Iron Regulates Nitric Oxide Synthase Activity by Controlling Nuclear Transcription

By Günter Weiss,* Gabriele Werner-Felmayer,‡ Ernst R. Werner,‡ Kurt Grünewald,* Helmut Wachter,‡ and Matthias W. Hentze§

*From the Departments of *Internal Medicine and ‡Medical Chemistry and Biochemistry, University of Innsbruck, A-6020 Innsbruck, Austria; and the §Gene Expression Programme, European Molecular Biology Laboratory, D-69117, Heidelberg, Federal Republic of Germany*

Summary

Recently, it was reported that nitric oxide (NO) directly controls intracellular iron metabolism by activating iron regulatory protein (IRP), a cytoplasmic protein that regulates ferritin translation. To determine whether intracellular iron levels themselves affect NO synthase (NOS), we studied the effect of iron on cytokine-inducible NOS activity and mRNA expression in the murine macrophage cell line J774A.1. We show here that NOS activity is decreased by about 50% in homogenates obtained from cells treated with interferon γ plus lipopolysaccharide (IFN-γ/LPS) in the presence of 50 μM ferric iron [Fe(3+)] as compared with extracts from cells treated with IFN-γ/LPS alone. Conversely, addition of the iron chelator desferrioxamine (100 μM) at the time of stimulation with IFN-γ/LPS increases NOS activity up to 2.5-fold in J774 cells. These effects of changing the cellular iron state cannot be attributed to a general alteration of the IFN-γ/LPS signal, since IFN-γ/LPS–mediated major histocompatibility complex class II antigen expression is unaffected. Furthermore, neither was the intracellular availability of the NOS cofactor tetrahydrobiopterin altered by treatment with Fe(3+) or desferrioxamine, nor do these compounds interfere with the activity of the hemoprotein NOS in vitro. We demonstrate that the mRNA levels for NOS are profoundly increased by treatment with desferrioxamine and reduced by Fe(3+). The half-life of NOS mRNA appeared not to be significantly altered by administration of ferric ion, and NOS mRNA stability was only slightly prolonged by desferrioxamine treatment. Nuclear run-off experiments demonstrate that nuclear transcription of cytokine-inducible NOS mRNA is strongly increased by desferrioxamine whereas it is decreased by Fe(3+). Thus, this transcriptional response appears to account quantitatively for the changes in enzyme activity. Our results suggest the existence of a regulatory loop between iron metabolism and the NO/NOS pathway.

Nitric oxide (NO)[1] is a short-lived messenger molecule involved in neurotransmission, regulation of blood pressure, and cytotoxicity (for reviews see references 1–3). Many biological effects of NO can be explained by its interaction with iron. Cytotoxicity of activated macrophages towards tumor target cells is characterized by inhibition of DNA synthesis, by inhibition of non-heme iron containing enzymes of the respiratory electron transfer chain, and by iron release (4). It has been demonstrated that the loss of activity of the critical Krebs-cycle enzyme aconitase in activated macrophages is a result of the formation of iron–nitrosyl complexes induced by the synthesis of nitric oxide which interacts with the iron-sulfur cluster of the enzyme (5, 6). It was also shown that non-heme iron of ferritin is a target of NO (7). Furthermore, the activation of soluble guanylyl cyclase by NO appears to be achieved by nitrosylation of the heme iron of the enzyme (for a review see reference 8). Thus, many of the biological effects of NO can be traced back to chemical interactions between NO and iron.

Control over cellular iron homeostasis is largely exerted by a genetic system that operates posttranscriptionally in the cytoplasm. A *cis*-acting RNA motif, the so-called iron-responsive element (IRE), and a *trans*-acting cytoplasmatic protein, known as iron regulatory protein (IRP, formerly known as IRF, IRE-BP, FRP, or p90) cooperate to coordinate the iron-dependent expression of the intracellular storage

[1] *Abbreviations used in this paper:* Fe(3+), ferric iron; iNOS, inducible nitric oxide synthase; IRE, iron responsive element; IRP, iron regulatory protein; NMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase.

G. Weiss and G. Werner-Felmayer contributed equally to the work presented here.

protein ferritin, of the receptor critical for cellular uptake of the iron carrier transferrin, and of erythroid 5-amino levulinic acid synthase, the first enzyme in the heme biosynthetic pathway. Iron deprivation stimulates IRE-binding by IRP, thereby repressing ferritin and e-ALAS-mRNA translation and protecting transferrin receptor mRNA from degradation, which consecutively results in increased transferrin receptor expression (for reviews see references 9, 10). Recently, it was found that not only iron deprivation, but also activation of NO synthase (NOS) can stimulate the IRE-binding activity of IRP (11, 12) in the murine macrophage cell lines J774, RAW264.7, and P338D1, as well as in primary macrophages. These results indicate involvement of NO in the intracellular regulation of iron metabolism (13). Stimulation of NO synthesis was shown to repress ferritin translation via IRP in the murine macrophage cell line J774 (12). Enhanced generation of NO resulting from stimulation of inducible NOS by cytokines may also play a role in iron disturbances characteristic in chronic immune activation states which are hallmarked by low levels of iron and high ferritin and cytokine concentrations in serum (14–16). Based on these recent discoveries pertaining to the function of NO in iron regulation, we investigated the effect of intracellular iron levels on NOS activity and expression in the murine macrophage cell line J774.

Materials and Methods

Cell Culture Techniques. The J774A.1 murine macrophage cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM medium supplemented with 10% heat-inactivated FCS, 1 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. 10^7 cells were supplemented with 50 μM ferric iron [$Fe(3^+)$] (applied as $Fe(NO_3)_3.9H_2O$), 100 μM desferrioxamine, or 250 μM N^G-monomethyl-L-arginine (NMA; all from Sigma Chemical Co., Munich, Germany) in culture medium. Cells were stimulated by combined treatment with 50 U/ml murine rIFN-γ (sp act 10^7 U/mg, from GIBCO BRL, Life Technologies, Vienna, Austria) and 10 μg/ml *Escherichia coli* LPS (055:B5; phenolic extract; Sigma Chemical Co.). These stimuli have been shown to stimulate inducible NOS (iNOS) in J774 cells (17).

After incubation for 20 h at 37°C in humidified air containing 5% CO_2, cells were harvested and washed with PBS, containing 130 mM NaCl, 2 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4. For determination of NOS activity in homogenates, 10^7 cells were frozen in 1 ml of distilled water at −80°C and treated further as described below.

Determination of NOS Activity. Enzyme activity in cell homogenates was determined by formation of [^{3}H]citrulline as previously done (18, 19) and modified as described (20). In brief, J774 cells frozen at −80°C were thawed rapidly, centrifuged for 15 min at 10,000 *g* at 4°C, and the supernatant was freed from low molecular mass compounds by Sephadex G-25 chromatography. The protein fraction was eluted with 40 mM Tris/HCl, pH 7.5, containing 100 μM PMSF. Standard reaction mixtures contained 40 mM Tris-HCl, pH 7.0, 100 μM L-arginine, 25 μM flavinadeninedinucleotide, 25 μM flavinmononucleotide, 2 mM NADPH, 5 μM 6R-tetrahydrobiopterin, 100 μM PMSF, 60,000-80,000 cpm of purified L-[2,3,4,5-^{3}H]-arginine (Amersham International, Amersham, Bucks, UK; for purification see reference 20) and 100 μl of cell extract (about 200 μg of cell protein) in a final volume of 200 μl. Samples were incubated for 30 min at 37°C, and the reaction was stopped by the addition of 800 μl of 200 μM sodium acetate, pH 5.0, containing 200 μM EDTA and 1 mM L-citrulline. [^{3}H]citrulline was quantified after separation from [^{3}H]arginine by Dowex 50W (21). Enzyme activity is expressed as pmol [^{3}H]citrulline formed per minute per milligram of protein in the cell extract.

Measurement of Nitrite in Supernatants. Cells were grown in culture medium and stimulated for 20 h as described above. Nitrite was determined in supernatants by the Griess reaction (22) using the stable Griess-Ilosvay's reagent from Merck (Darmstadt, Germany) and sodium nitrite as a standard. The detection limit was 1 μmol/liter.

Determination of MHC Class II Expression. Expression of mouse MHC class II antigens was estimated by use of the anti-I-$E^{k,d,p,r,u,v}$-IgG2a mAb (clone 13/4.R5; obtained from Serotec, Oxford, UK). For isotype control, a mouse IgG2a mAb anti-CD-10 (Becton Dickinson & Co., Mountain View, CA) was used. Both antibodies were applied as purified Ig (0.5 μg in 20 μl of PBS containing 0.1% azide). Cells were seeded at a density of 10^6 cells/ml in 24-well plates and supplemented and stimulated as described above for 48 h. After harvesting by scraping, cells were washed and resuspended in 50 μl of DMEM containing 2% FCS and 0.1% azide and incubated with the appropriate antibody for 30 min on ice. For reagent control, the first step was carried out only with culture medium. Incubation was stopped by addition of 1 ml of cold DMEM/2% FCS/0.1% azide. After two washes, cells were counterstained with FITC-conjugated anti-mouse IgG serum diluted 1:100 in DMEM/2% FCS/0.1% azide. For analysis, a fluorescence-activated cell sorter (FACScan®; Becton Dickinson & Co.) was used. Fluorescence intensities of 5×10^3 cells were measured for each determination. Data are expressed as mean fluorescence channel in relation to isotype and reagent control.

Determination of Intracellular Biopterin. J774 mouse macrophages were seeded at a density of 10^6/ml in 6-well plates (5 ml/well) and stimulated as described above. Cells were harvested after 20 h by means of a cell scraper, washed with PBS, and pellets were resuspended in 250 μl of distilled water and rapidly frozen in liquid nitrogen. After thawing, samples were centrifuged at 10,000 *g* for 2 min and subjected to oxidation with iodine at alkaline or acidic pH according to previous protocols (23). Briefly, 100 μl of cell extracts was mixed with 5 μl of 1 M HCl and 5 μl of 0.1 M KI/I_2 in order to determine the amount of total biopterin including 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin. Another 100 μl of cell extracts was mixed with 5 μl of 1 M NaOH and 5 μl of 0.1 M KI/I_2 for destroying the tetrahydro-derivative, thus detecting the sum of 7,8-dihydrobiopterin and biopterin. After incubation for 1 h in the dark at room temperature, 10 μl HCl were added to the $NaOH/KI_2$ treated extracts, samples were centrifuged for 2 min at 10,000 *g*, and the supernatant was mixed with 10 μl of 0.1 M ascorbic acid. Samples were then extracted with AASP-SCX cartridges (Analytichem International, Inc., Harbor City, CA), and pteridines were directly eluted on to a reversed-phase HPLC column (Lichrosorb, RP-18; Merck) and quantified by fluorescence detection (LS 4; Perkin-Elmer Corp., Baconsfield, UK) as described (24). Values are presented as picomole per milligram of total cell protein.

Northern Blot Analysis and Determination of mRNA Half Life. J774 cells were stimulated for 20 h as described above. For determination of mRNA half-life, actinomycin D (5 μg/ml) was added and cells were then harvested for RNA preparation every 1.5 h for 7.5 h (25). Cells were harvested by scraping, washed twice with

Table 1. *Influence of Fe(3+), Desferrioxamine and NMA on IFN-γ/LPS–induced NOS Activity and Relative and Absolute Concentrations of 5,6,7,8-tetrahydrobiopterin (BH4) in J774 Cells*

Supplementation	NOS activity (pmol/min^{-1} mg^{-1})	Intracellular BH4 (nmol/mg protein)	BH4/ (Biopterin + BH2)
Control	8.6 ± 0.3	73.4 ± 21.6	2.37
Iron	6.4 ± 0.4	75.7 ± 23.1	2.23
Desferrioxamine	5.6 ± 0.2	72.2 ± 17.8	2.28
IFN-γ/LPS	286.7 ± 8.3	58.6 ± 19.0	2.42
IFN-γ/LPS plus Fe(3+)	157.0 ± 23.5	61.6 ± 18.9	2.15
IFN-γ/LPS plus desferrioxamine	699.7 ± 39.1	56.3 ± 14.7	2.43
IFN-γ/LPS plus NMA	53.8 ± 6.4	64.2 ± 21.2	2.23
IFN-γ/LPS plus NMA plus Fe(3+)	29.5 ± 4.9	62.9 ± 13.7	2.41
IFN-γ/LPS plus NMA plus desferrioxamine	137.6 ± 14.9	59.0 ± 18.2	2.33

Confluent J774 cells were treated with Fe(3+) (50 μM), applied as ferric nitrate nonahydrate, desferrioxamine (100 μM), or NMA (250 μM) in the presence or absence of IFN-γ (50 U/ml) and LPS (10 μg/ml) for 20 h. NOS activity and intracellular concentrations of biopterin, 7,8-dihydrobiopterin (BH2), and 5,6,7,8-tetrahydrobiopterin (BH4) were determined as described in Materials and Methods. A ratio of BH4/(BH2 plus biopterin) was calculated in order to obtain a measure of relative BH4 availability in the cell. Values are means of triplicate cultures ± SD from one of five similar experiments.

PBS, and pellets were stored at −80°C until preparation of total RNA by acid guanidinium thiocyante-phenol-chloroform extraction as previously done (26). 10 μg of total RNA were separated on 1% agarose/2.2 M formaldehyde gels and RNA was blotted onto Duralon-UV membranes (Stratagene, La Jolla, CA). After UV-cross-linking and prehybridization for 6–8 h at 65°C, blots were hybridized overnight with 10^6 cpm/ml of α[^{32}P]dCTP-radiolabeled cDNA plasmid probes at 65°C. The hybridization solution contained 3 × SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 10% dextran sulfate, 10× Denhardt's solution (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, and 0.2% BSA), and 1 mg/ml of denaturated salmon sperm DNA. Blots were washed subsequently with 2 × SSC/0.5% SDS (twice for 30 min) and with 0.1 × SSC/0.5% SDS (twice for 30 min) at 65°C. Filters were exposed for up to 4 d to XRP-5 x-ray films (Kodak, X-OMAT RP) with intensifying screens at −80°C. Autoradiographs were densitometrically scanned using the Bio-Profil system for image analysis (Vilber Lourmat, Marne La Vallée, France).

cDNA Probes. The murine inducible macrophage NOS cDNA probe (clone piNOSL3 in pU19) was a generous gift of Drs. Q.-W. Xie and C. F. Nathan (Cornell University Medical College, New York) (27). For Northern hybridization, the 817-bp EcoRI/HincII insert was used. Murine m5β tubulin cDNA (in pUC9; 1.6-kb EcoRI/EcoRI insert) (28) and chicken β-actin cDNA (in pBR322; 1.9-kb HindIII/HindIII insert) (29) were kindly provided by Dr. D. W. Cleveland (Johns Hopkins University School of Medicine, Baltimore, MD). Probes were labeled with α[^{32}P]dCTP (DuPont New England Nuclear, Boston, MA) using the oligoprimer procedure (30) to a sp act of 1–5 × 10^9 cpm/μg DNA.

Nuclear Runoff Transcription. Nuclei of J774 cells treated as outlined above were prepared after 12 h of stimulation. Purification of nuclei and in vitro transcription were performed as described (31). Briefly, nuclei were isolated from 5 × 10^7 cells with NP-40 lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 and stored in 50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 5 mM EDTA in liquid nitrogen. For in vitro transcription, α[^{32}P]UTP (800 Ci/mmol, 100 μCi/5 × 10^7 nuclei) was used. Plasmids (piNOSL3 and mβ5-tubulin, see above), linearized with EcoRI, were spotted to Duralon-UV nylon membranes using a dot blot (2.5 μg/dot) apparatus and bound by UV-cross-linking. Freshly transcribed ^{32}P-labeled RNA was hybridized to membranes for 48 h at 65°C, applying equal amounts of TCA-precipitable counts. Filters were then washed twice in 2 × SSC at 65°C for 1 h, treated with RNase A, washed twice in 2 × SSC at 37°C, and exposed to x-ray films at −80°C.

Protein Determination. Protein concentration of cell lysates was estimated according to Bradford (32) using the protein dye reagent from BioRad Laboratories (Richmond, CA) and BSA as a standard.

Iron Determination. Concentration of low molecular weight iron in cell lysates was estimated by the ferrozine-ascorbic acid method at 550 nm modified from Siedel et al. (33) using a microplate reader (model 2001; Anthos Labtec Instruments, Salzburg, Austria).

Results

Iron Regulates NOS Activity in Induced J774 Cells. To study the effect of iron on induction of NOS, we determined NOS activities in homogenates from J774 cells treated with 50 μM Fe(3+), applied as ferric nitrate nonahydrate, or the iron chelator desferrioxamine (100 μM) in the absence or presence of IFN-γ/LPS. Administration of iron or desferrioxamine to unstimulated J774 cells did not significantly alter the low NOS activity present in otherwise untreated control cells ($p > 0.05$, Student's t test) (Table 1). As reported (17), stimulation with IFN-γ/LPS for 20 h strongly induced NOS activity in this cell line. Concurrent treatment with 50 μM Fe(3+) reduced enzyme activity by about 45% ($p < 0.001$). In contrast, 100 μM desferrioxamine added at the time of stimulation with IFN-γ/LPS yielded a 2.5-fold increase of NOS activity ($p < 0.0001$). The difference in enzyme activities of iron-supplemented cells as compared to cells treated with desferrioxamine was about fourfold ($p < 0.0001$) (Table 1). Further administration of NMA, a stereospecific inhib-

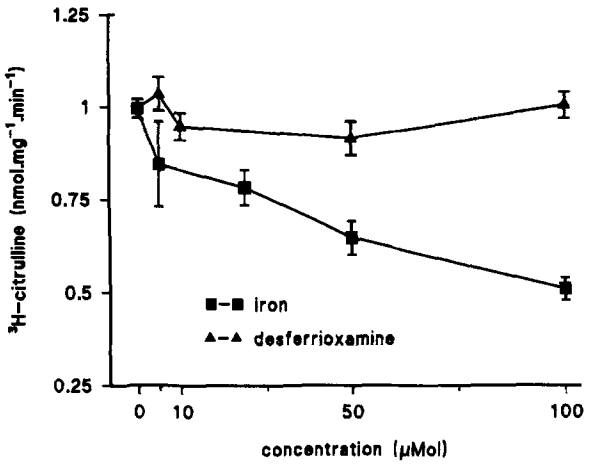

Figure 1. In vitro effects of iron and desferrioxamine on IFN-γ/LPS-induced NOS activity. Cells were stimulated with IFN-γ/LPS for 20 h and extracts were prepared as described under Materials and Methods. Increasing concentrations of [Fe(3+)] or desferrioxamine were added to the enzyme incubation mixture for determination of NOS activity which was measured as described in Materials and Methods. Values are means of triplicate incubations ± SD for one of three experiments.

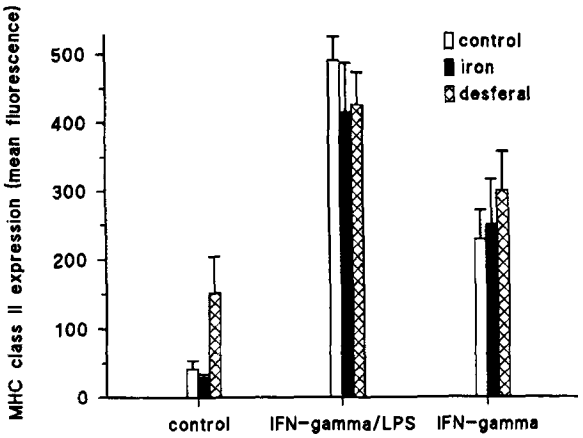

Figure 2. Expression of murine MHC class II antigens in J774 cells supplemented with iron and desferrioxamine and stimulated with IFN-γ and/or LPS. Confluent monolayers were treated with Fe(3+) (50 μM) or desferrioxamine (100 μM) and stimulated with IFN-γ/LPS for 20 h. Immunofluorescence staining of expressed murine MHC class II antigens (I-E^k) was carried out as described in Materials and Methods. Data are expressed as mean channel log fluorescence minus fluorescence of isotype control and reagent control for three different experiments performed in triplicate (mean ± SD).

itor of NOS, to J774 cells, strongly reduced cytokine-induced NOS activity but did not alter the modulating effect of iron supplementation or withdrawal on enzyme activity (Table 1). The regulation of NOS activity by cellular iron is also reflected in the amount of NO released into the supernatants of J774 cells, which was estimated by determination of nitrite (for nitrite levels see Fig. 3).

As expected, addition of Fe(3+) to J774 cells resulted in an increase of the intracellular concentration of this metal up to 195 μg/dl after 20 h of treatment, as compared to untreated cells (means 13 μg/dl), whereas supplementation with desferrioxamine reduced intracellular iron amounts below the limit of detection (<5 μg/dl) (details not shown). It is well established that 5,6,7,8-tetrahydrobiopterin is an essential cofactor of NOS (for a review see reference 1) and that its intracellular levels modulate NOS activity in various cell types (20, 34, 35). Since Fe(3+) is known to affect reduced pteridines (24), we examined whether the effect of iron on NOS activity could originate from alterations in intracellular tetrahydrobiopterin concentrations. No significant changes in intracellular levels of tetrahydrobiopterin or in its ratio to the oxidized species 7,8-dihydrobiopterin and biopterin could be observed after treatment with Fe(3+) or desferrioxamine (Table 1). The minor decrease in tetrahydrobiopterin levels in cells treated with IFN-γ/LPS as compared to controls may be caused by cell damage after prolonged exposure to LPS.

Iron Availability Does Not Influence NOS Activity In Vitro. Since NOS is a heme protein (36, 37), we studied whether iron salts or iron chelation could directly influence the enzyme itself. We therefore tested NOS activity in homogenates of IFN-γ/LPS-treated J774 cells in presence of increasing concentrations of Fe(3+) or desferrioxamine. As shown in Fig. 1, administration of desferrioxamine to the incubation mixture did not significantly alter NOS activity. In contrast, addition of Fe(3+) to the assay mixture caused a concentration-dependent inhibition of NOS activity showing half maximal effects at about 50 μM (Fig. 1). As expected, tetrahydrobiopterin was destroyed in these samples by Fe(3+) and no tetrahydroderivative was detectable, thus explaining the inhibitory effect of Fe(3+) in these assays (data not shown).

Influence of Fe(3+) and Desferrioxamine on MHC Class II Expression. To test whether Fe(3+) and desferrioxamine exert their effects on NOS activity by modulating the signals mediated by IFN-γ and/or LPS, we investigated whether iron availability influenced MHC class II antigen expression induced in J774 cells by either IFN-γ alone or by IFN-γ/LPS (38). As shown in Fig. 2, MHC class II antigen expression (determined as I-E^k) was not significantly altered with either supplement after stimulation with IFN-γ/LPS or with IFN-γ alone. Addition of desferrioxamine alone to otherwise untreated cells, which did not influence NOS activity (see Table 1), showed a significant ($p < 0.001$) increase of I-E^k expression which will be subject of further investigation.

Iron Modulates the Steady-state mRNA Levels of iNOS. Since our previous analyses argued against a posttranslational effect of iron on NOS expression, we examined the NOS mRNA levels in cells treated with Fe(3+) and desferrioxamine by Northern blotting. Low levels of NOS mRNA were detectable in untreated J774 cells which were strongly induced after treatment with IFN-γ/LPS for 20 h (Fig. 3). The high levels of NOS mRNA after IFN-γ/LPS stimulation were strongly reduced when Fe(3+) was present and notably enhanced by desferrioxamine. Addition of NMA to IFN-γ/LPS-stimulated cells did not alter the modulating effects of iron and desferrioxamine on iNOS-mRNA levels. As expected from the NOS activity determinations (Table 1), the low mRNA

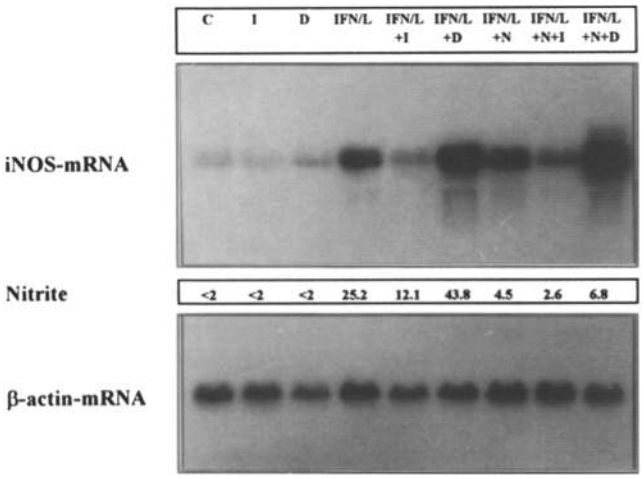

Figure 3. Modulation of mRNA levels for inducible NOS by Fe(3⁺), desferrioxamine, and IFN-γ/LPS. J774 cells were treated with ferric nitrate nonahydrate (*I*), desferrioxamine (*D*), NMA (*N*), and/or IFN-γ/LPS (*IFN/L*) for 20 h as described. Cellular mRNA was isolated and Northern blotting for inducible NOS mRNA and β-actin mRNA were carried out as detailed in Materials and Methods. Concentrations of nitrite (μmol/liter) in cell culture supernatants were determined by the Griess reaction (22) in order to ascertain whether modulations of cytoplasmatic concentration of NOS mRNA by different treatments correlated with alterations of nitrite formation, used as a measure for NO production. One of three similar experiments is shown.

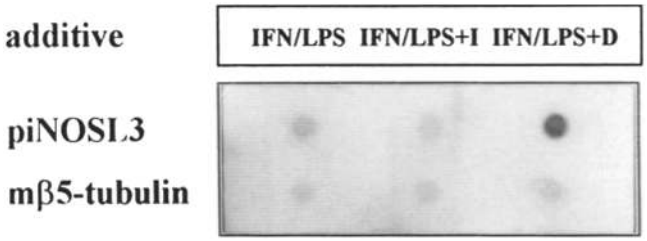

Figure 5. Modulation of inducible NOS gene transcription by iron and desferrioxamine. Nuclei of J774 cells treated with IFN-γ/LPS alone or in combination with 50 μM ferric nitrate nonahydrate (*I*)or 100 μM desferrioxamine (*D*)were prepared after 12 h of stimulation. Purification of nuclei and in vitro transcription were performed as described in Materials and Methods using the plasmid piNOSL3 and mβ5-tubulin. One of two similar experiments is shown. For densitometric evaluation of the runoff plots see Results.

levels observed in unstimulated cells were not changed by Fe(3⁺) or by desferrioxamine (Fig. 3).

Effect of Iron and Desferrioxamine on NOS mRNA Half-life. To assess the contribution of Fe(3⁺) or desferrioxamine to the regulation of NOS mRNA stability, we estimated the half-life of NOS mRNA in the presence of the transcription inhibitor actinomycin D. No pronounced differences in the decay patterns of NOS mRNA were obtained from cells treated with IFN-γ/LPS alone or in combination with Fe(3⁺) or desferrioxamine (Fig. 4). Densitometric evaluation showed that the half-life of NOS mRNA was 3.86 ± 0.77 h (mean ± SD from three independent experiments) for treatment with IFN-γ/LPS and 3.26 ± 0.81 h for further addition of Fe(3⁺). Supplementation with desferrioxamine results in prolongation of NOS mRNA half-life to 5.50 ± 1.3 h, which, however, was not significant as compared to treatment with IFN-γ/LPS alone ($p > 0.05$). The levels of β-actin mRNA did not change during the incubation period (Fig. 4).

Transcriptional Regulation of NOS Gene Expression by Iron and Desferrioxamine. Although a nonspecific effect of actinomycin D on the half-life of NOS mRNA cannot be excluded, the data suggested that the changes in iNOS mRNA levels were more likely be caused by transcriptional regulation. To test this hypothesis directly, we performed nuclear runoff analyses. NOS transcription induced by IFN-γ/LPS is indeed reduced by Fe(3⁺) as compared to IFN-γ/LPS treated controls. Similarly, nuclear transcription of NOS was strongly increased by combined treatment with IFN-γ/LPS and desferrioxamine (Fig. 5). In contrast to the iNOS gene, the transcription of mβ5-tubulin was not changed by Fe(3⁺) or desferrioxamine (Fig. 5). Densitometric evaluation of runoff experiments more clearly demonstrated that nuclear transcription for NOS was significantly reduced by supplementation of iron to 47.8 ± 8.3% (means ± SD for two independent experiments with two exposures scanned for each) as compared to IFN-γ/LPS treated control cells, which were estimated as 100% ($p < 0.01$), whereas NOS transcription was

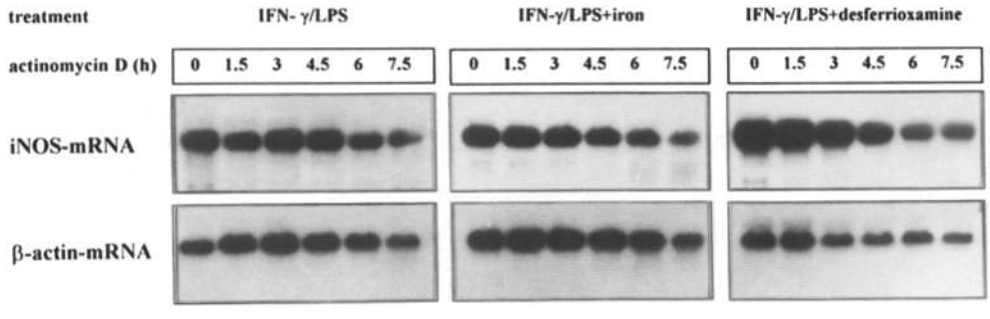

Figure 4. Effects of iron and desferrioxamine on NOS mRNA stability. In J774 cells, which were treated with ferric nitrate nonahydrate or desferrioxamine and IFN-γ/LPS for 20 h, transcription was inhibited by addition of actinomycin D (5 μg/ml) for up to 7.5 h. Cells were harvested every 1.5 h after addition of actinomycin D. RNA was isolated and Northern blotting for NOS mRNA was carried out as described in Materials and Methods. Filters were exposed between 8 and 72 h to XRP-5 x-ray films in order to obtain optimal conditions for densitometric evaluation of mRNA half-life. One of three similar experiments is shown.

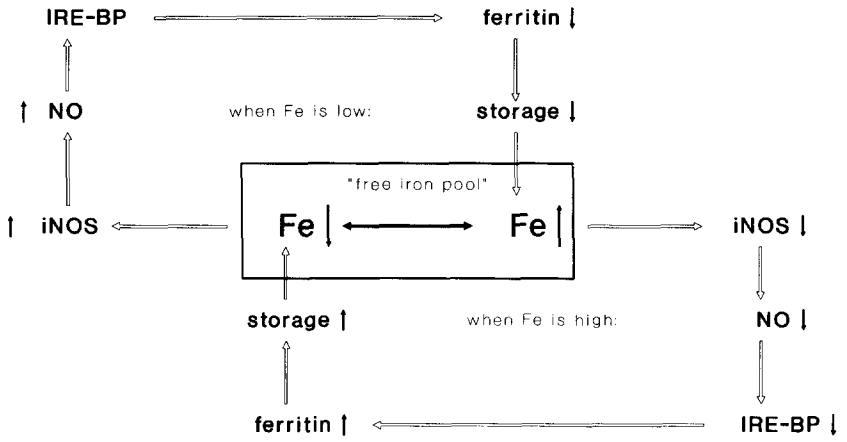

Figure 6. Proposed feedback regulation between iron metabolism and the NO/NOS pathway. Based on the data presented here and on previous results on NO–iron interactions (11, 12), a suitable model is designed demonstrating regulatory loops between iron and NOS. (↑) Increase or (↓) decrease of either free cellular iron concentration, transcription and activity of inducible NOS (*iNOS*), formation of NO, IRE-binding activity of IRP (*IRE-BP*), translation of ferritin, or storage of iron.

enhanced by desferrioxamine treatment to 284.0 ± 24.2% (p <0.001). As evidenced from Fig. 5 nuclear transcription for β-tubulin was not significantly altered by either treatment which was confirmed by densitometric evaluation (control, i.e. treatment with IFN-γ/LPS, estimated as 100%; iron supplementation 96.4 ± 11.3%; desferrioxamine 109.4 ± 18.6%). It is important to note that the changes in NOS transcription rates appear to account quantitatively for the observed alterations in iNOS mRNA levels and activity (compare with Table 1). We conclude that alterations (increases as well as decreases) in cellular iron availability cause regulation of NOS transcription in J774 cells.

Discussion

It was recently recognized that NO can directly control the activities of IRP, the central regulatory protein for cellular iron metabolism, and that NO thereby directly represses the synthesis of the iron storage protein ferritin (11–13). Based on these findings, we investigated in the present study whether NO production is conversely controlled by iron availability. We examined the effect of iron addition or withdrawal on NOS enzyme activity, mRNA levels, mRNA stability, and gene transcription in the murine macrophage cell line J774. Our results show that increased intracellular iron levels led to a decrease of NOS activity, whereas depletion of intracellular iron strongly enhances the enzyme activity in IFN-γ/LPS–stimulated cells. These differences in enzyme activity are due to altered NOS mRNA levels rather than to direct interference of $Fe(3^+)$ and desferrioxamine with the NOS protein. Determinations of mRNA half-life and nuclear runoff experiments indicate that the regulatory effects of $Fe(3^+)$ and desferrioxamine are primarily caused by influencing nuclear gene transcription for inducible NOS.

It was shown previously (39, 40) that heme and non-heme iron reduced IFN-γ–mediated effects on neopterin formation, trytophan degradation, and MHC class II expression in a human myelomonocytic cell line (THP-1), whereas iron chelation by desferrioxamine enhanced IFN-γ activity in these cells. In the case of the murine macrophage cell line J774, no such general effects of iron on pteridine formation or MHC class II expression could be observed. This may be explained by species-specific differences concerning cytokine-induced formation of pteridines and NO among human and murine cells. In contrast to human cells or murine fibroblasts (for a review see reference 41), the activity of GTP cyclohydrolase I, the first enzyme in the biosynthetic pathway of tetrahydrobiopterin, is already high in J774 cells and is not further enhanced by IFN-γ or IFN-γ plus TNF-α (42). On the other hand, THP-1 cells, like many other human cell lines, cannot be induced for NO formation in vitro. In the murine macrophage cell line J774, however, profound effects of intracellular iron concentration on NOS gene transcription can be observed. The generality of this regulation will have to be assessed in other cell lines and in human blood cells. Taken together, the results reported previously for the regulation of IRP and ferritin by NO (11, 12) and the results presented in this study suggest the existence of a regulatory loop between iron and NO/NOS (Fig. 6): iron deprivation of cells results in increased NOS gene transcription leading to enhanced NO formation. NO stimulates IRE-binding by IRP, the central regulatory factor of intracellular iron metabolism, possibly by withdrawing iron from the central iron-sulfur cluster of this protein (11, 12). High-affinity binding to the IREs in the 5′-untranslated region of ferritin represses ferritin translation (12) and thus iron storage, which in turn would lead to an increase in "free" cellular iron. Although an effect of NO on transferrin receptor mRNA stabilization has not yet been observed (13), increased stability of transferrin receptor mRNA and consequently, enhanced expression of transferrin receptors, would lead to increased cellular iron uptake and could thus augment the effect resulting from ferritin repression. Increasing intracellular concentrations of free iron could induce the opposing feedback response by inhibition of NOS transcription and NO formation as is indicated by the data presented here. This would consecutively result in reduction of IRE-binding activity of IRP and therefore lead to increased ferritin translation and iron storage (Fig. 6).

Formation of NO has been shown to be involved in the antitumor and antimicrobial cytotoxic effector function of activated macrophages (43–46). The mechanism suggested

Formation of NO has been shown to be involved in the antitumor and antimicrobial cytotoxic effector function of activated macrophages (43–46). The mechanism suggested here could therefore provide a tool for cytokine-activated macrophages to link maintenance of iron homeostasis within the effector cell with optimal NO formation for host defense. On the other hand, parasites consuming iron (14) would contribute to increased formation of NO and therefore to enhanced cytotoxicity towards themselves. In this respect, recent in vivo data which report that patients suffering from *Plasmodium falciparum* infection improved after treatment with desferrioxamine are of clinical interest (47, 48). Since NO was shown to be involved in the destruction of malaria parasites (49, 50), the beneficial effect of desferrioxamine may not only be due to limitation of iron availability for the parasites (as suggested by the investigators) but also be based on enhancement of NO production by desferrioxamine. Regulatory linkage of intracellular iron availability and the NO/NOS pathway might also account for altered iron traffic causing anemia in chronic inflammatory disorders which is characterized by low concentrations of nonferritin-bound iron and increased macrophage activity, caused by enhanced levels of circulating cytokines, such as IFN-γ (14–16, 51).

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Address correspondence to Dr. G. Weiss, Department of Internal Medicine, University Hospital, Anichstr. 35, A-6020 Innsbruck, Austria.

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