Effects of Interferon-γ and Lipopolysaccharide on Macrophage Iron Metabolism Are Mediated by Nitric Oxide-induced Degradation of Iron Regulatory Protein 2*

(Received for publication, September 24, 1999, and in revised form, November 12, 1999)

Sangwon Kim and Prem Ponka†

From the Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital and Departments of Physiology and Medicine, McGill University, 3755 Cote-St-Catherine Road, Montreal, Quebec H3T 1E2, Canada

Iron regulatory proteins (IRP-1 and IRP-2) control the synthesis of transferrin receptors (TfR) and ferritin by binding to iron-responsive elements, which are located in the 3′-untranslated region and the 5′-untranslated region of their respective mRNAs. Cellular iron levels affect binding of IRPs to iron-responsive elements and consequently expression of TfR and ferritin. Moreover, NO, a redox species of nitric oxide that interacts primarily with iron, can activate IRP-1 RNA binding activity resulting in an increase in TfR mRNA levels. Recently we found that treatment of RAW 264.7 cells (a murine macrophage cell line) with NO+ (nitrosourea ion, which causes S-nitrosylation of thiol groups) resulted in a rapid decrease in RNA binding of IRP-2 followed by IRP-2 degradation, and these changes were associated with a decrease in TfR mRNA levels (Kim, S., and Ponka, P. (1999) J. Biol. Chem. 274, 33035-33042). In this study, we demonstrated that stimulation of RAW 264.7 cells with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) increased IRP-1 binding activity, whereas RNA binding of IRP-2 decreased and was followed by a degradation of this protein. Moreover, the decrease of IRP-2 binding/protein levels was associated with a decrease in TfR mRNA levels in LPS/IFN-γ-treated cells, and these changes were prevented by inhibitors of inducible nitric oxide synthase. Furthermore, LPS/IFN-γ-stimulated RAW 264.7 cells showed increased rates of ferritin synthesis. These results suggest that NO+-mediated degradation of IRP-2 plays a major role in iron metabolism during inflammation.

Macrophages stimulated by cytokines, such as interferon-γ (IFN-γ),1 or microbial components, such as lipopolysaccharide (LPS), take part in the nonspecific resistance against pathogens (1). It is now well established that cytostatic and cytotoxic effects of murine macrophages are caused in part by nitric oxide (NO) produced enzymatically by the stepwise oxidation of l-arginine to NO and l-citrulline (2, 3). The cytotoxic function of NO requires that it is produced in relatively large quantities in macrophages and in other cells and this is accomplished by inducible NO synthase (iNOS) (3). Many of the cytotoxic effects of NO can be explained by its reactivity with iron in the active sites of critically important molecules such as mitochondrial aconitase, ribonucleotide reductase, and the [Fe-S] proteins of the mitochondrial respiratory system (reviewed in Refs. 3 and 4). Moreover, the identification of the [4Fe-4S] cluster of mitochondrial aconitase as a target susceptible to inactivation by NO suggested that NO may also be involved in the regulation of iron regulatory protein (IRP)-1, which is closely related to mitochondrial aconitase (5–7). Although many actions of NO are mediated by its binding to either non-heme or heme iron (8), this metal is not the only target for NO. The oxidized form of NO, the nitrosion ion (NO•), can S-nitrosylate proteins via their thiol groups, a modification that may have important regulatory functions (9–14).

IRP-1 and IRP-2 are cytoplasmic proteins known to interact with specific nucleotide sequences, known as iron-responsive elements (IREs) (reviewed in Refs. 15–19), which are located in the 5′-untranslated region of ferritin mRNA (20) and the 3′-untranslated region of transferrin receptor (TfR) mRNA (21). The interactions of IRPs with IREs control cellular iron metabolism in the following manner: When cellular iron becomes limiting, IRP-2 is present in the cytosol, and the IRP-1 recruited into the high affinity binding state. The binding of IREs to the IRP in the 5′-untranslated region of the ferritin mRNA blocks the translation of ferritin, whereas an association of IRPs with IREs in the 3′-untranslated region of TfR mRNA stabilizes this transcript. On the other hand, when intracellular iron is plentiful, IRP-1 is unable to bind to IREs, IRP-2 is degraded, and this results in an efficient translation of ferritin mRNA and rapid degradation of TfR mRNA (reviewed in Refs. 15–19).

IRP-1 has been shown to share 30% homology with mitochondrial aconitase (5), and 18 active-site residues of mitochondrial aconitase are conserved in IRP-1, including 3 cysteines (22, 23). In iron-starved cells, IRP-1 lacks the [4Fe-4S] cluster as well as aconitase activity and has the IRE binding activity. On the other hand, in iron-replete cells IRP-1 has an intact [4Fe-4S] cluster and aconitase activity, but lacks the IRE binding activity (16, 19, 24). The second IRE-binding protein, IRP-2, shares 62% amino acid sequence identity with IRP-1 (25) but differs in a unique 73-amino acid insertion in its N-terminal region (26), which is responsible for the degradation of the protein via the ubiquitin-proteasome pathway (26–28) in iron-replete cells. Because IRP-1 is homologous to mitochondrial aconitase (5–7), whose activity is modulated by NO (4, 18, 19, 29), it is not surprising that the biological activity of IRP-1 can also be affected by NO (29). Indeed, several studies have demonstrated that NO can activate IRP-1 RNA binding activity (reviewed in Refs. 18 and 19) (30–33), and it has been proposed
that NO can ligate to the [4Fe-4S] center, disrupt the cluster, and consequently activate RNA binding activity of IRP-1 (reviewed in Ref. 18).

Numerous investigators examined the effects of NO on ferritin synthesis and TfR mRNA or protein levels, but the results obtained are notably controversial. Whereas some investigators observed that NO caused a marked decrease in ferritin synthesis (31, 34) (as would be predicted based on NO-mediated activation of IRP-1 RNA binding activity), others reported that NO increased ferritin synthesis and accumulation (35–37). These latter findings are in agreement with numerous studies showing that inflammation and inflammatory cytokines stimulate ferritin synthesis (38, 39). Similarly, according to some reports (32, 33, 37), a NO-mediated increase in IRE binding activity of IRP-1 is associated with increases in TfR mRNA levels. However, these observations are in apparent conflict with studies showing that treatment of macrophages with IFN-γ and LPS (known to cause iNOS induction followed by increased NO production) leads to a dramatic decrease in TfR mRNA. Importantly, these changes occurred with increases in IRP-2 binding/levels was associated with a dramatic decrease in IRP-2 binding by disrupting the cluster (18, 19, 29–33), a condition known to stabilize TfR mRNA. On the other hand, we have concluded that treatment of K562 cells with an NO− donor resulted in S-nitrosylation of critical thiol groups that may prevent binding of IRP-1 to the IRE, leading to TfR mRNA degradation (32). Our more recent study yielded data indicating that IRP-2 may also be a very important and unexpected target for NO+ (43, 44). We found that a 1-h exposure of RAW 264.7 cells (a murine macrophage cell line) to a NO+ generator caused a significant decrease in IRP-2 binding to the IRE, followed by IRP-2 degradation, probably in proteasomes. The decrease in IRP-2 binding/levels was associated with a dramatic decrease in TfR mRNA. Importantly, these changes occurred at a time at which TfR binding activity of IRP-1 was not yet decreased by NO− treatment (43, 44), indicating that IRP-2 alone plays a crucial role in controlling TfR mRNA levels. In this study, we found that stimulation of RAW 264.7 cells with IFN-γ and LPS caused a strong reduction of IRP-2 activity, accompanied by a dramatic drop in TfR mRNA levels, changes similar to those occurring in NO−-treated RAW 264.7 cells (44). Hence, our results suggest that treatment of macrophages with IFN-γ and LPS may lead to S-nitrosylation of IRP-2 followed by its inactivation and degradation, a condition which, in turn, causes the decrease in TfR mRNA levels.

MATERIALS AND METHODS

Cells—RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Rockville, MD) and grown in 100-cm2 plastic culture flasks (Life Technologies, Inc.) in the humidified atmosphere of 95% air and 5% CO2 at 37 °C in Dulbecco’s modified essential medium containing 10% fetal calf serum, extra L-glutamine (300 µg/ml), sodium pyruvate (110 µg/ml), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Chemicals—Dulbecco’s modified essential medium was obtained from Mediatech (Washington, D.C.); fetal calf serum and penicillin-streptomycin-glutamine were from Life Technologies, Inc. 5′-[γ-32P]ATP was synthesized by the kinase reaction of 5′-AMP and [γ-32P]ATP. [3H]L-lysine mesylate from CIBA; ferric ammonium citrate (FAC), LPS, IFN-γ, dithiothreitol, β-mercaptoethanol were from Sigma; calyculin A and indomethacin from Calbiochem; N-(3-aminomethyl)-benzylacetamide and N5-(1-iminoethyl)-lysine, were from Alexis Biochemicals.

Gel Retardation Assay—The gel retardation assay was used to measure the interaction between IRPs and IREs using established techniques (21). Briefly, 4 × 106 cells were washed with ice-cold phosphate-buffered saline and lysed at 4 °C in 40 µl of extraction buffer (10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 0.2% Nonidet P-40). After lysis, the samples were centrifuged at 4 °C for 10 min at 10,000 × g to remove nuclei. Samples of cytoplasmic extract were diluted to a protein concentration of 3 µg/ml in lysis buffer without Nonidet P-40, and 10-µg aliquots were analyzed for IRP binding by incubating with 0.1 ng of 32P-labeled pSRE-ter RNA transcript (21). This RNA was transcribed in vitro from linearized plasmid template using T7 RNA polymerase in the presence of [α-32P]UTP. To form RNA-protein complexes, cytoplasmic extracts were incubated for 10 min at room temperature with 0.1 ng of labeled RNA. Heparin (5 mg/ml) was added for another 10 min to prevent nonspecific binding. Unprotected probe was degraded by incubation with 1 unit of RNase T1 for 10 min. RNA-protein complexes were analyzed in 6% nondenaturing polyacrylamide gels. In parallel experiments, samples were treated with 2% β-mercaptoethanol before the addition of the RNA probe.

Ribonuclease Protection Assay—RNase protections assays were performed using a kit from Pharmingen (Mississauga, ON) as described in the manufacturer’s manual. 32P-labeled antisense RNAs were generated using T7 polymerases. Actin mRNA was used as the control.

Western Blot Analysis—Approximately 5 × 106 cells were lysed with extraction buffer (see above), and 60 µg of protein was resolved using 6% SDS-polyacrylamide gel electrophoresis. The protein was transferred to a nitrocellulose membrane, which was subsequently incubated with rabbit anti-IRP-2 antibodies that were generously provided by Dr. E. Leibold. After 1 h of incubation, the membrane was washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) for 1 h. The protein was then visualized with an enhanced chemiluminescence Western blotting detection system (Bio-Rad) according to the manufacturer’s manual.

Iron Uptake—Equal numbers of cells (~1 million cells/ml) were incubated with diferric 56Fe-labeled Tf at 37 °C and harvested at different time intervals. Cells were washed three times with ice-cold phosphate-buffered saline, and the radioactivity of cell pellet was measured in a gamma scintillation counter.

RESULTS

LPS/IFN-γ Activates IRP-1 but Decreases RNA Binding of IRP-2 and Its Protein Levels—Murine macrophages treated with LPS/IFN-γ produce a large quantity of NO (3), which can modulate IRP-1 by a direct interaction with its [Fe-S] cluster (4, 18, 19, 29), but the effect of NO on IRP-2 is ill-defined. Therefore, we examined the effects of LPS and IFN-γ on IRP-1 and -2 binding activities in a macrophage cell line, RAW 264.7 cells. Fig. 1 shows that neither LPS nor IFN-γ alone was able to activate IRP-1, even though the cells generated significant amounts of NO. However, following a 10-h treatment of RAW 264.7 cells with LPS/IFN-γ, RNA binding activity of IRP-1 increased whereas that of IRP-2 decreased. The decrease in IRP-2 binding activity (Fig. 1A) in LPS/IFN-γ-treated cells was associated with a corresponding decrease in IRP-2 protein levels (Fig. 1B). As expected, the iron chelator deferoxamine caused a significant increase in IRP-1 binding activity and slightly increased IRP-2 binding activity and protein levels. On the other hand, treatment of RAW 264.7 cells with FAC reduced IRP-2 binding (Fig. 1B) as well as IRP-1 binding to the IRE (see Fig. 2A and Fig. 4A).

LPS/IFN-γ Decreases TfR mRNA and Increases Ferritin Synthesis in RAW 264.7 Cells—Because LPS/IFN-γ have distinct effects on IRP-1 and IRP-2 (see Fig. 1), we examined the effect of these agents on TfR mRNA levels and ferritin synthesis in RAW 264.7 cells. Fig. 1C shows that LPS/IFN-γ causes a dramatic decrease in TfR mRNA levels, which, however, is not associated with any appreciable decrease in IRP-1 binding activity (Fig. 1A). Importantly, the combination of LPS and IFN-γ caused an even more pronounced decrease in TfR mRNA levels although IRP-1 was fully activated (Fig. 1A). The comparison of results in Fig. 1, A–C, clearly indicates that the drop in TfR mRNA levels following LPS and IFN-γ treatment cor-
relates with the decrease in IRP-2 binding/levels and is unrelated to IRP-1 RNA binding activity. Similarly, ferritin synthesis in LPS/IFN-γ-treated cells is significantly increased (Fig. 1D) despite the high RNA binding activity of IRP-1; once again increased ferritin synthesis correlates with a decrease in IRP-2. However, ferritin mRNA levels did not change in RAW 264.7 cells incubated with LPS/IFN-γ for 10 h (not shown). In accord with these results, Recalcati et al. (36) recently reported that stimulation of J774 cells (a murine macrophage cell line) with cytokines caused a reduction of IRP-2 accompanied by increased ferritin synthesis. Iron-depleted (deferoxamine mesylate) and -replete (FAC) cells served as positive controls and increased ferritin synthesis correlates with a decrease in IRP-2. 

**Inhibitors of iNOS Prevent IRP-2 Degradation and Attenuate the Decrease in TFR mRNA Levels in LPS/IFN-γ-treated RAW 264.7 Cells**—We previously demonstrated that NO− caused a significant decrease in IRP-2 binding to the IRE, associated with a dramatic decrease in TFR mRNA levels in RAW 264.7 (44). Similar changes occur in RAW 264.7 cells following their treatment with LPS and IFN-γ (see Fig. 1), suggesting that their effects are NO-mediated. To test this, we examined the effect of specific iNOS inhibitors on IRP-2 and TFR mRNA in LPS/IFN-γ-treated cells. Fig. 2A shows that specific iNOS inhibitors prevented the LPS/IFN-γ-induced decrease in IRP-2 RNA binding, indicating that the iNOS inhibitors prevented IRP-2 degradation. This result strongly suggests that the LPS/IFN-γ-induced decrease in IRP-2 binding is due to a NO-related mechanism. This conclusion is in conflict with the one proposed by Bouton et al. (46), but these investigators did not use iNOS-specific inhibitors. Nω-Monomethyl-l-arginine did not completely block NO production in LPS/IFN-γ-stimulated macrophages (46), whereas iNOS-specific inhibitors used in this study did (Fig. 2A). Moreover, we also found that the iNOS inhibitors attenuated a LPS/IFN-γ-mediated decrease in TFR mRNA levels (Fig. 2B), suggesting that this decrease is also NO-mediated.

**LPS/IFN-γ-induced IRP-2 Degradation can be Prevented by an Iron Chelator**—It is well established that the depletion of intracellular iron by iron chelators activates the RNA binding activity of IRP-1 and increases the half-life of IRP-2. To examine whether iron is involved in IRP-2 deactivation and degradation following LPS and IFN-γ, we exploited the cell membrane permeable chelator, SIH (Ref. 45). In accord with the previous result (Fig. 1), IRP-2 activities (Fig. 3A) and levels (Fig. 3B) decreased in LPS- and IFN-γ-treated RAW 264.7 cells. Interestingly, this decrease was fully prevented when the iron chelator, SIH, was included in the incubation mixture (Fig. 3). Bouton et al. (46) failed to prevent IRP-2 deactivation in LPS/IFN-γ-treated RAW 264.7 cells using desferrioxamine, but this chelator seems to penetrate cell membranes less efficiently than SIH (45). Importantly, the LPS- and IFN-γ-mediated decrease in TFR mRNA levels in RAW 264.7 cells was prevented by the iron chelator SIH (Fig. 3C). These results suggest that iron is somehow involved in the LPS/IFN-γ-mediated degradation of IRP-2 and consequent decrease in TFR mRNA levels. To examine how an increase in intracellular iron levels affects both IRPs, we incubated RAW 264.7 cells with FAC for different time intervals. Fig. 4 shows that iron-mediated deactivation of IRP-1 and IRP-2 occurs with similar kinetics, confirming an earlier observation by Hanson and Leibold (47). As expected, LPS and IFN-γ increased iNOS mRNA levels in RAW 264.7 cells (see Fig. 3C).

**Tumor Necrosis Factor (TNF-α) Is Not Involved in the Modulation of IRPs in LPS/IFN-γ-treated RAW 264.7 Cells**—Macrophages activated by LPS/INF-γ can produce not only NO but also TNF-α (1) and, therefore, we investigated whether TNF-α could be involved in either the activation of IRP-1 or
degradation of IRP-2. RAW 264.7 cells were incubated in medium containing TNF-α (20 ng/ml) with or without LPS/IFN-γ. Fig. 5 shows that TNF-α had no effect on the RNA binding activity of either IRP-1 or IRP-2. Moreover, the pretreatment of RAW 264.7 cells with anti-TNF-α antibodies had no appreciable effects on IRP binding activities in cells subsequently treated with LPS/IFN-γ. These results indicate that TNF-α does not play any role in modulating IRPs.

LPS/IFN-γ-treated RAW 264.7 Cells Have Decreased Iron Uptake from Transferrin—As part of the inflammatory response, NO generated by activated macrophages could affect iron metabolism by modulating the IRPs (30–32) as well as by a direct interaction with Fe-Tf (48). Hence, it was of interest to examine 59Fe uptake from 59Fe-Tf by RAW 264.7 cells incubated without or with LPS/IFN-γ. Fig. 6 shows that following 6 h of incubation of RAW 264.7 cells with LPS/IFN-γ, iron uptake was significantly decreased and remained at very low levels at later time intervals. It seems highly likely that decreased iron uptake from transferrin in LPS/IFN-γ-treated cells is caused by a drop in TfR levels.

DISCUSSION

NO is a short lived messenger molecule that plays a crucial role in the function of diverse biological processes, including vasorelaxation, adhesion, and aggregation of platelets and neutrophils, neurotransmission, and macrophage-mediated cytotoxicity (3, 4, 8). Many of the functions of NO can be attributed to its binding in the heme prosthetic group of soluble guanylate cyclase, which activates this enzyme, consequently increasing cyclic GMP levels (8). Apart from its reaction with guanylate cyclase, much interest has focused on the reactivity of NO with the [Fe-S] centers in proteins such as mitochondrial aconitase, the [Fe-S] proteins of the mitochondrial respiratory system, and numerous other non-heme iron proteins with important regulatory functions (reviewed in Refs. 4, 18, and 19). Recent research has revealed that mitochondrial aconitase is highly homologous to IRP-1 (5–7), an important regulator of cellular iron homeostasis. The homology between IRP-1 and mitochondrial aconitase suggested that IRP-1 may also be a target of NO and, in fact, several studies have demonstrated that NO can activate IRP-1 RNA binding activity (30–33), associated with increase in TfR mRNA levels (32, 33). However, these results are in conflict with the finding that TfR levels are decreased in IFN-γ- and/or LPS-treated macrophages (40–42), a condition known to increase NO production (3). These paradoxical results may be explained by the fact that NO has markedly different biological effects depending upon its redox state (9, 10). NO in its reduced form, nitric oxide (NO•), binds to...
iron, whereas NO in its oxidized form, the nitrosonium ion \(\text{NO}^+\), results in S-nitrosylation of numerous proteins (9, 10). Considering this, it seems likely that \(\text{NO}^+\) affects the [Fe-S] cluster of IRP-1, whereas \(\text{NO}^+\) may regulate IRP-1 activity by S-nitrosylation of key sulfhydryl groups. In fact, we previously demonstrated that the \(\text{NO}^+\) generator, sodium nitroprusside (SNP), had the opposite effect of the \(\text{NO}^2\) donor, markedly decreasing IRP-1 RNA binding activity, Tf mRNA levels, TfR numbers, and iron uptake by K562 cells (32). More recently, we found that IRP-2 is also a very important target of \(\text{NO}^+\) (43, 44). Using RAW 264.7 cells we confirmed that the \(\text{NO}^+\) donor (SNP) caused a decrease in IRP-1 binding activity in ~10 h. Importantly, only a 1-h exposure of RAW 264.7 cells to SNP resulted in a significant decrease in IRP-2 binding, followed by a decrease in IRP-2 protein levels. The SNP-mediated degra-

![Figure 4: IRP binding activities in RAW 264.7 cells treated with FAC. A. The cells were incubated without or with FAC (F, 100 μg/ml) for indicated time intervals and washed. Cytosolic protein (C, 10 μg) extracts were incubated with \(^{32}\text{P}-\text{IRE} as described under “Materials and Methods.” B. Densitometric analysis of IRP-1 and IRP-2 binding activities in the absence of β-mercaptoethanol (α = 3); error bars represent standard deviations.](image1)

![Figure 5: The Effect of FAC on IRPs](image2)

![Figure 6: Effect of LPS/IFN-γ on iron uptake from diferric Tf in LPS/IFN-γ-treated RAW 264.7 cells. The cells were incubated without (CTL) or with LPS (5 μg/ml)/IFN-γ (100 units/ml) in the presence of \(^{59}\text{Fe-Tf} (50 μM), harvested at indicated time intervals, and washed following which their \(^{59}\text{Fe}-\text{radioactivity was measured.](image3)
diation of IRP-2 in RAW 264.7 cells could be prevented by MG-132 or lactacystin. We have concluded that a specific S-nitrosylation of critical thiol groups of IRP-2 may target this protein for degradation in proteasomes (44). Moreover, a decrease in IRP-2 binding/levels, following a short treatment of RAW 264.7 cells with SNP, was associated with a dramatic decrease in TfR mRNA that occurred hours before any effect on IRP-1 binding was detected. The changes in both of these proteins induced by SNP, were inhibitable by MG-132 (44).

In this study we found that LPS/IFN-γ treatment of RAW 264.7 cells caused changes that were strikingly similar to those seen following NO⁺ treatment. Specifically, LPS/IFN-γ caused deactivation and degradation of IRP-2 that was associated with the increase in ferritin synthesis and the decrease in TfR mRNA levels in RAW 264.7 cells. Collectively, our previous results (44) and those presented here suggest that S-nitrosylation of IRP-2 may occur in macrophages in response to LPS/IFN-γ. Moreover, it is tempting to speculate that S-nitrosylation of a “degradation domain” of IRP-2, which contains five free cysteines (26), may represent a signal for ubiquitination and consequent degradation by a proteasome-dependent mechanism. This conclusion is supported by the finding that IRP-2 degradation is completely blocked when three of five cysteines in the 73-amino acid degradation domain of IRP-2 are mutated (26).

We also found that stimulation of RAW 264.7 cells with LPS/IFN-γ caused a slight increase of IRP-1 activity that, however, was not associated with the increase in TfR mRNA levels. On the contrary, as described above, TfR mRNA levels decreased in LPS/IFN-γ-treated cells, and this change correlates with a selective decrease in IRP-2 binding and degradation. Hence, IRP-2 alone appears to play an important role in controlling TfR mRNA levels, a conclusion also supported by the recent report of Schalinske et al. (49). In addition, we demonstrated that iNOS-specific inhibitors prevented IRP-2 degradation and attenuated the TfR mRNA decrease (Fig. 2) in LPS/IFN-γ-treated RAW 264.7 cells, indicating that these agents exerted their effects via an NO-mediated mechanism. It is well documented that cytokine-activated RAW 264.7 cells have dramatically increased levels of nitrosoglutathione (GS-NO) (50), a species that is capable of inducing S-nitrosylations of proteins in activated macrophages (51). It is reasonable to expect that in LPS/IFN-γ-treated RAW 264.7 cells GS-NO causes S-nitrosylation of IRP-2. GS-NO can be formed from NO⁺ by an iron-dependent reaction (52) that is likely to occur in cytokine-activated macrophages. Hence, the presence of both NO⁺ and GS-NO (which has nitrosonium character) can explain activation of IRP-1, while IRP-2 is deactivated. Although our experiments strongly suggest that in the absence of IRP-2, IRP-1 is unable to maintain normal TfR mRNA levels (44), it can be argued that cytokines may cause a decrease in transcriptional activity of the TIR gene. However, INF-γ does not seem to regulate TIR gene transcription (53). Moreover, our experiments clearly demonstrated that inhibitors of iNOS prevented IRP-2 degradation and attenuated TfR mRNA decrease in LPS/IFN-γ-stimulated RAW 264.7 cells (Fig. 2). These results strongly support the idea that NO-mediated IRP-2 degradation is responsible for the decrease in TfR mRNA.

The membrane-permeable iron chelator, SIH, prevented not only the LPS/IFN-γ-mediated decrease in IRP-2 but also maintained normal TfR mRNA levels in activated RAW 264.7 cells. This finding provides further strong evidence that the decrease in TfR mRNA is causally related to the degradation of IRP-2. These results might also be interpreted as indicating that the decreases in IRP-2 and TfR mRNA levels are caused by the LPS/IFN-γ-mediated redistribution of intracellular iron and by an increase of iron levels in the labile pool. However, there are strong arguments against this possibility. First, LPS/IFN-γ treatment of RAW 264.7 cells does not decrease IRP-1 activity (Fig. 1), and this would have been expected if intracellular iron levels increased. Second, iron-mediated deactivation of IRP-1 and IRP-2 occurred with similar kinetics (Fig. 4, Ref. 47), whereas in LPS/IFN-γ-treated RAW 264.7 cells IRP-2 was inactivated while IRP-1 was activated (Fig. 1). Hence, it is likely that in LPS/IFN-γ-treated RAW 264.7 cells iron promotes formation of GS-NO (see above, Ref. 52) that can nitrosylate proteins.

IRPs are targets for phosphorylation by protein kinase C, and the phosphorylated IRPs have enhanced IRE binding activities (54, 55). Macrophages stimulated by LPS/IFN-γ can produce large quantities of TNF-α (1), which activate several kinases in the cells. Therefore, we investigated whether IRP-2 can be modulated by TNF-α, but this agent did not cause any effect (Fig. 5). Moreover, anti-TNF-α antibodies did not prevent LPS/IFN-γ effects and sautosporine, a protein kinase inhibitor, did not affect IRP binding activities in LPS/IFN-γ-treated RAW 264.7 cells (not shown).

When discussing regulation of cellular iron metabolism via the IRE/IRP system, it is relevant to point out that iron and NO are not the only players that modulate this system. Pantopoulos and Hentze (56) demonstrated that treatment of cultured cells with micromolar H₂O₂ concentrations caused a rapid induction of IRP-1, a finding that established a direct regulatory connection between iron metabolism and oxidative stress. As expected, based on the IRE/IRP paradigm, exposure of cells to H₂O₂ increased TfR mRNA levels and repressed ferritin synthesis (56). Because we found that LPS and IFN-γ led to a decrease in TfR mRNA levels and to an increase in ferritin synthesis, oxidative stress is unlikely to be involved in the above responses. Moreover, the H₂O₂ activates IRP-1 without affecting IRP-2 (57), whereas we observed that LPS/IFN-γ treatment increased IRP-1 activity and dramatically decreased IRP-2 binding, changes that could be prevented by specific iNOS inhibitors (Fig. 2). Because cellular production of reactive oxygen species is related to O₂ concentration, the effects of hypoxia on IRP-1 and IRP-2 RNA binding activity were investigated by several groups, but the results obtained are somewhat controversial. Hanson and Leibold (47) showed that hypoxia increased IRP-2 levels by post-translational mechanism involving protein stability (58); however, these authors did not measure TIR expression after hypoxia. On the other hand, Toth et al. (59) found that hypoxia enhanced IRE/IRP-1 binding that was associated with a significant increase in TfR mRNA levels. Moreover, recently we (60) and others (61) reported that the TIR gene contains a functional hypoxia response element that binds hypoxia-inducible factor-1, which transcriptionally enhances TIR expression under hypoxic conditions. However, the mechanism of action of LPS/INF-γ is clearly different, because treatment with these agents leads to a decrease in TfR mRNA levels.

In conclusion, recently we reported that chemically produced NO⁺, which causes S-nitrosylation of thiol groups of proteins, decreased RNA binding activity of IRP-2 followed by IRP-2 degradation, a subsequent decrease in TfR mRNA levels, and an increase in ferritin synthesis. Results in this study complement and further extend these findings. We have demonstrated that the decrease in TfR mRNA and increase in ferritin synthesis in IFN-γ- and LPS-treated macrophages is caused by a selective decrease in IRP-2. Moreover, our results together with

2 K. Pantopoulos, personal communication.
the observations of others suggest that NO\textsuperscript{+} from GS-NO may play a role in IRP-2 decrease in LPS/IFN-\gamma-stimulated macrophages. Furthermore, IRP-2 alone plays a significant role in controlling TR mRNA levels, because IRP-1, although available for RNA binding in LPS/IFN-\gamma-treated macrophages, was unable to prevent the decrease in TR mRNA levels. The current and previous study (44) from this lab provide further evidence for a dichotomy in NO effects, caused by the existence of two redox-related species of NO (NO\textsuperscript{-} and NO\textsuperscript{+}), which may explain at least some of the paradoxical observations regarding iron metabolism changes in cytokine-treated macrophages.

Acknowledgment—We are grateful to Dr. L. Kühn for CDhTR9, Dr. L. Neckers for TR cDNA, and Dr. B. Leibold for antibodies against IRP-2. We thank Joan Buss for reading this paper and many useful comments, and Sandy Fraiberg for excellent editorial assistance.

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Sangwon Kim and Prem Ponka

J. Biol. Chem. 2000, 275:6220-6226.
doi: 10.1074/jbc.275.9.6220

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