatic antioxidant defense mechanism (45–47). More recently, a functional "antioxidant response element" has been identified in the promoters of L- (48) and H-ferritin (49). This element is shared in promoter regions of several phase II detoxification genes and functions as a transcriptional enhancer in response to pro-oxidant stimuli. Treatment of mouse BNL CL.2 normal liver cells or Hepa1–6 hepatoma cells with >250 μM H2O2 stimulated a delayed (after 8 h) transcriptional activation of H- and L-ferritin mRNAs via the antioxidant response element, which gradually overcame the initial IRP1-mediated translational inhibition of ferritin synthesis (49). The data presented in Fig. 2A suggest that in B6 cells H2O2 fails to increase ferritin mRNA levels over 8 h (at least that of ferritin H-chain). Although it is conceivable that application of more stringent conditions of oxidative stress may stimulate ferritin mRNA transcription, it is apparent that low micromolar concentrations of H2O2 exhibit solely inhibitory effects on ferritin expression. These are reflected in the decrease in ferritin synthesis (Fig. 1) and the reduction of ferritin pool (Fig. 2B). In preliminary pulse-chase experiments, H2O2 did not appear to affect ferritin half-life (not shown), suggesting that H2O2-mediated translational inhibition of ferritin synthesis suffices to reduce dramatically (~50% of control levels) the intracellular ferritin pool for at least 8 h.

It is well established that the regulation of TfR expression is more complex in erythroid cells, the major iron consumers in the body, and involves transcriptional as well as post-transcriptional mechanisms (51–54). Thus, it is conceivable that additional, IRP1-independent pathways regulate TfR in various cell types in response to H2O2. Nevertheless, it would be interesting to investigate the effects of H2O2 on IRP1 activity in K562 and HL-60 cells.

**FIG. 5.** Treatment with H2O2 does not affect steady-state levels of m-aconitase, DMT1 mRNA, and DMT1. A, 30 μg total cell extracts as in Fig. 2C were analyzed by Western blotting. The membrane was probed with 1:500 diluted rabbit antibody against bovine heart m-aconitase (a generous gift of Dr. Rick Eisenstein) and reprobed with 1:200 diluted rabbit antibody against β-actin; m-aconitase and β-actin were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Lanes are as in Fig. 2C. B, analysis of DMT1 and β-actin mRNAs by Northern blotting. 15 μg of total RNA from B6 cells, treated as indicated, was resolved on an agarose gel (1%), electrotransferred onto a nylon membrane, and hybridized to the respective 32P-radiolabeled cDNA probes. Radioactive bands were visualized by autoradiography. C, analysis of DMT1 by Western blotting. 30 μg total cell extracts from B6 cells, treated as indicated, were resolved by SDS-PAGE on a 10% gel and electrotransferred onto a nitrocellulose membrane. The membrane was probed with 1:200 diluted rabbit antibody against DMT1, and DMT1 was detected by a chromogenic (alkaline phosphatase) reaction. Lane 1, untreated control; lane 2, 100 μM DFO (1 h); lane 3, 100 μM DFO (4 h); lane 4, 100 μM DFO (4 h) and 30 μg total cell extracts from B6 cells, treated as indicated, were resolved by SDS-PAGE on a 10% gel and electrotransferred onto a nitrocellulose membrane. The membrane was probed with 1:200 diluted rabbit antibody against DMT1, and DMT1 was detected by a chromogenic (alkaline phosphatase) reaction. Lane 1, untreated control; lane 2, 100 μM DFO (1 h); lane 3, 100 μM DFO (4 h); and lane 4, untreated control, probed in the presence of 10 ng of DMT1 immunogenic peptide.
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