Recycling of RNA Binding Iron Regulatory Protein 1 into an Aconitase after Nitric Oxide Removal Depends on Mitochondrial ATP*

Iron regulatory proteins (IRPs) control iron metabolism by specifically interacting with iron-responsive elements (IREs) on mRNAs. Nitric oxide (NO) converts IRP-1 from a [4Fe-4S] aconitase to a trans-regulatory protein through Fe-S cluster disassembly. Here, we have focused on the fate of IRE binding IRP1 from murine macrophages when NO flux stops. We show that virtually all IRP-1 molecules from NO-producing cells dissociated from IRE and recovered aconitase activity after re-assembling a [4Fe-4S] cluster in vitro. The reverse change in IRP-1 activities also occurred in intact cells no longer exposed to NO and did not require de novo protein synthesis. Likewise, inhibition of mitochondrial aconitase via NO-induced Fe-S cluster disassembly was also reversed independently of protein translation after NO removal. Our results provide the first evidence of Fe-S cluster repair of NO-modified aconitases in mammalian cells. Moreover, we show that reverse change in IRP-1 activities and repair of mitochondrial aconitase activity depended on energized mitochondria. Finally, we demonstrate that IRP-1 activation by NO was accompanied by both a drastic decrease in ferritin levels and an increase in transferrin receptor mRNA levels. However, although ferritin expression was recovered upon IRP-1-IRE dissociation, expression of transferrin receptor mRNA continued to rise for several hours after stopping NO flux.

In the early nineties, the notion that iron-sulfur cluster proteins may play an important role in regulating gene expression began to emerge (1). Iron regulatory protein 1 (IRP-1),1 which controls intracellular iron homeostasis at a post-transcriptional level, is one of the best illustrations. Once properly activated, IRP-1 specifically binds to one or several iron-responsive element(s) (IRE) located in the untranslated regions (UTRs) of several mRNAs, including those encoding ferritin, mitochondrial aconitase, and transferrin receptor (Tf-R). The binding of IRP-1 to IRE in the 5′-UTR of ferritin or mitochondrial aconitase mRNA blocks its translation, whereas binding of IRP-1 to at least three of the five IREs in the 3′-UTR of Tf-R mRNA stabilizes it. Remarkably, IRP-1 is a bifunctional protein that in resting cells displays a [4Fe-4S] cluster at the active site and consequently exhibits an aconitase activity in the cytosol. When cellular iron becomes limiting, cytosolic aconitase activity disappears, whereas IRP-1 progressively gains the capacity to bind IREs. A second IRP, named IRP-2, also binds to IRE motifs in response to iron depletion but does not have aconitase activity because of its incapacity to assemble an Fe-S cluster (for review, see Ref. 2).

It is now well known that nitric oxide (NO) can also modulate IRP-1 (3, 4). Moreover, several lines of evidence indicate that activation of IRP-1 by NO is followed by repression of ferritin translation and an increase in Tf-R mRNA levels (4, 5). In contrast to iron depletion, NO quickly modulates IRP-1 activity at a post-translational level (6, 7). NO rapidly converts IRP-1 from aconitase to its trans-regulatory form without requiring de novo protein synthesis (6). Its primary target site is the Fe-S cluster, because it has no effect on IRP-1 from cytosol that had been pretreated with an excess of aconitase substrates (e.g. citrate or cis-aconitate) known to interact directly with the labile iron of the cluster (8, 9). Subsequently, electron paramagnetic resonance studies have definitively established that NO promotes the cluster disassembly of the protein (10). At this juncture, one of the challenging issues ahead was to investigate the fate of the trans-regulatory form of IRP-1 once NO flux stops. This question addresses the still ill-defined cell machinery involved in iron-sulfur cluster (ISC) assembly in mammals. Recent results using yeast indicate that mitochondria are most probably the primary site of the biogenesis of both mitochondrial and cytosolic Fe-S cluster proteins (11–13). Yet in mammalian cells, components of the ISC machinery have recently been characterized in extra-mitochondrial compartments (14, 15). Taking these recent data into consideration and to understand better the impact of NO on proteins whose mRNAs are under the control of the IRP/IRE system, we have investigated the possible Fe-S cluster repair of NO-modified IRP-1 when NO flux stops. In parallel, we have analyzed the consequences of this regulation for ferritin and mitochondrial aconitase expression as well as Tf-R mRNA level. In addition to IRP-1, we have also explored the fate of the related mitochondrial Fe-S aconitase when cells were no longer exposed to NO and studied the participation of energized mitochondria in the recovery of their aconitase activity.

EXPERIMENTAL PROCEDURES

Materials—Spermine NONOate (SPER/NO), DPTA NONOate (DPTA/NO), and DETA NONOate (DETA/NO) were from Cayman Chemical (Ann Arbor, MI). Cycloheximide (CHX), carbonyl cyanide m-chlorophenylhydrazone (CCCP), LD-L 10 kit, and oligomycin were from Sigma.
Cell Culture and Treatments—The RAW 264.7 macrophage cell line was cultured in high glucose Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (Invitrogen). Cells were incubated with different NONOates for the times indicated in the figures and washed twice with phosphate-buffered saline. Cells were then treated with iron, sulfide, or CHX or CCCP as indicated in the figure legends. At the indicated times, cells were harvested, and both cytosol and mitochondria-enriched fractions were prepared. To determine the expression of Tf-R mRNA, total RNAs were extracted in parallel experiments.

Preparation of Mitochondria-enriched Fraction—RAW 264.7 cells (5 × 10^6/ml) were harvested and supernatants were kept at 4°C. The cytosol-enriched pellet was treated with a lysis buffer composed of 100 mM Tris, pH 7.5, 0.5% Triton X-100, and 20 μg/ml phenylmethylsulfonyl fluoride. After 20 min on ice, the lysate was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was immediately treated for mitochondrial aconitase activity and aliquots were kept at −80°C for Western blot analysis. Cytosolic contamination of mitochondria-enriched fractions was determined by measuring the amount of lactate dehydrogenase activity associated with the mitochondria-enriched fraction (kit LD-L 10, Sigma Diagnostics). This amount value did not exceed 5%. In control experiments, we also checked by Western blot that IRP-1 protein was not detectable in mitochondria-enriched fractions.

Preparation of Cytoplasmic Extracts—RAW 264.7 cell monolayers were harvested in 250 μl of 0.25 M sucrose buffered with 100 mM HEPES, pH 7.4. The cell suspension was then treated with 0.007% digitonin as described (16). The mitochondria-enriched pellet was treated with a lysis buffer composed of 100 mM Tris, pH 7.5, 0.5% Triton X-100, and 20 μg/ml phenylmethylsulfonyl fluoride. After 20 min on ice, the lysate was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was immediately treated for mitochondrial aconitase activity and aliquots were kept at −80°C for Western blot analysis. Cytosolic contamination of mitochondria-enriched fractions was determined by measuring the amount of lactate dehydrogenase activity associated with the mitochondria-enriched fraction (kit LD-L 10, Sigma Diagnostics). This amount value did not exceed 5%. In control experiments, we also checked by Western blot that IRP-1 protein was not detectable in mitochondria-enriched fractions.

Preparation of RNA Transcripts—Transcription reactions were performed in vitro with 1 or 2 μg of either linearized pSPT-TR11 (corresponding to the 2.3-kilobase 3′-UTR of the human Tf-R) or linearized pSPT-fer (corresponding to the human ferritin H-chain IRE (kindly provided by Dr. L. C. Kuhn, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland), which was labeled by the random hexamer priming method (Invitrogen). Blots were washed twice in 2× standard saline phosphate/EDTA (0.15 m NaCl/10 mM phosphate, pH 7.4, 1 mM EDTA) for 15 min at room temperature and then twice in 0.1× standard saline phosphate/EDTA at 55°C for 15 min before exposing to phosphorimaging. Blots were also re-probed with a 1.3-kilobase PstI fragment of glyceraldehyde-3-phosphate dehydrogenase cdNA as a loading control and washed as above except for the last two washes, which were performed at 65°C.

Aconitase Activity—Mitochondrial and cytosolic aconitase activities were determined spectrophotometrically at 240 nm by following the disappearance of cis-aconitate, which is the intermediate substrate of aconitases, at 37°C. Fifty micrograms of protein were used in 100 mM Tris, pH 7.4, 2.5 mM magnesium chloride, and DTT further attenuated IRP-1-IRE binding activity and aliquots were kept at −80°C for further measurements.

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Measurement of Protein Synthesis—Cells were cultivated with various concentrations of CHX (250, 500, and 1000 ng/ml) in leucine-starved Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum at 37°C in 5% CO2 humidified atmosphere and treated with the uncoupler of oxidative phosphorylation CCCP for 6 h. Control or CCCP-treated cells (10 × 10^6/ml) were treated with a pulse-chased two wave experiment, sampling, washing, and rescaling of 10 μg/ml H-chain ferritin, pH 7.2, 20 mM sucrose, 1.2 mM KH2PO4, 118 mM NaCl, 4.8 mM KCl, 1 mM CaCl2, and then centrifuged at 232 × g for 10 min. The cell pellet was resuspended in 1 ml of pre-warmed respiration buffer and homogenized with constant stirring in the respiration chamber at 37°C during measurements. During the oxygen consumption measurement, the mitochondrial ATPase inhibitor oligomycin (10 μM) was added to the chamber.

RESULTS

In Vitro Reconstitution of the [4Fe-4S] Cluster into IRE Binding IRP-1 from NO-producing Macrophages—We first investigated the possible in vitro repair of the [4Fe-4S] cluster in IRE binding IRP-1 from RAW macrophages previously stimulated by IFN-γ/LPS to produce NO. Nitrite production was verified by nitrite accumulation in the culture medium (data not shown). Cytosols from control or NO-producing cells were extracted and incubated with a source of ferrous iron and sulfide in the presence of DTT. After 20 min in anaerobic atmosphere, cytosols were tested for their IRP-1-IRE binding and aconitase activities. In the IRP-1-IRE binding assay, samples were continuously treated with 2% 2-ME in a syringe experiment to express full IRE binding activity (Fig. 1A, lower panel). Untreated cytosols from control cells presented low IRP-1-IRE binding activity and only a chicken polyclonal antibody raised against purified human recombinant IRP-1 (Agro-Bio, La Ferté Saint-Aubin, France).

Northern Blot Analysis—Total RNA from RAW 264.7 macrophages was extracted using the TRIzol® reagent (Invitrogen). Equal amounts of RNA (20 μg) were electrophoresed at 61 mV for 2–3 h in a 1% agarose gel and transferred to a positively charged nitrocellulose membrane (Pall Corp. Ann Arbor, MI). After cross-linking of the RNA to the membrane by UV light and prehybridization for at least 1 h with Hybrizol 1 solution (Intergen Co., NY), membranes were probed overnight with the 2.3-kilobase EcoRI fragment of the murine Tf-R cDNA (kindly provided by Dr. L. C. Kuhn, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland), which was labeled by the random hexamer priming method (Invitrogen). Blots were washed twice in 2× standard saline phosphate/EDTA (0.15 m NaCl/10 mM phosphate, pH 7.4, 1 mM EDTA) for 15 min at room temperature and then twice in 0.1× standard saline phosphate/EDTA at 55°C for 15 min before exposing to phosphorimaging. Blots were also re-probed with a 1.3-kilobase PstI fragment of glyceraldehyde-3-phosphate dehydrogenase cdNA as a loading control and washed as above except for the last two washes, which were performed at 65°C.

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Reverse Change in IRP-1 Activities after Stopping NO Flux

Fig. 1. In vitro reconstitution of the [4Fe-4S] cluster in IRP-1 from IFN-γ/LPS-treated RAW 264.7 macrophages. Cells were exposed to 20 units/ml IFN-γ and 50 ng/ml LPS for 16 h. Cytosols from control or IFN-γ/LPS-treated cells were then extracted. A and B, reconstitution of the [4Fe-4S] cluster in IRP-1 was carried out by the addition of 125 μM ferrous ammonium sulfate (Fe²⁺), 125 μM sodium sulfide (S²⁻), and 10 mM DTT to 100 μg of control or IFN-γ/LPS-treated cell cytosol in 300 μl of 20 mM HEPES, pH 7.5, 5% glycerol, and 20 mM KCl. All reactions were performed at room temperature under anaerobic atmosphere for 20 min. Two μg of protein of each reaction were then analyzed for IRP-1-IRE binding activity (panel A) as described under “Experimental Procedures.” In panel B, IRP-1-IRE binding activity was quantified by phoshorimaging and expressed as percent of the value obtained with 2% 2-ME (open bars). In parallel, 50 μg of protein were transferred to an open-top cuvette for UV spectroscopy to measure aconitase activity (filled bars). C, IRP-1 levels were analyzed by Western blotting as described under “Experimental Procedures” and quantified by densitometry. Purified recombinant IRP-1 (rIRP-1) was used as the positive control. The experiments were performed four times, and a representative result is shown.

increased aconitase activity to 41.1 units/mg (Fig. 1A, lane 2, and B). In contrast, cytosols from NO-producing cells showed full IRP-1-IRE binding activity and no aconitase activity (Fig. 1A, lane 3, and B). Interestingly, we observed that treatment of these cytosols with chelators, sulfide, and DTT almost completely abolished IRP-1 affinity toward IRE (Fig. 1A, compare lane 4 to lane 3). This drastic decrease in IRE binding was not observed if one of the components used for the Fe-S cluster reconstitutition was missing (data not shown). In parallel to the loss of IRE binding by IRP-1, cytosolic aconitase activity resumed and reached up to 50% that of control cytosols (Fig. 1B, black bars). Based on recent data showing down-regulation of IRP-1 gene expression by NO (19), we measured the IRP-1 expression level under our experimental conditions. In fact, the IRP-1 protein level was decreased by 57% in NO-producing cells (Fig. 1C), explaining why only half of aconitase activity was recovered. These data show that virtually every IRP-1 from NO-producing cells had their Fe-S cluster repaired. We also showed that in vitro repaired Fe-S clusters were stable for at least 2 h in air, as indicated by the constant level of aconitase activity (data not shown).

Change in IRP-1 Activities after Stopping Exposure of Cells to Exogenous NO—To investigate the change in IRP-1 structure/activities in intact cells when NO flux stops, we used NONOates because of their predictable and various rates of NO release. To activate IRP-1, we first exposed cells to three different NONOates, SPER/NO, DPTA/NO, and DETA/NO. Under our experimental conditions, they released NO linearly at different rates, 3–4, 1.5, and 0.4 μM/min, respectively. We showed that the fast NO-releasing SPER/NO led to a rapid activation of IRP-1 within 2 h (Fig. 2A, upper row, compare lane 6 to lane 1). Aconitase activity was strongly decreased, corresponding to 38% of that of the control (right panel of A). DPTA/NO triggered significant activation of IRP-1 within 4 h (Fig. 2B, compare lane 5 to lane 1) with a remaining aconitase activity of 22% of the control (right panel of B). Finally, DETA/NO, whose rate of NO release is similar to that of inducible NO synthase (20), allowed strong activation of IRP-1 within 16 h (Fig. 2C, compare lane 4 to lane 1) and showed a remaining aconitase activity of 19% of the control (right panel of C). After removing NO donors by extensive washing, we measured both aconitase and IRE binding activities of IRP-1 at the indicated times. IRP-1-IRE binding activity progressively decreased (Fig. 2, left panels) while the cells were recovering cytosolic aconitase activity (Fig. 2, right panels). This reverse change in IRP-1 was apparent within the first hours after NO removal whatever the rate of NO release previously generated to activate IRP-1. NO also inhibits mitochondrial aconitase through disruption of its [4Fe-4S] cluster (10, 21). We also showed that DPTA/NO as a source of NO gave the same results (data not shown).

To investigate whether resumption of IRP-1 and mitochondrial aconitase activities requires de novo protein synthesis, CHX was added to cell culture medium. As previously shown, a 16-h preincubation of macrophages with DETA/NO was accompanied by loss of cytosolic aconitase activity (5% of control, black bars) and showed a remaining aconitase activity of 19% of the control (right panel of Fig. 2A) while the cells were recovering cytosolic aconitase activity (Fig. 2A, right panels). This reverse change in IRP-1 was apparent within the first hours after NO removal whatever the rate of NO release previously generated to activate IRP-1. NO also inhibits mitochondrial aconitase through disruption of its [4Fe-4S] cluster (10, 21). Therefore, we also tested the possible restoration of its activity under the same experimental conditions. Mitochondrial aconitase from NO-treated cells was quickly recovered after stopping NO flux (data not shown).

Based on recent data showing down-regulation of IRP-1 gene expression by NO (19), we measured the IRP-1 expression level under our experimental conditions. In fact, the IRP-1 protein level was decreased by 57% in NO-producing cells (Fig. 1C), explaining why only half of aconitase activity was recovered. These data show that virtually every IRP-1 from NO-producing cells had their Fe-S cluster repaired. We also showed that in vitro repaired Fe-S clusters were stable for at least 2 h in air, as indicated by the constant level of aconitase activity (data not shown).
Effect of Mitochondria De-energization on IRP-1 and Mitochondrial Aconitase Activity Repair—Bio-synthesis of extra-mitochondrial Fe-S cluster proteins in yeast has been found to depend on an ATP binding cassette (ABC) transporter located in the inner membrane of mitochondria (22). Accordingly, we analyzed whether the reverse change in IRP-1 activities as well as the activity repair of the mitochondrial aconitase after DETA/NO removal required an ATP-dependent mechanism from mitochondria. As described above, DETA/NO was removed, and the cells were then treated with an uncoupler of oxidative phosphorylation, CCCP. In a preliminary experiment, we showed that it instantaneously de-energized mitochondria. We then performed a time-course experiment to follow the activity of both IRP-1 and mitochondrial aconitase. We showed that recovery of aconitase activity was markedly delayed in the cytosol of CCCP-treated cells as compared with untreated cells (left panel of Fig. 4A, compare the white bars to the black bars). Conversely, the high IRE binding activity of IRP-1 resulting from the treatment by DETA/NO remained elevated in CCCP-treated cells at least 6 h after stopping NO flux (data not shown). Recovery of mitochondrial aconitase activity was also delayed in CCCP-treated cells but to a lesser extent (right panel of Fig. 4A, compare white bars to black bars). In parallel experiments, the uncoupler CCCP was tested for its efficiency on mitochondria de-energization (Fig. 4B). Results show that CCCP was effective during the 6-h recovery phase, as indicated by the unchanged oxygen consumption of CCCP-treated cells after inhibition of ATP synthesis by oligomycin.

Recovery of Initial IRP-1 Activities Correlates with Resumption of High Levels of Ferritin—We investigated whether inactivation of IRP-1 trans-regulatory activity was followed by a change in expression of proteins whose mRNA contains an IRE sequence in their 5′-UTR. We first incubated RAW macrophages with DETA/NO for 16 h and analyzed their cytosols for aconitase activity and IRE binding by IRPs using the ferritin IRE as a probe. In parallel, we measured the ferritin expression level. We showed that both down-regulation of IRP-2-IRE binding and activation of IRP-1-IRE binding in DETA/NO-treated cells resulted in severe reduction of ferritin expression (Fig. 5). Six hours after NO donor removal, loss of IRP-1 in its capacity to bind ferritin IRE was directly correlated with resumption of ferritin translation (Fig. 5C, compare lane 4 to lane 2). Noteworthy, loss of IRE binding activity of IRP-2 in response to NO was not reversed after stopping NO flux. In parallel, the level of mitochondrial aconitase, whose mRNA also has an IRE in its 5′-UTR (23, 24), was measured. Despite activation of IRP-1 to bind IRE, the level of this protein was not reduced in response.
Reverse Change in IRP-1 Activities after Stopping NO Flux

...to an overnight stimulation by NO and remained unchanged 6 h after stopping NO flux once IRP-1 was inactivated (Fig. 5C, lower panel).

Resumption of IRP1 Activities and Consequences for Tf-R mRNA Level—Tf-R mRNA contains five IRE sequences in its 3’-UTR (25). We therefore studied the regulation of its expression after the change in IRP-1 activities induced by the “on/off” NO flux signal described above. It is worth noting that in this set of experiments, IRP-1 and IRP-2-IRE binding capacity was assessed using the entire 3’-H11032-UTR that contained the five IRE sequences of the Tf-R as a probe. In parallel to cytosolic extraction, total mRNAs were extracted and analyzed for Tf-R expression. We first observed that the capacity of IRP-2 to bind Tf-R IREs was not significantly modified in control and DETA/NO-exposed cells (Fig. 6A, compare lane 2 to lane 1). In contrast, we observed that exposure of macrophages to DETA/NO resulted in a strong increase in IRP-1 binding capacity toward Tf-R IRE sequences that was accompanied by an enhanced Tf-R mRNA expression (Fig. 6, panel A and C). After 6 additional hours without DETA/NO, IRP-1 no longer interacted with Tf-R IREs and recovered aconitase activity (Fig. 6A, compare lane 4 to lane 2, and panel B), but surprisingly, this was not accompanied by a decrease in Tf-R mRNA level. Actually, Tf-R mRNA expression continued to increase even 10 h after exposure to NO was ended (data not shown).

DISCUSSION

Whereas the mechanism of IRP-1 activation in response to NO has been progressively disclosed, the inactivation process of IRP-1 when cells are no longer exposed to NO has never been characterized. Investigating this pathway is crucial to understanding the real impact of NO on proteins whose mRNAs contain the IRE motif(s). In this report, we first showed that in addition to iron and DTT, sulfide was required to ensure both IRP-1-IRE dissociation and recovery of aconitase activity. As documented earlier by Kennedy et al. (26), this observation strongly suggests that NO-modified IRP-1 from activated macrophages is an apoprotein that can be readily reactivated through repair and reinsertion of its [4Fe-4S] cluster. Interestingly, we have observed that recovery of IRP-1 aconitase activity also occurred in intact cells as soon as they were relieved of the NO burden, whatever the time course and the rate of NO formerly generated. Because recovery did not require de novo protein synthesis, we propose that IRE binding IRP-1 could be recycled into an aconitase through reassembly and reinsertion...
of the Fe-S cluster into the backbone of the protein upon NO removal (Fig. 7). Along the same lines, we noticed that the presence of a labile protein(s) may be necessary to keep the IRP-1-IRE interaction, because the translation inhibitor cycloheximide favored the IRP-1-IRE dissociation in cells relieved of NO (Fig. 3B). Taking into account this result, we are now seeking new partner proteins and/or chaperones that could participate in the IRP-1 activation/deactivation process in response to the on/off NO flux signal. We also investigated the repair activity of the related mitochondrial aconitase after stopping NO exposure. This enzyme, whose Fe-S cluster and in turn enzymatic activity are altered by NO (10, 18, 21), regained its function easily as soon as NO flux stopped without requiring protein translation. Again, these in vivo experiments point to a reinsertion of a fully assembled [4Fe-4S] cluster into NO-modified mitochondrial aconitase. Altogether, our results also indicate that cellular factors that participate in aconitase recovery were left intact during NO exposure and were therefore available to reconstitute IRP-1 and mitochondrial aconitase initial functions once NO flux stopped.

It is now clear that Fe-S cluster formation and repair do not occur spontaneously in vivo. In yeast and bacteria, the ISC (iron-sulfur cluster assembly) machinery, which comprises some 10 specific proteins, is responsible for both iron and elemental sulfur delivery and (re)assembly of Fe-S clusters (11). Interestingly, it was recently reported that the ISC machinery, in particular the NifS-like IscS protein, could efficiently repair the NO-modified ferredoxin [2Fe-2S] cluster in Escherichia coli (27). It is, thus, reasonable to assume that the recently identified mammalian NifS-like proteins (14, 15, 28) may participate in the Fe-S cluster repair of NO-modified IRP-1 and mitochondrial aconitase. As previously mentioned, Kissel et al. (22) also found that maturation of cytosolic Fe-S cluster-containing proteins of Saccharomyces cerevisiae depends on an ABC transporter, named Atm1p, located in the inner membrane of mitochondria. It has been reported that this transporter exports Fe-S clusters previously constituted in the matrix of mitochondria to the cytosolic compartment. Importantly, a mitochondrial ABC transporter named ABC7 has also been identified in mouse and human as the functional ortho-

**FIG. 5.** Effect of IRP-1 deactivation on ferritin and mitochondrial aconitase levels. Cells were pretreated with 0.5 mM DETA/NO for 16 h. Cytosols from control (lane 1) or DETA/NO-treated cells (lane 2) were then extracted. In a parallel experiment, the medium of control (lane 3) or DETA/NO-treated cells (lane 4) was replaced with fresh medium for an additional 6 h before extracting cytosols. A, analysis of IRP-1 binding activity using the ferritin IRE probe (IRE-Ft). B, measurement of cytosolic aconitase activity. The enzymatic activity is expressed as a percent of the appropriate control (%/control). C, expression of cytosolic ferritin and mitochondrial aconitase (mt-aco) levels. Equal amounts of protein (10 μg) were subjected to Western blot analysis using an anti-ferritin and anti-mitochondrial aconitase antisera. Purified mouse liver ferritin and mitochondrial aconitase (Sigma) were used as positive control (lanes 5). The experiments were performed four times, and a representative result is shown.

**FIG. 6.** Effect of IRP-1 deactivation on Tf-R mRNA expression. Cells were pretreated with 0.5 mM DETA/NO for 16 h. Control (lane 1) and DETA/NO-pretreated cell cytosols (lane 2) were then extracted. In a parallel experiment, the medium of control cells (lane 3) and DETA/NO-pretreated cells (lane 4) was replaced with fresh medium for an additional 6 h before extracting cytosols. A, analysis of IRP-1 and IRP-2-IRE binding activity using the entire 3′-UTR of Tf-R mRNA as probe (TR 5-IREs). B, cytosolic aconitase activity was measured spectrophotometrically at 240 nm as described under “Experimental Procedures.” C, in a parallel experiment, cells were treated as in panel A, and total mRNA was extracted. Twenty µg of total mRNA extracts were probed with the 2.3-kilobase fragment of the Tf-R cDNA (upper panel of C). The membranes were rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (lower panel of C). The experiments were performed four times, and a representative result is shown.
ferritin is rapidly restored (5). As a result, a high level of dependent process (5) and, via a mitochondrial ATP-removal, IRP1 dissociates from IRE (protein(s). As a consequence, ferritin translation is repressed. Upon NO fermentation of the [4Fe-4S] cluster allows IRP-1 to function as an aconitase. In the cytosol of resting cells, a fully assembled [4Fe-4S] cluster is intact, NO in this context proves an authentic regulator rather than a determinant role of IRP-1 in this regulation versus IRP-2. Moreover, in macrophages re-incubated in fresh culture medium after 16 h of exposure to DETA/NO, the inability of IRP-1 to bind the ferritin IRE motif perfectly correlated with reappearance of ferritin expression as illustrated in Fig. 7. Because inhibition of ferritin translation increases sensitivity to oxidative stress (37–38), it is conceivable that the NO-dependent modulation of IRPs strengthens the macrophage array of weapons against intracellular pathogens.

In contrast to ferritin and despite a functional IRE in its mRNA (24, 39), the level of mitochondrial aconitase was not modulated in response to overnight exposure to DETA/NO. This could be explained by the fact that mitochondrial aconitase is a very stable protein with a half-life >16 h in RAW 264.7 cells (data not shown). Therefore, regulation of mitochondrial aconitase via the IRP/IRE system, if any, could be visible only after several days, in line with what has been described in mice maintained for several weeks on a low iron diet (39).

Lastly, to investigate whether IRP-1 activation/deactivation is correlated with regulation of T-F-R expression level, we analyzed changes in IRP activities in macrophages exposed to our experimental on/off NO flux signal. As expected, IRP-1-IRE binding greatly increased in response to NO, but surprisingly, binding of IRP-2 to T-F-R-specific IREs was unchanged. This exclusive IRP-1 activation was accompanied by a noticeable increase in T-F-R mRNA levels. Strikingly, the level of this mRNA continued to increase even after 10 h of cell culture in the absence of NO and despite the fact that IRP-1 had long been dissociated from IREs and had recovered an aconitase activity. This unexpected high expression of T-F-R mRNA indicates that it is not solely controlled post-transcriptionally by IRPs. This NO-dependent steady up-regulation requires additional mechanisms that deserve further investigation, but some clues are already appealing. Recently, it has been shown that NO induces accumulation of the transcription factor hypoxia-inducible factor 1 (40). Assuming that a hypoxia response element, the binding site for hypoxia-inducible factor 1, has been identified in the T-F-R promoter region (41), the continuous increase in T-F-R mRNA could be explained by a long-lasting activation of hypoxia-inducible factor 1 by NO. However, preliminary studies using transcription inhibitors suggest that T-F-R mRNA remains stable after DETA/NO removal. It is, thus, tempting to speculate that NO may interfere with the machinery involved in the degradation of T-F-R mRNA. Unfortunately, this process is still largely unknown, and to date only polymerase III transcripts have been suspected to be involved (42).

In conclusion, our results demonstrate that fluctuation of NO levels regulates IRP-1 by inducing reversible post-translational modifications that allow the same molecule of IRP-1 to commute quickly between two activities. One function, aconitase, is lost, but another, IRE binding capacity, is gained and lasts as long as NO flux is sustained. Upon removal of the latter, the process is reversible, and initial activities are recovered. By leaving the protein undamaged, ready to be reactivated as aconitase after [4Fe-4S] cluster re-insertion by a machinery left intact, NO in this context proves an authentic regulator rather than a somewhat toxic effector molecule. Like a hormone, NO has a short half-life. In addition, NOS dimerization, which is necessary to synthesize NO, can be interrupted under various pathophysiological conditions affecting availability of heme.

To understand better the role of NO in iron and energy metabolism, we then analyzed whether the expression of ferritin and mitochondrial aconitase, whose mRNAs contain an IRE motif, matched the IRP-1 activation/deactivation process induced by the on/off NO flux signal. Interestingly, we noticed that DETA/NO mimicked the effect of stimulation by IFN-γ/LPS on macrophage IRP-1 and IRP-2 activities (6, 35, 36). Therefore, use of DETA/NO by avoiding interference due to the immunological stimuli proved a relevant approach to examining the real impact of both IRPs on the control of iron metabolism in response to NO. We showed that IRP-1 activation combined with concomitant IRP-2 inactivation was accompanied by a complete inhibition of ferritin expression. This points to a determinant role of IRP-1 in this regulation versus IRP-2. Moreover, in macrophages re-incubated in fresh culture medium after 16 h of exposure to DETA/NO, the inability of IRP-1 to bind the ferritin IRE motif perfectly correlated with reappearance of ferritin expression as illustrated in Fig. 7. Because inhibition of ferritin translation increases sensitivity to oxidative stress (37–38), it is conceivable that the NO-dependent modulation of IRPs strengthens the macrophage array of weapons against intracellular pathogens.

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\[\text{Fe-S reassembly-reinsertion}\]

\[\text{mitochondrial pool}\]

\[\text{ATP}\]

\[\text{cytosol}\]

\[\text{Fe-S reassembly-reinsertion}\]

\[\text{mitochondrial pool}\]

\[\text{ATP}\]

\[\text{cytosol}\]

\[\text{Fe-S reassembly-reinsertion}\]

\[\text{mitochondrial pool}\]

\[\text{ATP}\]

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\[\text{Fe-S reassembly-reinsertion}\]

\[\text{mitochondrial pool}\]

\[\text{ATP}\]

\[\text{cytosol}\]
tetrahydrobiopterin, or L-arginine (43). It is thus well established that in vivo, L-arginine is depleted in inflammatory settings or microbial infection as a consequence of arginase activity (44–46). Cells must adjust to NO rise and fall, and some flexible transducer molecules have to cope with this obligate metabolic challenge. Our data support such a special role for IRP-1.

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Recycling of RNA Binding Iron Regulatory Protein 1 into an Aconitase after Nitric Oxide Removal Depends on Mitochondrial ATP

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