Nitrogen Monoxide Activates Iron Regulatory Protein 1 RNA-Binding Activity by Two Possible Mechanisms: Effect on the [4Fe-4S] Cluster and Iron Mobilization from Cells†

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ABSTRACT: The iron-regulatory protein 1 (IRP1) regulates the expression of several molecules involved in iron (Fe) metabolism by reversibly binding to iron-responsive elements (IREs) in the untranslated regions (UTR) of particular mRNA transcripts. Several studies have indicated that nitrogen monoxide (NO) may influence IRP1 RNA-binding activity by a direct effect on the [4Fe-4S] cluster of the protein. It has also been suggested that NO may act indirectly on IRP1 by affecting the intracellular Fe pools that regulate the function of this protein [Pantopoulous et al. (1996) *Mol. Cell. Biol. 16*, 3781−3788]. There is also the possibility that NO may S-nitrosate sulfhydryl groups that are crucial for mRNA binding and decrease IRP1 activity by this mechanism. We have examined the effect of a variety of NO donors [e.g., *S*-nitroso-*N*-acetylpenicillamine (SNAP), spermine-NONOate (SperNO), and *S*-nitrosoglutathione (GSNO)] on IRP1 RNA-binding activity in both LMTK$^-$ fibroblast lysates and whole cells. In cell lysates, the effects of NO at increasing RNA-binding activity were only observed when cells were made Fe-replete. Under these circumstances, IRP1 contains an [4Fe-4S] cluster that was susceptible to NO. In contrast, when lysates were prepared from cells treated with the Fe chelator desferrioxamine (DFO), NO had no effect on the RNA-binding activity of IRP1. The lack of effect of NO under these conditions was probably because this protein does not have an [4Fe-4S] cluster. In contrast to the NO generators above, sodium nitroprusside (SNP) decreased IRP1 RNA binding when cells were incubated with this compound. However, SNP had no effect on IRP1 RNA-binding activity in lysates, suggesting that the decrease after incubation of cells with SNP was not due to S-nitrosation of critical sulfhydryl groups. Apart from the direct effect of NO on IRP1 in Fe-replete cells, we have shown that NO generated by SNAP, SperNO, and GSNO could also mobilize Fe from cells. When NO generation was induced in RAW 264.7 macrophages, an increase in IRP1 RNA-binding activity occurred but there was only a small increase in Fe release. Our results suggest that NO could activate IRP1 RNA-binding by two possible mechanisms: (1) its direct effect on the [4Fe-4S] cluster and (2) mobilization of ^{59}Fe from cells resulting in Fe depletion, which then increases IRP1 RNA-binding activity.

The iron regulatory proteins (IRP1 and IRP2)[1] posttranscriptionally regulate the expression of several proteins that play key roles in cellular iron metabolism (*1*−*4*). IRP1 binds to conserved hairpin loop structures called iron-responsive elements (IREs) found in the 5′ and 3′ untranslated regions (UTRs) of several mRNAs including those encoding the ferritin H- and L-subunits (*5*, *6*) and transferrin receptor (TfR) (*7*, *8*). The binding of IRP1 to the single IRE in the 5′-UTR of ferritin mRNA inhibits translation, while the binding of IRP1 to the five IREs in the 3′-UTR of TfR mRNA confers stability against endonucleolytic degradation (*2*).

The binding of IRP1 and IRP2 to IRE motifs is regulated by intracellular Fe levels (*3*). A high intracellular Fe concentration promotes the assembly of a [4Fe-4S] cluster in IRP1, with concomitant loss of IRE-binding activity (*9*, *10*). Under these conditions, IRP1 has aconitase activity and is identical to cytoplasmic aconitase (c-acon) (*2*, *3*). In Fe-deficient cells, IRP1 loses its [4Fe-4S] cluster and gains the ability to bind the IRE (*2*, *3*). The details of how cells regulate the assembly and disassembly of the [4Fe-4S] cluster to modulate RNA-binding activity remains unclear (*2*). Several cysteine residues, particularly cysteine 437, also appear to be involved in the binding of IRP1 to the IREs (*11*−*13*). Indeed, cysteine 437 must remain in its free and

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[1] Abbreviations: c-acon, cytosolic aconitase; DFO, desferrioxamine; FAC, ferric ammonium citrate; GSH, glutathione; IFN-γ, interferon γ; IRE, iron-responsive element; IRP, iron-regulatory protein; GSNO, *S*-nitrosoglutathione; LPS, lipopolysaccharide; NAP, *N*-acetylpenicillamine; L-NMMA, N^G-monomethyl-L-arginine; NEM, *N*-ethylmaleimide; NO, nitrogen monoxide; NO$^\bullet$, nitric oxide; ONOO$^-$, peroxynitrite; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SperNO, spermine-NONOate; Tf, transferrin; UTR, untranslated region.

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reduced form to allow the protein to bind to the IRE (*12*, *13*). In contrast to IRP1, IRP2 does not contain a [4Fe-4S] cluster and is rapidly degraded in cells that are Fe-replete via proteasome-dependent degradation (*14*, *15*).

As well as decreasing intracellular Fe levels, nitrogen monoxide (NO) can also increase IRP1 RNA-binding activity, although the mechanism by which this occurs is yet to be determined (*16*−*19*). Both the [4Fe-4S] cluster and sulfhydryl groups are potential target sites of NO or its redox-related species (*2*, *3*, *16*−*22*). It has been suggested that NO increases IRP1 RNA binding by direct coordination to the [4Fe-4S] cluster and its subsequent destruction (*16*, *17*). There is also evidence that peroxynitrite ($ONOO^-$) formed from the reaction of $NO^\bullet$ with superoxide can interact with the [4Fe-4S] cluster, although to a more limited extent than $NO^\bullet$ (*22*, *23*).

It has also been suggested that NO may have an indirect effect on IRP1 RNA-binding activity by influencing the intracellular Fe pool (*18*, *24*). These studies demonstrated that the NO-mediated increase in the IRP1 RNA-binding activity occurred by similar kinetics as that found with DFO (*24*). While no direct evidence of an interaction between NO and intracellular Fe pools has been described, this hypothesis is supported by evidence demonstrating the high affinity of NO for Fe and other transition metal ions (*20*). Furthermore, Fe−nitrosyl complexes have been shown to exist in activated macrophages and their tumor cell targets (*25*−*28*), and NO can diffuse quickly into cells to exert its effects (*29*).

In the present study we have investigated the mechanism by which NO affects IRP1 RNA-binding activity. The effect of NO on lysates obtained from cells that had been either Fe-loaded with ferric ammonium citrate (FAC) or Fe-depleted with the Fe chelator desferrioxamine (DFO) was determined. Under these conditions, IRP1 is predominantly present either with its [4Fe-4S] cluster or without, respectively. We also examined the effect of NO on its ability to mobilize Fe from cells. The results demonstrate that the NO generators *S*-nitroso-*N*-acetylpenicillamine (SNAP), spermine-NONOate (SperNO), and *S*-nitrosoglutathione (GSNO) increase RNA-binding activity of IRP1 in cell lysates derived from Fe-loaded cells only, suggesting direct interaction with the [4Fe-4S] cluster. The NO generators described above were also shown to increase ^{59}Fe release from cells, having an efficacy that was greater than or similar to that found for DFO. These results suggest that NO could increase IRP1 RNA-binding activity by the direct interaction of NO with the [4Fe-4S] cluster and also by decreasing intracellular Fe levels.

EXPERIMENTAL PROCEDURES

Cell Treatments and Reagents. The NO generator *S*-nitroso-*N*-acetylpenicillamine (SNAP) was synthesized by established techniques (*30*) from the precursor compound *N*-acetylpenicillamine (NAP; Sigma Chemical Co., St. Louis, MO). Bovine catalase, bovine methemoglobin, bovine superoxide dismutase (SOD), FAC, glutathione (GSH), heparin, lipopolysaccharide (LPS; from *Salmonella minnesota* Re 595), L-glutamine, ferricyanide, ferrocyanide, mouse interferon γ (IFN-γ), N^G-monomethyl-L-arginine (L-NMMA), *S*-nitrosoglutathione (GSNO), sodium cyanide, sodium nitroprusside (SNP), and transferrin (Tf) were obtained from Sigma. Spermine-NONOate (SperNO) and 3-morpholinosydnonimine (SIN-1) were obtained from Molecular Probes. DFO was obtained from Ciba-Geigy Pharmaceutical Co. (Summit, NJ). All other chemicals were of analytical reagent quality. The NO generators and other compounds were dissolved in medium immediately prior to an experiment and used on that day. In studies lasting more than 4 h with an NO generator, the medium was replaced with fresh solutions to maintain NO levels (*24*).

Cell Culture. The mouse fibroblast cell line, $LMTK^-$, was obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, U.K.). The human SK-Mel-28 melanoma and human SK-N-MC neuroepithelioma cell lines were obtained from the American Type Culture Collection (Rockville, MD). The human HepG2 cell line was kindly provided by Dr. Greg Anderson (Queensland Institute of Medical Research, 300 Herston Rd., Brisbane, Queensland, Australia). The RAW 264.7 mouse macrophage cell line was obtained from Dr. David Cestor (Centre for Molecular and Cellular Biology, University of Queensland, St. Lucia, Brisbane, Queensland). The $LMTK^-$, SK-Mel-28, and SK-N-MC cell lines were grown in Eagle's modified minimum essential medium (MEM) containing 10% fetal calf serum (FCS; CSL Limited, Brisbane, Australia), 1% (v/v) nonessential amino acids (Gibco BRL, Sydney, Australia), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine, 100 μg/mL streptomycin (Gibco), 100 units/mL penicillin (Gibco), and 0.28 μg/mL fungizone (Squibb Pharmaceuticals, Montréal, Canada). The RAW 264.7 and HepG2 cell lines were maintained in RPMI-1640 with the same additions as described above for MEM, except that RAW 264.7 cells were grown using 10% Serum Supreme (Biowhittaker, Walkersville, MD) instead of FCS. Activation of RAW 264.7 cells was achieved by incubation for 20 h with either LPS (100 ng/mL) and/or IFN-γ (50 units/mL) (*29*). Cells were grown in an incubator (Forma Scientific) at 37 °C in a humidified atmosphere of 5% CO_2/95% air and subcultured as described previously (*31*). Cellular growth and viability were monitored by phase-contrast microscopy, cell adherence to the culture substratum, and trypan blue staining.

Nitrite Determination. The accumulation of nitrite in cell culture supernatants is commonly used as a relative measure of NO production (*16*, *24*, *32*, *33*). Nitrite was assayed with the Griess reagent, which gives a characteristic spectral peak at 550 nm (*34*).

Aconitase Activity Determination. Briefly, cytosolic aconitase was measured after treatment of cells with digitonin. This detergent selectively permeabilizes the plasma membrane and leaves inner mitochondrial membrane intact (*35*). Digitonin stock solution (5%) was prepared in DMSO and then added at a final concentration of 0.007% to 40×10^6 cells that were resuspended in 10 mL of 0.25 M sucrose buffered with 50 mM Hepes (pH 7.4). The tube was gently shaken and left for 5 min on ice prior to centrifugation at 1800*g*/8 min/4 °C. The supernatant was then taken and centrifuged at 230000*g*/20 min/4 °C to remove mitochondria. The cytosolic supernatant was then desalted and concentrated with an Amicon concentrator (M_r cutoff = 30 000) so that the protein concentration was 3−4 mg/mL. The cytoplasmic aconitase activity of extracts was determined spectrophotometrically by measuring the disappearance of *cis*-aconitate at 240 nm as described previously *(35)*. Units represent

nanomoles of substrate consumed per minute at 37 °C (ϵ_{240nm} = 3.6 mM^{-1} cm^{-1}) (*35*).

Protein Preparation and Labeling. Apotransferrin was labeled with ^{59}Fe (Dupont NEN) or ^{56}Fe to produce Fe_2-transferrin (Tf) by standard procedures (*31*). In all studies, fully saturated diferric Tf was used. Unbound ^{59}Fe or ^{56}Fe was removed by exhaustive dialysis against 0.15 M NaCl adjusted to pH 7.4 with 1.4% $NaHCO_3$ (*31*).

Oxyhemoglobin (oxyHb) was prepared from bovine methemoglobin by the method of Murphy and Noack (*36*). The concentration of oxyHb was determined spectrophotometrically with ϵ_{541nm} = 13.8 mM (*33*).

Preparation and Treatment of Cytosolic Extracts. Cellular extracts were prepared by incubating cells with medium alone (control) or medium containing FAC (100 μg/mL), DFO (100 μM), or the NO generators for 20 h at 37 °C. This incubation procedure with FAC and DFO has been shown to successfully deplete and load $LMTK^-$ cells with Fe, respectively, as indicated by IRP−IRE binding, TfR mRNA levels, and Tf-bound-Fe uptake (*37*). With this protocol, IRP1 was predominantly present with or without the [4Fe-4S] cluster, respectively, and this was confirmed by the gel-retardation assay (Figures 1−3), c-acon activity, and the susceptibility of IRP1 RNA binding to the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM; *12*). Considering the assay with NEM, a similar method using cells treated with DFO or hemin (an Fe donor) has been implemented to examine the effects of sulfhydryl-binding agents on IRP1 RNA-binding activity (*12*).

To examine the binding of the IRPs to the IRE, approximately 2−5 × 10^6 cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in ice-cold Munro extraction buffer (*6*). The samples were centrifuged at 10000*g* for 3 min at 4 °C to remove nuclei and the supernatant was stored at −70 °C. The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Ltd.). In some experiments, the cytosolic extracts (2 μg) were directly incubated with the NO-generating compounds for 60 min at 37 °C or NEM (1 mM) for 30 min at 4 °C in 25 mM Tris/40 mM KCl (pH 8). Lysates were derived from cells treated with either FAC or DFO (as described above).

RNA−Protein Gel-Retardation Assays. The gel-retardation assay was used to measure the interaction between IRP1 and IREs by established techniques (*6*, *8*). Briefly, aliquots containing 1 μg of protein were incubated with 0.1 ng (approximately 1 × 10^5 cpm) of ^{32}P-labeled pGL66 RNA transcript containing 118 bp of the ferritin H-chain (pGL66 was kindly provided by Dr. Elizabeth Leibold, University of Utah, UT). With this protocol, the level of RNA added was saturating. The riboprobe was transcribed in vitro from linearized plasmid templates with SP6 RNA polymerase in the presence of [α-^{32}P] UTP (Dupont NEN) with the Promega Riboprobe in vitro transcription kit (Promega, Madison, WI) and purified on a 6% urea/polyacrylamide gel. Unprotected probe was degraded by incubation with 1 unit of RNAse T1 for 10 min at room temperature. Heparin (5 mg/mL) was then added for another 10 min to exclude nonspecific binding. The IRE−protein complexes were analyzed in 6% nondenaturing polyacrylamide gels that were run for 3 h at 4 °C.

In parallel experiments, samples were treated with β-mercaptoethanol (β-ME) at a final concentration of 2%, prior to the addition of the IRE probe to allow full expression of IRE binding activity. The IRE-protein complexes were quantified by scanning densitometry using a laser densitometer and analyzed by Kodak Biomax I software (Kodak Ltd.).

Efflux Assay of Radioactive Labels from Cells. Standard techniques were used to examine the effect of NO and other agents on the efflux of ^{59}Fe, ^{65}Zn (Dupont NEN), ^3H-thymidine (Dupont NEN), or ^3H-leucine (Dupont NEN) from cells (*31*, *33*). Briefly, $LMTK^-$ cells were labeled with either ^{59}Fe-Tf (0.75 μM), ^{65}Zn-citrate ([Zn] = 2.5 μM; molar ratio of Zn:citrate 1:100), ^3H-thymidine (1 μCi/plate), or ^3H-leucine (1 μCi/plate) for various incubation periods at 37 °C in medium containing all supplements except FCS. After this incubation, the cell culture dishes were placed on a tray of ice, the medium was aspirated, and the cell monolayer was washed four times with ice-cold balanced salt solution (BSS). The cells were then reincubated for various times at 37 °C with serum-free medium in the presence or absence of the agents to be tested. All compounds examined for their effect on ^{59}Fe release were prepared just prior to an experiment and used immediately. After this incubation, the overlying medium was removed and placed into γ-counting tubes. The cells were removed from the Petri dishes after addition of 1 mL of BSS by using a plastic spatula to detach them. Radioactivity was measured in both the cell pellet and supernatant by using a γ-scintillation counter (LKB Wallace 1282 Compugamma, Finland).

RESULTS

Effect of NO Generators on IRP1 RNA-Binding Activity in Cell Lysates and Whole Cells. There are at least two potential sites on IRP1 that can react with redox-related species of NO (*20*), namely, the [4Fe-4S] cluster and/or sulfhydryl groups (*19*, *22*, *38*). Our initial experiments examined the effect of NO produced by a variety of NO generators on the RNA-binding activity of IRP1 derived from $LMTK^-$ cell lysates. Lysates were prepared from cells incubated for 20 h with either DFO (100 μM) or FAC (100 μg/mL). In DFO-treated cells, IRP1 is present predominantly as an active RNA-binding protein (see Figures 1−3) without an [4Fe-4S] cluster and little c-acon activity. In FAC-treated cells, IRP1 is present predominantly as c-acon that does not readily bind mRNA but has c-acon activity.

In all experiments, densitometry demonstrated that when compared to the relevant control, a marked increase in IRP1 RNA-binding activity was observed after treatment with DFO (3−8-fold) while a decrease was observed after preincubation with FAC (0.5−2-fold). This was further confirmed by incubation of cell lysates with *N*-ethylmaleimide (NEM; 1 mM) that binds to free sulfhydryl groups and inhibits binding of IRP1 to the IRE (*12*). In lysates derived from FAC-treated cells, NEM had relatively little effect on the binding of IRP1 to the IRE, reducing it by 25% compared to untreated samples. In contrast, in lysates derived from DFO-treated cells, NEM inhibited IRP1 binding to the IRE, reducing it by 98% (data not shown). Furthermore, after a 20 h incubation with DFO or FAC, the c-acon activity was 5 ± 1.1 and 29 ± 1.2 units/mg (3 determinations), respectively. Hence, these latter results agree with our NEM data plus gel-retardation assays and indicate that, in DFO- and FAC-

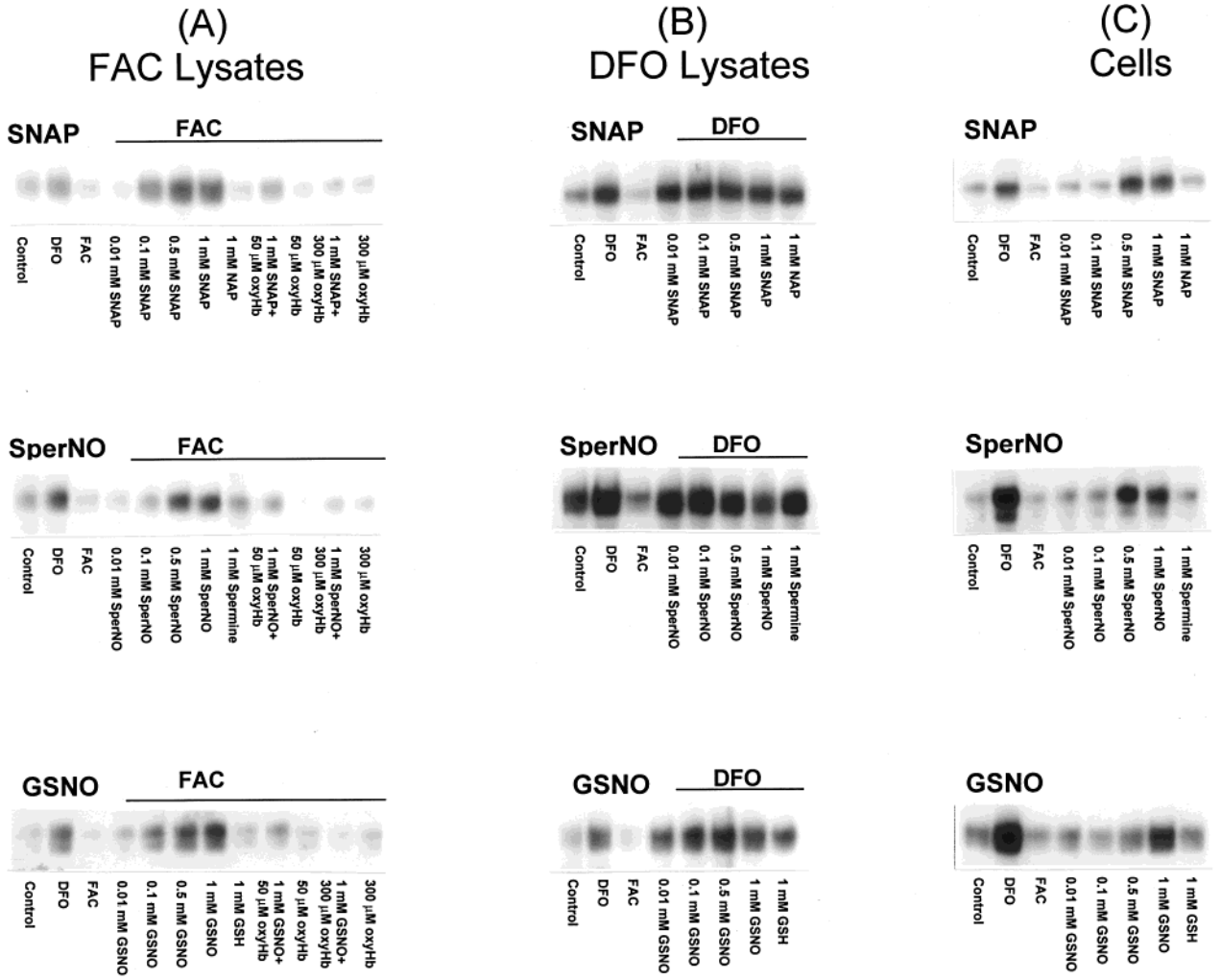

FIGURE 1: Effect of the NO-generating agents *S*-nitroso-*N*-acetylpenicillamine (SNAP), spermine-NONOate (SperNO), and *S*-nitrosoglutathione (GSNO) on IRP1 RNA-binding activity. (A, B) The NO generators were incubated for 60 min at 37 °C with lysates derived from LMTK$^-$ cells treated with either (A) ferric ammonium citrate (FAC; 100 μg/mL) or (B) desferrioxamine (DFO; 100 μM) for 20 h at 37 °C. (C) LMTK$^-$ cells were incubated with the NO generators for 20 h at 37 °C. These results are typical of 3−4 independent experiments performed.

treated cells, IRP1 is present predominantly in its RNA-binding and c-acon forms, respectively. Considering these data collectively, examination of the effect of NO on the RNA binding of IRP1 from DFO- and FAC-treated lysates will provide clues on whether the sulfhydryl groups or [4Fe-4S] cluster are targeted by NO congeners. These results have been compared to when NO generators were incubated for 20 h with LMTK$^-$ cells in culture.

Lysates derived from LMTK$^-$ cells incubated with FAC were treated with increasing concentrations of three NO-generating agents, SNAP, SperNO, or GSNO (Figure 1A). When compared to lysates derived from cells treated with FAC alone, the three NO generators markedly increased IRP1 RNA-binding activity (Figure 1A). In contrast, these NO donors had no appreciable effect on the RNA binding of IRP1 in lysates derived from DFO-treated cells when compared to DFO alone (Figure 1B). Addition of β-ME to the cell lysates demonstrated that these agents had no effect on the total amount of IRP1 and confirmed the equal loading of protein in all lanes (data not shown). The results for lysates from FAC-treated cells (Figure 1A) suggest that NO derived from these agents may have reacted with the [4Fe-4S] cluster of IRP1 to increase RNA binding. In addition, since NO had no marked effect on RNA-binding activity in DFO-treated cells (Figure 1B), this suggests that it did not S-nitrosate the critical sulfhydryl groups required for mRNA binding or affect RNA-binding activity by some allosteric mechanism. The precursor compounds for SNAP, SperNO, and GSNO that do not have the NO group, namely, *N*-acetylpencillamine (NAP), spermine, and glutathione (GSH) respectively, had no effect on IRP1 RNA-binding activity (Figure 1A,B). In addition, oxyHb at either 50 μM or 300 μM added to all three NO generators (1 mM) prevented the increase in IRP1 RNA-binding activity in FAC-treated lysates, whereas oxyHb by itself had no effect compared to FAC alone (Figure 1A). Since oxyHb is an efficient NO scavenger (*3*), these results suggest that NO released from these donors was responsible for the increase in IRP1 RNA-binding activity (Figure 1A). The increase in RNA-binding activity of IRP1 observed in lysates after treatment with SNAP, SperNO, or GSNO was also seen when LMTK$^-$ cells were incubated for 20 h with these agents (Figure 1C). A faint IRP2 band is observed in some samples but not others (Figures 1−3), and the low level of expression of this protein in LMTK$^-$ cells impeded detailed investigation of its response to NO.

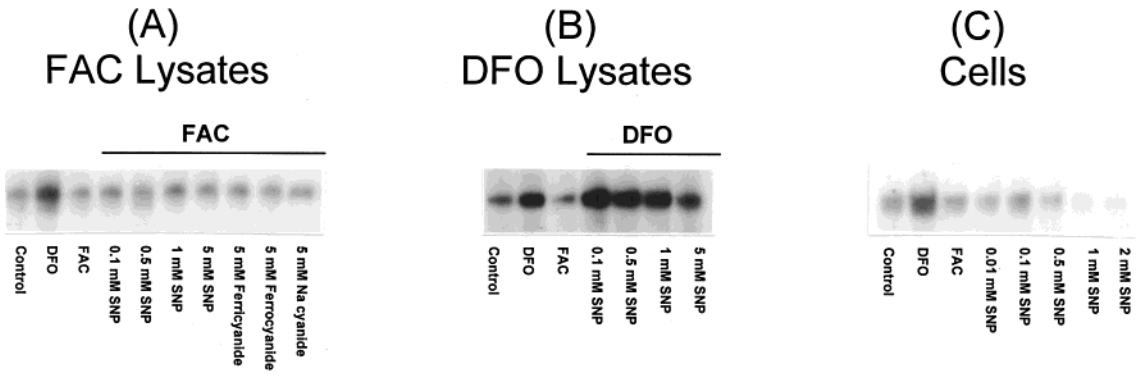

FIGURE 2: Effect of sodium nitroprusside (SNP) concentration on IRP1 RNA-binding activity. (A, B) SNP was incubated for 60 min at 37 °C with lysates derived from LMTK$^-$ cells treated with either (A) ferric ammonium citrate (FAC; 100 μg/mL) or (B) desferrioxamine (DFO; 100 μM) for 20 h at 37 °C. (C) LMTK$^-$ cells were incubated with SNP for 20 h at 37 °C. These results are typical of 3−4 independent experiments performed.

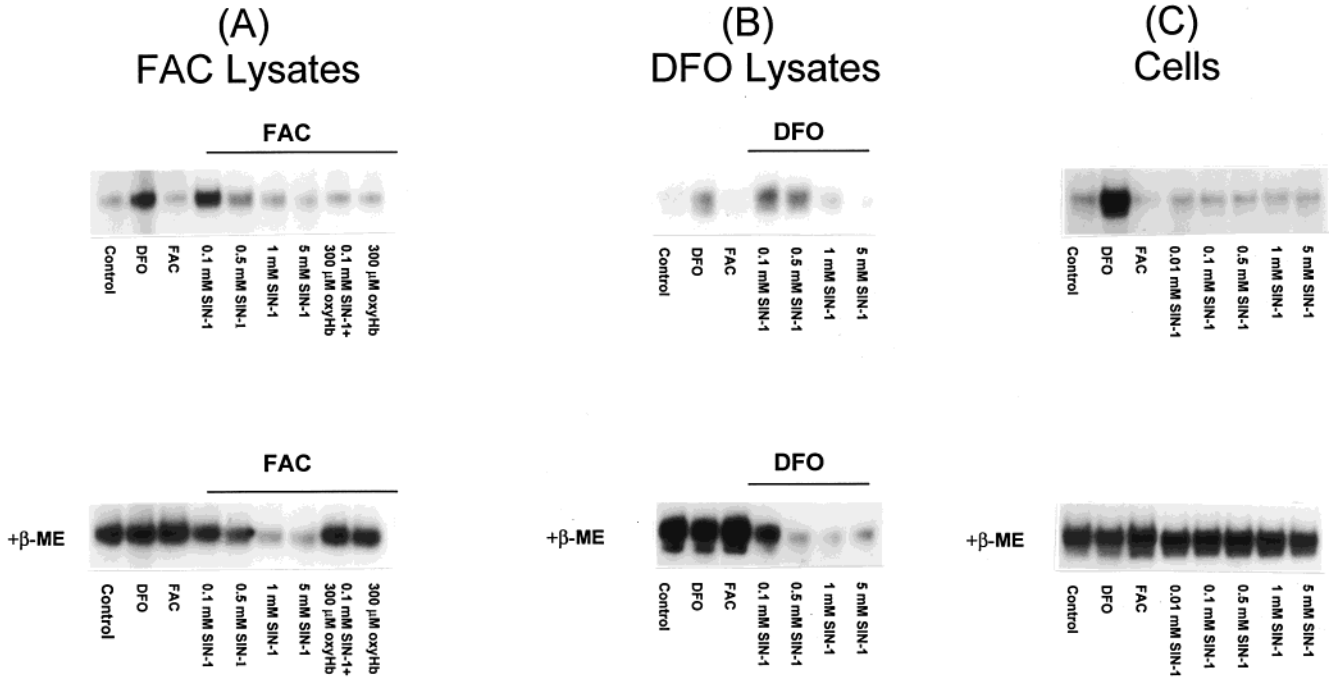

FIGURE 3: Effect of 3-morpholinosydnonimine (SIN-1) concentration on IRP1 RNA-binding activity. (A, B) SIN-1 was incubated for 60 min at 37 °C with lysates derived from LMTK$^-$ cells treated with either (A) ferric ammonium citrate (FAC; 100 μg/mL) or (B) desferrioxamine (DFO; 100 μM) for 20 h at 37 °C. (C) LMTK$^-$ cells were incubated with SIN-1 for 20 h at 37 °C. In panels A and B, lysates were incubated with or without 2% β-ME. These results are typical of 3−4 independent experiments performed.

The effect of SNAP, SperNO, or GSNO on IRP1 RNA-binding activity was also compared to that of SNP (Figure 2). In a previous study we showed that when SNP (1 mM) was incubated for 18 h with K562 cells it decreased IRP1 RNA-binding activity (*19*). Similar results with SNP have also been obtained by other investigators using HepG2 hepatoma cells (*39*). From our data we suggested that the effect of SNP may be due to its ability to S-nitrosate the critical sulfhydryl groups required for mRNA binding (*19*). To test this hypothesis, SNP at concentrations up to 5 mM were incubated with lysates derived from LMTK$^-$ cells exposed to either FAC (Figure 2A) or DFO (Figure 2B). However, no effect of SNP was observed. To ensure that the products of SNP decomposition (CN^-, ferricyanide, ferrocyanide) did not affect IRP1 RNA-binding activity, lysates were also incubated with these compounds (*19, 20, 40*). However, no effect was observed in either lysates from FAC-treated cells (Figure 2A) or DFO-treated cells (data not shown). In contrast to the results obtained with lysates, when LMTK$^-$ cells were incubated for 20 h with increasing concentrations of SNP, this compound decreased IRP1 RNA-binding activity compared to the control (Figure 2C). Addition of β-ME to cell lysates demonstrated that SNP did not affect the total amount of protein (data not shown).

We also examined the effect on IRP1 RNA-binding activity of SIN-1 (Figure 3), a compound that generates both $NO\cdot$ and superoxide, which react together to form $ONOO^-$ (*21, 22, 41*). Previous studies by Bouton et al. (*21*) using RAW 264.7 macrophages showed that the addition of SIN-1 to lysates increased IRP1 RNA-binding activity but only at high concentrations (1−10 mM) in the presence of SOD. In our experiments, in the absence of SOD, increasing concentrations of SIN-1 added to LMTK$^-$ cell lysates resulted in the opposite effect (Figure 3A). At a SIN-1 concentration of 0.1 mM, an increase in IRP1 RNA-binding activity was observed in both FAC- and DFO-treated cells (Figure 3A,B). As the concentration of SIN-1 was increased from 0.5 mM up to 5 mM, the RNA-binding activity markedly decreased

(Figure 3A,B). The concentration-dependent effect of SIN-1 on IRP1 RNA-binding activity in LMTK$^-$ cells may relate to the endogenous level of thioredoxin (*38*), although other factors may be involved.

Addition of oxyHb (300 μM) prevented the effect of SIN-1 (0.1 mM) at increasing RNA-binding activity, suggesting that NO$^\bullet$ or ONOO$^-$ may be responsible for the increase observed (Figure 3A). Both of these latter redox-related species of NO have been shown to interact with the [4Fe-4S] cluster of c-acon, although NO$^\cdot$ appeared to be more efficient (*23*). Treatment of the FAC- and DFO-treated lysates with β-ME resulted in a decrease in total IRP1 RNA-binding activity with increasing concentrations of SIN-1 (Figure 3A,B). These data may indicate possible damage to IRP1 by the oxidizing effects of ONOO$^-$. Alternatively, SIN-1 and/or its byproducts may prevent the effect of β-ME at increasing RNA-binding activity by oxidation of critical thiol residues.

In contrast to the results obtained with lysates, SIN-1 incubated for 20 h with LMTK$^-$ cells at concentrations from 0.01 to 5 mM had little effect on IRP1 RNA-binding activity compared to the controls (Figure 3C). The addition of β-ME showed there was no appreciable change in the total amount of protein (Figure 3C). The inability of SIN-1 to increase RNA-binding activity when incubated with cells may be due to the high reactivity of ONOO$^-$ with plasma membrane lipids (*42*), effectively quenching the influence of this molecule on IRP1.

Effect of NO Generators on Iron Release from Cells. The results above for SNAP, SperNO, and GSNO in LMTK$^-$ cell lysates derived from FAC-treated cells (Figure 1A) indicate that NO may interact with the [4Fe-4S] cluster of c-acon. However, it has also been suggested that NO acts indirectly by interfering with intracellular Fe metabolism (*18*, *24*), since the kinetics of NO activation of IRP1 RNA-binding activity were very similar to that found with DFO (*24*). Considering this, we decided to examine the ability of SNAP, GSNO, SperNO, SNP, and SIN-1 to release ^{59}Fe from LMTK$^-$ cells prelabeled for 3 h with ^{59}Fe-Tf (0.75 μM; Figure 4A). Interestingly, a 3 h reincubation with SNAP, GSNO, or SperNO at 0.5 mM markedly increased ^{59}Fe release from 14% in control cells to 36%, 44%, and 46%, respectively (Figure 4A). Increasing the concentrations of these NO generators up to 1 mM had relatively little effect on ^{59}Fe mobilization. The control compounds without the NO group, namely, NAP, spermine, and GSH, had no effect on ^{59}Fe mobilization (Figure 4A). In addition, the NO scavenger oxyHb also prevented the effect of SNAP, GSNO, and SperNO on ^{59}Fe efflux from cells (data not shown). These controls strongly suggest that NO was the agent responsible for inducing ^{59}Fe release. In contrast, neither SNP nor SIN-1 increased ^{59}Fe release compared to the relevant control (Figure 4A). The production of NO by these agents was estimated by the nitrite assay (Figure 4B), and it was of interest to note that nitrite production was not directly correlated with ^{59}Fe efflux. For example, SIN-1 produced more nitrite than either SNAP or GSNO (Figure 4B) but had no appreciable effect on ^{59}Fe mobilization (Figure 4A). This effect may be related to the fact that SIN-1 generates ONOO$^-$ that rapidly decomposes to produce nitrite (*43*, *44*). Thus, in the case of SIN-1, the formation of nitrite may not be a reliable measure of biologically active NO$^\bullet$ levels (*33*).

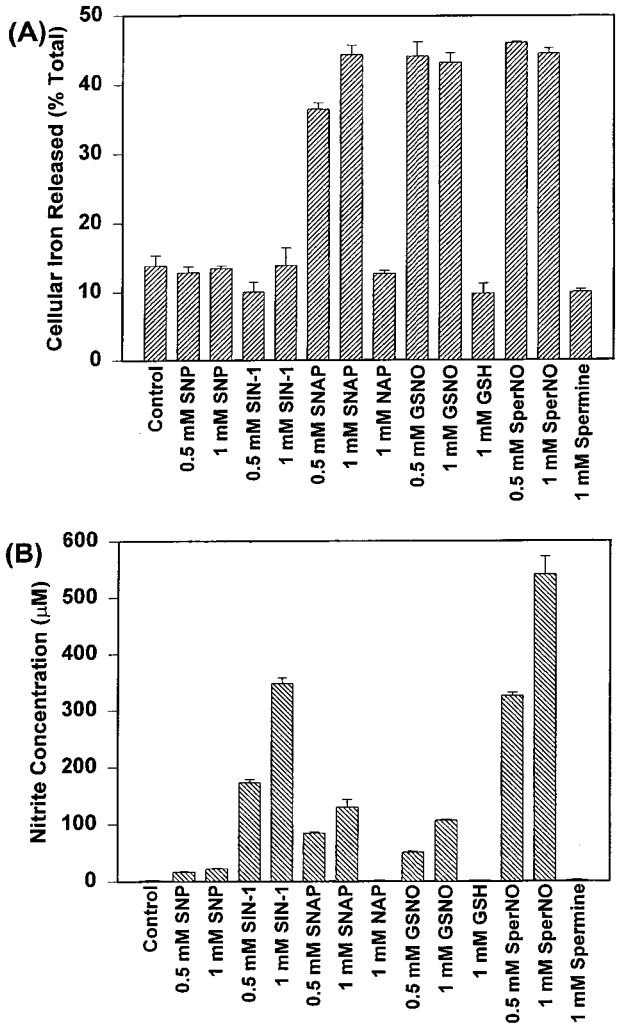

FIGURE 4: Effect of sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), *S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione (GSNO), and spermine-NONOate (SperNO) on (A) ^{59}Fe mobilization from LMTK$^-$ fibroblasts and (B) nitrite concentrations in the overlying medium. Cells were labeled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37 °C, washed, and then reincubated for 3 h at 37 °C in the presence or absence of the NO generators. The overlying medium was then removed for estimation of ^{59}Fe release and nitrite concentration. The results are mean ± SD of 3 replicates in a typical experiment of 2 experiments performed.

Further studies examined if the increase in ^{59}Fe release was due to a nonspecific increase in cellular permeability resulting from NO damaging the cell membrane. To determine this, cells were labeled for 3 h at 37 °C with either ^{3}H-leucine (1 μCi/plate), ^{3}H-thymidine (1 μCi/plate), ^{59}Fe-Tf (0.75 μM), or ^{65}Zn-citrate (2.5 μM), and the effect of SNAP and GSNO at 0.5 mM on the efflux of these labels was examined during a 3 h reincubation at 37 °C. Neither SNAP nor GSNO had any effect on ^{3}H-leucine or ^{3}H-thymidine release, but they markedly increased ^{59}Fe mobilization from 4% in control cells to 29% and 26%, respectively (data not shown). These results suggest that the increase in ^{59}Fe release was not a nonspecific effect due to NO-mediated cell death or an increase in membrane permeability. Furthermore, after incubation with SNAP or GSNO, there was no change in the percentage of trypan blue stained cells (viability >97%). Both GSNO and SNAP only slightly increased ^{65}Zn release from 20% ± 6% (control) to 30% ± 2% and 28% ± 2% (3 determinations), respectively (data

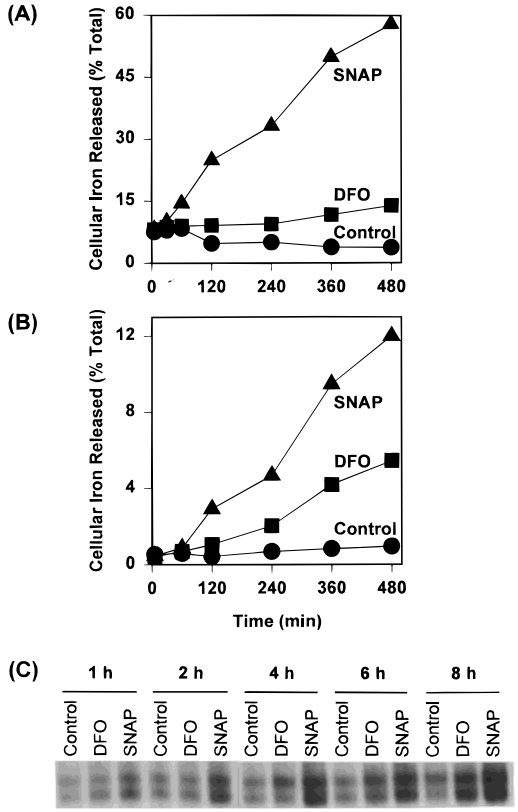

FIGURE 5: Effect of incubation time with desferrioxamine (DFO) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) on (A, B) ^{59}Fe release from LMTK$^-$ cells and (C) IRP1 RNA-binding activity. Cells were incubated for either (A) 1 h or (B) 20 h at 37 °C with ^{59}Fe-transferrin (0.75 μM), washed, and then reincubated for 5 min−8 h at 37 °C with DFO (0.5 mM) or SNAP (0.5 mM). (C) The LMTK$^-$ cells were incubated for 1−8 h with DFO (0.5 mM) or SNAP (0.5 mM) at 37 °C and the IRP1 RNA-binding activity was assessed. The results in panels (A) and (B) are means of duplicate determinations in a typical experiment of 2 experiments performed. The results in panel C are representative of 10 independent experiments.

not shown). The fact that NO slightly increased ^{59}Zn release was not totally unexpected, as NO can bind to Zn(II) and other transition metals (*20*, *45*).

To investigate if the NO-mediated increase in ^{59}Fe release from cells was not just a property unique to LMTK$^-$ fibroblasts, we examined a range of cell types regularly used in our laboratory, including HepG2 hepatoma cells, SK-Mel-28 melanoma cells, and SK-N-MC neuroepithelioma cells. In these experiments, cells were incubated with ^{59}Fe-Tf (0.75 μM) for 5.5 h at 37 °C and the ability of 1 mM DFO, SNAP, NAP, or SNP to increase ^{59}Fe release was examined over a 2 h reincubation at 37 °C. Using all cell types, SNAP increased ^{59}Fe release over that observed for control cells and was equally or more effective than DFO (data not shown). In contrast, NAP and SNP were not effective.

The ability of NO generators to mobilize ^{59}Fe from cells was further explored by investigating the kinetics of ^{59}Fe release from LMTK$^-$ cells mediated by SNAP compared to DFO. In these experiments, cells were labeled for either 1 h (Figure 5A) or 20 h (Figure 5B) at 37 °C with ^{59}Fe-Tf (0.75 μM), washed, and then reincubated for up to 8 h at 37 °C with SNAP (0.5 mM) or DFO (0.5 mM). Two labeling times with ^{59}Fe-Tf were used to examine the effects of NO on cellular Fe pools, as we have previously shown that a greater proportion of Fe can be released by some chelators after short rather than long labeling periods (*45*). This latter effect may be because after long incubation periods a greater proportion of Fe is found in ferritin (*45*, *46*), which is generally more difficult to access (*45*). SNAP was more effective than DFO at increasing ^{59}Fe mobilization after both incubation times, being far more effective after a 1 h label (Figure 5A) compared to a 20 h label (Figure 5B). Considering these results, we then examined the ability of these concentrations of DFO and SNAP to increase IRP1 RNA-binding activity in LMTK$^-$ cells under the same conditions (Figure 5C). Interestingly, SNAP increased IRP1 RNA-binding activity after only a 1 h incubation, while a 4 h incubation with DFO was required before an increase in RNA-binding activity occurred (Figure 5C). In contrast to SNAP, its control NAP did not have any effect on IRP1 RNA-binding activity (data not shown). The addition of β-ME to the cell lysates demonstrated that neither DFO nor SNAP affected the total amount of protein (data not shown). These results demonstrating the ability of SNAP to increase IRP1 RNA-binding activity more rapidly than DFO are in contrast to those obtained by Pantopoulos et al. (*24*), where DFO and SNAP displayed similar kinetics. The reason for the difference in results may be because these authors compared DFO and SNAP at lower concentrations (0.1 mM) than those used in the present study (viz. 0.5 mM).

Effect of NO Generated Intracellularly on Fe Mobilization and IRP1 RNA-Binding Activity. Following on from the previous experiments, the effect of intracellular NO generation on ^{59}Fe efflux was examined in RAW 264.7 macrophages. Incubation of these cells with LPS or LPS/IFN-γ results in a marked increase in iNOS synthesis that produces significant amounts of NO (*29*). In our hands, incubation of RAW 264.7 cells for 20 h with LPS alone (100 ng/mL) slightly increased nitrite production, while incubation with both LPS (100 ng/mL) and IFN-γ (50 units/mL) resulted in high levels of nitrite (Figure 6, inset). To determine whether intracellular NO generation increased ^{59}Fe release, cells treated with LPS or LPS/IFN-γ for 20 h were incubated for 3 h with ^{59}Fe-Tf (0.75 μM), washed four times, and then reincubated for 3 h at 37 °C with medium. To ensure that ^{59}Fe release was not due to a general increase in membrane permeability, cells were also incubated in the same way with ^{3}H-leucine (1 μCi), ^{3}H-thymidine (1 μCi), or ^{65}Zn-citrate (2.5 μM) (Figure 6). The mobilization of ^{59}Fe increased from 2% in control cells to 4% and 11% in LPS and LPS/IFN-γ-treated cells, respectively (Figure 6). This increase in ^{59}Fe release was not as marked as that observed when cells were exposed to the NO generators SNAP, GSNO, or SperNO (see Figure 4A). In addition to an increase in ^{59}Fe efflux, there was also an increase in the release of ^{3}H-leucine, ^{3}H-thymidine, and ^{65}Zn in RAW 264.7 cells exposed to LPS or especially LPS/IFN-γ compared to the control (Figure 6). The marked increase in ^{3}H-thymidine release could be related to the NO-mediated inhibition of ribonucleotide reductase (*47*). Inhibition of this enzyme may prevent the incorporation of ^{3}H-thymidine into DNA, allowing it to diffuse from the cell during reincubation. Interestingly, while there was an increase in the release of ^{3}H-leucine and ^{65}Zn from LPS- or LPS/IFN-γ-treated cells, the increase was less than that observed for ^{59}Fe (Figure 6). However, similar experiments using the iNOS inhibitor L-NMMA (1 mM) had no effect on ^{59}Fe release from RAW 264.7 cells, suggesting that the

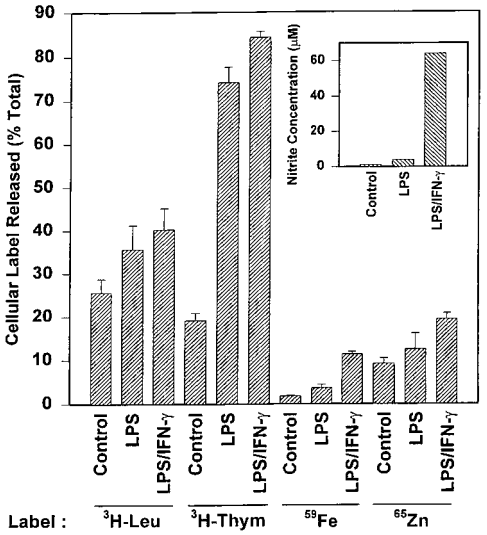

FIGURE 6: Effect of incubating the RAW 264.7 macrophage cell line with lipopolysaccharide (LPS) or LPS and interferon γ (IFN-γ) on the efflux of ^{3}H-leucine, ^{3}H-thymidine, ^{59}Fe, and ^{65}Zn. The macrophages were incubated for 20 h at 37 °C with LPS (100 ng/mL) or LPS (100 ng/mL) and IFN-γ (50 units/mL), washed, and then labeled for 3 h at 37 °C with either ^{3}H-leucine (1 μCi/plate), ^{3}H-thymidine (1 μCi/plate), ^{59}Fe-transferrin (0.75 μM), or ^{65}Zn-citrate ([Zn] = 2.5 μM; Zn:citrate ratio 1:100). After this, the cells were washed four times and then reincubated with medium for 3 h at 37 °C. The inset shows the concentration of nitrite in the overlying medium after the 20 h incubation of RAW cells with LPS or LPS/IFN-γ. The results are mean ± SD of 3 replicates in a typical experiment of 2 separate experiments performed.

small increase in mobilization may be a nonspecific effect due to increased membrane permeability after activation (data not shown).

While the combination of LPS/IFN-γ had little effect on ^{59}Fe mobilization from the RAW 264.7 cells (Figure 6), there was an increase in the RNA-binding activity of IRP1 (upper band) but no significant effect on the RNA-binding activity of IRP2 (lower band) (Figure 7A). Incubation of RAW 264.7 cells with IFN-γ alone slightly increased IRP1 RNA-binding activity, but to a lesser extent than that found with both IFN-γ and LPS (Figure 7A). Addition of β-ME to the lysates demonstrated that none of the agents had any appreciable effect on total protein. The increase in IRP1 RNA-binding activity in RAW 264.7 cells could be partially prevented by incubation with IFN-γ alone or LPS/IFN-γ in the presence of the iNOS inhibitor L-NMMA (Figure 7A). The inhibition of iNOS by L-NMMA was suggested by the decreased nitrite concentration in the overlying medium (Figure 7B).

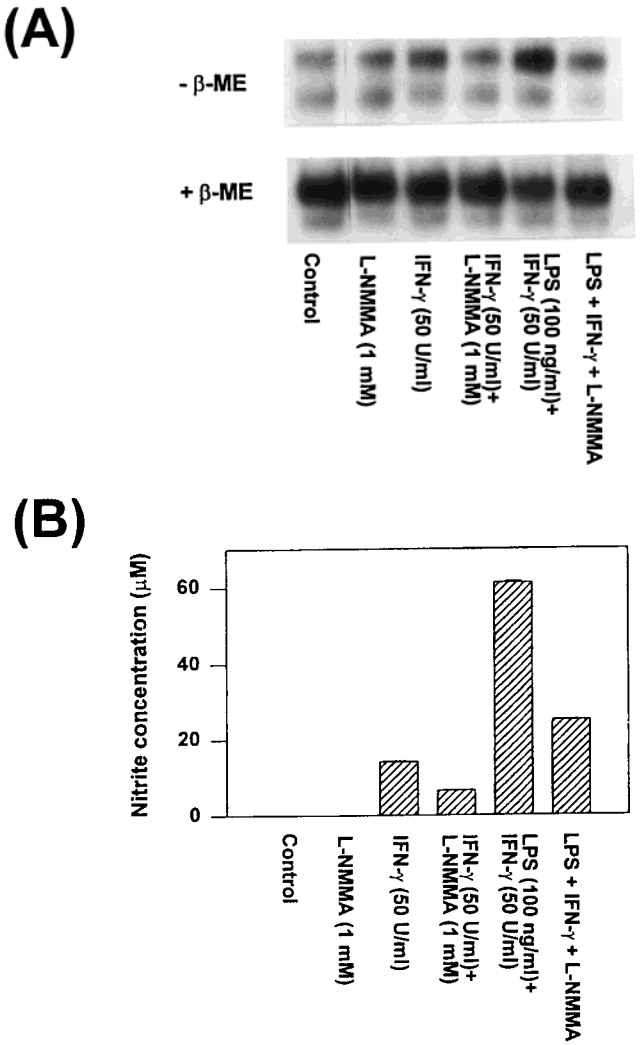

FIGURE 7: Effect of interferon γ (IFN-γ) alone or lipopolysaccharide (LPS) and IFN-γ on (A) IRP1 RNA-binding activity and (B) nitrite production of RAW 264.7 macrophages. The macrophages were incubated for 20 h at 37 °C with IFN-γ alone (50 units/mL) or IFN-γ (50 units/mL) and LPS (100 ng/mL) in the presence and absence of the inducible nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 1 mM). In panel A, cell lysates were incubated with or without 2% β-mercaptoethanol (β-ME) and the IRP1 RNA-binding activity was assessed by the gel-retardation assay. Panel B shows the nitrite concentration in the overlying medium after a 20 h incubation with the agents (mean ± SD of 3 replicates). The results are typical from 2 independent experiments.

DISCUSSION

Considering the present active interest in the role of NO in the modulation of c-acon function (*16*−*19*, *21*−*23*, *29*, *39*), we have examined the mechanisms by which NO can increase the RNA-binding activity of this protein. Previous studies have suggested that NO can act directly on the [4Fe-4S] cluster to destabilize it and increase IRP1 RNA-binding activity (*16*, *17*, *23*). Apart from this mechanism, there is also kinetic evidence to suggest that NO produced by SNAP acts to increase RNA-binding activity by an indirect effect on Fe pools that regulate this molecule (*18*, *24*). However, one of these latter studies (*24*) did not directly demonstrate any effect of NO on intracellular Fe pools (*2*, *3*). In the present investigation, we have examined whether both or either mechanism may be responsible for the increase in IRP1 RNA-binding activity that is observed after exposure of cells to NO-generating agents or when cellular NO generation occurs.

Initially, we used lysates derived from LMTK$^-$ fibroblasts incubated with FAC or DFO for 20 h. Under these conditions, IRP1 is present predominantly in its holoform with a [4Fe-4S] cluster that does not bind mRNA (FAC-treated) or in its active RNA-binding state (DFO-treated). By use of these lysates, the two potential target sites of NO congeners could be examined, namely, the [4Fe-4S] cluster and/or sulfhydryl groups (*11*−*13*, *19*, *20*, *22*). A similar protocol using lysates derived from cells incubated with DFO and hemin has been used to successfully study the interaction of NEM and other sulfhydryl-binding agents with IRP1 in its holo and apo states (*12*). While previous studies have

assessed the effect of NO generators on IRP1 RNA-binding activity (*16*−*19*, *21*, *22*), none have simultaneously compared the results between lysates prepared from FAC- and DFO-treated cells and whole cells incubated with these agents.

Our experiments demonstrated that three NO generators, namely, SNAP, SperNO, and GSNO, act to increase IRP1 RNA-binding activity only in lysates derived from cells pretreated with FAC but not DFO (Figure 1A). These studies suggest that NO produced by these donors acts on c-acon that contains a [4Fe-4S] cluster but does not inhibit mRNA-binding by S-nitrosation of critical cysteine residues that are required for mRNA binding (*11*−*13*). We have also clearly shown that these three NO generators are effective at increasing ^{59}Fe mobilization from cells (Figure 4A) at concentrations shown to markedly enhance IRP1 RNA-binding activity (Figure 1C). Collectively, these results suggest that the increase in RNA-binding activity of IRP1 observed after incubation of cells with NO generators (Figure 1C) may be due to both a direct effect on the [4Fe-4S] cluster and an indirect effect on the intracellular pools that supply Fe for cluster formation. Indeed, on a concentration basis, SNAP was found to be more effective than DFO in terms of its ability to increase Fe mobilization from cells (Figure 5A,B). In contrast to the work of Pantopoulos et al. (*24*), we have found that NO produced from SNAP increased IRP1 RNA-binding activity more rapidly than DFO (Figure 5C). There may be several reasons for this difference in activity, including that NO may diffuse more quickly than DFO across the membrane and deprive cells of Fe at a faster rate (see Figure 5A,B) and/or that NO not only may function to deplete cellular Fe pools but also may act directly to destabilize the [4Fe-4S] cluster to increase RNA-binding activity (Figure 1A). The combination of these latter two effects by NO may result in more rapid destruction of the cluster than that seen with DFO, which can only chelate Fe (*2*, *3*). However, further studies are required to clearly establish which mechanism predominates. It should be noted that while both NO and DFO can deplete cells of Fe that can induce IRP1 RNA-binding activity, it is known that this alone is probably not sufficient to induce the loss of the [4Fe-4S] cluster from IRP1. The depletion of intracellular Fe is an initiating factor that results in some agent or mechanism eliminating the [4Fe-4S] cluster.

While three NO generators (SNAP, GSNO, and SperNO) were effective at increasing Fe mobilization, SNP and SIN-1 were not (Figure 4A). The ability of the NO generators to increase Fe release may be related to the different forms of NO produced by these agents (*19*, *20*, *40*, *41*). SIN-1 produces both NO$^{\bullet}$ and superoxide that react together to form $ONOO^-$ (*21*, *22*, *41*). At low SIN-1 concentrations of 0.1 mM, marked activation of IRP1 RNA-binding activity was observed, and this decreased as the SIN-1 concentration was increased up to 5 mM in lysates from both FAC- and DFO-treated cells (Figure 3A,B). In contrast, SIN-1 had little effect on IRP1 RNA-binding activity when incubated with whole cells (Figure 3C). These results were in contrast to what was observed with agents that produce NO (viz SNAP, SperNO, and GSNO; *24*, *41*) that increased IRP1 RNA-binding activity in cell lysates and also in whole cells (Figure 1). These data may be explained by the ability of NO to diffuse through cell membranes while $ONOO^-$ may be effectively quenched due to its high reactivity (*42*). Indeed, the fact that SIN-1 did not mobilize Fe from cells while the three NO generators could (Figure 4A) substantiates this proposal. The results for LMTK$^-$ cell lysates with SIN-1 appear different to than reported previously by others, where SIN-1 increased RNA-binding activity in lysates from RAW 264.7 macrophages but only in the presence of SOD (*21*). These authors used high concentrations of SIN-1 (1 mM), which in our hands had little influence on IRP1 RNA binding when compared to the control (Figure 3C), and this may explain the apparent contradiction.

When cells were exposed to SNP there was a decrease in IRP1 RNA-binding activity as a function of concentration (Figure 2C). In contrast, SNP added to lysates had little effect (Figure 2A). At present, it remains unclear how SNP decreases RNA-binding activity in whole cells. The experiments using lysates (Figure 2A,B) suggest that it was not due to a direct effect of SNP S-nitrosating critical sulfhydryl groups of IRP1. It is known that SNP added to cellular systems releases a number of species, including Fe (*20*, *40*). As discussed in our previous study (*19*), the decrease in IRP1 RNA binding observed in SNP-treated cells could be due to it donating Fe. However, the addition of the impermeable chelator EDTA to SNP did not have any effect on the ability of this NO generator to prevent the decrease in IRP1 RNA-binding activity (*19*). These studies suggest that Fe donation may not be the mechanism involved. Clearly, further studies on the mechanism of action of SNP on IRP1 are necessary.

In contrast to the effect of NO-generating agents, intracellular NO generation in induced RAW 264.7 macrophages increased IRP1 RNA-binding activity (Figure 7A) in the absence of significant changes in Fe mobilization (Figure 6). Moreover, the increase in Fe mobilization in RAW 264.7 macrophages appeared to be independent of NO and due to a general increase in membrane permeability (Figure 6). These results suggest that, under the experimental conditions used, intracellular NO generation was not causing Fe depletion due to increased Fe efflux. Since SNAP, SperNO, and GSNO produced more nitrite per unit time after a 3 h incubation compared to RAW 264.7 macrophages after a 20 h incubation [cf. Figures 4B and 6 (inset)], it can be suggested that this is the reason for the lack of Fe efflux. Hence, under these experimental conditions in RAW 264.7 macrophages, NO generated intracellularly may be acting primarily to destabilize the [4Fe-4S] cluster.

In the present study, by using pharmacological NO generators and their relevant control compounds, we have been able to directly demonstrate the effect of NO on Fe mobilization. At present, the form of Fe that is mobilized from cells by NO remains unknown. Since NO appears to act somewhat like an Fe chelator (Figures 4 and 5), and considering its high affinity for Fe (*20*), it is likely that an NO−Fe complex was being released from the cells. It is well-known that NO has a rich coordination chemistry and can form complexes with a variety of transition metal ions (*20*). Previous studies by Vanin (*48*) indicated that NO in cells forms a complex with Fe and thiol ligands such as cysteine. It was further suggested that the Fe may be derived from a non-heme-containing Fe pool, rather than mitochondrial Fe-containing proteins (*49*). NO may remove Fe from a number of sites in cells; in fact, it can remove Fe from ferritin in vitro (*50*), and Lee et al. (*51*) have found EPR-active Fe−nitrosyl complexes in this molecule. Other Fe-containing

targets of NO could include ribonucleotide reductase, mitochondrial aconitase, Fe−S proteins of the mitochondrial respiratory system, and ferrochelatase, all of which are Fe-containing proteins affected by NO (*47*, *52*−*54*).

Additional evidence that NO can enter cells and release Fe is supported by the work of Hibbs and colleagues using activated macrophages (*52*, *55*, *56*). In these studies, when tumor target cells were cocultivated with activated mouse macrophages, there was a significant loss of Fe (64% of cell Fe/24 h) from target cells associated with inhibition of mitochondrial respiration and DNA synthesis. Further support for an interaction between NO and Fe in activated macrophages has come from studies demonstrating Fe−nitrosyl complexes in these cells (*25*, *26*) and their tumor targets (*27*, *28*).

In conclusion, it is possible that NO can increase IRP1 RNA-binding activity by several mechanisms including destabilization of the [4Fe-4S] cluster and also via an indirect effect by mobilizing Fe from intracellular pools. The relative contribution of each mechanism to the increase in IRP1 RNA-binding activity may be related to the concentration, source, and redox-related state of the NO generated. Finally, it should also be noted that physiological or pharmacological levels of NO may be sufficient to activate IRP1 irrespective of the level of Fe in the cell.

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REFERENCES

1. Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993) *Cell 72*, 19−28.
2. Hentze, M. W., and Kühn, L. C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8175−8182.
3. Richardson, D. R., and Ponka, P. (1997) *Biochim. Biophys. Acta 1331*, 1−40.
4. Theil, E. C. (1998) *Metal Ions Biol. Syst. 35*, 403−434.
5. Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., Harford, J. B., and Klausner, R. D. (1987) *Science 238*, 1570−1573.
6. Leibold, E. A., and Munro, H. N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2171−2175.
7. Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L. N., Klausner, R. D., and Harford, J. B. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3574−3578.
8. Müllner, E. W., Neupert, B., and Kühn, L. C. (1989) *Cell 58*, 373−382.
9. Kaptain, S., Downey, W. E., Tang, C., Philpott, C., Haile, D., Orloff, D. G., Harford, J. B., and Klausner, R. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10109−10113.
10. Haile, D. J., Rouault, T. A., Tang, C. K., Chin, J., Harford, J. B., and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7536−7540.
11. Hentze, M. W., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1989) *Science 244*, 357−359.
12. Philpott, C. C., Haile, D., Rouault, T. A., and Klausner, R. D. (1993) *J. Biol. Chem. 268*, 17655−17658.
13. Hirling, H., Henderson, B. R., and Kühn, L. C. (1994) *EMBO J. 13*, 453−461.
14. Samaniego, F., Chin, J., Iwai, K., Rouault, T. A., and Klausner R. D. (1994*) J. Biol. Chem. 269*, 30904−30910.
15. Guo, B., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) *J. Biol. Chem. 270*, 21645−21651.
16. Drapier, J.-C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kühn, L. C. (1993) *EMBO J. 12*, 3643−3649.
17. Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., and Hentze, M. W. (1993) *EMBO J. 12*, 3651−3657.
18. Oria, R., Sanchez, L., Houston, T., Hentze, M. W., Liew, F. Y., and Brock, J. H. (1995) *Blood 85*, 2962−2966.
19. Richardson, D. R., Neumannova, V., Nagy, E., and Ponka, P. (1995) *Blood 86*, 3211−3219.
20. Stamler, J., Singel, D. J., and Loscalzo, J. (1992) *Science 258*, 1898−1902.
21. Bouton, C., Raveau, M., and Drapier, J.-C. (1996) *J. Biol. Chem. 271*, 2300−2306.
22. Bouton, C., Hirling, H., and Drapier, J. C. (1997) *J. Biol. Chem. 272*, 19969−19975.
23. Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997) *J. Biol. Chem. 272*, 20340−20347.
24. Pantopoulos, K., Weiss, G., and Hentze, M. W. (1996) *Mol. Cell. Biol. 16*, 3781−3788.
25. Lancaster, J. R., and Hibbs, J. B., Jr. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1223−1229.
26. Pellat, C., Henry, Y., and Drapier, J.-C. (1990) *Biochem. Biophys. Res. Commun. 166*, 119−125.
27. Drapier, J.-C., Pellat, C., and Henry, Y. (1991) *J. Biol. Chem. 266*, 10162−10167.
28. Bastian, N. R., Yim, C. Y., Hibbs, J. B., Jr., and Samlowski, W. E. (1994) *J. Biol. Chem. 269*, 5127−5131.
29. Bouton, C., Oliveira, L., and Drapier, J.-C. (1998) *J. Biol. Chem. 273*, 9403−9408.
30. Field, L., Dilts, R. V., Ravichandran, R., Lenhert, P. G., and Carnahan, G. E. (1978) *J. Chem. Soc., Chem. Commun.*, 249−250.
31. Richardson, D. R., and Baker, E. (1990) *Biochim. Biophys. Acta 1053*, 1−12.
32. Knowles, R. G., and Moncada, S. (1994) *Biochem. J. 298*, 249−258.
33. Richardson, D. R., Neumannova, V., and Ponka, P. (1995) *Biochim. Biophys. Acta 1266*, 250−260.
34. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem. 126*, 131−138.
35. Drapier, J. C., and Hibbs, J. B., Jr. (1996) *Methods Enzymol. 269*, 26−36.
36. Murphy, M. E., and Noack, E. (1994) *Methods Enzymol. 233*, 240−250.
37. Wardrop, S. L., and Richardson, D. R. (1999) *Eur. J. Biochem. 263*, 41−49.
38. Oliveira, L., Bouton, C., and Drapier, J.-C. (1999) *J. Biol. Chem. 274*, 516−521.
39. Barisani, D., Cairo, G., Ginelli, E., Marozzi, A., and Conte, D. (1999) *Hepatology 29*, 464−470.
40. Butler, A. R., and Glidewell, C. (1987) *Chem. Soc. Rev. 16*, 361−380.
41. Feelisch, M. (1991) *J. Cardiovasc. Pharmacol. 17* (Suppl. 3), S25−S33.
42. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *Arch. Biochem. Biophys. 288*, 481−487.
43. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1620−1624.
44. Hogg, N., Darley-Usmar, V. M., Wilson, M. T., and Moncada, S. (1992) *Biochem. J. 281*, 419−424.
45. Richardson, D. R., and Milnes, K. (1997) *Blood 89*, 3025−3038.
46. Roberts, S., and Bomford, A. (1988) *J. Biol. Chem. 263*, 19181−19187.
47. Lepoivre, M., Fieschi, F., Coves, J., Thelander, L., and Fontecave, M. (1991) *Biochem. Biophys. Res. Commun. 179*, 442−448.
48. Vanin, A. F. (1991) *FEBS Lett. 289*, 1−3.
49. Vanin, A. F., Men'shikov, G. B., Moroz, I. A., Mordvintcev, P. I., Serezhenkov, V. A., and Burbaev, D. S. (1992) *Biochim. Biophys. Acta 1135*, 275−279.

50. Reif, D. W., and Simmons, R. D. (1990) *Arch. Biochem. Biophys. 283*, 537−541.
51. Lee, M., Arosio, P., Cozzi, A., and Chasteen, N. D. (1994) *Biochemistry 33*, 3679−3687.
52. Drapier, J.-C., and Hibbs, J. B., Jr. (1986) *J. Clin. Invest. 78*, 790−797.
53. Drapier, J.-C., and Hibbs, J. B., Jr. (1988) *J. Immunol. 140*, 2829−2838.
54. Kim, Y.-M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D., and Lancaster, J. R., Jr. (1995) *J. Biol. Chem. 270*, 5710−5713.
55. Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1984) *Biochem. Biophys. Res. Commun. 123*, 716−723.
56. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988) *Biochem. Biophys. Res. Commun. 157*, 87−94.

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