Sustained Hydrogen Peroxide Induces Iron Uptake by Transferrin Receptor-1 Independent of the Iron Regulatory Protein/Iron-responsive Element Network*

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Local and systemic inflammatory conditions are characterized by the intracellular deposition of excess iron, which may promote tissue damage via Fenton chemistry. Because the Fenton reactant H$_2$O$_2$ is continuously released by inflammatory cells, a tight regulation of iron homeostasis is required. Here, we show that exposure of cultured cells to sustained low levels of H$_2$O$_2$ that mimic its release by inflammatory cells leads to up-regulation of transferrin receptor 1 (TfR1), the major iron uptake protein. The increase in TfR1 results in increased transferrin-mediated iron uptake and cellular accumulation of the metal. Although iron regulatory protein 1 is transiently activated by H$_2$O$_2$, this response is not sufficient to stabilize TfR1 mRNA and to repress the synthesis of the iron storage protein ferritin. The induction of TfR1 is also independent of transcriptional activation via hypoxia-inducible factor 1α or significant protein stabilization. In contrast, pulse experiments with $^{35}$S-labeled methionine/cysteine revealed an increased rate of TfR1 synthesis in cells exposed to sustained low H$_2$O$_2$ levels. Our results suggest a novel mechanism of iron accumulation by sustained H$_2$O$_2$, based on the translational activation of TfR1, which could provide an important (patho)physiological link between iron metabolism and inflammation.

Systemic iron homeostasis undergoes typical changes during inflammatory or infectious conditions. A decrease in plasma iron concentration limits the availability of the metal for erythropoiesis, ultimately leading to the so-called anemia of chronic disease (1). In addition to iron retention within the reticuloendothelial system, parenchymal cells such as hepatocytes also accumulate iron under inflammatory conditions (2–8), and this iron deposition has been identified as an important factor in tissue damage by free radicals (9). In addition, hepatic iron accumulation appears to be an important cofactor in the development of fibrosis and end stage liver disease in such common chronic liver pathologies such as hepatitis C or alcoholic steatohepatitis (4–8).

Significant progress has been made toward understanding the molecular basis of iron retention within the reticuloendothelial system during inflammation (10–12). The mechanism involves the interleukin-6-mediated induction of the iron-regulatory peptide hepcidin (13, 14), which inhibits iron efflux from macrophages and intestinal enterocytes (15, 16) by binding to and promoting the degradation of the transporter ferroportin 1 (IREG1 or MTP1) (17). The ensuing hypoferremia is thought to be part of a physiological defense strategy to deplete invading bacteria from the growth-essential iron. Thus far, the possibility that inflammation-mediated accumulation of iron in parenchymal cells may also contribute to hypoferremia has not received much attention. Nevertheless, the expression of transferrin receptor 1 (TfR1), the major iron uptake protein, is induced in several models of inflammation (2, 18).

Upon activation, inflammatory cells such as neutrophils and macrophages undergo an “oxidative burst” that results in the release of large amounts of reactive oxygen species to kill invading bacteria (19). The membrane-associated NADPH-oxidase (NOX2) first generates superoxide that is rapidly dismutated to the more stable H$_2$O$_2$ by superoxide dismutases (20). Thus, during inflammation, cells and tissues are exposed to sustained concentrations of H$_2$O$_2$ demanding a tight regulation of iron homeostasis to prevent tissue damage via Fenton and Fenton-like reactions. The activation of iron regulatory protein 1 (IRP1) by H$_2$O$_2$ has been proposed as a regulatory link between cellular iron homeostasis and inflammation. IRP1 regulates the expression of several proteins by post-transcriptional mechanisms. In iron-deficient cells, the mRNA of TfR1 is stabilized upon binding of IRP1 and IRP2 to iron-responsive elements (IREs) within

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2 The abbreviations used are: TfR1, transferrin receptor 1; CAT, catalase; EMSA, electrophoretic mobility shift assay; GOX, glucose oxidase; IR, iron-responsive element; IRP1, iron regulatory protein 1; LIP, labile iron pool; HIF, hypoxia-inducible factor; PBS, phosphate-buffered saline; MT, 3-[(4,5-dimethoxiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription.
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its 3′-untranslated region (21). Earlier work showed that the IRE binding activity of IRP1 is induced by H$_2$O$_2$ (22, 23). Thus, exposure of cultured cells or intact rat liver to H$_2$O$_2$ at quantities that are commonly released by inflammatory cells rapidly activate IRP1 within 30–60 min (24, 25). Importantly, IRP1 activation by H$_2$O$_2$ was sufficient to increase TfR1 expression in B6 fibroblasts (26). TfR1 expression is also controlled transcriptionally; one mechanism involves the hypoxia-inducible factor 1α (HIF-1α), which binds to a conserved binding site within the TfR1 promoter (27, 28). HIF-1α is activated in response to hypoxia, but up-regulation of HIF-1α has also been linked to mitochondria-derived oxidative stress (29).

Transient pulses of H$_2$O$_2$ are commonly employed to study the effects of this reactive oxygen intermediate in biochemical pathways. Such conditions, however, hardly mimic the continuous release of H$_2$O$_2$ from inflammatory cells because H$_2$O$_2$ is degraded rapidly by cultured cells (30). We have previously employed an enzymatic system for H$_2$O$_2$ generation at steadystate levels based on glucose oxidase (GOX) and catalase (CAT) (25, 30–32). For methodological reasons, however, these studies were restricted to relatively short time intervals (24, 25). In an optimized setting, we expose here cultured cells to a sustained flux of H$_2$O$_2$ at low, nontoxic concentrations that mimic the H$_2$O$_2$ release by inflammatory cells in terms of time and dose response. We show that such conditions induce the expression of TfR1, which is associated with increased transferrin-mediated iron uptake and intracellular iron accumulation. Neither IRP1 nor HIF-1α are involved in the up-regulation of TfR1 in response to such sustained low levels of H$_2$O$_2$. Moreover, H$_2$O$_2$ does not block TfR1 turnover but significantly stimulates TfR1 expression at the translational level. We suggest that H$_2$O$_2$-mediated iron uptake via translational induction of TfR1 could be a general mechanism that contributes to iron accumulation and tissue damage under conditions of inflammation.

EXPERIMENTAL PROCEDURES

Reagents—Luminol, NaOCl, phosphate-buffered saline (PBS), Hanks’ buffer, H$_2$O$_2$, catalase, tetrazolium salt (MTT), glucose oxidase, and sodium azide were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM), McCoy’s 5A medium, and penicillin/streptomycin were purchased from Gibco (Paisley, Scotland). Human colon carcinoma HT29 cells were grown in McCoy’s 5A medium, and murine B6 fibroblasts were purchased from ATCC (Manassas, VA). Human endothelial cell cultures were maintained in supplemented DMEM with 10% fetal calf serum. Human fibroblast cell cultures were maintained in McCoy’s 5A medium, and murine B6 fibroblasts were grown in supplemented DMEM containing 1000 mg/liter of glucose. The cells were maintained in an incubator at 37 °C with 5% CO$_2$.

Cell Culture—Human HepG2, HeLa, and HT29 cells were grown in DMEM supplemented with 2 mM glutamine, 4.5 g/liter glucose, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum. Human colon carcinoma HCT116 cells were grown in McCoy’s 5A medium, and murine B6 fibroblasts were grown in supplemented DMEM containing 1000 mg/liter of glucose. The cells were maintained in an incubator at 37 °C with 5% CO$_2$.

Determination of Enzymatic Activities for Catalase and GOX—Enzymatic activities of GOX and catalase were determined at submicromolar H$_2$O$_2$ concentrations prior to the experiment using a sensitive chemiluminescence technique (31, 33). Continuous measurements on supernatants of cultured cells confirmed the maintenance of steady-state concentrations of H$_2$O$_2$ during each experiments (30).

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as described previously using a radiolabeled human ferritin H-chain IRE probe (34). RNA–protein complex formation was quantified by densitometric scanning of the depicted autoradiographs.

Western Blotting—The cells were solubilized directly in radioimmune precipitation assay lysis buffer, and lysates were immediately boiled for 10 min. Equal aliquots were resolved by SDS/PAGE on 8% gels, and proteins were transferred on to nitrocellulose filters. The blots were saturated with 5% nonfat milk in PBS and probed with 1:4000 TfR1 (Zymed Laboratories Inc., San Francisco, CA), 1:250 HIF-1α mouse (Biosciences, Heidelberg, Germany), or 1:500 β-actin (Sigma) antibodies. After washing with Tris-buffered saline containing 0.05% (v/v) Tween 20, the blots were further incubated with horseradish peroxidase-conjugated secondary antibodies using the following dilutions: TfR monoclonal antibodies with rabbit anti-mouse IgG 1:6000, β-actin antibodies with goat anti-rabbit IgG 1:10000, and HIF-1α mouse antibodies with goat anti-mouse IgG 1:10000 dilution. Glucose oxidase was detected with a previously developed anti-GOX polyclonal antibody (35) in conjunction with a horseradish peroxidase-conjugated anti-guinea pig secondary antibody (Dianova, Hamburg, Germany) at a dilution of 1:3000. Detection of the horseradish peroxidase-coupled secondary antibodies was performed with the ECL® method (Amersham Biosciences). The blots were quantified by densitometric scanning using the TotalLab software version 1.11 (Nonlinear Dynamics Inc., Durham, NC).

Determination of the Labile Iron Pool (LIP)—The LIP was measured using the metal-sensitive fluorescence probe calcein (36, 37). We modified the technique to allow LIP detection of attached cells. Briefly, the cells were first treated for over 24 h in the presence of H$_2$O$_2$ or the membrane-impermeable iron chelator desferal (desferrioxamine). The cells were then washed twice with PBS and loaded with calcein-acetoxymethyl ester at a final concentration of 5 μM for 30 min (from a 10 mM stock solution in dimethyl sulfoxide). First fluorescence readings (F1) were taken using a Fluostar (BMG Labtechnologies GmbH, Offenburg, Germany) in bottom read technique with a fluoroscein optical filter (excitation, 465–495 nm; emission, 505 nm). Subsequently, the cells were depleted of iron in the presence of the membrane-permeable iron chelator salicylaldehyde isonicotinoyl hydrazone for 30 min (38), and a second measurement of fluorescence was performed (F2). After background subtraction, this protocol allowed us to determine the F2/F1 ratio as relative indicator of LIP independent of the cell number and distribution within the wells.

Northern Blotting—RNA prepared with the TRizol® reagent (Invitrogen) was analyzed by Northern blotting with 32P–radio labeled mouse TfR1, hepcidin, or rat β-actin cDNA probes (23).

Oxygen Measurements and Induction of Hypoxia—Oxygen was measured using a computer-driven oxygen electrode OxI 325-B (WTW, Weilheim, Germany). The electrode was calibrated with air-saturated water (21%). Permanent magnetic stirring was necessary to facilitate oxygen exchange at the membrane side of the oxygen electrode. To induce hypoxia, an
custom-made oxygen chamber was used and equilibrated with a prepared gas mixture of 3% oxygen, 5% carbon dioxide, and 92% nitrogen (Lifegas).

Iron Uptake Experiments—Transferrin-mediated iron uptake experiments were carried out as described in Ref. 39. Briefly, uptake experiments of $^{55}$Fe-labeled dipher ferritin (holo transferrin) were performed in 6-cm dishes at 37 °C for 30 min in 1 ml of buffer A (140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM Na$_2$HPO$_4$, and 5 mM HEPES, pH 7.4). The cells were washed five times with ice-cold PBS and then lysed with 1 ml 1 M NaOH. Radioactivity of lysates was determined using a Cobra II Auto Gamma counter (Canberra Packard, Meriden, CT). For all of the uptake experiments, the controls were incubated on ice to determine nonspecific binding of holotransferrin to the cell membrane. The 0 °C values were subtracted from the 37 °C values to determine net uptake rates.

Analysis of Tfr1 mRNA Levels by Quantitative RT-PCR—Total RNA was isolated from cell culture using the TRIzol® reagent (Invitrogen). Tfr1 and control β-actin mRNA transcripts were quantified by a two-step RT-PCR with the Applied Biosystems 7500 real time PCR system. cDNA synthesis was performed with a first strand cDNA synthesis kit for RT-PCR (Invitrogen), according to the manufacturer’s instructions. Transcripts were amplified in duplicates with specific sense and antisense QuantiTect® primers (Qiagen). The thermal cycler profile consisted of a total reaction volume of 50 μl that underwent a 95 °C activation for 10 min, followed by 40 repetitions of the following three steps: 95 °C denaturation for 15 s, annealing at 55 °C for 30 s, and a 33-s extension period at 72 °C. Transcripts were detected with the QuantiTect SYBR® Green kit. Tfr1/β-actin ratios were calculated using LightCycler sequence detection software v1.2.

Tfr1 Protein Stability and Synthesis—For protein stability, the cells were metabolically labeled for 1 h with (50 μCi/ml) trans-$^{35}$S label, a mixture of 70:30 [35S]methionine/cysteine (ICN, Irvine, CA) in RPMI methionine/cysteine-free medium prior to GOX treatment. Following the 1-h pulse, the cells were chased with cold medium in the absence or presence of GOX treatment. In contrast, the rate of protein synthesis was determined by first incubating the cells in cold medium with or without GOX and subsequently metabolically labeling the cells for 1 h. Following both the protein stability and synthesis studies, the cells were lysed with radioimmune precipitation assay buffer, and 1 mg of lysates was subjected to quantitative immunoprecipitation with 2 μl of mouse monoclonal Tfr (Zymed Laboratories Inc., San Francisco, CA). Immunoprecipitated proteins were analyzed by SDS/PAGE and visualized by autoradiography. Radioactive bands were quantified by phosphorimaging.

Cytotoxicity Studies—Cell viability was determined with the MTT assay (40). Briefly, conversion of the tetrazolium salt (MTT) into a blue formazan product was detected using a 96-well plate reader (Fluostar, BMG Labtechnologies GmbH, Offenburg, Germany) at 570 nm. The cells were treated with different activities of glucose oxidase and catalase in culture medium for 24 h in 96-well plates at 37 °C. After two washing steps with PBS, MTT was added to each well (0.5 mg/ml), the cells were incubated for further 4 h at 37 °C and, finally, 10% SDS in 0.01 M HCl was added to lyse the cells. The samples were incubated overnight, and the absorbance was measured.

RESULTS

Generation of Sustained H$_2$O$_2$ in Cultured Cells under Nontoxic Conditions—Optimization of the previously developed GOX/CAT system (30–32) allowed us to expand H$_2$O$_2$ exposure times up to several days, thus mimicking, with respect to oxidative stress, a chronic inflammatory response. Levels and flux of H$_2$O$_2$ by GOX are lower or resemble those of activated leukocytes in vivo (0.2 μm/s or <10 μm) (41). Using an ultrasensitive H$_2$O$_2$ assay (30, 41), we show (Fig. 1A) that H$_2$O$_2$ is maintained at a constant concentration of ~5 μm over the entire time interval of 24 h, indicating that GOX remains stable under these experimental conditions and the substrates glucose and oxygen are not depleted. Cell cultures could be exposed to GOX/CAT over several days, and such experiments were only limited by cellular confluence and eventual growth arrest. The media were always replaced every 12 or 24 h to prevent glucose depletion. We then examined whether under the above experimental conditions toxicity/growth inhibition was associated with H$_2$O$_2$ concentrations or flux by varying GOX amounts or ratios of GOX/CAT (32, 42). As demonstrated in Fig. 1B, toxicity depended solely on the levels of H$_2$O$_2$ and was independent of the flux. Both GOX 1× and 5× inhibited cell growth when H$_2$O$_2$ levels exceeded a concentration of 10 μM H$_2$O$_2$. Interestingly, B6 cells that were incubated with increasing GOX without the additional external catalase also showed growth inhibition at a steady-state concentration of 10 μM H$_2$O$_2$ because of the low cellular catalase activity of 0.001 s$^{-1}$ (31). Thus, toxicity of a GOX/CAT system only depends on the concentration of H$_2$O$_2$ (defined by the ratio of GOX/CAT) and not the H$_2$O$_2$ generation rate (defined by the GOX activity), which could be linked to consumption or other metabolites. No changes in cell growth were observed with H$_2$O$_2$ concentrations below 5 μM, and these nontoxic conditions are tolerated well over 24 h.

Sustained Exposure of Cultured Cells to Nontoxic H$_2$O$_2$ Concentrations Up-regulates Tfr1—Long term exposure of B6 fibroblasts over 48 h to low H$_2$O$_2$ concentrations was accompanied by a strong induction of Tfr1 expression (Fig.
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A. B6 fibroblasts were exposed to stable concentrations of 5 μM H₂O₂ similar as described in the legend to Fig. 1A, and expression of TfR1 was analyzed by Western blotting (top panel). The middle panel shows TfR1 expression of the untreated control. The bottom panel shows the ratio of H₂O₂-treated versus untreated controls as obtained by densitometry. The depicted experiment is representative of three independent measurements. B. B6 fibroblasts were treated for 24 h with the iron chelator desferrioxamine (D), hemine (H), two different sustained concentrations of H₂O₂, and expression of TfR1, ferritin, and β-actin was determined by Western blotting. In contrast to iron depletion by desferrioxamine, sustained H₂O₂ slightly increases ferritin levels in addition to TfR1 induction. The depicted experiment is representative of three independent measurements.

FIGURE 2. Sustained H₂O₂ up-regulates TfR1 without repression of ferritin. A, B6 fibroblasts were exposed to stable concentrations of 5 μM H₂O₂ similar as described in the legend to Fig. 1A, and expression of TfR1 was analyzed by Western blotting (top panel). The middle panel shows TfR1 expression of the untreated control. The bottom panel shows the ratio of H₂O₂-treated versus untreated controls as obtained by densitometry. The depicted experiment is representative of three independent measurements. B. B6 fibroblasts were treated for 24 h with the iron chelator desferrioxamine (D), hemine (H), two different sustained concentrations of H₂O₂, and expression of TfR1, ferritin, and β-actin was determined by Western blotting. In contrast to iron depletion by desferrioxamine, sustained H₂O₂ slightly increases ferritin levels in addition to TfR1 induction. The depicted experiment is representative of three independent measurements.

2A). A slight increase (40%) of TfR1 steady-state levels was observed after 8 h, whereas maximal activation (∼3.5-fold) was manifested after 24 h and sustained up to 48 h. Excess of catalase completely blocked TfR1 up-regulation (not shown). Thus, H₂O₂ not only antagonized the previously reported inhibiting effect of cell growth on TfR1 expression (43) but further increased TfR1 expression. The ratios of TfR1 densities from H₂O₂-treated and control cells are shown in the bottom panel of Fig. 2A. In contrast to iron-depleting conditions, the iron storage protein ferritin was slightly up-regulated in H₂O₂-treated cells and more so with iron loading with hemin (Fig. 2B). Thus, prolonged exposure of B6 cells to nontoxic H₂O₂ concentrations strongly activates TfR1 expression and slightly up-regulates ferritin. Importantly, these data were corroborated in additional cell lines of various tissue origin (HeLa, HepG2, HCT116, and HT29).

FIGURE 3. GOX induces expression of TfR1 via H₂O₂ in the absence of hypoxia. A, GOX activities that are sufficient to induce TfR1 (GOX 1×) do not change oxygen levels, whereas high activities of GOX (GOX 10×) are able to induce mild hypoxia in the cell culture medium. The oxygen levels were measured with an oxygen electrode in the presence of varying GOX activities in a 10-cm culture dish containing 45 ml of medium and 25 mM glucose. GOX 1× corresponds to k₆₅₈ = 3.0 × 10⁻₈ M s⁻¹. B, expression of HIF-1α was measured in HepG2 cells that were exposed to different levels of sustained H₂O₂. An oxygen chamber (3% oxygen) was used as positive control. HIF-1α was rapidly induced within 6 h. No HIF-1α induction was observed with the high GOX/CAT system (GOX 10×) generating 1 or 5 μM of sustained H₂O₂, although TfR1 is induced under these conditions (see Fig. 2). Catalase was used to adjust H₂O₂ levels. Lanes (medium volume, k₆₅₈; R₅₆₈; lane 1, 10 ml, 0, 0; lane 2, 10 ml, 3% oxygen; lane 3, 10 ml, 3 × 10⁻₈ M s⁻¹; lane 4, 10 ml, 3 × 10⁻₈ M s⁻¹; 4.8 × 10⁻₃ M s⁻¹). The depicted experiment is representative of three independent measurements.

TfR1 Up-regulation in Cells Exposed to GOX/CAT Is Mediated Solely by H₂O₂ and Not Hypoxia—In the GOX/CAT system, the ratio of GOX/CAT activities determines the concentration of H₂O₂, whereas GOX defines the consumption rate of oxygen (30). At higher concentrations, GOX significantly decreases oxygen levels in the culture medium (Fig. 3A). Because hypoxia (27, 28) and oxidative stress (29) have been shown to induce TfR1 expression via the transcription factor HIF-1α, we next studied whether exposure of cells to sustained H₂O₂ at our conditions increases expression of HIF-1α. Hepatoma HepG2 cells that express HIF-1α in response to hypoxia (44) were exposed for 6 h to different concentrations of H₂O₂ generated by the GOX/CAT system that are known to induce TfR1 (Fig. 2), and HIF-1α was determined by Western blotting (Fig. 3B). The cells that were incubated with 3% oxygen using an oxygen chamber served as a positive control. As expected, HIF-1α expression is only induced at 3% oxygen (lane 2), whereas the application of GOX/CAT at an H₂O₂ flux that results in TfR1 activation (as in Fig. 2) did not stimulate HIF-1α expression (lane 3 and 4). We conclude that sustained H₂O₂ activates TfR1 specifically and in the absence of hypoxia.

H₂O₂-mediated Up-regulation of TfR1 Is Functional and Results in Intracellular Accumulation of Iron—We next addressed the question of whether TfR1 up-regulation in response to sustained H₂O₂ is physiologically significant. Transferrin-mediated iron uptake was measured by exposing B6 cells to various steady-state H₂O₂ concentrations over 24 h in the presence of ⁵⁷Fe-loaded and purified transferrin. Fig. 4A demonstrates a significant H₂O₂-dependent increase in ⁵⁷Fe uptake 2.4-fold. We then assessed the effects of the prolonged H₂O₂ treatment on the LIP using a modified calcine assay (36, 37). Iron depletion by desferrioxamine was used as negative control. Fig. 4B shows that the LIP is clearly increased in B6 fibroblasts upon exposure to a H₂O₂ flux over 24 h. Similar responses of LIP were found with all other cell lines (data not shown).
Stimulated Iron Uptake by Sustained H$_2$O$_2$ Is Independent of the IRE/IRP Regulatory System—Earlier studies had shown that H$_2$O$_2$ rapidly activates IRP1 in cultured cells (22, 23) and in perfused rat liver (24) and that H$_2$O$_2$-mediated activation of IRP1 was sufficient to increase TfR1 mRNA and protein levels (26). A threshold of ~10 $\mu$M was defined as the minimal concentration required for IRP1 activation within 30–60 min (25); however, methodological constrains did not allow H$_2$O$_2$ exposure times longer than 60 min. The optimized H$_2$O$_2$ models presented herein enabled us to evaluate IRP1 activity by EMSA after prolonged H$_2$O$_2$ treatments. Exposure of B6 cells to steady-state concentrations of ~5 $\mu$M H$_2$O$_2$ was associated with an initial modest activation of IRP1 that was detectable up to 6 h (Fig. 5A, lanes 1 and 2). However, IRP1 activity declined within the next 24 h of H$_2$O$_2$ treatment (lanes 3–4), to the same degree observed in response to iron-loading with hemin (lane 5). As expected, the iron chelator desferrioxamine drastically activated IRP1 (lane 6). In contrast to iron overload with hemin, however, incubation of cell lysates with 2-mercaptoethanol that activates IRP1 in vitro indicated also decreased IRP1 protein levels. Moreover, TR1 mRNA levels were not increased in response to H$_2$O$_2$ as determined by quantitative RT-PCR (Fig. 5B). Thus, it appears that the sustained exposure of cells to such low, nontoxic H$_2$O$_2$ concentrations only modestly and transiently induces IRP1 activity, and this response is not sufficient to stabilize TfR1 mRNA.

Increased Levels of TfR1 during Sustained H$_2$O$_2$ Is Independent of TfR1 Stability but Involves Stimulation of TfR1 Synthesis—Expression of TfR1 is typically controlled at the level of transcription or stabilization of its mRNA via IRPs. Because these mechanisms were excluded, we next studied whether TfR1 levels were affected by either protein stability or synthesis. To measure protein stability in cells with or without sustained H$_2$O$_2$ treatment, B6 cells were pulsed with [35S]methionine/cysteine for 1 h (time 0) and chased for 1, 6, 5, and 24 h (Fig. 6). The cells were then lysed, and TfR1 levels were determined by quantitative immunoprecipitation and autoradiography. After 1 and 6 h of chase, TfR1 expression appeared to be slightly higher in cells treated with H$_2$O$_2$ than in control cells. However, a 24-h treatment with H$_2$O$_2$ did not affect the stability of TfR1.

We conclude that the surge of TfR1 levels during H$_2$O$_2$ treatment is independent of TfR1 stabilization. We next addressed whether the rate of protein synthesis played a role in the elevated levels of TfR1 during H$_2$O$_2$ treatment. The cells were treated with GOX for 1, 6, 12, and 24 h and subsequently pulsed with the [35S]methionine/cysteine for 1 h (Fig. 7). The cells were then lysed, and TfR1 synthesis was analyzed by quantitative RT-PCR and expressed relative to $\beta$-actin mRNA. Data from three independent experiments are shown (means ± S.D.). Iron depletion in the presence of 100 $\mu$M desferrioxamine served as positive control strongly inducing TfR1 mRNA via IRP1 as shown in Fig. 5A.
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**FIGURE 6.** TfR1 up-regulation by H$_2$O$_2$ is independent of protein stability. B6 cells were pulsed with H$_2$O$_2$ for 1, 6, 12, and 24 h and subsequently pulsed with [35S]methionine/cysteine for 1 h. Immunoprecipitated TfR1 was analyzed by SDS/PAGE and visualized by autoradiography (top panel). Radioactive bands were quantified by phosphorimaging (bottom panel). Densitometry data indicate the ratio of TfR synthesis of H$_2$O$_2$ treated versus control from three independent experiments.

**FIGURE 7.** Sustained H$_2$O$_2$ induces TfR1 synthesis via direct stimulation of translation. B6 cells were treated with H$_2$O$_2$ for 1, 6, 12, and 24 h and subsequently pulsed with [35S]methionine/cysteine for 1 h. Immunoprecipitated TfR1 was analyzed by SDS/PAGE and visualized by autoradiography (top panel). Radioactive bands were quantified by phosphorimaging (bottom panel). Densitometry data indicate the ratio of TfR synthesis of H$_2$O$_2$ treated versus control from three independent experiments.

...treatment. In contrast, TfR1 mRNA translation was dramatically up-regulated in H$_2$O$_2$-exposed cells after 12 h of GOX treatment. These findings indicate a novel mechanism by which sustained H$_2$O$_2$ increases iron uptake via translational stimulation of TfR1.

**DISCUSSION**

The typical changes of iron homeostasis during acute inflammation are considered to be part of the defensive immune response. In chronic inflammation, however, parenchymal cells often show increased deposits of iron that may aggravate disease progression and promote further tissue damage. Under these conditions, iron becomes especially harmful because it catalyzes the generation of free radicals from reactive oxygen species released by inflammatory cells. Here, we show that the exposure of various cell types to sustained H$_2$O$_2$ results in the up-regulation of TfR1 expression and accumulation of iron. We also provide evidence that the mechanism for TfR1 activation by H$_2$O$_2$ is translational.

The levels of sustained H$_2$O$_2$ utilized in our experiments are nontoxic and mimic inflammatory conditions. Thus, our experimental system provides a tool to study the alterations of iron homeostasis observed in chronic inflammation. A diversion of iron from circulation into intracellular compartments is well established. This is associated with impaired erythropoiesis, which eventually leads to the anemia of chronic disease (1, 3, 45, 46). The antimicrobial peptide hepcidin inhibits iron efflux from macrophages via ferroportin 1 (17, 47). Considering that hepcidin expression is induced by the pro-inflammatory cytokine interleukin-6, this pathway is expected to promote tissue iron accumulation and thereby plays a key role in the development of anemia of chronic disease. It is, however, possible that in addition to inhibition of iron efflux, increased iron uptake may also contribute to tissue iron accumulation in anemia of chronic disease. Our results suggest that the H$_2$O$_2$-mediated increase of TfR1 may account for this response.

Importantly, increased expression of TfR1 has been observed in animal models of inflammation (2, 18). In humans, direct evidence of TfR1 up-regulation has been recently found in patients with acute respiratory distress syndrome (18). In addition, levels of soluble TfR receptor are associated with inflammation independent of the degree of erythropoiesis, the hypoxic response and iron status (48). During acute inflammation, the up-regulation of TfR1 may reduce the availability of essential iron to invading bacteria and is probably beneficial for the host. In chronic inflammation, however, accumulation of iron in tissues is associated with toxicity (9) and seems to drive progression of fibrosis and end stage liver disease in such common liver pathologies such as chronic hepatitis C or alcoholic liver disease (4–8).

What is the mechanism for TfR1 activation by low, nontoxic doses of H$_2$O$_2$? We first explored the role of HIF-1α that is known to transcriptionally activate TfR1 (27, 28) and also responds to reactive oxygen species independently of hypoxia (29, 49). However, our data clearly show that the GOX system can be calibrated to generate H$_2$O$_2$ steady-state levels in the absence of hypoxia (Fig. 3). Moreover, the H$_2$O$_2$-mediated activation of TfR1 expression does not require the induction of HIF-1α. Second, we hypothesized that the mechanism may involve TfR1 mRNA stabilization by IRP1, which is rapidly activated by H$_2$O$_2$ within 30 min to bind to IREs (22, 23, 34). As shown previously in B6 fibroblasts, IRP1 is activated by a bolus of 100 μM H$_2$O$_2$ (26). In the present study, B6 and other cells were exposed to sustained ~5 μM H$_2$O$_2$ (Fig. 1), and IRP1 was only partially and temporarily activated (Fig. 5A), very likely because of H$_2$O$_2$ signaling. The absence of full IRP1 activation under these conditions is fully consistent with previous findings, showing that a concentration of ~10 μM H$_2$O$_2$ was a minimum requirement to elicit the complete response (25).
Notably, IRP1 activity decreased at later time intervals (24 h) of GOX treatment, possibly because of increased intracellular iron accumulation. The observed modest alterations in ARE binding activity did not affect TfR1 mRNA levels (Fig. 5B), suggesting that the increase in TfR1 expression in GOX-treated B6 cells (Fig. 2A) is independent of IRPs. H$_2$O$_2$ has been shown to differentially affect protein degradation in mammalian cells (50, 51). However, pulse-chase experiments with [35S]methionine/cysteine indicate that H$_2$O$_2$ does not affect the stability of TfR1 (Fig. 6). Studies of [35S]methionine/cysteine incorporation rather demonstrate that H$_2$O$_2$ directly and significantly stimulates TfR1 synthesis.

The direct stimulation of TfR1 protein synthesis by low levels of H$_2$O$_2$ is somewhat unexpected. Proteomics data showed that H$_2$O$_2$ induces the expression of proteins that are important for translation and RNA processing (52). However, only a few studies on the oxidative modulation of translation exist, and they show a complex response depending on cell type and conditions (52–57). Thus, H$_2$O$_2$ was shown to inhibit translation (52, 53) by mechanisms such as the inhibition of the 70-kDa ribosomal protein S6 kinase (52), dephosphorylation of the eukaryotic initiation factor 4E-binding protein 1, or increased binding of this repressor protein to eukaryotic initiation factor 4E and initiation factor 4E-binding protein 1, or increased binding of protein S6 kinase (52), dephosphorylation of the eukaryotic initiation factor 4E (52, 57). Translation can also be inhibited by H$_2$O$_2$ via phosphorylation of the elongation factor eukaryotic elongation factor 2 (eEF2) (52), which was either mediated by activation of the eEF-2 specific, Ca$^{2+}$/calmodulin-dependent protein kinase III (55) or reversible inhibition of the protein phosphatase 1 (56). On the other side, H$_2$O$_2$ has been shown to stimulate translation in cell-free systems (58), plants (59), bacteria (60), and mammalian cells (54). Importantly, the latter study indicated that the effect of H$_2$O$_2$ on translation depends on the levels of H$_2$O$_2$ and the cell sensitivity toward H$_2$O$_2$. Thus, in Huh7 cells that are moderately sensitive toward H$_2$O$_2$, translation was up-regulated even at a low level of H$_2$O$_2$.

In summary, our study establishes that sustained, nontoxic concentrations of H$_2$O$_2$ stimulate TfR1 expression by a novel translational mechanism. Hence, H$_2$O$_2$ is able to stimulate TfR1 expression both at the post-transcriptional level via IRP1 and at the translational level, which points to a potentially general mechanism of iron internalization in the presence of H$_2$O$_2$. In addition to the role of hepcidin and ferroportin on iron homeostasis during inflammation (17, 47), H$_2$O$_2$-mediated iron uptake could provide an alternative local mechanism that removes iron from the inflammatory battle field and prevents unspecific collateral tissue damage. Under conditions of chronic inflammation, however, the continued accumulation of iron could itself impose a threat to cells and tissues (2–9). In addition, translational stimulation of TfR1 expression by H$_2$O$_2$ could participate in the toxicity of chemotherapeutic anticancer agents such as doxorubicin that are known to increase TfR1 expression (61, 62). Our work underlines the importance of studying the expression of proteins in clinical and animal trials that are commonly focusing on the mRNA levels because of limited tissue availability. Our novel tool of a sustained exposure of cultured cells to H$_2$O$_2$ will help to better understand molecular mechanisms related to oxidative stress in future studies that should also include the complex redox-sensitive regulation of translation.

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