Nitric Oxide and Peroxynitrite Activate the Iron Regulatory Protein-1 of J774A.1 Macrophages by Direct Disassembly of the Fe−S Cluster of Cytoplasmic Aconitase†

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ABSTRACT: Posttranscriptional regulation of iron homeostasis involves, among other factors, a reversible conversion of the Fe−S enzyme cytoplasmic aconitase to a mRNA-binding iron regulatory protein (IRP-1) that lacks an Fe−S cluster. Previous studies have shown that aconitase/IRP-1 may be a target of •NO or peroxynitrite ($ONOO^-$), formed after reaction of •NO with superoxide anion ($O_2^{\bullet -}$); however, the mechanisms and consequences of such interactions have remained uncertain. In this study, recombinant aconitase/IRP-1 was exposed to SIN-1, whose thermal decomposition releases •NO and $O_2^{\bullet -}$. Results showed that SIN-1 was able to induce concomitant inactivation of aconitase and activation of IRP-1, attributable to cluster disassembly induced by $ONOO^-$. SIN-1 was used also in lysates of J774A.1 mouse macrophages grown under control conditions, or subjected to iron loading or starvation by treatment with hemin or desferrioxamine, respectively. Three lines of evidence confirmed that $ONOO^-$ activated IRP-1 by removing iron from the Fe−S cluster of cytoplasmic aconitase. First, IRP-1 activation was accompanied by iron release and loss of aconitase activity. Second, aconitase activity was recovered by reassembling Fe−S clusters with cysteine and ferrous ammonium sulfate. Third, iron release and IRP-1 activation were observed in lysates from control or iron-loaded macrophages, containing increasing levels of Fe−S clusters, but not in lysates from iron-starved macrophages, in which aconitase had already undergone cluster disassembly and switched to IRP-1. •NO was less efficient than $ONOO^-$ in attacking the Fe−S cluster of cytoplasmic aconitase; in fact, SIN-1-dependent iron release and IRP-1 activation were diminished by superoxide dismutase, which scavenged $O_2^{\bullet -}$ before it reacted with •NO to form $ONOO^-$. Under comparable conditions, however, both •NO and $ONOO^-$ inactivated an IRP-2 unable to assemble an Fe−S cluster. These results indicate that •NO and $ONOO^-$ may activate IRP-1 by attacking the Fe−S cluster of cytoplasmic aconitase, while also inactivating the cluster-deficient IRP-2. Such divergent actions offer clues to explain links between iron homeostasis and reactive nitrogen species in macrophages involved in inflammation or other pathophysiologic conditions.

Cells must acquire sufficient iron for metabolic needs while also preventing excess iron from catalyzing formation of toxic free radicals. A major mechanism for the regulation of iron homeostasis relies on divergent but coordinate control of transferrin receptor-mediated iron uptake and ferritin-mediated iron sequestration. Iron regulatory proteins (IRPs)[1] 1 and 2 play an important role in these settings (*1*−*4*). When iron levels are low, the two IRPs bind to iron responsive elements (IRE) in untranslated regions of mRNAs for transferrin receptor and ferritin, blocking degradation of the former while decreasing translation of the latter (*1*−*4*). These processes result in simultaneous increase of iron uptake and decrease of iron sequestration, forming a pool of iron that is available for metabolic utilization. When iron levels are high, IRP-1 assembles a [4Fe-4S] cluster and acquires aconitase activity, while also losing its function as a posttranscriptional regulator. IRP-2 does not assemble an Fe−S cluster but responds to iron repletion by undergoing degradation through the ubiquitin−proteasome pathway (*5*−*7*). Thus, iron homeostasis is regulated through reversible switches between aconitase and IRP-1 and through the control of IRP-2 levels.

IRPs can be regulated also by factors other than iron levels in the cells. For example, studies in macrophages, fibroblasts,

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[1] Abbreviations: IRP, iron regulatory proteins; SIN-1, 3-morpholinosydnonimine; NO, nitric oxide; $ONOO^-$, peroxynitrite; DFO, desferrioxamine; PBS, phosphate buffered saline; SOD, CuZn superoxide dismutase; IRE, iron regulatory elements; 2-ME, 2-mercaptoethanol.

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and erythroleukemic cells, or in their cytoplasmic extracts, have shown that both IRP-1 and -2 are highly sensitive to •NO generated by endogenous synthases or by exogenous donors (reviewed in refs *4* and *8*−*10*). In most of these studies, •NO was shown to decrease IRP-2 activity, an effect attributed to redox modifications of -SH residues that mediate RNA binding, followed by a degradation of this protein (*7*). In contrast, •NO was often shown to increase IRP-1 activity, by mechanisms as yet unclear. Some investigators have proposed, but not conclusively shown, that •NO might directly attack the Fe−S cluster of cytoplasmic aconitase, inducing its disassembly and switching the enzyme to IRP-1 (*11*−*13*). Others have suggested that •NO would induce cluster disassembly indirectly, that is, by modifying the size of the pool of free iron in the cell (*13*−*15*). This picture is further complicated by the fact that in some studies •NO was shown to decrease aconitase activity while not increasing IRP-1 activity, unless IRP-1 assays were conducted in the presence of reducing agents to regenerate -SH groups (*16*, *17*). Conflicting results also have been obtained in regard to the modulation of IRPs by peroxynitrite ($ONOO^-$), produced after reaction of •NO with superoxide anion ($O_2^{\bullet-}$). Whereas IRP-2 activity was almost invariably shown to decrease after exposure of cell lysates to $ONOO^-$ (*17*, *18*), or in macrophages which likely produced $ONOO^-$ after stimulation with cytokines (*7*, *19*−*22*), IRP-1 activity was reported to increase (*19*, *20*, *23*), to remain unaltered (*12*, *18*), or to decrease (*24*). In hepatoma cells, •NO activated IRP-1 but had no effect on the binding activity of IRP-2 (*25*).

In light of the above reports, we designed experiments to further characterize interactions between reactive nitrogen species and IRPs. In a first set of experiments, we incubated SIN-1 with recombinant protein samples exhibiting only IRP-1 activity or both aconitase and IRP-1 activities. These experiments were aimed at establishing how the absence or presence of a labile Fe−S cluster influenced direct interactions between $ONOO^-$ and IRP-1 or aconitase, respectively. In a second set of experiments, we used lysates derived from J774A.1 mouse macrophages that had been grown under control conditions or had been subjected to iron starvation or supplementation, to obtain varying ratios of aconitase to IRP-1 and to further elucidate whether changes in IRP-1 activity depended on the presence or absence of Fe−S clusters possibly prone to disassembly by $ONOO^-$. Experiments on lysates were also used to (i) compare •NO with $ONOO^-$, (ii) monitor changes in IRP-2 activity, and (iii) characterize how $ONOO^-$ and •NO modulate an iron-sensitive protein which responds to iron loading or deprivation by mechanisms other than cluster assembly or disassembly.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant IRP-1. Recombinant human IRP1 (rIRP-1) with an N-terminal His_6 tag was expressed in *Escherichia coli* BL21 DE3 transformed with the plasmid pT7-His-hIRF (kindly provided by Dr. Lukas Kuhn). Optimal expression of soluble rIRP-1 was obtained from overnight culture at 25 °C in Luria broth without induction. The bacterial lysate obtained by sonication was centrifuged and the supernatant loaded on a Ni^+ nitrilotriacetic acid-agarose column. The protein was eluted with 100 mM imidazole and dialyzed against 20 mM Tris-HCl pH 8.0, 15 mM KOAc, 0.15 mM $MgCl_2$, 7.2 mM 2-ME, and 5% glycerol. The sample was then applied to an ionic exchange 5-mL Hi-Trap Q column (Amersham Pharmacia Biotech, Cologno Monzese, Italy), and the protein was eluted with a linear KCl gradient (0−150 mM). Purified rIRP-1 was then dialyzed against 20 mM Tris-HCl pH 7.4 and stored in aliquots at −80 °C. Where indicated, Fe−S cluster reconstitution was performed by incubating the protein in the presence of 140 mM KOAc, 1.3 mM $MgCl_2$, 70 mM dithiothreitol, 5% glycerol, 2 μM Na_2S and 400 μM iron citrate for 30 min at room temperature. The mixture was desalted by chromatography on a PD10 column (Amersham Pharmacia Biotech, Cologno Monzese, Italy), and the purified protein was stored in aliquots at −80 °C.

Cell Culture and Preparation of Lysates. J774A.1 mouse macrophages were grown in MEM at 37 °C in 5% CO_2 as previously described (*19*). Iron loading and deprivation were obtained as described (*19*, *26*) by incubating cells for 16 h in the presence of 50 μM hemin or 50 μM desferrioxamine (DFO), respectively. No apparent toxicity was observed by the trypan blue exclusion assay under all experimental conditions employed in this study. Cells were harvested, washed with PBS, and homogenized in the buffer originally described by Leibold and Munro (*27*), as reported (*28*).

Treatment of Recombinant IRP-1 and Lysates with •NO or $ONOO^{\bullet-}$. rIRP-1 (5 μg/mL) and cytosolic lysates (0.75 mg of protein/mL) were incubated at 37 °C for 60 min in the presence of 0.25−0.5 μM or 3 mM, respectively, 3-morpholinosydnonimine (SIN-1) (Sigma, Milan, Italy), whose thermal decomposition generates •NO and $O_2^{\bullet-}$, which then react to form $ONOO^-$. Where indicated, incubations also contained bovine erythrocyte CuZn superoxide dismutase (SOD, 500 units/mL) (Sigma, Milan, Italy) to scavenge $O_2^{\bullet-}$ and monitor the effect of •NO.

RNA−Protein Gel Retardation Assay. At the end of incubations with SIN-1, IRP activities were measured by RNA−protein gel retardation. For this purpose, the pSPT-fer plasmid containing the IRE of human ferritin H chain (*29*) was linearized with BamHI and transcribed in vitro with T7 RNA polymerase in the presence of 100 μCi of [α^{32}-P] UTP (800 μCi/mmol; Amersham Pharmacia Biotech, Cologno Monzese, Italy). Two microgram protein samples of lysates or 50 ng of rIRP-1 were incubated with a molar excess of IRE probe, digested with Rnase T1 and treated with heparin as described previously (*28*). After separation on 6% nondenaturing polyacrylamide gels, IRP/IRE complexes were visualized by autoradiography and quantified by direct nuclear counting using an IstantImager (Packard Instruments Co., Milano, Italy).

Assay for Iron Release from the Fe−S Cluster of Cytoplasmic Aconitase. The release of iron from the Fe−S cluster of cytoplasmic aconitase was monitored spectrophotometrically in 1-mL incubations containing lysates from control and DFO- or hemin-treated J774A.1 cells (0.3−0.6 mg of protein), plus or minus SOD (500 units/mL), in 10 mM HEPES, pH 7.0, at 37 °C. Reactions were started by the addition of 1.5−3 mM SIN-1, reproducing SIN-1/protein ratios comparable to those used in the incubations for IRP assay. •NO or $ONOO^-$-induced Fe(II) delocalization was detected by chelation with 0.25 mM ferrozine; the formation of ferrozine-Fe(II) complexes [$\epsilon_{564\ nm} = 27.9\ mM^{-1}\ cm^{-1}$] (*30*) was routinely corrected against samples that also

contained 0.25 mM *cis*-aconitate to protect the Fe−S cluster of cytoplasmic aconitase from attack by SIN-1 derived species (*18*). The difference between [ferrozine-Fe(II) (− *cis*-aconitate)] and [ferrozine-Fe(II) (+ *cis*-aconitate)] therefore gave a net measurement of Fe(II) ions removed from the Fe−S cluster. Experiments were carried out in a Hewlett-Packard 8453 spectrophotometer equipped with cuvette stirring apparatus and computer-assisted corrections for turbidity and scatter.

Assay for Aconitase. Aconitase activity of control and DFO- or hemin-treated J774A.1 cells was determined spectrophotometrically by monitoring the disappearance of *cis*-aconitate (ϵ_{240} = 3.6 mM^{-1} cm^{-1}) in 1-mL incubations containing lysates (25−100 μg of protein) and *cis*-aconitate (0.1 mM) in 0.3 M NaCl, pH 7.0 at 37 °C. Authentic aconitase activity was confirmed by suppression of *cis*-aconitate consumption after addition of the aconitase pseudo-substrate-inhibitor D,L-fluorocitrate (0.1 mM) (*31*, *32*). To determine the effect of •NO or $ONOO^-$ on aconitase activity, lysates were exposed to SIN-1 plus or minus SOD (500 units/mL) as described for iron delocalization. After 60 min, lysates were supplemented with 0.25 mM *cis*-aconitate to protect residual clusters from the action of SIN-1; unreacted *cis*-aconitate or SIN-1 were eventually removed prior to aconitase assay by means of sequential dialysis against 0.1 mM *cis*-aconitate/0.3 M NaCl and 0.3 M NaCl. Where indicated, lysates were assayed for aconitase activity after 5 min preincubation at 37 °C with cysteine and ferrous ammonium sulfate at the final ratios of 1000 and 50 nmol/mg of protein, respectively. This treatment was previously shown to reconstitute [4Fe-4S] clusters, achieving maximal activation of aconitase (*32*).

RESULTS

We studied the effects of SIN-1, which generates $ONOO^-$ by decomposing to •NO and $O_2^{\bullet -}$, on recombinant cytoplasmic aconitase expressed in *E. coli*. The IRP-1 preparation obtained from *E. coli* had no aconitase activity but exhibited strong RNA-binding activity (Figure 1A,B). This suggested that the recombinant protein lacked an Fe−S cluster and exhibited only IRP-1 activity (r-IRP-1). Following in vitro treatment with iron and reducing agents, rIRP-1 acquired aconitase activity but showed lower RNA-binding activity. This suggested Figure 1A,B, indicating that the recombinant protein had been converted to a mixture of cluster-containing aconitase and cluster-free IRP-1 (rIRP-1/aconitase). As shown in Figure 2A, SIN-1 concentration-dependently decreased the RNA-binding activity of rIRP-1 but had different effects on rIRP-1/aconitase. SIN-1 (0.25 μM) increased the RNA-binding activity of rIRP-1/aconitase while also blunting its enzymatic activity, an effect consistent with cluster disassembly and consequent conversion of the pool of aconitase into a new pool of IRP-1. However, 0.5 μM SIN-1 not only blunted aconitase activity but also decreased IRP-1 activity to levels similar to those of untreated samples. Treatment with cysteine and ferrous ammonium sulfate, known to achieve maximal reconstitution of [4Fe-4S] clusters, recovered and increased the aconitase activity of rIRP-1/aconitase exposed to the lower concentration of SIN-1. This provides more evidence that low concentrations of $ONOO^-$ switched aconitase to IRP-1 by inducing cluster disassembly. Nonetheless, cysteine and ferrous ammonium sulfate failed to rescue aconitase activity in rIRP-1/aconitase exposed to the higher concentration of SIN-1, perhaps because the effects of higher fluxes of $ONOO^-$ extended beyond inducing cluster disassembly and eventually damaged -SH residues that are needed for both RNA binding and cluster reassembly. Thus, the higher concentration of SIN-1 converted r-IRP-1/aconitase into a more extensively oxidized protein; this form is sometimes called "null protein" to remark that it lacks both enzymatic and RNA binding activity (*18*). Collectively, experiments with recombinant proteins

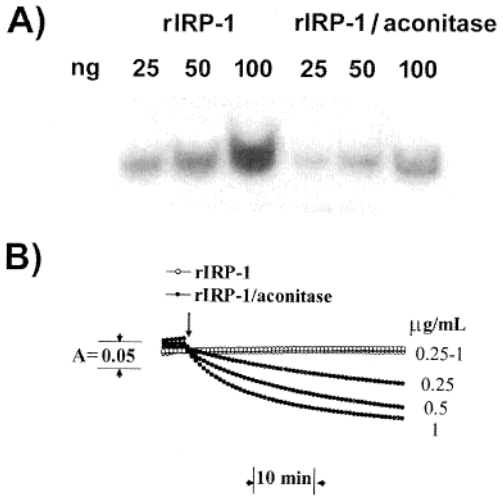

FIGURE 1: RNA-binding and aconitase activities of rIRP-1 or rIRP-1/aconitase. Panel A shows concentration-dependent RNA-binding activities of rIRP-1 or rIRP-1/aconitase, obtained as described under Experimental Procedures. In panel B, increasing concentrations of the same recombinant samples were assayed for aconitase activity (monitored as *cis*-aconitate consumption at 240 nm). rIRP-1 had essentially no enzymatic activity when assessed in a 0.25−1 μg/mL range; rIRP-1/aconitase, assessed in the same range of concentrations, exhibited increasing enzymatic activity.

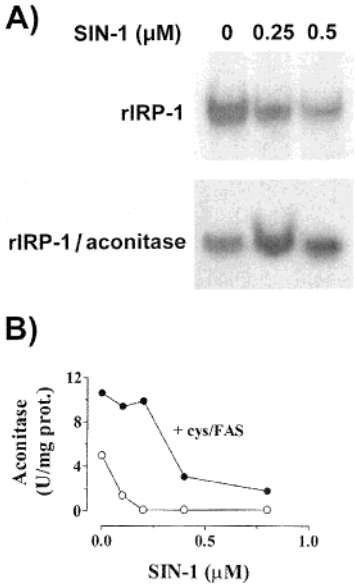

FIGURE 2: Effects of SIN-1 on rIRP-1 or rIRP-1/aconitase activities. In panel A, rIRP-1 or rIRP-1/aconitase (5 μg/mL) were exposed to 0.25 or 0.5 μM SIN-1 for 30 min and then assayed for IRE binding. In panel B, rIRP-1/aconitase, previously exposed to SIN-1 under comparable SIN-1/protein ratios, was assayed for aconitase activity before or after treatment with cysteine (Cys) and ferrous ammonium sulfate (FAS). See also Experimental Procedures and Results, for further details.

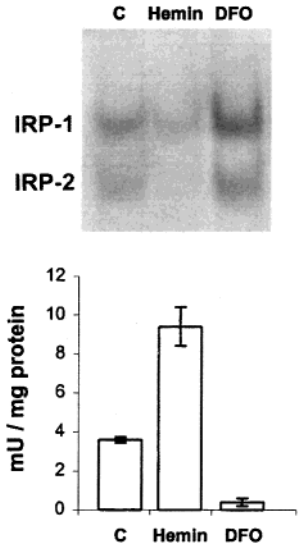

FIGURE 3: Effect of modulation of iron availability on IRP and aconitase activities in J774A.1 cells. Cells were grown for 16 h under control conditions or in the presence of hemin or DFO, and then assayed for IRP and aconitase activities, as described under Experimental Procedures. The gel shows a representative experiment. Columns are means of aconitase activities ± SE of 3−4 experiments.

showed that $ONOO^-$ was able to attack aconitase/IRP-1 by direct mechanisms that seemed to be influenced by the presence or absence of an Fe−S cluster. Moreover, minor changes in the concentration of SIN-1 determined whether $ONOO^-$ switched aconitase to IRP-1 or converted it into a null protein.

After these characterizations, we studied the effects of SIN-1 on lysates derived from J774A.1 macrophages. In a first set of experiments, we measured IRP and aconitase activities in lysates of macrophages which had been grown under normal conditions, or had been subjected to iron supplementation or starvation by treatment with hemin or the iron chelator DFO, respectively (Figure 3). In comparison to control samples, lysates from hemin-treated cells were characterized by decreased IRP-1 and increased aconitase activities, suggesting that iron supplementation favors the assembly of [4Fe-4S] clusters and switches some IRP-1 to aconitase. Conversely, DFO-treated cells were characterized by a concomitant increase in IRP-1 and decrease in aconitase activities, suggesting that iron starvation caused disassembly of [4Fe-4S] clusters and had made aconitase switch to IRP-1. The RNA-binding activity of IRP-2 responded to iron supplementation or starvation in a fashion similar to IRP-1 (see also Figure 3). The aconitase/IRP-1 and IRP-2 activities of J774A.1 macrophages therefore exhibited typical responses to modifications of iron availability. Having shown this, we determined how aconitase/IRP-1 and IRP-2 responded to SIN-1 as a source of $ONOO^-$. As reported in Figures 4−6, SIN-1 decreased IRP-2 activity in both control and hemin- or DFO-treated samples. •NO had the same effects as $ONOO^-$; in fact, IRP-2 decreased also when SOD was included for scavenging $O_2^{\bullet -}$ before it reacted with •NO. Control experiments (not shown) gave a similar pattern also when lysates were assayed in the presence of 1% 2-ME, a concentration known to be optimal for maximal activation of both IRP-1 and IRP-2 (*18*). These results demonstrated that $ONOO^-$ and •NO were both able to irreversibly

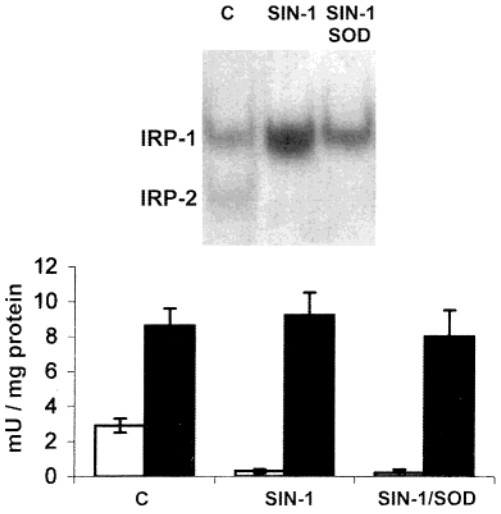

FIGURE 4: Effect of SIN-1 on IRP and aconitase activities in lysates from control J774A.1 cells. Lysates from cells grown under control conditions were exposed to 3 mM SIN-1 for 1 h in the presence or absence of SOD and then assayed for IRP and aconitase activities, as described under Experimental Procedures. The gel shows a representative experiment. Columns are means of aconitase activities ± SE of 3−4 experiments. Aconitase was assayed before (open columns) or after (solid columns) preincubation of lysates with cysteine and ferrous ammonium sulfate, as also described under Experimental Procedures.

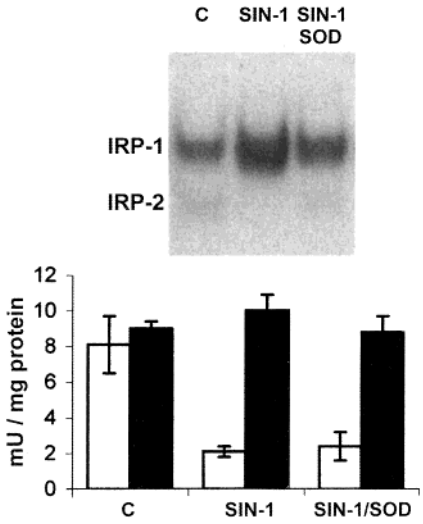

FIGURE 5: Effect of SIN-1 on IRP and aconitase activities in lysates from hemin-treated J774A.1 cells. Lysates from cells grown in the presence of hemin were exposed to 3 mM SIN-1 for 1 h in the presence or absence of SOD and then assayed for IRP and aconitase activities. The gel shows a representative experiment. Columns are means of aconitase activities ± SE of 3−4 experiments. Aconitase was assayed before (open columns) or after (solid columns) preincubation of lysates with cysteine and ferrous ammonium sulfate. See also Experimental Procedures for further details.

inactivate IRP-2, regardless of the iron status of the cells. In contrast, the effects of SIN-1 on aconitase/IRP-1 were strongly influenced by the iron status of the cells. In the lysates from control (Figure 4) and hemin-treated (Figure 5) macrophages, which contained mixed pools of aconitase and IRP-1, SIN-1 was found to increase IRP-1 activity while abolishing aconitase activity, an effect consistent with the disassembly of [4Fe-4S] clusters. The addition of SOD only partially prevented the activation of IRP-1 and had no effect on the inactivation of aconitase induced by SIN-1 (see also

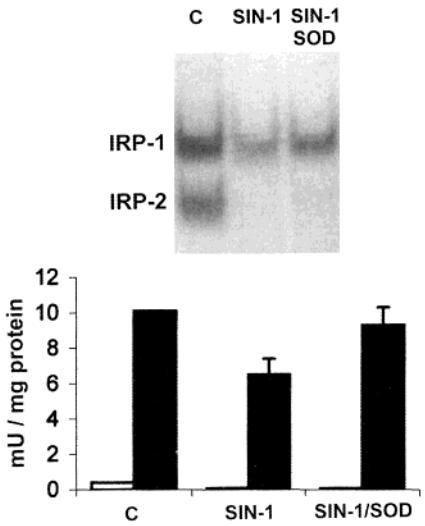

FIGURE 6: Effect of SIN-1 on IRP and aconitase activities in lysates from DFO-treated J774A.1 cells. Lysates from cells grown in the presence of DFO were exposed to 3 mM SIN-1 for 1 h in the presence or absence of SOD and then assayed for IRP and aconitase activities. The gel shows a representative experiment. Columns are means of aconitase activities ± SE of 3−4 experiments. Aconitase was assayed before (open columns) or after (solid columns) preincubation of lysates with cysteine and ferrous ammonium sulfate. See also Experimental Procedures for further details.

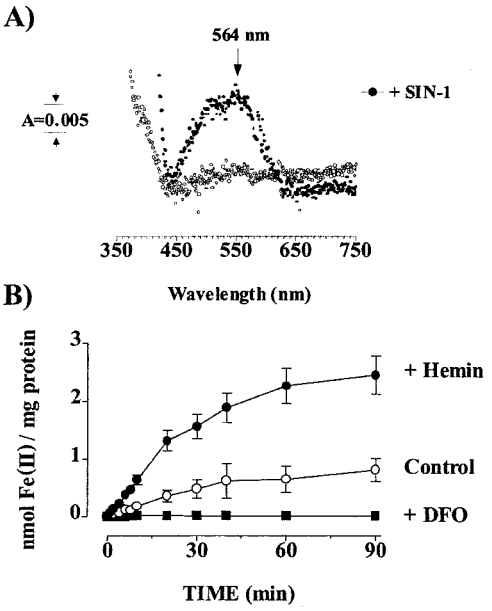

FIGURE 7: Effect of SIN-1 on cluster iron mobilization. In panel A, lysates from J774A.1 cells, grown for 16 h under control conditions, were assayed spectrally for [ferrozine-Fe(II) minus *cis*-aconitate]-[ferrozine-Fe(II) plus *cis*-aconitate], an index of cluster iron release, as described under Experimental Procedures. Where indicated, 3 mM SIN-1 was included in the incubations. Spectra are those recorded at 60 min. In panel B, the time course of SIN-1 dependent iron release was monitored in lysates from control, DFO-, or hemin-treated J774A.1 macrophages. Values are means ± SE of three experiments.

Figures 4 and 5). Again, 2-ME did not modify the responses of IRP-1 to SIN-1 or SIN-1/SOD (not shown). Both •NO and $ONOO^-$ were therefore able to induce converse activation of IRP-1 and inactivation of aconitase, although $ONOO^-$ seemed to be more active than •NO in increasing IRP-1. It is worth noting that all samples (control, SIN-1, and SIN-1/SOD) acquired maximal aconitase activity after exposure to cysteine and ferrous ammonium sulfate, suggesting that neither $ONOO^-$ nor •NO precluded cluster reassembly. Different results were obtained in lysates from DFO cells. Whereas SIN-1 did not increase but actually decreased IRP-1 activity in these samples, SIN-1/SOD had essentially no effect (Figure 6). 2-ME did not appreciably modify this pattern (not shown), but the decrease of IRP-1 activity was less pronounced (20% instead of 35%); moreover, all samples gained aconitase activity after treatment with cysteine and ferrous ammonium sulfate, but it was noted that lysates exposed to SIN-1 recovered less activity than control or SIN-1/SOD samples (see also Figure 6). Because samples from DFO-treated cells were deficient in [4Fe-4S] clusters, these results provided one more evidence that converse increase of IRP-1 and decrease of aconitase by SIN-1 or SIN-1/SOD were mediated by processes of cluster disassembly, induced by $ONOO^-$ or •NO. These results also confirmed that the absence of an Fe−S cluster enables $ONOO^-$ to modify protein -SH residues that are required for both RNA binding and cluster assembly, yielding a null protein with lower levels of IRP-1 activity and of cysteine/ferrous ammonium activatable aconitase (cfr. data on rIRP-1 exposed to either low or high concentrations of SIN-1 and data on rIRP-1/aconitase exposed to high concentrations of SIN-1; Figure 2).

We performed experiments to see whether SIN-1 or SIN-1/SOD removed iron ions from the [4Fe-4S] cluster of cytoplasmic aconitase in a manner that explained their ability to switch this enzyme to IRP-1. These experiments were performed by exposing lysates to SIN-1 and by monitoring formation of the ferrozine-Fe(II) complex in the absence or presence of *cis*-aconitate, which is known to stabilize the Fe−S cluster of aconitase and to prevent its attack by SIN-1-derived species (*18*). Therefore, the difference between [ferrozine-Fe(II) minus *cis*-aconitate] and [ferrozine-Fe(II) plus *cis*-aconitate] could be assumed as an index of Fe(II) ions released from the Fe−S cluster of cytoplasmic aconitase. Figure 7A shows typical spectra obtained by monitoring cluster iron release in lysates derived from control macrophages and incubated for 60 min in the absence or presence of SIN-1. No ferrozine-Fe(II) complex could be detected when lysates were incubated in the absence of SIN-1; however, the addition of SIN-1 clearly increased mobilization and detection of Fe(II), indicated by the very good signal/noise ratio relative to samples not exposed to SIN-1. Cluster iron release proceeded in a time-dependent manner and was influenced by the iron status and by the cluster content of the samples; hence, it became more evident in lysates from hemin-treated macrophages, but became nearly undetectable in lysates from DFO-treated cells (Figure 7B). Experiments in lysates from hemin-treated macrophages also showed that cluster iron release was partially inhibited by SOD (Figure 8). It is worth noting that iron release was in a nanomolar range on average in contrast to previous reports that IRE-binding activity was in the picomolar range in various cell types (*33*). Although iron release was routinely determined by comparing *cis*-aconitate sensitive vs insensitive values, this discrepancy suggests that the assay was confounded by mobilization of iron sources other than cytoplasmic aconitase. The contribution of mitochondrial aconitase, possibly detached in lysates during cell manipulations, cannot be ruled out. In addition, one should consider that the IRE-binding activity usually accounted for less than 25% of the total pool

Table 1: Summary of Discrepant Findings Observed in Various Studies on the Effects of •NO and $ONOO^-$ on the Activity of Iron Regulatory Proteins[a]

authors	cell	challenge	IRP-2	IRP-1	IRP-1 + reducing agents
Drapier et al. (1993), ref *11*	macrophages	IFN-γ/LPS, SIN-1, •NO gas	ND	↑	↔
Weiss et al. (1993), ref *23*	macrophages	IFN-γ/LPS	↑	↑	↔
Pantopoulos and Hentze (1995), ref *14*	fibroblasts	endogenous •NO	↑	↑	↑
Richardson et al. (1995), ref *24*	erythroleukemia	SIN-1 ± SOD, SNAP, SNP	ND	↑ or ↓	ND
Phillips et al. (1996), ref *25*	hepatoma	IFN-γ/LPS, SNAP	↔	↑	ND
Bouton et al. (1996), ref *12*	macrophages	SIN-1 ± SOD	ND	↑ or ↔	↔
Bouton et al. (1997), ref *18*	macrophages	$ONOO^-$	↓	↔	↑
Recalcati et al. (1998), ref *19*	macrophages	IFN-γ/LPS	↓	↑	↑
Bouton et al. (1998), ref *20*	macrophages	IFN-γ/LPS	↓	↑	↔
Oliveira et al. (1999), ref *17*	macrophages	SIN-1 + SOD	↓	↑	↑↑
Mulero and Brock (1999), ref *22*	macrophages	IFN-γ/LPS	↓	↑	↑
Wardrop et al. (2000), ref *13*	fibroblasts	SIN-1	ND	↑ at low conc ↓ at high conc	↓
Kim & Ponka (2000), ref *7*	macrophages	IFN-γ/LPS	↓	↑	↔
this work	macrophages	SIN-1	↓	↑	↑

[a] ND: not determined; ↑: upregulation; ↓: downregulation; ↔: no change; SNAP: *S*-nitroso-*N*-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; conc: concentration.

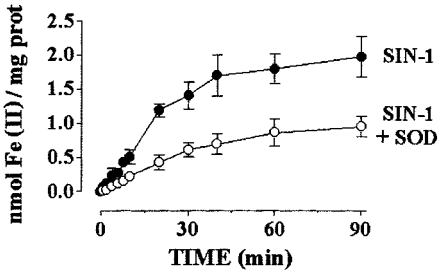

FIGURE 8: Relative effect of $ONOO^-$ and •NO on cluster iron mobilization. Lysates from J774A.1 cells grown for 16 h in the presence of hemin, were exposed to 3 mM SIN-1 for the indicated time periods in the presence or absence of SOD, and iron mobilization was monitored by chelation with 0.25 mM ferrozine. Values are means ± SE of three experiments. See also Experimental Procedures for further details.

of aconitase/IRP-1 and that lysate preparation and removal of gross membrane fractions may have produced a sizable enrichment in aconitase/IRP-1. All such factors may have contributed to producing nanomolar levels of iron detection. Nevertheless, iron release was in good agreement with the greater efficacy of $ONOO^-$ over •NO in activating IRP-1, providing at least qualitative correlate to experiments in which aconitase and IRP-1 had been assayed after lysate exposure to SIN-1 or SIN-1/SOD.

DISCUSSION

A close link exists between iron homeostasis and •NO, but conflicting results have been reported in regard to the effect of reactive nitrogen species on IRP (see refs *4* and *8*−*10* for reviews). Changes in IRP-1 and IRP-2 activities were shown to be quite variable, depending on the cell type used, the concentration and redox characteristics of nitrogen species (•NO versus $ONOO^-$), and the experimental system used for producing •NO and related species (exogenous donors versus inducers of •NO synthase). Despite such variability, the response pattern characterized in these studies usually consisted of a converse activation of IRP-1 and inactivation of IRP-2. What also has remained undefined is whether •NO and/or $ONOO^-$ activated IRP-1 through direct interactions with the labile Fe−S cluster of cytoplasmic aconitase. Moreover, it is unclear whether IRP-1 activation requires also the presence of reductants that regenerated -SH groups in this protein. Table 1 summarizes reports that have addressed all such issues. In the present study, the mechanism(s) of IRP-1 activation were investigated in two cell-free systems that were suitable for characterizing direct interactions of •NO or $ONOO^-$ with IRP-1 while avoiding indirect effects due to cellular iron release possibly induced by the two nitrogen species. One system involved rIRP-1 or rIRP-1/aconitase. Experiments with rIRP-1/aconitase showed that $ONOO^-$ switched aconitase to IRP-1 while not precluding aconitase reactivation by cysteine and ferrous ammonium sulfate, effects consistent with cluster disassembly and preservation of -SH residues needed for both RNA binding and Fe−S coordination. These experiments also showed that the concentration of SIN-1 was quite critical, as excess $ONOO^-$ not only induced cluster disassembly but also damaged the newly formed IRP-1 in a manner that reduced its RNA binding activity as well as its ability to recover aconitase activity after cluster reassembly. That cluster-free IRP-1 is highly sensitive to the damaging effects of $ONOO^-$ was further confirmed in experiments with rIRP-1, which underwent inactivation by both low and high concentrations of SIN-1. Data obtained with rIRP-1 or rIRP-1/aconitase were confirmed and extended by experiments performed on lysates of J774A.1 mouse macrophages, previously subjected to manipulations of their iron status by treatment with hemin or DFO. This model was chosen because macrophages contain sizable levels of both aconitase/IRP-1 and IRP-2 and are known to form •NO and $ONOO^-$ in several pathophysiologic conditions. Moreover, treatment with hemin or DFO gave us an opportunity to modulate the content of Fe−S clusters and to establish how their levels influenced responses of IRP-1 to •NO or $ONOO^-$. Possible modifications in the expression of iron-related genes could not confound the interpretation of experiments, as •NO or $ONOO^-$ were delivered to lysates in which interactions of the nitrogen species with IRP could be studied in a direct manner. Under such defined conditions, both •NO or $ONOO^-$ seemed to activate IRP-1 by inducing disassembly of the Fe−S cluster of cytoplasmic aconitase. This interpretation was supported by the following lines of evidence: (i) $ONOO^-$ and •NO activated IRP-1 in lysates from control or iron-loaded

macrophages, containing mixed pools of aconitase/IRP-1; (ii) IRP-1 activation was accompanied by cluster iron release and loss of aconitase activity; (iii) $ONOO^-$ proved more effective than •NO in releasing cluster iron, and consistently exhibited a greater efficacy in activating IRP-1. A potential caveat to this picture is given by the fact that $ONOO^-$ was superior to •NO in delocalizing iron and activating IRP-1 but not in abolishing aconitase activity, which was blunted equally well by the two species (cfr. Figures 4 and 5). This inconsistency may be explained by keeping in mind that aconitase and IRP-1 are regulated in a different fashion during the course of cluster iron delocalization. Whereas limited removal of the fourth labile iron of the cluster (Fe_a) is sufficient to inactivate aconitase, converse activation of IRP-1 only occurs when the process of delocalization extends to the remaining iron centers of the cluster (Fe_{b1-3}). It is therefore possible that $ONOO^-$ and •NO shared the reactivity necessary for delocalizing Fe_a, proving equally effective in abolishing aconitase activity; however, $ONOO^-$ probably was superior to •NO in removing also Fe_{b1-3}, favoring the switch of aconitase to IRP-1. The reduced efficacy of $ONOO^-$ in activating IRP-1 may not be attributed to an interfering action of H_2O_2, produced through the coincubation of SIN-1 with SOD and the consequent enhanced dismutation of $O_2^{\bullet-}$; in fact, H_2O_2 does not affect the RNA binding activity of IRP-1 (*4*, *28*, *34*, *35*). Hydrogen peroxide could, in principle, contribute in inactivating aconitase by removing Fe_a, but this would not explain the activation of IRP-1 that undoubtedly occurs with SIN-1/SOD. Adding catalase to decompose H_2O_2 would have only introduced an additional confounding factor, due to the avidity of •NO for the heme group of this enzyme (*36*). It is also worth noting that activation of IRP-1 did not require the presence of -SH reductants in the bandshift assay. This indicates that $ONOO^-$ or •NO had not damaged -SH residues that mediate the binding of IRP-1 to RNA (e.g., cys^{437}), perhaps because the concentration of SIN-1 used in our study was significantly lower than that used in preceding reports (*18*). Accordingly, lysates exposed to $ONOO^-$ or •NO readily gained aconitase activity upon reconstitution with cysteine and ferrous ammonium sulfate.

Neither •NO nor $ONOO^-$ activated IRP-1 in lysates derived from DFO-treated iron-depleted macrophages, in which aconitase had already undergone cluster disassembly and conversion to IRP-1 (cfr. Figure 6). In these samples, $ONOO^-$, but not •NO, actually decreased IRP-1 activity in a fashion that was only partially rescued by 2-ME; moreover, $ONOO^-$ precluded complete recovery of aconitase activity after treatment with cysteine and ferrous ammonium sulfate (cfr. Figure 6). While confirming data obtained with rIRP-1 exposed to SIN-1, these results had several implications. On one hand, these results reaffirmed that $ONOO^-$, and •NO, could activate IRP-1 through the disassembly of the Fe−S cluster of aconitase, a process that could not occur in cluster-deficient samples. On the other hand, these results indicated that the absence of Fe−S clusters allowed $ONOO^-$ to approach and to damage a significant fraction of -SH residues in the apoprotein. While extending earlier evidence for a "protective" action of the Fe−S cluster against chemical agents directed toward Cys^{437} (*18*, *37*), these observations also demonstrate that reactive nitrogen species can both activate and inactivate IRP-1, depending on whether they attack the Fe−S cluster of aconitase or -SH groups exposed by the cluster-free apoprotein, respectively. This dual mode of action, which depends on cell iron content, clearly provides new clues to reconcile previous diverging reports in the literature. In fact, unrecognized differences in cellular iron content, caused by different growth conditions, may result in important changes in the ratio of holo- to apo-protein. These changes, in turn, influence the mode of action of •NO and $ONOO^-$, explaining how IRP-1 modifications may vary from increase to decrease or no effect (see Table 1), even when reactive nitrogen species are delivered to cell types belonging to the same lineage, as in the case of J774A.1 and RAW 264.7 macrophages. Interestingly, •NO did not induce the concomitant loss of IRP-1 and of cysteine ferrous ammonium sulfate-activatable aconitase, which was induced by $ONOO^-$. This latter finding may be explained by the higher sulfhydryl-reactivity of $ONOO^-$ over •NO, producing sulfenic or sulfinic moieties that could not be converted back to -SH by reducing agents (*10*).

Whereas the effects of $ONOO^-$ and •NO on IRP-1 were strongly influenced by the iron status of the cell, concomitant changes in IRP-2 were insensitive to iron levels and resulted in a consistent pattern of irreversible inactivation by either nitrogen species. The unique susceptibility of IRP-2 to inactivation by both $ONOO^-$ and •NO probably reflects its inability to build Fe−S clusters, a factor favoring direct and extensive modifications of its -SH by chemical agents.

In summary, we have shown that $ONOO^-$ and •NO activate IRP-1 by disassembling the Fe−S cluster of cytoplasmic aconitase. Activation is replaced by inactivation in iron-depleted cells, in which the absence of Fe−S motifs facilitates direct attack of IRP-1 by reactive nitrogen species, most notably by $ONOO^-$. The cluster-free IRP-2 is constantly inactivated by both $ONOO^-$ and •NO. Therefore, our results provide new biochemical correlates to consider the experimental factors that govern modifications of IRPs by reactive nitrogen species. While providing information of a biochemical nature, the results described in this study may be of relevance also to pathophysiologic conditions. For example, recent studies in murine macrophages exposed to cytokines have shown that •NO formation is accompanied by an increase of the RNA binding activity of IRP-1 in face of a concomitant decrease of its levels of mRNA and immunoreactive protein (*38*). This paradoxical response suggests that processes of cluster disassembly, such as those described in our present study, probably are sufficient for switching a critical pool of aconitase to IRP-1, producing an increase of RNA binding activity that otherwise would be difficult to reconcile with a reduced level of protein expression. The functional consequences of IRP-1 activation are less obvious. In principle, an enhanced binding activity would repress ferritin translation and expand a cellular pool of free iron able to promote oxidative damage. The above-mentioned down-regulation of IRP-1 expression, induced by •NO, was therefore suggested to represent a compensatory mechanism, aimed at preventing the accumulation of potentially toxic-free iron (*38*). However, we and others have demonstrated that in mouse macrophages treated with cytokines the increased binding activity of IRP-1 was counteracted also by a strong inhibition of IRP-2 activity, leading to an increased ferritin content (*19*, *21*). The loss of IRP-2 activity, which is particularly abundant in macro-

phages, may therefore represent an additional important determinant of iron homeostasis in cells exposed to NO and related species. Accordingly, down-regulation of IRPs activity is observed also when cytokines are given to human monocytes/macrophages, an experimental model that resembles the downregulation of IRPs activity in patients with inflammation (*39*). The biologic ramifications of such a complex balance between IRP-1 activation versus IRP-2 inactivation clearly remain open to investigation. Here we have illustrated mechanisms that produce converse regulation of the two IRPs and may help to improve appraisal of the regulatory links between •NO or $ONOO^-$ and iron metabolism.

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