Iron-regulatory proteins (IRP1 and IRP2) are RNA-binding proteins that bind to stem-loop structures known as iron-responsive elements (IREs). IREs are located in the 5'- or 3'-untranslated regions (UTRs) of specific mRNAs that encode proteins involved in iron homeostasis. The binding of IRPs to 5' IREs represses translation of the mRNA, whereas the binding of IRPs to 3' IREs stabilizes the mRNA. IRP1 and IRP2 IRE binding activities are regulated by intracellular iron levels. In addition, nitric oxide (NO) increases the affinity of IRP1 for IREs. The role of NO in the regulation of IRP1 and IRP2 in rat hepatoma cells was investigated by using the NO-generating compound S-nitroso-N-acetylpenicillamine (SNAP), or by stimulating cells with multiple cytokines and lipopolysaccharide (LPS) to induce NO production. Mitochondrial and IRP1 aconitase activities were decreased in cells producing NO. NO increased IRE binding activity of IRP1, but had no effect on IRE binding activity of IRP2. The increase in IRE binding activity of IRP1 was coincident with the translational repression of ferritin synthesis. Transferrin receptor (TfR) mRNA levels were increased in cells treated with NO-generating compounds, but not in cytokine-and LPS-treated cells. Our data indicate that IRP1 and IRP2 are differentially regulated by NO in rat hepatoma cells, suggesting a role for IRP1 in the regulation of iron homeostasis in vivo during hepatic inflammation.

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binding activity by NO correlated with decreased ferritin synthesis and increased TIR mRNA levels. These data indicate that NO' activation of IRP1 IRE binding activity is sufficient for regulation of ferritin and TIR mRNAs.

MATERIALS AND METHODS

Cell culture. The rat hepatoma cell line FTO2B was grown at 37°C in an 8% CO2 atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. FTO2B cells (1 x 10^6) were grown in 60-mm dishes in the presence of either 50 μg/mL ferric ammonium citrate (FAC), 10 ng/mL tumor necrosis factor-α (TNF-α), 100 μM interferon-γ (IFN-γ), and 5 μg/mL lipopolysaccharide (LPS) or a combination of various cytokines and LPS. The choice and doses of cytokines and LPS were based on cytokine studies in hepatocytes grown in culture.43 Briefly, TNF-α and IFN-γ were purchased from GIBCO BRL (Grand Island, NY). M^2-monomethyl l-arginine (NMA) Sigma, St Louis, MO) was added at 125 μg/mL either alone or in the presence of IFN-γ, TNF-α, and LPS. In some experiments, cells were treated with 0.5 mmol/L S-nitroso-N-acetylpenicillamine (SNAP) or 0.5 mmol/L N-acetylpenicillamine (NAP) Sigma. SNAP was a gift from Dr Neil Bastian of the University of Utah.

Separation of mitochondrial and cytoplasmic cell fractions and aconitase assays. FTO2B cells were grown in the presence of FAC, IFN-γ/TNF-α/LPS, and NMA or NMA in the presence of IFN-γ/TNF-α/LPS (NMA, C + L) for 16 hours. Cytoplasmic and mitochondrial fractions were separated as previously described.3 Briefly, 0.01% digitonin was added to a dish of cells for 10 minutes on ice. The digitonin solution containing the cytoplasmic fraction was then removed and stored at 4°C. The remaining cellular components left on the dish contained the mitochondrial fraction. After solubilization with 0.2% Triton X-100 in 0.15 mol/L NaCl and 20 mmol/L HEPES, pH 7.5, the lysate was centrifuged at 5,000 g for 15 minutes. The purity of the cytoplasmic fraction was determined by immunoblotting with rabbit antiimotochondrial aconitase antibody. Aconitase activity was measured by determining the change in absorbance (as-as) at 240 nm. Immunoblotting with rabbit anticytoplasmic aconitase antibody and assaying for the mitochondrial enzyme, glutamate dehydrogenase.42 The purity of mitochondrial fractions was assayed by RNA--band shift assays and by measuring the cytoplasmic enzyme lactate dehydrogenase.

Aconitase was assayed in each fraction by the addition of 0.2 μmol/L cis-aconitate to 50 μg protein in 1 mL 50 mmol/L Tris-HCl, pH 7.5, from each fraction and monitoring the disappearance of cis-aconitate at 240 nm over time.

Nitrite assay. After incubation of cells with IFN-γ/TNF-α/LPS, NMA, or FAC for 8, 12, 16, 20, and 24 hours, samples of media were assayed for the end product of NO production, nitrite, using the Griess reaction.40 Culture media (100 μL) were mixed with 200 μL of a 1:1 solution of 1% sulfanilamide in 30% acetic acid and 0.1% naphthylethylenediamine in 60% acetic acid and shaken for 3 minutes, and absorbance at 543 nm was measured.

RNA--band shift assays and northern blotting. FTO2B cells were lysed in 0.3 mL buffer A (20 mmol/L HEPES, pH 7.5, 25 mmol/L KCl, 5% glycerol, 1 mmol/L DTT) containing 0.5% Nonidet P-40, and the lysates were centrifuged at 13,000 g for 10 minutes. The concentration of protein in the lysates was quantified using the BCA protein assay (Pierce, Rockford, IL). RNA--band shift assays were performed using 10 μg protein incubated with ^32P-labeled ferritin IRE RNA as previously described. RNA complexes were separated by 3% native polyacrylamide gels, and the gels were dried and subjected to autoradiography. IRP1 and IRP2 IRE binding activities were quantified by densitometry.

Total RNA was prepared from treated FTO2B cells using TR1zol (GIBCO BRL). Total RNA (10 μg) was analyzed on 1% formaldehyde-agarose gels in MOPS buffer, pH 7.0. The RNA was transferred to nylon membranes and hybridized with random-primed ^32P-labeled mouse TIR cDNA obtained from Dr Prem Ponka of the Lady Davis Institute for Medical Research in Montreal, Quebec. Hybridization was performed using Rapid-hyb buffer (Amersham, Arlington Heights, IL) for 1 hour at 65°C, and the filters were washed at 2 x SSC (20 x SSC = 3 mol/L NaCl, 0.3 mol/L sodium acetate) containing 0.1% SDS at 25°C for 15 minutes, followed by a wash at 1 x SSC at 55°C for 10 minutes.

Ferritin and IRP2 immunoprecipitation. Ferritin and IRP2 were immunoprecipitated from FTO2B cells that were stimulated with IFN-γ/TNF-α/LPS or FAC for 12 or 16 hours. Trans ^35S-label (100 μCi/mL; ICN Biomedicals, Costa Mesa, CA) was added to the cells for the last 2 hours of treatment. The cells were then washed with PBS and lysed by lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L dithiothreitol (DTT). Ferritin and IRP2 were immunoprecipitated from lysates using 5 x 10^5 cpmp with rabbit antihamster antisemur (ICN Biomedicals) or rabbit anti-IRP2 antisemur44 and protein A-agarose (GIBCO BRL). The lysates were washed four times with RIPA buffer (150 mmol/L NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 50 mmol/L Tris-HCl, pH 8.0, 1 mol/L PMSF, and 1 mol/L DFP) followed by one wash in 20 mmol/L Tris-HCl, pH 6.8, and 0.2% NonidetP-40. Immunoprecipitated ferritin and IRP2 was dissociated from protein A-agarose by boiling for 5 minutes in SDS sample buffer (65 mmol/L Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, and 0.001% bromophenol blue) and analyzed by 15% and 8% SDS-PAGE, respectively. The protein was electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and the membrane was subjected to autoradiography.

Protein synthesis measurements. Total protein synthesis was measured by trichloroacetic acid (TCA) precipitation. For each sample, 10 mL lysate was added to 500 mL 0.1% albumin in lysis buffer on ice, followed by addition of 500 μL ice-cold 20% TCA. The samples were vortexed and incubated on ice for 30 minutes. The samples were then filtered through glass filters (GF/C; Whatman) and washed two times with 5 mL cold 10% TCA and two times with 5 mL cold 100% ethanol. Filters were dried and counted in 3 mL Opti-Fluor (Puckard, Meriden, CT).

RESULTS

IRP1 and mitochondrial aconitase activities are decreased in FTO2B cells stimulated to produce NO. Cultured rat hepatocytes express inducible nitric oxide synthase (iNOS) in response to multiple cytokines and endotoxin stimulation, resulting in the formation of large amounts of NO.48 To determine if the rat hepatoma cell line FTO2B produced NO in response to cytokines and endotoxin, FTO2B cells were treated for 4 to 24 hours with a variety of cytokines and LPS used either individually (data not shown) or in combination (Fig 1). NO production was measured by assaying culture supernatant NO^2 and NO^3 levels in cytokine-and LPS-stimulated cells. Maximal NO^2 formation was observed using a combination of IFN-γ, TNF-α, and LPS, whereas IFN-γ, TNF-α, or LPS used individually did not induce significant NO^2 production (data not shown). NO^2 levels increased to 2.5 μmol/L after 4 hours of IFN-γ/TNF-α/LPS treatment, with further accumulation to 45 μmol/L at 24 hours. Treatment of cells with NMA, a competitive inhibitor of NO synthase, in the presence of IFN-γ,
TNF-α and LPS abolished NO production. In the experiments described below, a combination of TNF-α, IFN-γ, and LPS was used for NO production in FTO2B cells.

Previous studies indicated that aconitase activity of IRP1 is inhibited in macrophage and nonmacrophage cell lines stimulated with cytokines to produce NO,31,32 but not in hepatocytes.51 To determine the effect of NO on cytoplasmic IRP1 and mitochondrial aconitase activities in hepatoma cells, FTO2B cells were treated with IFN-γ/TNF-α/LPS for 16 hours and aconitase activity was measured in mitochondrial and cytoplasmic fractions. Aconitase activity was measured spectrophotometrically by following the rate of disappearance of cis-aconitate measured at 240 nm.49 In untreated cells, mitochondrial and cytoplasmic fractions exhibited aconitase activity that was fully inhibited in IFN-γ/TNF-α/LPS-treated cells (Fig 2). These data indicated that mitochondrial and cytoplasmic aconitase activities are both inhibited by NO production in rat hepatoma cells.

**IRE binding activities of IRP1 and IRP2 are differentially regulated in cytokine- and LPS-stimulated FTO2B cells.**

To determine if NO production modulates IRP2 IRE binding activity, FTO2B cells were treated with IFN-γ/TNF-α/LPS for 4 to 24 hours and cytoplasmic extracts were incubated with a 32P-labeled IRE, followed by resolution of the RNA-protein complexes by 5% native polyacrylamide gels (Fig 3A). IRE binding activity of IRP1 increased twofold after 8 hours of treatment, with an eightfold increase after 24 hours. The increase in IRP1 IRE binding activity correlated with an increase in NO production (Figs 1 and 3A). Increased IRE binding activity is not due to increased IRP1 protein levels, since immunoblots using anti-IRP1 antibody demonstrated that the amounts of IRP1 remain constant during IFN-γ/TNF-α/LPS treatment (Fig 3B). Treatment of cells with NMA in the presence of IFN-γ/TNF-α/LPS prevented the increase in IRP1 IRE binding activity observed with IFN-γ/TNF-α/LPS, indicating that the change in IRE binding activity is due to NO. In NMA-treated cells, IRP1 IRE binding activity decreased slightly as compared with untreated control cells; however, the significance of this observation is unclear (Fig 3A). In contrast to IRP1, IRP2 IRE binding activity (Fig 3A) and protein levels (Fig 3C) increased only slightly (<0.5-fold) in cells treated with IFN-γ/TNF-α/LPS.

To confirm the data indicating that IRP2 is not affected by NO, the capacity of the NO-generating compound, SNAP, to modulate IRP1 and IRP2 IRE binding activities in FTO2B cells was also examined. Addition of SNAP to medium causes spontaneous release of NO,52 Figure 4 shows that the IRE binding activity of IRP1 increased twofold after 4 hours of SNAP treatment and increased about eightfold by 20 hours. In contrast, IRP2 IRE binding activity was not affected by SNAP. The nonnitrosylated compound, NAP, which does not generate NO, used at the same concentration had no effect on IRP1 IRE binding activity, indicating that NO release from SNAP was responsible for the activation in IRP1 IRE binding activity (Fig 4A). Since NO targets proteins containing Fe-S clusters, these data suggested that cells do not contain a pool of IRP2 containing Fe-S clusters that can be activated by NO. IRP2 IRE binding activity can be increased in cells by treatment with desferroxamine. However, this increased activity can be accounted for by increased IRP2 protein levels due to stabilization of iron-depleted cells31,33,34 (Fig 4B and C). This is in contrast to IRP1, where NO activates a pool of IRP1 containing 4Fe-4S clusters, occurring without changes in IRP1 protein levels31,34 (Fig 3A and B). These data are consistent with the
results obtained in IFN-γ/TNF-α/LPS-treated cells, indicating that IRP2 is not a target for NO in rat hepatoma cells.

**Ferritin synthesis is decreased in FTO2B cells stimulated with cytokines and LPS**. Ferritin synthesis is translationally regulated by iron by the binding of IRPs to the IRE in the 5' UTR of ferritin mRNA. Since the production of NO in IFN-γ/TNF-α/LPS–stimulated cells resulted in activation of the IRE binding activity of IRP1 but had no effect on IRE binding activity of IRP2, we wanted to determine the effect of increased IRP1 IRE binding activity on ferritin synthesis. FTO2B cells were treated with IFN-γ/TNF-α/LPS, NMA in the presence of IFN-γ/TNF-α/LPS, NMA alone or ferric ammonium citrate for 12 and 16 hours, and pulsed with [35S]methionine for the last 2 hours of each treatment. Ferritin was immunoprecipitated from extracts using anti–human ferritin antibody, and [35S]-labeled proteins were separated by an 8% SDS-polyacrylamide gel (Fig 5A). Iron administration resulted in a fivefold increase in ferritin synthesis, as previously shown for other cell types. IFN-γ/TNF-α/LPS–stimulated cells resulted in a twofold decrease and a fivefold decrease in ferritin synthesis by 12 hours and 16 hours, respectively. Treatment of cells with NMA in the presence of IFN-γ/TNF-α/LPS prevented the decrease in ferritin synthesis observed with cytokines plus LPS treatment, indicating that the decrease in ferritin synthesis is due to NO. Ferritin synthesis was increased slightly in NMA-treated cells; however, the significance of this observation is unclear. Two 46-kD immunoreactive bands also immunoprecipitated with the anti–human ferritin antisera. Whether these bands represent ferritin subunit dimers or nonspecific proteins is unclear. We conclude from these experiments that the decrease in ferritin synthesis is due to translational repression of ferritin mRNA, since ferritin heavy- and light-subunit mRNA levels were unaffected by NO production (data not shown).
Fig 4. Effect of a NO-generating compound on IRE binding activity of IRP1 and IRP2 in FTO2B cells. Cells were incubated in the presence or absence of (A) 0.5 mmol/L SNAP or 0.5 mmol/L NAP for 4 to 24 hours or (B) 100 μmol/L desferrioxamine for 4 and 16 hours. IRE binding activities of IRP1 and IRP2 were assayed in cytoplasmic extracts with a 32P-labeled IRE RNA and the IRE:IRP complexes resolved by 5% native polyacrylamide gels. Positions of IRP1:IRE and IRP2:IRE complexes and free RNA are indicated. (C) IRP2 protein levels were measured in extracts from B by SDS-PAGE and immunoblot analysis using anti-IRP2 antibodies. The position of IRP2 is indicated.

Fig 5. Effect of NO production on ferritin synthesis in cytokine- and LPS-stimulated FTO2B cells. Cells were incubated in the presence of 50 μg/mL FAC, IFN-γ/TNF-α/LPS (C + L), NMA, or NMA in the presence of C + L for 12 or 16 hours as described in Fig 1. For the last 2 hours of treatment, 100 μCi/mL Tran35S-label was added to each culture. Cells were lysed, and 5 × 10^6 cpm were immunoprecipitated separately with rabbit antiferritin antisera or rabbit anti-IRP2 antisera. 35S-labeled ferritin (A) and 35S-labeled IRP2 (B) were analyzed by 15% and 8% SDS-polyacrylamide gels, respectively. The protein was transferred to a PVDF membrane, and the membrane was subjected to autoradiography. Positions of ferritin, IRP2, and molecular weight standards are indicated.
Since NO production is associated with decreased protein synthesis in rat hepatocytes grown in culture, we measured total protein synthesis in FTO2B cells treated with IFN-γ/TNF-α/LPS. A decrease in total protein synthesis was observed with all treatments; however, protein synthesis was 36% of levels in untreated control cells at 12 hours after IFN-γ/TNF-α/LPS treatment (Table 1). This inhibition was partially reversed by treatment with NMA, increasing to 62% of untreated control levels. To confirm that the decrease in ferritin synthesis in cells producing NO is due to an increase in the translational repression of ferritin mRNA by IRP1 and not due to a decrease in total protein synthesis, we measured the rate of synthesis of IRP2 in IFN-γ/TNF-α/LPS–stimulated cells at 12 hours (Fig 5B). Our previous data indicated that IRP2 IRE binding activity and protein levels were unaffected in NO–producing cells (Fig 3C), and therefore, IRP2 synthesis rate should remain constant. Figure 5B shows that IRP2 synthesis is not affected in FTO2B cells producing NO∗.

Thus, our data indicate that the inhibition of ferritin synthesis in NO∗-producing cells is likely due to the translational repression of ferritin mRNA by IRP1.

TfR mRNA levels do not change in FTO2B cells stimulated with cytokines and LPS. IRP1 modulates TfR expression by binding to IREs located in the 3′-UTR of the TfR mRNA, resulting in the stabilization of TfR mRNA in iron-depleted cells. To determine the effect of NO∗ on TfR mRNA in hepatoma cells, FTO2B cells were stimulated with IFN-γ/TNF-α/LPS, NMA alone, or NMA in the presence of IFN-γ/TNF-α/LPS or ferric ammonium citrate for 16 hours and the amount of TfR mRNA was measured (Fig 6). As expected, TfR mRNA levels were decreased fivefold in iron-treated cells. In cells treated with IFN-γ/TNF-α/LPS in the presence or absence of NMA, TfR mRNA levels were slightly decreased as compared with untreated cells. Since treatment of cells with IFN-γ/TNF-α/LPS did not result in the expected increase of TfR mRNA levels, this suggested that IFN-γ/TNF-α/LPS affected TfR mRNA levels independent of NO∗.

To eliminate cytokine- and LPS-mediated effects, we treated cells with SNAP for 16 hours. In SNAP-treated cells, TfR mRNA levels increased about twofold as compared with untreated cells. An unexpected result was that TfR mRNA levels were also increased in NMA-treated cells. NMA should abolish basal levels of NO∗ production in untreated cells (Fig 1), which would presumably decrease TfR mRNA levels. Thus, NMA may be affecting other pathways in cells that result in TfR mRNA accumulation. These data indicate that TfR mRNA levels can be increased by exogenously produced NO∗ and that this increase is correlated with activation of IRP1 IRE binding activity (Fig 4), suggesting that IRP1 mediates stabilization of TfR mRNA in NO∗-producing cells. We conclude from these data that cytokines and LPS may regulate TfR mRNA levels in hepatoma cells independent of NO∗, and may therefore counteract NO∗-mediated effects on IRP1 regulation.

**DISCUSSION**

Although IRP1 and IRP2 are similar proteins sharing 61% overall amino acid identity, they exhibit different biochemical properties. IRP1 is a bifunctional protein acting either as an aconitase when iron is abundant or as an RNA-binding protein when iron is scarce. IRP1 accomplished these roles by the assembly of a 4Fe-4S cluster that results in the switch between RNA binding and aconitase active forms. The 4Fe-

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**Table 1. Total Protein Synthesis Rates in FTO2B Cells Stimulated With Cytokines and LPS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>12-hour cpm x 10⁶/µg</th>
<th>% Control</th>
<th>16-hour cpm x 10⁶/µg</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.43 ± 0.34</td>
<td>100.0</td>
<td>3.73 ± 0.13</td>
<td>100.0</td>
</tr>
<tr>
<td>FAC</td>
<td>5.58 ± 0.09</td>
<td>86.5</td>
<td>2.80 ± 0.20</td>
<td>75.7</td>
</tr>
<tr>
<td>C + L</td>
<td>2.28 ± 0.09</td>
<td>35.5</td>
<td>0.97 ± 0.11</td>
<td>27.0</td>
</tr>
<tr>
<td>NMA</td>
<td>5.11 ± 0.39</td>
<td>79.3</td>
<td>2.93 ± 0.18</td>
<td>78.4</td>
</tr>
<tr>
<td>NMA, C + L</td>
<td>3.98 ± 0.19</td>
<td>61.9</td>
<td>2.80 ± 0.23</td>
<td>75.7</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of triplicate samples. Results shown for 12 and 16 hours are from 1 of 3 independent experiments.

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**Fig 6. Effect of NO∗ on TfR mRNA levels in FTO2B cells stimulated with cytokines and LPS.** Total RNA was isolated from FTO2B cells incubated in the presence of 50 µg/mL FAC, IFN-γ/TNF-α/LPS (C + L), NMA, or NMA in the presence of C + L for 12 hours as described in Fig 1. The RNA was fractionated on a 1% formaldehyde-agarose gel and transferred to a nylon membrane. The RNA was hybridized to a 32P-labeled mouse TfR cDNA. The position of the TfR mRNA is indicated. The ethidium bromide–stained gel, prior to transfer, is shown below for normalization of RNA loading, and positions of 28S and 18S RNAs are indicated.
4S cluster in IRP1 may serve as a target for NO, leading to the disruption of the cluster with subsequent activation of IRE binding activity. In contrast to IRP1, IRP2 lacks aconitase activity and functions solely as an RNA binding protein. Our data reported here indicate that hepatoma cells do not contain a pool of IRP2 that can be activated by NO. These data suggest that IRP2 exists predominately as an apoprotein in cells and consequently is not a target for NO. This idea is consistent with data demonstrating that treatment of extracts with β-mercaptoethanol activates IRE binding activity of IRP2, but has no effect on IRE binding activity of IRP2. Although the mechanism of β-mercaptoethanol activation of IRP1 binding activity is unclear, these data suggest that a pool of activatable IRP1 is present in cells, unlike IRP2, which is always found in a high-affinity RNA-binding form. IRE binding activity of IRP2 can be increased in hepatoma cells treated with the iron chelator, desferrioxamine. However, this increase is correlated with increased IRP2 protein levels. Based on these data, a model of IRP2 regulation by iron can be proposed whereby increases in cytoplasmic iron levels result in the assembly of a 4Fe-4S cluster in IRP2. Whether IRP2 contains a 4Fe-4S cluster is unknown. However, since IRP2 contains the three conserved cysteines that coordinate the 4Fe-4S cluster in IRP1, this suggests that a cluster might also form in IRP2. Formation of a cluster might lead to a conformational change in IRP2 and a loss in RNA binding. The dissociation of IRP2 from the IRE results in its rapid proteolysis by the proteasome.

The functional significance of two IRPs is uncertain. However, recent data suggest that IRP1 and IRP2 may have a preference for different IREs in vitro, suggesting that they may regulate different IRE-containing mRNAs in vivo. Alternatively, IRP1 and IRP2 may bind to the same IRE-containing mRNAs, but respond to different levels of cytoplasmic iron. This model would allow for IRP regulation to occur over a wide range of cytoplasmic iron concentrations. Other studies have demonstrated that IRP2 IRE binding activity is increased in regenerating rat liver and is decreased in livers from rats subjected to oxidative stress. Whether this is due to changes in cytoplasmic iron levels or to other effectors has not been determined.

The differential regulation of IRP1 and IRP2 in rat hepatoma cells by NO allows us to correlate changes in IRE binding activities of IRP1 and IRP2 with changes in ferritin and TfR expression. Our data indicate that the increase in ferritin synthesis in IFN-γ/TNF-α/LPS–stimulated FTO2B cells correlates with an increase in IRE binding activity of IRP1, indicating that IRP1 is sufficient for translational repression of ferritin mRNA. Our results are consistent with other data indicating that ferritin synthesis is decreased concomitantly with an increase in the IRE binding activity of IRP1 in macrophages and nonmacrophage cell lines stimulated to produce NO. In these studies, IRP2 regulation was not reported. In one study using murine B6 fibroblasts stably transfected with a cDNA encoding the mouse macrophage iNOS synthase, constitutive synthesis of NO resulted in the activation of both IRP1 and IRP2 IRE binding activities as compared with untransfected cells. The discrepancies in IRP2 activation by NO between the study reported here and the one reported by Pantopoulos and Hentze may be due to variation in IRP2 regulation in different cell types. Alternatively, cells that are constitutively producing NO may have altered intracellular iron metabolism, so they may be chronically iron-depleted, resulting in IRP2 stabilization. This idea is consistent with studies demonstrating that NO causes increased iron efflux from cells.

Some of the same cytokines that induce NO synthesis in hepatocytes are also responsible for the induction of proteins during the acute-phase response that occurs during inflammation. Ferritin synthesis increases in the liver and spleen of rats during inflammation caused by turpentine injection. The increase in ferritin synthesis is due to the translational shift of ferritin mRNA from the free ribonucleoprotein pool to the polysomes. Similarly, in a cultured cell model of the acute-phase response, ferritin synthesis increased in human HepG2 hepatoma cells treated with IL-1β. This increase was due to the translational activation of ferritin mRNA, which occurred without changes in iron uptake. Our studies reported here differ from the latter cytokine study in that multiple cytokines and LPS were used to induce iNOS synthesis and subsequent NO production. Induction of iNOS in hepatocytes grown in culture requires the presence of multiple cytokines and/or LPS; cytokines used individually were less effective in inducing iNOS. Geller et al. have shown that hepatocyte iNOS is not synthesized as part of the hepatic acute-phase response induced by turpentine injection in rats, but is synthesized in hepatocytes isolated from rats injected with LPS to cause endotoxemia and chronic liver inflammation. These studies indicate that iNOS is differentially regulated from other acute-phase proteins during chronic hepatic inflammation, suggesting that in vivo specific combinations of cytokines may be required to induce the iNOS and NO production associated with endotoxemia.

Treatment of FTO2B cells with IFN-γ/TNF-α/LPS in the presence or absence of NMA resulted in an unexpected two-fold decrease in TfR mRNA levels, indicating that cytokines and LPS were modulating TfR mRNA levels independently of NO. Cytokines have been shown to modulate TfR expression by both transcriptional and posttranscriptional processes that may be distinct from IRP1 regulation. To avoid the pleiotropic effects of cytokines, we treated cells for 16 hours with SNAP to generate NO, and demonstrated that NO resulted in a twofold increase in TfR mRNA levels. This increase was coincident with an increase in IRP1 IRE binding activity, suggesting that IRP1 can regulate TfR mRNA expression in rat hepatoma cells. These data are consistent with recent studies indicating that TfR mRNA is increased by SNAP in K562 human erythroleukemia cells and in B6 fibroblasts stably overexpressing an iNOS cDNA. Therefore, it is likely that in vivo TfR regulation is complex, such that direct effects of NO may be counteracted by the pleiotropic effects of cytokines on either TfR transcription or TfR mRNA stabilization.

Although the role of NO production in hepatocytes is not fully understood, studies have shown that when NO
production is inhibited in rats injected with Corynebacterium parvum and LPS to induce chronic hepatic inflammation, liver damage was enhanced. These data suggest a protective role for NO during chronic hepatic inflammation. NO production in cultured hepatocytes is correlated with decreased total protein synthesis and aconitase activity and increased guanosine 3',5'-cyclic monophosphate synthesis and release; however, the implication of these changes in cellular metabolism during hepatic inflammation are unclear. The data presented here indicate that NO modulates cellular iron metabolism via activation of IRP1, suggesting a role for iron in the host immune response during liver inflammation.

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Differential regulation of IRP1 and IRP2 by nitric oxide in rat hepatoma cells

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