Zinc Deficiency-induced Iron Accumulation, a Consequence of Alterations in Iron Regulatory Protein-binding Activity, Iron Transporters, and Iron Storage Proteins*

Brad J. Niles‡, Michael S. Clegg‡, Lynn A. Hanna‡, Susan S. Chou‡, Tony Y. Momma‡, Heeok Hong‡, and Carl L. Keen*‡

From the Departments of ‡Nutrition and Internal Medicine, University of California, Davis, California 95616-8669 and the *Department of Food Service Management and Nutrition, Sangmyung University, Seoul 110–743, Korea

One consequence of zinc deficiency is an elevation in cell and tissue iron concentrations. To examine the mechanism(s) underlying this phenomenon, Swiss 3T3 cells were cultured in zinc-deficient (D, 0.5 μM zinc), zinc-supplemented (S, 50 μM zinc), or control (C, 4 μM zinc) media. After 24 h of culture, cells in the D group were characterized by a 50% decrease in intracellular zinc and a 35% increase in intracellular iron relative to cells in the S and C groups. The increase in cellular iron was associated with increased transferrin receptor 1 protein and mRNA levels and increased ferritin light chain expression. The divalent metal transporter 1 (+) iron-responsive element isoform mRNA was decreased during zinc deficiency-induced iron accumulation. Examination of zinc-deficient cells revealed increased binding of iron regulatory protein 2 (IRP2) and decreased binding of IRP1 to a consensus iron-responsive element. The increased IRP2-binding activity in zinc-deficient cells coincided with an increased level of IRP2 protein. The accumulation of IRP2 protein was independent of zinc deficiency-induced intracellular nitric oxide production but was attenuated by the addition of the antioxidant N-acetylcysteine or ascorbate to the D medium. These data support the concept that zinc deficiency can result in alterations in iron transporter, storage, and regulatory proteins, which facilitate iron accumulation.

Zinc deficiency can result in multiple biochemical abnormalities and tissue pathology (1). Although many of these effects can be attributed to reductions in the activities of select zinc-dependent enzymes, and reductions in circulating growth factors, some pathology can be a consequence of zinc deficiency-induced alterations in the metabolism of other nutrients. A number of studies indicate that a diet deficient in zinc can result in the accumulation of iron in numerous tissues, including testes, liver, kidney, and spleen, as well as in fetuses of zinc-deficient dams (2–4). Elevated tissue iron is also associated with a number of hereditary disease states, including hemochromatosis and aceruloplasminemia, as well as disease states that require frequent blood transfusions (e.g. β-thalassemia) (5–7).

Iron homeostasis is a tightly regulated process given that iron is both essential and deleterious to living organisms. Cellular iron levels are primarily regulated by intake, recycling, and conservation of body iron stores, and to a limited extent, by excretion. Cellular iron uptake is predominantly controlled by the transferrin (Tf)2/transferrin receptor 1 (TfR1) uptake system. When cellular iron is needed, TfR1 protein levels increase, allowing the cell to import iron as diferric Tf. Subsequently, iron is released from Tf in acidic endosomes where it is utilized, or stored in ferritin (Ft). In response to an increased iron load, cellular Ft levels are increased by a combination of transcriptional and translational mechanisms (8), thus minimizing the labile iron pool (LIP), or the amount of “free” iron (9). High LIP levels can be detrimental to the cell’s macromolecules via the ability of iron to participate in Fenton-mediated free radical damage (10).

The LIP is tightly regulated by post-translational control of mRNAs for iron carriers/transporters and storage proteins that contain either 5’- or 3’-untranslated (UTR) base sequences, which form hairpin loops, and are referred to as iron-responsive elements (IREs). Post-translational control of IRE-containing mRNAs is conferred by iron regulatory proteins (IRP) 1 and 2, which “sense” LIP levels of the cell. When cellular iron levels decrease, the affinities of IRPs for IREs increase, while conversely, if cellular iron levels rise, the affinities of IRPs for IREs decrease. The decreased affinity of IRPs for IREs under high iron conditions is a result of conformational alterations to IRP1 (11) and increased degradation of IRP2 (12–15). In the former, increased cellular iron results in the formation of a [4Fe-4S] cluster, consequently decreasing the affinity of IRP1 for IREs and thereby switching the function of this protein to a cytosolic aconitase. In the latter case, increased cellular oxygen, and/or

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1 To whom correspondence should be addressed: Dept. of Nutrition, University of California, One Shields Ave., Davis, CA 95616-8669. Tel.: 530-752-6331; Fax: 530-752-8966; E-mail: cokieen@ucdavis.edu.

2 The abbreviations used are: Tf, transferrin; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; DAF-2 DA, 4,5-diaminofluorescein diacetate; ASC, ascorbate; β-ME, β-mercaptoethanol; DFO, deferoxamine mesylate; DMT1, divalent metal transporter 1; FAC, ferric ammonium citrate; Ft, ferritin; FHT, ferritin heavy chain; Flt, ferritin light chain; IRE, iron-responsive element; ICP-AES, inductively coupled plasma-atomic emission spectrophotometry; IRP, iron regulatory protein; LIP, labile iron pool; NAC, N-acetylcysteine; RN5, reactive nitrogen species; ROS, reactive oxygen species; SEIT, S-ethylisothiourea; TfR1, transferrin receptor 1; UTR, untranslated region; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; D, zinc-deficient medium; S, zinc-supplemented medium; C, control medium; INOS, inducible nitric oxide synthase.
iron derived from increased non-heme iron, the breakdown of heme iron, or heme itself, result in enhanced proteasomal degradation of IRP2 (12, 16, 17). In addition to iron, the activities of IRP1 and IRP2 can be modulated by phosphorylation (18), hypoxia (19), and by reactive nitrogen species (RNS) such as NO+, NO2−, and peroxynitrite, and by reactive oxygen species (ROS) such as H2O2 and O2·− (20–22).

Through the 3′-UTR IRE of the Ft transcript, translation is repressed by IRPs under low iron conditions. Conversely, if the cellular iron load increases, the Fe-S cluster assembly in IRP1 decreases its affinity for the IRE, releasing inhibition of translation of the mRNA for the Ft protein. Concomitantly, increased iron load enhances IRP2 degradation, which also contributes to increased Ft synthesis (23). mRNAs for the iron transport proteins TfR1 and the divalent metal transporter 1 (DMT1) (26), IRP2, TfR1, and 18 S rRNA were purchased from Applied Biosystems (Foster City, CA). The FtL TaqMan assay was synthesized by Integrated DNA Technology (Coralville, IA). The RETROscript kit, RNase inhibitor, Moloney murine leukemia virus reverse transcriptase, inducible nitric-oxide synthase (iNOS) Gene Specific RT-PCR kit, and 18 S rRNA primer set were purchased from Ambion (Austin, TX). QIAshredder and RNeasy kits were purchased from Qiagen. 4,5-Diamidino-2-phenylindole (DAPI), Hoechst 33342, 1400W, and 5-ethyglycolic acid (SEIT) were purchased from Calbiochem. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). All other reagents were obtained from Sigma-Aldrich.

Preparation of Zinc-free Buffers and Zinc Treatment Media—Zinc was removed from buffers by batch washing with 5% (w/v) Chelex-100 for 1 h. The first batch of supernatant was discarded, and subsequent batches were pooled. Removal of spurious Chelex-100 resin particles from buffer supernatants was ensured by filtration (0.22 μm). Zinc-deficient medium (D, 0.5 μM zinc) was prepared from FBS that had been subjected to a relatively short dialysis (12 h) against the chelator diethylene-triaminepentaacetic acid. To remove the chelator after zinc binding, the FBS was subsequently dialyzed exhaustively (72 h) against several changes of 20 mM HEPES, pH 7.4. The zinc-depleted FBS was diluted with DMEM, HEPES (final concentration, 20 mM), and penicillin/streptomycin antibiotics. Zinc-supplemented medium (S, 50 μM zinc) was prepared directly from D medium by adding a stock solution of ZnSO4 to obtain a final concentration of 50 μM zinc. Thus, D and S media differed only by their respective concentrations of zinc. Control medium (C, 4 μM zinc) was prepared from undialyzed FBS and had a final zinc concentration of 4 μM. All media contained 3 μM iron, 0.2 μM copper, and a protein concentration of 3 mg/ml (26).

Cell Culture—Swiss 3T3 cells were obtained from American Type Culture Collection (Manassas, VA) and were propagated under log phase growth conditions in C medium at 37 °C in 5% CO2. Cells (passages 3–12) were plated at a density of 1.5 million cells per 20 × 100-mm dish. After 24 h, C medium was removed, and cells were synchronized by incubation in DMEM for 24 h (t = 0 h). Subsequently, DMEM was removed, experimental media were added, and cells were grown for the indicated time frames (t = 16 h, t = 24 h, and t = 32 h).

Media and Cell Mineral Analysis—Media and buffer trace element concentrations were determined directly by inductively coupled plasma-atomic emission spectrophotometry (ICP-AES, Trace Scan, Thermo Elemental, Wilmington, MA). For the analysis of cell trace elements, medium was removed by vacuum suction, and the cells were then rinsed with two successive volumes of ice-cold phosphate-buffered saline. Excess phosphate-buffered saline was removed from the dishes by vacuum suction, and the culture dishes with attached cells were placed directly in a −80 °C freezer to promote membrane disruption. After freezing, the cells were thawed and harvested by scraping them into buffer containing 20 mM HEPES and 2 mM EDTA, pH 8.0. The cell milieu from each dish was transferred by plastic transfer pipette into a micro-
centrifuge tube and vortexed at high speed for 1 min. An aliquot of each extract was removed for protein analysis, and the balance of each extract was acidified with Ultrex nitric acid (final concentration of 2 M). Insoluble material was removed by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant fraction was subjected to ICP-AES for the determination of zinc and iron. The ICP-AES was calibrated with a series of multielement standards diluted in 2 M nitric acid. Values were corrected for appropriate blank readings, dilution, and starting protein content. Data are expressed as pmol of metal/µg of cell protein.

Total Protein, RNA, and DNA Quantification—Protein concentrations were determined using the Bradford assay with the Bio-Rad protein assay reagent (27). RNA concentrations were determined with the Ribogreen Assay. Cellular DNA was determined by the Cyquant DNA assay (26).

Preparation of Cell Extracts and Western Immunoblot Analysis—Cytosolic fractions were prepared as described (26). Equal amounts of protein were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membranes. A solution of 5% (w/v) skim milk in TTBS (0.5 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% Tween 20) was used for blocking and antibody dilution. The antibody-antigen complexes were visualized by chemiluminescence. Membranes were stripped using Restore Western blot stripping buffer, following manufacturer’s specifications, and blotted for β-actin. Densitometry analysis was performed by Quantity One software after capturing the image in a ChemiDoc XRS gel documentation system (Bio-Rad).

Semiquantitative Analysis of IRP1, IRP2, TfR1, FtL, iNOS, and DMT1 Isoform Transcripts—A real-time RT-PCR strategy was employed to measure relative transcript levels of IRP1, IRP2, TfR1, and FtL. RNA was isolated with QiAmp RNA kit following by quantification. Reverse transcription was performed with RETROscript on 0.2–0.9 μg of total RNA. Serial dilutions of cDNA were used to verify equivalent efficiency of real-time PCR reactions for each target compared with separate reactions for 18 S rRNA cDNA before multiplexing. The same cDNA was then used to measure each of the transcripts in multiplex with 18 S rRNA. Tripletate real-time PCR was conducted on 1/50 dilutions of cDNA in PCR buffer, 0.2 μM 18 S rRNA TaqMan, and 2 μM target TaqMan in an iCycler instrument (Bio-Rad). The threshold of detection was determined by iCycler software and triplicates were averaged for ΔCt (target Ct – 18 S rRNA Ct).

DMT1 isoform and iNOS transcripts were measured by semiquantitative RT-PCR. cDNA was generated by RETROscript and PCR was done using 5% of that reaction, 0.2 mM dNTPs, 1.25 μg AmpliTaq DNA polymerase, and 20 μM each of the primers DMT1f(+)/IRE forward 5′-CTGCTGGAGGCAAGATACGGAC-3′ and reverse 5′-CTCAGGAGCTTAGCTCAG-AAG-3′ and DMT1r(−)/IRE forward 5′-CGCGCAATTTTTACACAGTG-3′ and reverse 5′-AGGTCCACTACCTGCA-CAC-3′ (24), and the iNOS primer set obtained from Ambion. Amplification was normalized with 18 S rRNA primer set from Ambion, using a 3:7 ratio with 18 S RNA competitors. PCR reactions were performed at 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 40 s for 35 cycles in the GeneAmp PCR System 9700 (PE Applied Biosystems). PCR products were resolved on 10% TBE gels, and stained with ethidium bromide. Densitometry was quantified as described for Western analysis.

IRP-RNA Shift Assay—An IRE probe was made by first annealing 20 μM synthetic oligonucleotides 5′-CAATTCCTGGCAGAAGACG-3′ and 5′-ATCCAGCTATAACCCTTCACAGTGC-3′ corresponding to a sequence shown to recognize both IRP1 and IRP2 (IRE loop sequence underlined) (28). The pGEM-IRE was generated by cloning this sequence into pGEM. For confirmation of results, another vector was used that encoded the FtL IRE (generously provided by Dr. Elizabeth Leibold, University of Utah). Labeled RNA probes were transcribed from 0.5 μg of linearized templates using T7 RNA polymerase, 40 units of RNase inhibitor, 50 μCi of [α-32P]CTP, 10 mM ATP, GTP, UTP, and 100 μM CTP in a 20-μl reaction volume at 37 °C for 1 h. The DNA templates were digested for 15 min with RNase-free DNase at 37 °C, and free nucleotides were removed using a ProbeQuant G-50 micro column.

Cells were washed twice with phosphate-buffered saline and scraped down in lysis buffer (10 mM HEPES, pH 7.4, 40 mM KCl, 3 mM MgCl2, 5% glycerol, 0.2% Igepal, 1× complete protease inhibitor without EDTA, 1 mM dithiothreitol, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride) and homogenized in a glass homogenizer for 30 strokes. Nuclei and cellular debris were removed by centrifugation at 3,300 × g at 4 °C for 15 min. The resulting supernatant fraction was stored at −80 °C until use.

Binding activity of IRPs in cytosolic fractions was measured by diluting 10 μg of protein in binding buffer (10 mM HEPES, pH 7.4, 40 mM KCl, 3 mM MgCl2, 5% glycerol, 0.2% Igepal, 1 mM dithiothreitol) and incubating for 10 min at room temperature. Then, after addition of 60,000 cpm of RNA probe, the reaction was incubated for another 30 min, with 5 μg/µl heparin added for the last 10 min, in a final 20-μl reaction volume. To determine total IRP1 content, duplicate samples were incubated with 2% β-mercaptoethanol (β-ME) for 5 min before addition of the probe. After addition of 2 μl of loading buffer (0.5% (w/v) bromphenol blue, 80% glycerol), samples were loaded onto a 6% non-denaturing polyacrylamide gel and run for 3 h at 150 V. The gel was exposed to a phosphorimaging screen, imaged (Molecular Imager FX, Bio-Rad), and quantified using Quantity One software.

Live Cell NO Imaging and Conditioned Media Nitrite Levels—Synchronized 3T3 cells were grown for 24 h in 8-well glass chamber slides under 200 μl of the various zinc treatment media. At t = 24 h, medium was removed and the cells were carefully washed three times with rinse solution (phenol red-free DMEM, 20 mM HEPES, pH 7.4). Cells were incubated in 200 μl of DAF-2 DA solution (5 μM DAF-2 DA in 0.5% FBS, DMEM, 20 mM HEPES, pH 7.4) for 20 min at 37 °C, 5% CO2. The solution was removed and replaced with 200 μl of DAF-2 DA-Hoechst solution (0.5% FBS, DMEM, 20 mM HEPES, pH 7.4, 5 μM DAF-2 DA, 3 μg/ml Hoechst 33342). After 10 min of further incubation at 37 °C, the cells were washed three times with rinse solution. Cells were imaged immediately for DAF-2 DA and Hoechst 33342 fluorescence.
under 200-μl rinse solution. Images were captured, processed, and stored via an imaging system composed of an Olympus IMT-2 inverted microscope (Melville, NY) with epi-fluorescence (mercury arc excitation source), and trichroic emission cube (4′,6-diamidino-2-phenylindole/fluorescein isothiocyanate/Texas Red), connected to an automated LEP filter wheel (4′,6-diamidino-2-phenylindole/fluorescein isothiocyanate/Texas Red excitation filters) controlled by a Mac 2002 controller (Ludl, New York, NY). A Hamamatsu Orca II charge-coupled device camera (Bridgewater, NJ) captured fluorescent images through a 10× UV/340 lens. Captured images were processed and stored on a Dell workstation running ISee imaging software (Inovision Corp., Raleigh, NC).

Nitrite levels were determined in conditioned medium from synchronized 3T3 cells cultured for 24 h in the various treatment media. Nitrite was converted to NO in the purge vessel containing a reducing agent (1% (w/v) potassium iodide in acetic acid), which is carried by N2 gas and subsequently mixed with ozone to generate a chemiluminescence signal that was detected by a nitric-oxide analyzer 280i (Sievers Instruments Inc., Boulder, CO). The system was calibrated with a series of sodium nitrite standards ranging from 10 to 600 nM. Under the conditions used for nitrite determination, nitrite cannot be converted to NO. This outcome was verified by injecting a 10 μM solution of sodium nitrate into the reaction chamber, which yielded no detectable chemiluminescence.

Statistics—All culture experiments were performed at least three times. Data were analyzed using one-way analysis of variance (SPSS version 10.0), and treatments generating a significant F-value were subjected to post-hoc analysis using the Fisher’s least significant difference test. p ≤ 0.05 was considered significant. Data are presented as means ± S.E. In the case of real-time RT-PCR data, natural log ΔCt, and ΔCt relative to t = 0 h (2^ΔΔCt) were used in one-way analysis of variance with least significant difference or Kruskal-Wallis post-hoc analysis. Outcomes of these statistical tests were confirmed to be similar for both types of data transformation for all targets. Data are expressed as -fold difference from t = 0 h (2^ΔΔCt).

RESULTS

Zinc Deficiency Results in Decreased Cellular Zinc and Increased Cellular Iron Levels—Our first experiment was to demonstrate that, similar to whole animal models fed zinc-deficient diets, cells cultured in zinc-deficient medium would accumulate iron. Thus, 3T3 cells were cultured in various experimental media to examine potential changes in cellular zinc and iron as a function of time. Beginning at t = 16 h, 3T3 cells cultured in D medium showed a significantly decreased zinc concentration relative to cells cultured in S or C medium (Fig. 1A). At t = 16 h, no significant differences in iron concentrations were evident among groups (Fig. 1B). However, by t = 24 h, the iron concentration in the D group was significantly and persistently elevated over that found in the S and C groups (Fig. 1B). Thus, cells cultured in D medium first displayed a decline in cellular zinc, which was followed temporally by an increase in cellular iron concentrations.

Zinc Deficiency Alters Levels of Iron Transport Proteins—Increased cellular iron concentration in cells could be a result of increased transport into cells, increased storage, decreased export of iron, or a combination of these events. Initially, we examined transcript and protein levels of the major iron transport proteins, TfR1 and DMT1. At t = 16 h and prior to iron accumulation, no significant changes in the level of TfR1 protein could be detected among the groups; however, after t = 24 h, TfR1 expression significantly increased in the D group relative to the S and C groups (Fig. 2A). The change in TfR1 expression coincided with the increase in cellular iron concentrations. We then examined mRNA transcript levels of TfR1. As depicted in Fig. 2B, there was a significant elevation in TfR1 mRNA in the D group at t = 16 h when compared with the S and C groups. Although still significantly elevated, TfR1 mRNA in the D group relative to the control groups is tempered at t = 24 h (Fig. 2B), perhaps as a consequence of the increased iron in the cell. At t = 32 h, although the levels of TfR1 did not significantly differ among groups, the trend (p < 0.08) was for higher TfR1 mRNA in the D group compared with the S and C groups. Contrary to the results observed with a zinc deficiency, a positive control for iron accumulation (100 μM FAC supplementation of C medium for t = 24 h) resulted in the predicted decrease in TfR1 mRNA, whereas iron chelation (100 μM DFO in C medium for t = 24 h) resulted in higher TfR1 mRNA levels (Fig. 2B).

FIGURE 1. Zinc and iron mineral analysis. 3T3 fibroblasts were cultured in C (control), S (zinc-supplemented), or D (zinc-deficient) media for the indicated time periods. Acid-extracted supernatants of 3T3 cells were analyzed for total (A) zinc or (B) iron content by ICP-AES, and normalized to protein content. Data for three individual experiments are expressed as mean ± S.E. *, significantly different within the time point (p ≤ 0.05).
Given the up-regulation of cellular TfR1, we examined a second iron transporter, DMT1. Although this transporter is found primarily in intracellular acidic endosomes, it is also expressed to a lesser extent on the plasma membrane of several cell types where it can transport non-heme or low molecular weight iron (29). To examine the different isoforms of DMT1, mRNA levels were examined by RT-PCR. The mRNA for the IRE-regulated DMT1(+)IRE was similar in all groups at t = 16 h of culture, although as iron accumulated at t = 24 h and t = 32 h, DMT1(+)IRE mRNA was significantly lower in the D group relative to the S and C groups (Fig. 3A). The transcript level for the DMT1(−)IRE was similar among all groups at all time points (Fig. 3B).

Zinc Deficiency Alters Levels of Iron Storage Proteins—Given the accumulation of iron observed in the current study and increased ROS noted in zinc-deficient 3T3 cells in a previous study (30), we investigated the potential effects of these conditions on the expression of the iron storage protein Ft. At t = 24 h, and coinciding with the increased iron accumulation, the D group had significantly higher FtL expression relative to the S and C groups (Fig. 4A). FtH levels did not differ among the groups (Fig. 4B). The increase in FtL protein most likely was due to post-transcriptional regulation, because no significant difference in FtL mRNA was found among the groups (data not shown).

Zinc Deficiency Alters IRP1- and IRP2-binding Activity—Differences in post-translational regulation conferred by IRP binding could potentially explain the altered levels of iron transporters and storage proteins noted during zinc deficiency. Consistent with the increase in iron in the D group, IRP1-binding activity significantly decreases at t = 24 h, relative to the S and C groups, with no decrease in total IRP1-binding capacity, evaluated by β-ME treatment of cell homogenates (Fig. 5, A and B). No differences in the levels of IRP1 protein or mRNA were noted among groups (data not shown). To test the possibility that the decrease in IRP1 binding was a consequence of increased ROS production in zinc-deficient cells, D medium was supplemented with either NAC (5 mM) or ASC (100 μM ascorbate plus 800 units/ml catalase). However, neither of these antioxidant treatments restored IRP1-binding activity to control levels after 24 h of cell culture (data not shown).

At t = 16 h of culture, and prior to the increase in cellular iron, IRP2-binding activity significantly increased in the D group relative to the S and C groups and remained so through-
out the culturing period (Fig. 5, A and C). Contrary to the results observed with a zinc deficiency, a positive control for iron accumulation (100 μM FAC supplementation of C medium for \( t = 24 \) h) resulted in the predicted decrease in IRP1- and IRP2-binding activity toward the IRE consensus probe (Fig. 5, A–C). Iron chelation (100 μM DFO in C medium for \( t = 24 \) h) led to the predicted increases in both IRP1- and IRP2-binding activity (Fig. 5, A–C).

**Increased IRP2-binding Activity Is Due to Increased IRP2 Protein Levels through a Mechanism That Is NO-independent**—The increase in IRP2 binding observed in the zinc-deficient group could be a result of an increased affinity to the consensus IRE, or more likely, a result of increased IRP2 protein levels. To examine the latter possibility in detail, we examined the time course of IRP2 expression. Preceding iron and TfR1 accrual, IRP2 was significantly elevated at \( t = 16 \) h in the D group only, and remained elevated at \( t = 24 \) and 32 h (Fig. 6). The increase in IRP2 levels was not likely caused by increased gene transcription, because message levels of IRP2 did not significantly differ among groups (data not shown).

Increased NO produced by iNOS has been shown to inhibit the degradation of IRP2 (31). As increased iNOS expression has previously been observed in rats fed a zinc-deficient diet (32), we investigated whether treatment with D medium would increase iNOS expression in 3T3 cells (endothelial and neuronal nitric-oxide synthases are not expressed in 3T3 cells). Start-
ing at t = 12 h (Fig. 7), and prior to the increase in IRP2-binding activity, we found increased levels of iNOS mRNA in the D group, which continued throughout the remainder of the culturing period (i.e. t = 32 h). We next examined the functional response of increased iNOS mRNA by examining the level of NO produced in 3T3 cells under the different culture conditions. NO, measured with the specific probe DAF-2 DA, was increased in the D group compared with the S and C groups (Fig. 8). These data suggest that increased NO production in the zinc-deficient cells might be responsible for the IRP2 protein accumulation. To further test this hypothesis, we cultured cells for 24 h in D medium supplemented with either 100 μM 1400W or 1 mM SEIT, both potent iNOS inhibitors. Although these inhibitors attenuated DAF-2 DA fluorescence (data not shown), they did not attenuate IRP2 levels in the D group, but rather, the iNOS inhibitors actually increased the levels of IRP2 (Fig. 9A), an event that was associated with a significant increase in TfR1 levels (Fig. 9B). Finally, if a significant amount of NO is generated during the 24-h culturing period, medium nitrite levels should rise concomitantly. However, no significant differences in nitrite levels among the conditioned media were detected (data not shown).

Redox Regulation of IRP2 Protein Accumulation during Zinc Deficiency—IRP2 levels are also regulated by ROS, as the antioxidants NAC and ASC have been shown to reduce the levels of IRP2 (33), perhaps by enhancing its proteasomal degradation. Therefore, we reasoned that oxidative stress inherent in zinc-deficient cells (30) might decrease proteasomal degradation of IRP2. Thus, if rising IRP2 levels in zinc-deficient 3T3 cells are responsible for stabilizing TfR1 message and increasing TfR1 protein levels, antioxidants should mitigate this effect. The addition of NAC (5 mM) or ASC (100 μM ASC plus 800 units/ml catalase) to the D medium resulted in lower levels of IRP2 (Fig. 10, A and B) and TfR1 (Fig. 10, A and C) compared with 3T3 cells cultured in D medium alone. To further define the importance of ROS on IRP2 accumulation, we attempted to increase IRP2 levels in the C group with the addition of 4 mM TEMPOL, a pro-oxidant when used at high concentrations (34). The addition of TEMPOL to C medium significantly increased the levels of both IRP2 and TfR1 (Fig. 10, A–C).

DISCUSSION

Zinc-deficient animals accumulate iron in several organs, including testis, thymus, kidney, and liver (35). Furthermore, fetuses from zinc-deficient dams also accumulate iron, and native molecular sieve chromatography of fetal liver labeled with 59Fe suggests that the iron is sequestered in ferritin (36). In this study, we used an in vitro cell culture model to determine the mechanisms of the underlying zinc deficiency-induced iron accumulation. Consistent with whole animal studies, we observed a time-dependent increase in cellular iron concentrations in zinc-deficient 3T3 cells. Furthermore, this iron accumulation occurred in a culture medium that contained relatively low iron concentrations (i.e. 3 μM). The increase in cellular iron was preceded by an increase in IRP2 protein levels and binding activity, suggesting altered regulation of IRP2 turnover plays a causative role in the increase in
cellular iron noted in the zinc-deficient cells. Unlike IRP2, IRP1 levels remained unchanged by treatments modulating redox status (i.e. NAC and ASC) and thus, the decreased IRP1 binding in zinc-deficient cells most likely results from its conversion from IRP to an aconitase in the face of rising cellular iron levels.

In concordance with normal iron homeostasis, the decreased IRP1-binding activity resulting from increased cellular iron load in zinc-deficient cells may explain the decreased level of the DMT1(+)IRE mRNA. Not surprisingly, DMT1(−)IRE mRNA, which lacks the iron regulated IRE structure, was not affected by the zinc deficiency-induced changes in IRP activity. At odds with normal iron homeostasis, however, is the continued up-regulation of TfR1 mRNA and protein levels during zinc deficiency, despite increasing cellular iron concentrations and decreasing IRP1-binding activity. Both TfR1 and DMT1 are iron transporters, which are regulated by 3′-UTR IREs. Typically, when iron levels in the cell increase, decreased binding to the IRE by IRPs occurs, which destabilizes the TfR1 and DMT1(+)IRE mRNAs and enhances their degradation, resulting in subsequent decreases in protein synthesis and iron transport into the cell. Although our study did not examine the underlying mechanism for the discordance in the regulation of the two iron transporters, we suggest that this phenomenon could be explained by the structural differences inherent in TfR1 and DMT1 3′-UTR IREs, which ultimately affect their affinity toward the two IRP proteins. It has been shown that DMT1(+)IRE is regulated by a single 3′ IRE, whereas the TfR1 mRNA is synergistically regulated by five 3′ IREs and intervening base sequences (37). A C-bulge in the DMT1(+)IRE provides for an 8-fold higher affinity toward IRP1 compared with IRP2 (25), whereas a distorted helix structure within the five IREs of the TfR1 3′-UTR enhances IRP2 binding over engineered constructs containing two or three TfR1-IREs (37). Hence, decreased IRP1 binding would likely enhance DMT1(+)IRE turnover relative to that of the TfR1 transcript, whereas increased IRP2-binding activity noted at all time points during zinc deficiency would preferentially enhance stabilization of TfR1 mRNA relative to that of DMT1(+).IRE. To our knowledge, the in vivo stability of DMT1(+).IRE relative to TfR1 transcripts, under differing physiological conditions that result in inversely regulated IRP1 versus IRP2 binding, has not been examined in detail and thus, this supposition needs experimental confirmation. Nevertheless, supporting the concept of the importance of IRP2 binding in TfR1 stabilization, is the demonstration that IRP2 knockouts present reduced levels of TfR1 despite the presence of normal IRP1 levels (38). Additionally, cell lines lacking IRP1 have normal TfR1 levels (39). The data presented in this study further support the importance of IRP2 binding in regulating TfR1 mRNA stability. For example, all conditions that raised IRP2 levels (D medium or treatment of the C medium with TEMPOL) also raised TfR1 levels, whereas conversely, all treatments that reduced IRP2 levels (S and C medium, or NAC and ASC addition to D medium) reduced TfR1 levels. The data presented in this study cannot rule out the possibility that the increase in TfR1 mRNA is a consequence of zinc deficiency-induced up-regulation of transcription independent of IRP2 binding.

Given the increased cellular iron in zinc-deficient 3T3 cells noted in this report, and the changes in LIP that are likely to occur as a consequence, the increase in cellular FtL levels noted for the D group is consistent with normal homeostatic iron regulation. Ft can be regulated by both transcriptional and translational mechanisms (8). However, analysis of FtL mRNA revealed no differences among treatment groups (data not shown), suggesting that the increase in FtL protein expression during zinc deficiency is likely due to post-transcriptional regulation through IRP-IRE binding events. Structural and binding studies confirm that both IRP1 and IRP2 can bind to the Ft 5′ IRE with approximately equal affinity, which in vivo, results in blocking the ribosomal initiation complex from docking and thereby inhibiting translation of the Ft mRNA (11, 28). Hence, in the case of zinc deficiency, the decreased influence of IRP1-binding activity may compensate for the increase in IRP2-binding activity. This outcome would result in an overall decrease in translational inhibition of Ft mRNA, leading to the observed increase in FtL protein.

The observed differential regulation of IRP1 and IRP2-binding activity during zinc deficiency does not typically occur in

Zinc Deficiency-Induced Iron Accumulation

FIGURE 10. Redox regulation of IRP2 and TfR1. 3T3 fibroblasts were cultured in C, S, D, D plus NAC (zinc-deficient plus 5 mM NAC), D plus ASC (zinc-deficient plus 100 μM ascorbate plus 800 units/ml catalase), or C plus TEMPOL (control plus 4 mM TEMPOL) medium for 24 h. A, representative images of three individual Western blot analyses of cytosolic fractions. Densitometry was normalized to actin and expressed as mean ± S.E. for IRP2 (B) and TfR1 (C). Bars labeled with different letters are significantly different (p < 0.05).
otherwise normal 3T3 cells. Given that IRP1 binding during zinc deficiency was responding normally to the increase in cellular iron, we focused our attention on factors that might result in increased IRP2 levels. After ruling out that changes in IRP2 transcription were occurring, we examined a number of possibilities that might affect IRP2 degradation including RNS and ROS.

The effects of NO on IRP2 binding affinity have been controversial (40, 41). In part, the controversy can be attributed to the specific NO donor used in each of the studies as well as whether the NO form is added directly to the culture medium or generated by endogenous means. Thus, it has been reported that endogenously generated NO stabilizes or destabilizes IRP2 in J774.A1 cells (42, 43) or has no effect on IRP2 in other cell lines (41). Similar discrepancies exist when comparing the effects of exogenous NO donors on IRP2-binding activity. For example, incubation of cells or cell extracts with NO donors SNAP, SNP, GSNO, and DEANO, increases, decreases, or leaves IRP2-binding activity unaffected (22, 40, 41, 44–46). In this study, we found evidence of increased NO production in the D group as measured by DAF-2 DA fluorescence. However, the inability to prevent the increase in IRP2 by potent iNOS inhibitors implies that increased NO is not the responsible mechanism. Additionally, IRP1 activity is almost always increased in cells and cell extracts treated with NO donors or endogenously generated NO (41, 44, 45). Conversely, IRP1-binding activity is decreased in zinc-deficient cells and thus, we conclude that NO generated in our system is likely insufficient to alter IRP-binding activity.

Similar to the results in this report, differential regulation of IRPs has also been observed during hypoxia (19). Although frank hypoxic conditions are not likely to exist under the culturing conditions used in this study, many of the results observed during hypoxia can be attributed to increased ROS, which is also seen in zinc-deficient cells. As with a zinc deficiency, ROS has been shown to result in both increased FeT (47) and increased TfR1 levels (20). In a previous study, we noted that zinc-deficient 3T3 cells are characterized by an elevation in ROS detected by dichlorodihydrofluoresceindiacetate fluorescence and an elicitation of a potent AP-1 response (30). Recent work has elucidated a mechanism for IRP2 degradation, which is similar to HIF-1α degradation and is dependent on hydroxylation of critical proline residues by the 2-oxoglutarate-dependent family of dioxygenases (12, 33). Increased cellular ROS levels have also been shown to inactivate the prolyl hydroxylase, decreasing HIF-1α degradation (48). Conversely, mitigating the effects of ROS with glutathione and ASC decreases the level of HIF-1α protein (49). It has been demonstrated previously that certain antioxidants can induce IRP2 degradation (33) while pro-oxidants may enhance IRP2 accumulation (31). Thus, the increased ROS in 3T3 cells subjected to zinc deficiency might lead to inactivation of the IRP2 prolyl hydroxylase leading to the observed accumulation of IRP2. Supporting this notion, addition of antioxidants to D medium prevented the accumulation of IRP2 and TfR1 protein.

Finally, iron accumulation in zinc-deficient cells may be partially mediated by zinc transporters as well as the Tf/TfR1 system. In a recent report, Zip14, a zinc transporter, was able to mediate the uptake of iron from culture medium in HEK 293H cells (50). Further studies are warranted to examine the relative ability of these two systems to transfer iron into cells as a consequence of zinc deficiency and/or other pathological conditions.

In summary, we show that similar to whole animal systems, zinc deficiency in isolated cells leads to the accumulation of iron. An unexpected rise in IRP2 levels provides a mechanism for increased expression of TfR1, and this outcome is most likely mediated by ROS and not RNS. Further studies are warranted to address if this model is consistent in other cell types and most importantly, if it holds true in the whole animal.

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