

Functional Requirement of the Hypoxia-responsive Element in the Activation of the Inducible Nitric Oxide Synthase Promoter by the Iron Chelator Desferrioxamine*

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Giovanni Melillo^{‡§}, Lynn S. Taylor[‡], Alan Brooks[¶], Tiziana Musso[¶], George W. Cox[‡], and Luigi Varesio^{‡¶}

From the [‡]Laboratory of Experimental Immunology, Division of Basic Sciences, and [¶]Intramural Research Support Program, SAIC Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland 21702-1201

We have previously reported that a 19-base pair element of the 5'-flanking region of the inducible nitric oxide synthase (iNOS) gene containing a sequence homology to a hypoxia-responsive enhancer (iNOS-HRE) mediates picolinic acid (PA)- or hypoxia-induced activation of the iNOS promoter in interferon- γ (IFN- γ)-treated murine macrophages. The iron chelator desferrioxamine (DFX) induces the activity of the human erythropoietin enhancer in Hep3B cells. We have investigated the influence of DFX on the activation of the iNOS promoter and iNOS gene expression in ANA-1 macrophages. We have found that DFX induced DNA-binding activity to the hypoxia-inducible factor 1 (HIF-1) consensus sequence of the iNOS promoter and activated the iNOS-HRE in murine macrophages. These activities of DFX were associated with a synergistic induction of iNOS mRNA expression and iNOS transcription in IFN- γ -treated ANA-1 macrophages. Functional analysis of the 5'-flanking region of the iNOS gene demonstrated that IFN- γ plus DFX activated the full-length iNOS promoter and that the iNOS-HRE was required for DFX-induced iNOS transcriptional activity. We also investigated the role of iron metabolism in the DFX- or PA-dependent induction of HIF-1 activity and iNOS expression. We demonstrate that addition of iron sulfate completely abolished DFX or PA induction of HIF-1 binding and iNOS-HRE activation and abrogated IFN- γ plus either DFX- or PA-induced iNOS expression. These data establish that DFX is a co-stimulus for the transcriptional activation of the iNOS gene in IFN- γ -treated macrophages, and they provide evidence that the iNOS-HRE is required for the DFX-dependent activation of the iNOS promoter. Furthermore, our results indicate that the iNOS-HRE is a regulatory element of the iNOS promoter responsive to iron chelation.

Tissue hypoxia is a condition of decreased oxygen (O_2) levels that elicits homeostatic responses aimed at counteracting the negative effects of O_2 depletion (1). Hypoxia induces the ex-

pression of several genes, such as erythropoietin (Epo)¹ (2), vascular endothelial growth factor (3), and glycolytic enzymes (4, 5) that favor the adaptation of the organism to the decreased availability of O_2 . Significant progress has been made in the understanding of the molecular mechanism underlying hypoxia-inducible gene expression. A hypoxia-responsive enhancer (HRE) has been identified in the 3'-flanking region of the Epo gene in a region required for Epo transcriptional activation (6, 7). This regulatory region, which is highly conserved between human and mouse, also plays a critical role in the hypoxia-induced transcriptional activation of genes encoding glycolytic enzymes (4, 5). The HRE contains the consensus sequence for the binding of a transactivating factor, which is inducible by hypoxia (hypoxia-inducible factor 1, HIF-1) (7–9). Induction of HIF-1 binding and functional activation of the HRE was demonstrated in a variety of mammalian cell lines, regardless of their ability to express Epo, suggesting a general involvement of HIF-1 in the regulation of gene expression by O_2 tension (10, 11).

Nitric oxide (NO), a free radical gas generated by the enzyme nitric oxide synthase, is involved in the antimicrobial and antitumor activities of murine macrophages (12–14). Macrophage nitric oxide synthase is the product of a transcriptionally inducible gene (iNOS), whose maximal expression requires stimulation by IFN- γ plus a second signal (15–17). Three regulatory elements of the 5'-flanking region of the iNOS gene play a functional role in the control of its transcriptional activation (18, 19). The interferon regulatory factor 1 binding site, located at position –923 to –913, is required for the synergistic activity of IFN- γ , as shown *in vivo* in interferon regulatory factor 1 knock-out mice (20) and *in vitro* in the RAW 264.7 cell line (21). The consensus sequence for the binding of NF- κ B family members, located at position –85 to –76, mediates iNOS transcriptional activation in response to bacterial lipopolysaccharide (LPS) (22). We recently reported that the promoter region of the iNOS gene contains a sequence homology to the HRE (referred to hereafter as iNOS-HRE) (23). Functional studies in murine macrophages demonstrated that the iNOS-HRE was required for the activation of the iNOS promoter by IFN- γ plus picolinic acid, a catabolite of L-tryptophan, and by IFN- γ plus hypoxia (1% O_2). These results provided the first indication that iNOS is a hypoxia-inducible gene. The iNOS-HRE contained a 100% homology to the consensus sequence for the

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§ To whom correspondence should be addressed at present address: NCI, National Institutes of Health, Medicine Branch, Bldg. 10, Rm. 12N226, Bethesda, MD 20892. Tel.: 301-496-4916; Fax: 301-402-0172.

¶ Present address: Laboratory of Molecular Biology, Institute G. Gaslini, Genova, Italy.

¹ The abbreviations used are: Epo, erythropoietin; EMSA, electrophoretic mobility shift assay; HRE, hypoxia-responsive enhancer; HIF-1, hypoxia-inducible factor 1; iNOS, inducible nitric oxide synthase; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; CAT, chloramphenicol acetyltransferase; DFX, desferrioxamine; PA, picolinic acid; bp, base pair(s); CHX, cycloheximide.

binding of HIF-1 (5'-TACGTGCT-3') (7), and the HIF-1 binding site was essential for the induction of iNOS promoter activity by picolinic acid or hypoxia in IFN- γ -treated ANA-1 macrophages (23). Furthermore, we demonstrated that a CAT construct containing three copies of the iNOS-HRE was inducible by hypoxia or picolinic acid and that mutation of the HIF-1 binding site abolished hypoxia- or picolinic acid-dependent transcriptional activation (23). These data established that a hypoxia-responsive element mediates a novel pathway of iNOS transcriptional activation induced by picolinic acid or hypoxia that is different from that mediated by NF- κ B induced by LPS. Thus, at least two distinct pathways of iNOS expression are elicited in response to different signals and multiple regulatory elements control its transcriptional activation.

Little is known about the signals that can modulate the inducibility of iNOS by these different pathways. It has been recently shown that iron can down-regulate iNOS transcription induced by IFN- γ plus LPS, whereas desferrioxamine (DFX), an iron chelator, can augment such a response (24). These results suggested the existence of a regulatory loop between iron metabolism and iNOS induction by the IFN- γ plus LPS pathway, although analysis of the activation of the iNOS promoter was not performed in these experiments. However, the discovery of the hypoxia-dependent activation of iNOS expression supports the hypothesis of a different mechanism of action of DFX on iNOS expression, independent from the IFN- γ plus LPS pathway. In fact, recent evidence has been provided that DFX induces HIF-1 binding activity in Hep3B cells (25). DFX also induces the expression of reporter genes carrying the human Epo enhancer, and mutations in the HIF-1 binding site eliminated DFX-inducible transcriptional activation (25). These results suggest that DFX can directly activate the HRE, at least in Hep3B cells. It follows, then, that DFX itself could be a co-stimulus for the activation of iNOS expression in IFN- γ -treated macrophages along the hypoxia pathway. It was important to test this possibility because it would change our perspective on the connections between iron metabolism, iNOS induction, and macrophage activation. Therefore, we studied the functional activation of the iNOS-HRE and iNOS gene by DFX in macrophages.

We found that DFX induced binding activity to the HIF-1 consensus sequence of the iNOS promoter and expression of the iNOS-HRE in the murine macrophage cell line ANA-1. DFX, although ineffective by itself, was also a potent co-stimulus of iNOS transcription and enhanced iNOS mRNA expression in IFN- γ -treated cells. Functional analysis of the 5'-flanking region of the iNOS gene in macrophages stimulated with IFN- γ plus DFX demonstrated that the integrity of the HIF-1 binding site was required for the induction of iNOS promoter activity. Finally, addition of iron sulfate completely abrogated the expression of HIF-1 activity and iNOS mRNA in response to either DFX or PA. These results demonstrate that DFX is in itself a co-stimulus for the transcriptional activation of iNOS expression in IFN- γ -treated murine macrophages, and they identify the iNOS-HRE as a regulatory sequence of the iNOS promoter that is activated by iron deprivation.

MATERIALS AND METHODS

Cells and Reagents—The mouse macrophage cell line ANA-1 was established by infecting fresh bone marrow-derived cells from C57BL/6 mice with the J2 (*v-raf/v-myc*) recombinant retrovirus (26, 27). ANA-1 macrophages were cultured in Dulbecco's modified Eagle's medium (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin (Life Technologies, Inc.) (complete medium). Cells were maintained at 37 °C in a humidified incubator containing 5% CO₂ in air. Mouse rIFN- γ (specific activity $\geq 10^7$ units/mg) was purchased from Life

Technologies, Inc. Desferrioxamine, ferrous sulfate, and cycloheximide were purchased from Sigma. Picolinic acid was purchased from Sigma (purity ~ 99%) and was prepared as described previously (28).

Preparation of Nuclear Extracts—Nuclear extracts were prepared by modification of a standard protocol (29) as described previously (23). Briefly, cells were washed twice with cold Dulbecco's phosphate-buffered saline and pelleted by centrifugation at 1,200 rpm for 5 min at 4 °C. The cell pellet was washed with buffer A (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl) and incubated on ice for 10 min in buffer A. The cell suspension was homogenized with a glass Dounce homogenizer, and the nuclei were pelleted by centrifugation at 2,000 rpm for 5 min at 4 °C and resuspended in buffer C (0.42 M KCl, 20 mM Tris-HCl, pH 7.5, 20% glycerol, 1.5 mM MgCl₂). The suspension was mixed on a rotor at 4 °C for 30 min, and nuclear debris was pelleted by centrifugation for 30 min at 13,000 rpm. The supernatant was dialyzed against two changes of buffer D (25 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA, 0.1 M KCl) and clarified by centrifugation at 15,000 rpm for 10 min at 4 °C. Aliquots were frozen and stored at -70 °C for subsequent analysis of DNA binding proteins.

Electrophoretic Mobility Shift Assay (EMSA)—Probes were generated using Klenow fragment of DNA polymerase and [α -³²P]dCTP. The following oligonucleotide probes were used: AB.2 (5'-GTGACTACGTGCTGCCTAG-3') encompassing the consensus sequence for the binding of HIF-1, shown in bold; AB.1 (5'-TGAGTCCCAGTTTGAAGTG-3') from an unrelated sequence of the iNOS promoter; Mu-AB.2 (5'-GTGACTAAAGCTGCCTAG-3'), with the three mutated bases underlined; Epo (5'-GCCCTACGTGCTGCCTCA-3') from the 3'-flanking region of the human Epo gene (7). Binding reactions were performed in buffer containing 25 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA, 0.1 M KCl, with 5 μ g of nuclear extract, and 0.4 μ g of denatured calf thymus DNA on ice. After incubation for 10 min, probe (1 \times 10⁴ cpm) was added, and the incubation was continued for an additional 20 min. Samples were loaded onto 5% nondenaturing polyacrylamide gel, and electrophoresis was performed at 180 V in 0.3 \times TBE at 4 °C.

Plasmids—The 1,749-bp fragment of the 5'-flanking region of the murine iNOS gene inserted upstream of the promoterless chloramphenicol acetyltransferase (CAT) gene in pCAT-Basic (Promega Corp.) (*i.e.* p1-iNOS-CAT) (18) was kindly provided by Q. Xie and C. Nathan (Cornell University Medical College, New York, NY). Deletion mutants of the iNOS promoter were obtained as described previously (23). For functional studies in the context of a heterologous promoter, three tandem copies of a 21-base pair (bp) double-strand oligonucleotide encompassing the iNOS-HRE (-231 to -211) were subcloned in the *Hind*III/*Bam*HI sites of pBLCAT2 plasmid, containing the CAT reporter gene under the control of a herpes simplex virus thymidine kinase promoter fragment spanning from -105 to +51. The constructs were sequenced using Sequenase version 2.0 (United States Biochemical Corp.).

Transient Transfection of ANA-1 and CAT Assay—ANA-1 macrophages were transfected by a modification of the DEAE-dextran method (30), as described (23). Briefly, 10 μ g of plasmid DNA were added to 10⁷ cells in 1 ml of Dulbecco's modified Eagle's medium without serum and containing 250 μ g/ml DEAE-dextran and 50 mM Tris-HCl, pH 7.5. The cells were incubated at 37 °C for 1 h followed by a 2-min shock with 10% Me₂SO at room temperature. The cells were washed, plated in 6-well plates at 1 \times 10⁶/ml in 2.5 ml of complete medium, and incubated at 37 °C in 5% CO₂. Twenty-four hours later, the cells were incubated with the appropriate stimulus for an additional 18 h. The cells were then washed, resuspended in 0.25 M Tris-HCl, pH 7.5, and lysed by three cycles of rapid freezing and thawing. The lysates were centrifuged (11,000 \times g for 10 min), and the supernatants were assayed for CAT activity by TLC (31). Protein content was determined as described by Bradford (32), using the Bio-Rad Protein Assay. To control for differences in the uptake of transfected DNA, cells were cotransfected with 5 μ g of pGL2 plasmid (pGL2 control, Promega), which contains the luciferase reporter gene under control of SV40 promoter and enhancer. Cell lysates were then assayed for luciferase activity.

Northern Blot Analysis—Total cellular RNA was harvested and processed as described previously (28). Briefly, cells were solubilized with guanidine isothiocyanate, and the total cellular RNA was purified by centrifugation through a cushion of cesium chloride. Twenty μ g of RNA were size-fractionated in a 1.2% agarose gel, blotted onto a Nytran membrane (Schleicher & Schuell), and incubated overnight at 42 °C in Hybrisol I hybridization solution (Oncor, Gaithersburg, MD). The cDNA probes that were specific for mouse macrophage nitric oxide synthase (33) or for human glyceraldehyde-3-phosphate dehydrogenase (CLONTECH Laboratories Inc.) were radiolabeled with [³²P]dCTP (Amersham Corp.) by using an RTS RadPrime DNA labeling system (Life Technol-

ogies, Inc.) according to the manufacturer's procedure. The blot was hybridized individually with the radiolabeled probes (1 to 2×10^6 cpm/ml) during an overnight incubation and washed three times for 10 min at room temperature with $2 \times$ SSC, 0.1% SDS and two times for 20 min at 60°C with $0.2 \times$ SSC, 0.1% SDS. The blot was autoradiographed

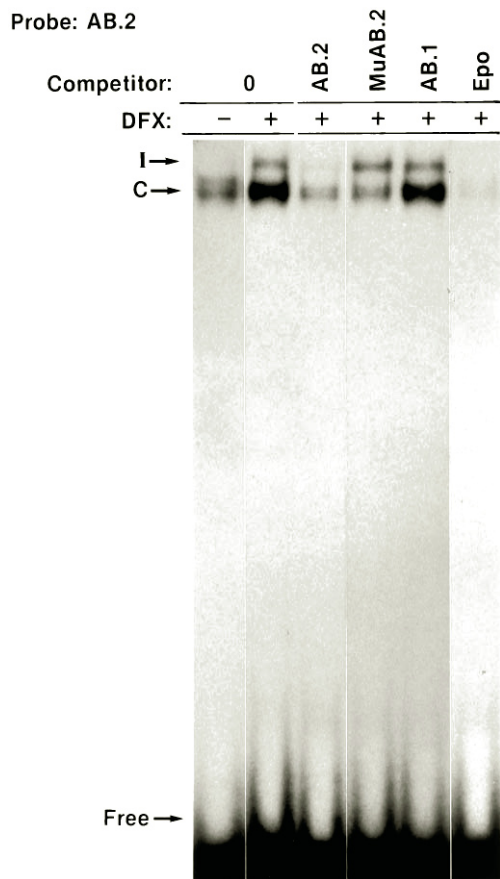
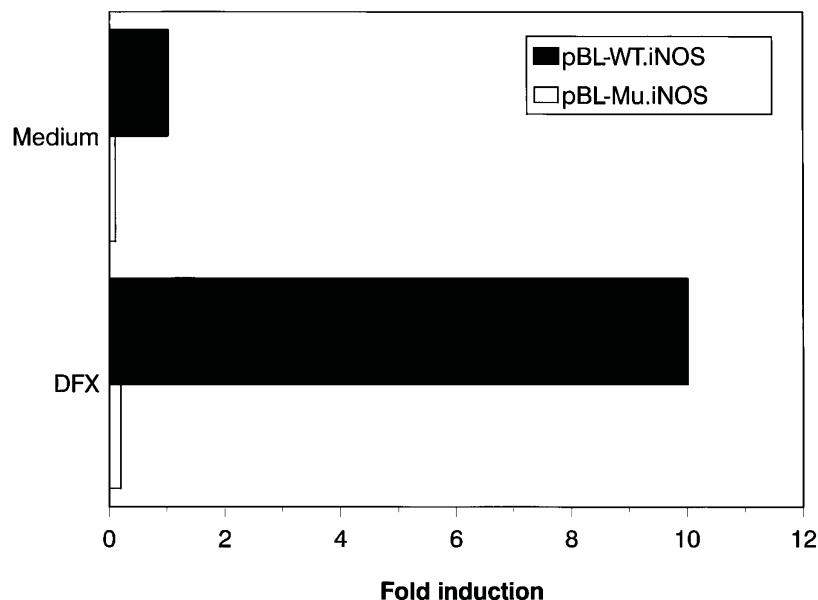


FIG. 1. DFX induces a DNA-binding activity to the HIF-1 binding site of the iNOS-HRE. Nuclear extracts from ANA-1 macrophages treated with medium or DFX ($400 \mu\text{M}$) for 12 h were incubated with AB.2 probe in the absence (0) or presence of 100-fold molar excess of the indicated unlabeled competitor oligonucleotide (described under "Materials and Methods") and analyzed by EMSA. Competitor DNAs were incubated with nuclear extracts for 10 min on ice prior to addition of labeled probe. Binding activities are labeled as follows: C, constitutive; I, induced; Free, free labeled probe.

FIG. 2. DFX induces iNOS-HRE activity in murine macrophages. ANA-1 macrophages were transfected with the indicated plasmids containing three copies of the iNOS-HRE either wild type ($5'$ -AGTGACTACGTGCTGCCTAGG- $3'$, pBL-WT.iNOS) or mutated ($5'$ -AGTGACTAAAAGCTGCCTAGG- $3'$, pBL-Mu.iNOS) in the pBLCAT2 vector. *Bold type* indicates the HIF-1 binding site, with the mutation *underlined*. Twenty-four hours after the transfection, ANA-1 cells were treated with medium or DFX ($400 \mu\text{M}$) for additional 18 h, and CAT activity was assayed. Results are expressed as fold increase of CAT activity (% acetylation) relative to that expressed by pBL-WT.iNOS in untreated cells (arbitrarily considered to be equal to 1). Results were normalized for the expression of the parental vector (pBLCAT2) and are from one representative experiment.



at -70°C on XAR-5 film (Eastman Kodak) with the use of Lightning Plus intensifying screens (DuPont NEN).

Nuclear Run-on Analysis—ANA-1 cells were treated as indicated for 12 h, and nuclei were isolated as described (28). Thawed nuclei were mixed with $150 \mu\text{l}$ of $2 \times$ transcription mix ($1 \times = 100 \text{ mM}$ sucrose, 10% glycerol, 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl_2 , 2.5 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP) and incubated with $100 \mu\text{Ci}$ of [^{32}P]UTP (800 Ci/mmol; Amersham Corp.) at 30°C for 30 min. Twenty μl of 100 mM CaCl_2 and 20 units of RNase-free DNase I were added and incubated 10 min at 30°C with gentle mixing every 2 min. The nuclei were then lysed with 1 ml of Trizol (Life Technologies, Inc.), and the RNA was isolated according to the manufacturer's procedure. Approximately 2×10^6 cpm of RNA were used in hybridization for 48 h with 2.5 μg of each slot-blotted denatured plasmid (pGEM-3Z vector alone, vector containing the 3.9-kilobase cDNA insert of macrophage iNOS (33), and vector containing a chicken β -actin cDNA insert). Filters were then washed and autoradiographed as for the Northern blot analysis.

RESULTS

DFX Induces DNA-binding Activity to the iNOS-HRE—To address whether DFX induces DNA-binding activity to the iNOS-HRE, EMSA was performed on nuclear extract prepared from ANA-1 macrophages treated with medium or DFX, using as radiolabeled probe a 19-base pair (bp) oligonucleotide (AB.2) containing the HIF-1 binding site of the iNOS promoter. As shown in Fig. 1, ANA-1 macrophages expressed a constitutive binding activity. Treatment with DFX caused the appearance of an inducible complex that was specifically inhibited by excess unlabeled probe (AB.2) but not by an unrelated probe (AB.1). In addition, the DFX-inducible binding complex was not competed for by a probe mutated in three bases within the putative HIF-1 binding site (Mu.AB2). Both the constitutive and the DFX-inducible binding activities were completely inhibited by a probe encompassing the canonical HIF-1 binding site (Epo) of the Epo enhancer, which differs from the AB.2 probe in five flanking bases. These results demonstrate that DFX induces in macrophages a specific DNA-binding activity to the HIF-1 binding site of the iNOS promoter.

DFX Activates the iNOS-HRE in Murine Macrophages—To establish whether DFX was a stimulus for the activation of the iNOS-HRE in macrophages, ANA-1 cells were transiently transfected with two CAT constructs containing three tandem copies of the iNOS-HRE, either wild type (pBL-WT.iNOS) or mutated at the HIF-1 binding site (pBL-Mu.iNOS), 5' of the herpes simplex virus thymidine kinase minimal promoter. The pBL-WT.iNOS construct was constitutively expressed in macrophages (Fig. 2). Treatment with DFX caused a 10-fold

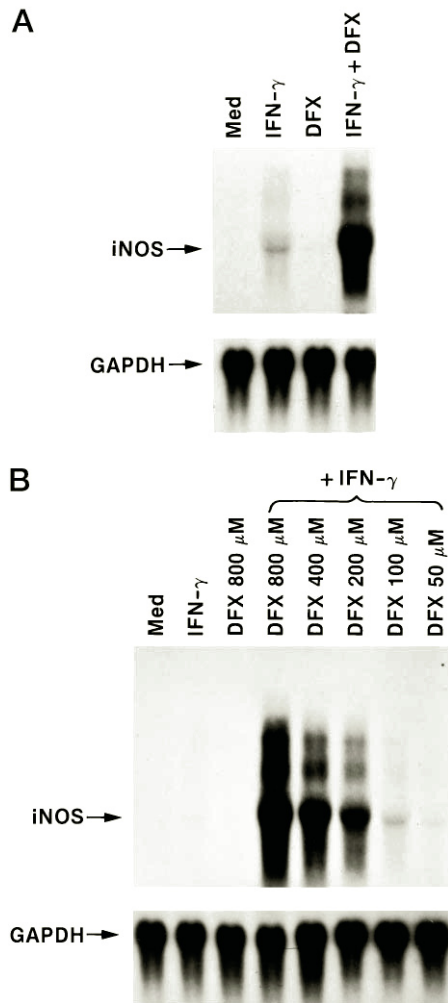


FIG. 3. DFX synergizes with IFN- γ for induction of iNOS mRNA expression in murine macrophages. ANA-1 macrophages were cultured at 1×10^6 cells/ml in 60-mm tissue culture plates and treated with medium (Med), IFN- γ (100 units/ml), DFX (400 μ M) alone or in combination with IFN- γ (A) or with medium, IFN- γ (100 units/ml) alone, DFX (800 μ M) alone, or IFN- γ plus the indicated concentrations of DFX (B) for 18 h. Total cellular RNA was examined for iNOS mRNA expression as described under "Materials and Methods." The same filters were hybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to monitor RNA loading. Results shown are from one representative experiment.

increase in the expression of CAT activity, relative to untreated cells. In contrast, plasmid pBL-Mu.iNOS was not inducible by DFX. These results, consistent with the data obtained in EMSA, demonstrate that the iNOS-HRE is sufficient to confer inducibility by DFX in murine macrophages and that mutation of the HIF-1 binding site abolishes DFX inducible transcriptional activation.

DFX Synergizes with IFN- γ in the Induction of iNOS mRNA Expression—To determine whether DFX can be a stimulus for the expression of iNOS mRNA, ANA-1 macrophages were incubated with medium, IFN- γ (100 units/ml), and DFX (400 μ M) alone or in combination, and total RNA was harvested after 18 h. No expression of iNOS mRNA was observed in cells treated with medium or DFX alone, and very low levels of iNOS mRNA were detected in macrophages stimulated with IFN- γ alone (Fig. 3A). In contrast, addition of DFX caused a major increase of IFN- γ -induced iNOS mRNA expression that was detectable after 6 h of treatment (see Fig. 5) and increased up to 18 h (Fig. 3A). To determine the dose dependence of DFX effects, ANA-1 macrophages were incubated with increasing

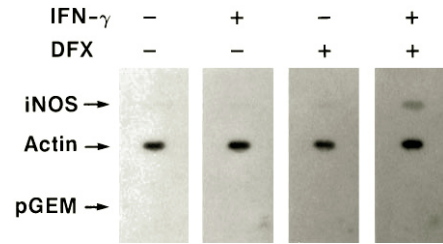


FIG. 4. DFX synergistically induces iNOS transcription in IFN- γ -treated ANA-1 macrophages. ANA-1 macrophages (2.5×10^7) were treated with medium, IFN- γ (100 units/ml), DFX (400 μ M) alone or in combination with IFN- γ . Nuclei were isolated after 12 h, and the rate of transcription of the iNOS gene was then determined by nuclear run-on analysis as described. The following plasmid DNA were used: piNOS, pGEM vector containing a 3.9-kilobase cDNA insert of mouse macrophage nitric oxide synthase; p-actin, vector containing a chicken β -actin cDNA insert; pGEM, vector lacking any cDNA insert.

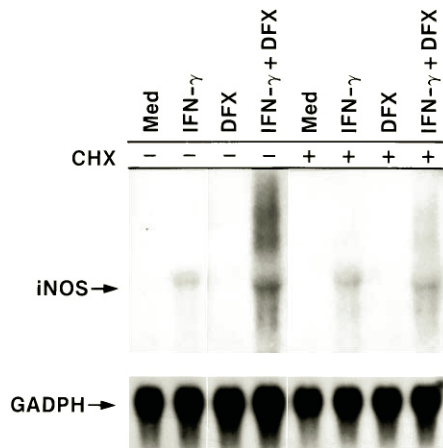


FIG. 5. Protein synthesis is required for IFN- γ plus DFX induction of iNOS mRNA expression. ANA-1 macrophages (1×10^6 cells/ml) were incubated with medium, IFN- γ (100 units/ml), DFX (400 μ M) alone or in combination with IFN- γ , in the absence or presence of 7.5 μ g/ml CHX. Total cellular RNA was harvested 6 h later and examined for iNOS mRNA expression as described. Data are from one of two similar experiments.

concentrations of DFX in the presence or absence of IFN- γ for 18 h. DFX alone, at doses ranging from 50 μ M (data not shown) to 800 μ M, failed to induce iNOS mRNA. The combination of IFN- γ and DFX increased the levels of iNOS mRNA at a dose of 100 μ M DFX (3–4-fold above IFN- γ alone) (Fig. 3B) and reached its maximum (15–20-fold above IFN- γ alone) at a dose of 800 μ M DFX. These data demonstrate that DFX acts synergistically with IFN- γ in a dose-dependent fashion for the induction of iNOS mRNA expression in murine macrophages.

DFX and IFN- γ Synergistically Induce iNOS Transcription—Nuclear run-on experiments were performed to demonstrate that the increase in iNOS mRNA in response to IFN- γ plus DFX was due to transcriptional activation of the gene. ANA-1 macrophages were treated with medium, IFN- γ , DFX alone or in combination with IFN- γ . The iNOS gene was not constitutively transcribed in ANA-1 macrophages, and treatment with IFN- γ or DFX alone failed to induce iNOS transcription (Fig. 4). In contrast, ANA-1 cells treated with IFN- γ plus DFX showed a significant increase in the rate of transcription of the iNOS gene. These data establish that DFX synergistically induces iNOS transcriptional activation in IFN- γ -treated macrophages.

Transcriptional activation of the iNOS gene is blocked in the presence of protein synthesis inhibitors (17, 28). To establish whether the induction of iNOS mRNA expression by DFX was dependent on *de novo* protein synthesis, ANA-1 macrophages

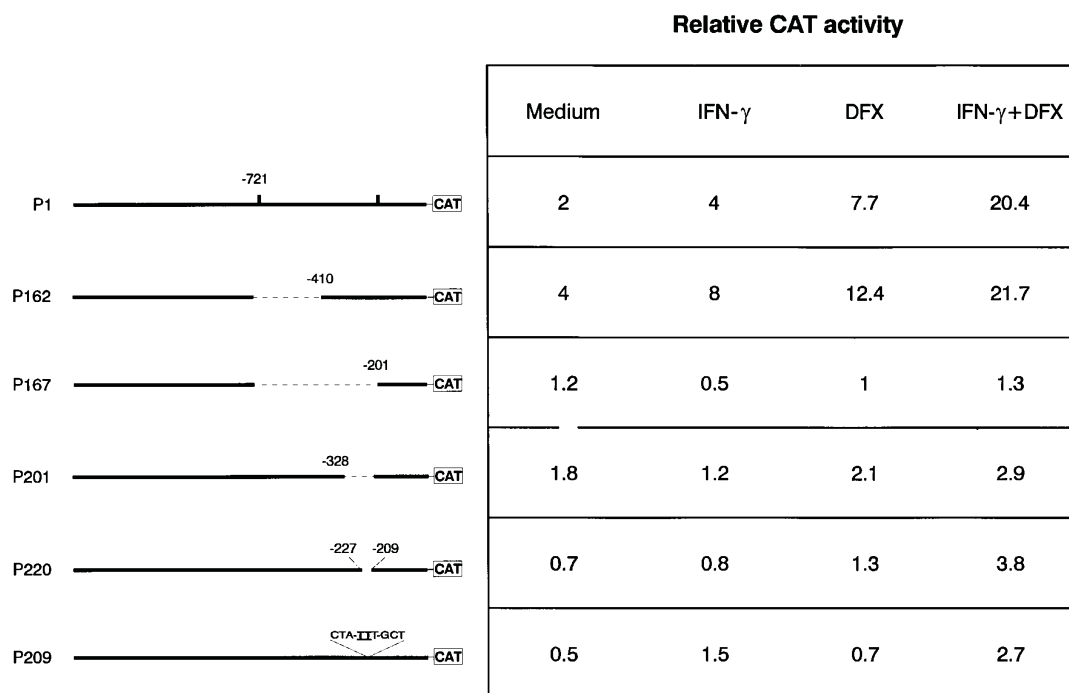


FIG. 6. Functional requirement of the iNOS-HRE for DFX-dependent induction of iNOS promoter activity. ANA-1 macrophages were transfected with the indicated plasmids as described under "Materials and Methods." Twenty-four hours later, cells were treated with medium, IFN- γ (100 units/ml), DFX (400 μ M) alone or in combination with IFN- γ for an additional 18 h, and CAT activity was assayed by TLC. Plasmid p220 contains a 19-bp deletion encompassing the iNOS-HRE (5'-aCTACGTGCTGCCTaGggg-3'); the sequence homology between iNOS promoter and erythropoietin (Epo) gene is shown in *uppercase*, and the binding site of the HIF-1 is *underlined*. In plasmid p209 a 2-bp mutation (5'-TATTTGCT-3', *underlined*) was created at position -223 -222 internal to the binding site for HIF-1. Results are expressed as relative CAT activity, obtained by dividing CAT activity (% acetylation) by relative light units for luciferase. Data shown are from one representative experiment.

were incubated in medium, IFN- γ , DFX alone or in combination with IFN- γ , in the presence or absence of CHX (7.5 μ g/ml) for 6 h. As shown in Fig. 5, the addition of CHX almost completely abrogated the synergistic induction of iNOS mRNA expression by IFN- γ plus DFX, indicating that *de novo* protein synthesis was required for iNOS mRNA induction by IFN- γ plus DFX.

Functional Requirement of the iNOS-HRE for the Induction of iNOS Promoter Activity by DFX—To identify the region(s) of the iNOS promoter responsible for the DFX-dependent transcriptional activation of the iNOS gene and, in particular, to investigate the functional role of the iNOS-HRE, ANA-1 macrophages were transiently transfected with plasmids containing the full-length or deletion mutants of the 5'-flanking region of the iNOS gene linked to the CAT reporter gene. Cells were stimulated with medium, IFN- γ (100 units/ml), or DFX (400 μ M) alone or in combination. ANA-1 cells transfected with plasmid p1, containing the full-length iNOS promoter, did not express significant levels of CAT activity either constitutively or after treatment with IFN- γ alone (Fig. 6). Low but consistent induction of CAT expression was induced by treatment with DFX alone. However, a strong synergistic induction of CAT activity was observed following stimulation with IFN- γ and DFX. Deletion of the upstream region of the iNOS promoter (-1588 to -721, plasmid p3), containing the IFN- γ -responsive region, completely abrogated the synergistic interaction between DFX and IFN- γ in the induction of iNOS promoter activity (data not shown). These data indicated that maximal activation of the iNOS promoter required the presence of the IFN- γ -responsive region and was induced by the combination of IFN- γ plus DFX. Transfection of plasmid p162, deleted of the region from -721 to -410, did not affect the induction of CAT activity by IFN- γ plus DFX. In contrast, plasmids p167 (deleted from -721 to -201) and p201 (deleted from -328 to -201)

were no longer inducible following stimulation of ANA-1 macrophages with the combination of IFN- γ and DFX (Fig. 6). To establish whether DFX-dependent activation of the iNOS promoter required the presence of a functional iNOS-HRE, ANA-1 macrophages were transfected with two iNOS promoter-CAT reporter gene constructs (23), containing either a deletion of the iNOS-HRE (-227 to -209, plasmid p220) or a mutation of two bases required for HIF-1 binding to DNA (-223 -222, plasmid p209) (7). A reduction greater than 80% relative to the wild type iNOS promoter was observed in the induction of CAT expression with either plasmid. These data demonstrate that the synergistic interaction between IFN- γ and DFX in the transcriptional activation of the iNOS promoter is mediated, at least in part, by the iNOS-HRE.

Chelation of Iron Is Involved in the DFX-dependent Induction of iNOS-HRE Activity and iNOS mRNA Expression—It has been previously reported that induction of HIF-1 binding activity by DFX in the Hep3B cell line was due to chelation of iron (25). Therefore, we investigated the role of iron chelation in the DFX-dependent induction of HIF-1 binding and iNOS-HRE activity in murine macrophages. ANA-1 macrophages were treated with medium or DFX in the presence or absence of iron sulfate (FeSO_4), and the induction of HIF-1 binding (Fig. 7A) or iNOS-HRE activity (Fig. 7B) was measured. The addition of FeSO_4 completely blocked the appearance of the DFX inducible complex to the HIF-1 binding site of the iNOS-HRE (Fig. 7A) and dramatically inhibited the inducibility of the pBL-WT.iNOS construct (Fig. 7B). We then tested whether the iron-dependent inhibition of iNOS-HRE activation also impaired the ability of IFN- γ plus DFX to induce the activity of the full-length iNOS promoter and the expression of iNOS mRNA. We measured the inducibility of plasmid p1 (Fig. 7C) or the levels of iNOS mRNA (Fig. 7D) in ANA-1 macrophages treated with IFN- γ plus DFX in the presence or absence of

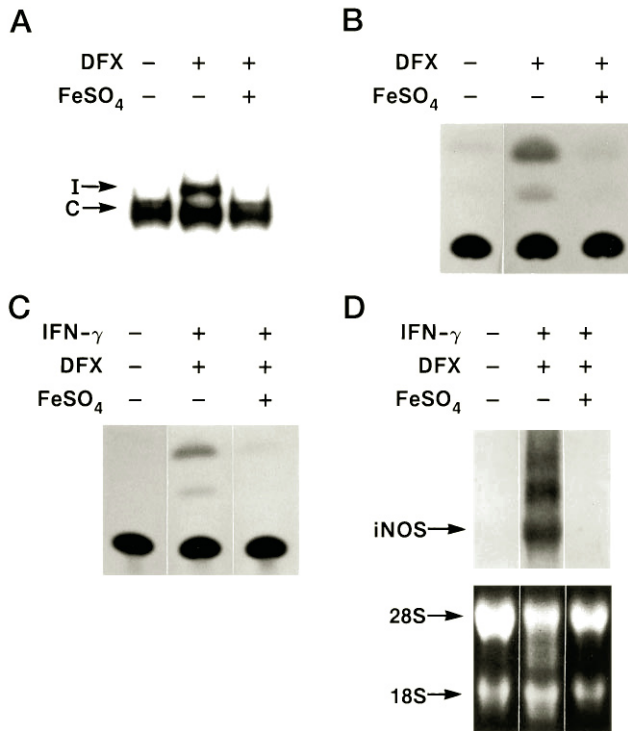


FIG. 7. Ferrous sulfate inhibits DFX-dependent induction of HIF-1 binding and iNOS expression. A, electrophoretic mobility shift assay. ANA-1 macrophages were treated with medium or DFX (200 μ M) in the absence or presence of FeSO₄ (300 μ M) for 12 h. Nuclei were isolated, and EMSA was performed with radiolabeled AB.2 probe as described. B, iNOS-HRE activity. ANA-1 macrophages were transfected with plasmid pBL-WT.iNOS. Twenty-four hours after the transfection cells were treated with medium or DFX (400 μ M) in the absence or presence of FeSO₄ (300 μ M) for an additional 18 h, and CAT activity was assayed as described. C, iNOS promoter activity. ANA-1 cells were transfected with plasmid p1, containing the full-length iNOS promoter. Cells were treated with medium, IFN- γ (100 units/ml) plus DFX (400 μ M) in the presence or absence of FeSO₄ (300 μ M) for 18 h, and CAT activity was assayed. D, Northern blot analysis. ANA-1 macrophages were treated with medium, IFN- γ (100 units/ml) plus DFX (200 μ M) in the presence or absence of FeSO₄ (300 μ M) for 18 h, and total cellular RNA was examined for iNOS mRNA expression as described.

FeSO₄. Iron sulfate caused a greater than 90% reduction in the IFN- γ plus DFX-dependent induction of iNOS promoter activity (Fig. 7C) and completely inhibited the synergistic activation of iNOS mRNA expression (Fig. 7D). We conclude that iron was involved in the mechanism by which DFX induced iNOS-HRE activity and iNOS mRNA expression in murine macrophages.

PA-dependent Activation of iNOS-HRE and Induction of iNOS mRNA Is Dependent on Chelation of Iron—The mechanism by which PA activates the iNOS-HRE is unknown. However, PA shares with DFX the property of being an iron chelator (34, 35), and this property seems to account for its antiproliferative effects on tumor cell lines (34). To address whether PA induces HIF-1 binding and iNOS-HRE activation via chelation of iron, ANA-1 macrophages were treated with medium or PA in the presence or absence of FeSO₄, and HIF-1 binding (Fig. 8A) or iNOS-HRE activity (Fig. 8B) was measured. PA induced a DNA-binding activity to the iNOS-HRE that was completely abolished by addition of FeSO₄ (Fig. 8A). PA also induced a 10-fold expression of the pBL-WT.iNOS construct over untreated cells that was almost completely abrogated in the presence of FeSO₄ (Fig. 8B). As previously reported, PA alone does not induce the expression of iNOS promoter or iNOS mRNA (data not shown). However, the PA-dependent induction of iNOS promoter activity (Fig. 8C) and iNOS mRNA expression (Fig. 8D) in IFN- γ -treated ANA-1

macrophages was dramatically inhibited in the presence of FeSO₄. As shown in Fig. 8D, FeSO₄ either alone or in combination with IFN- γ did not induce the expression of iNOS mRNA. These results indicate that the mechanism by which PA activates iNOS expression in murine macrophages is dependent, at least in part, on chelation of iron.

DISCUSSION

We have investigated the role of DFX in the regulation of iNOS promoter transcriptional activity in murine macrophages. We have found that DFX induced HIF-1 binding and iNOS-HRE expression. The presence of a functional iNOS-HRE was required for IFN- γ plus DFX-dependent iNOS promoter activation in ANA-1 macrophages. These activities were paralleled by a synergistic induction of iNOS transcription and iNOS mRNA expression in cells treated with IFN- γ plus DFX. These data demonstrated that DFX is in itself an activator of the iNOS-HRE in murine macrophages and that the hypoxia-responsive element is involved in the DFX-dependent transcriptional activation of the iNOS gene. Thus, DFX is a co-stimulus with IFN- γ for iNOS induction along the hypoxia/picolinic acid pathway.

Wang and Semenza (25) have provided evidence that DFX induces HIF-1 activity in Hep3B cells. HIF-1 binds to a hypoxia-inducible enhancer that was originally described in Hep3B cells as a 50-nucleotide functionally tripartite element (7). However, the homology between the human 50-nucleotide element and the murine iNOS-HRE is limited to the HIF-1 binding site plus six flanking bases. We have found that DFX induced a specific DNA complex to an oligonucleotide probe (AB.2) encompassing the HIF-1 binding site of the iNOS promoter in ANA-1 macrophages. The appearance of the binding complex was completely inhibited by an oligonucleotide containing the canonical HIF-1 consensus sequence of the Epo enhancer (Epo) (7) but not by an oligonucleotide mutated in 3 bp within the HIF-1 binding site (Mu.AB.2), indicating that the integrity of the HIF-1 binding site was critical for the occurrence of the binding activity. Accordingly, DFX also induced in macrophages the expression of a tk promoter CAT-reporter gene construct containing three copies of the wild type (pBL-WT.iNOS) but not mutated (pBL-Mu.iNOS) iNOS-HRE. The same 3-bp mutation within the HIF-1 binding site inhibited hypoxia- or DFX-induced transcriptional activation of the Epo enhancer in Hep3B (7, 25) and abolished the hypoxia or picolinic acid inducibility of the iNOS-HRE in murine macrophages (23). These results suggest that the HIF-1 binding site, conserved between the Epo enhancer and the iNOS-HRE, is sufficient to confer inducibility by DFX in murine macrophages.

Consistent with the finding that DFX was a potent activator of the iNOS-HRE in macrophages, DFX synergistically induced the expression of the full-length iNOS promoter in IFN- γ -treated macrophages. Although DFX alone did not induce detectable iNOS transcription, as assessed by nuclear run-on analysis, we found that DFX alone induced low but consistent levels of expression of the iNOS promoter in transient transfection assays. This discrepancy has been reported previously for PA- or LPS-induced activation of iNOS promoter and might be explained by negative regulatory regions that control the expression of the endogenous gene or by differences in the sensitivity of the assays (19, 23). Induction of iNOS promoter activity by DFX was the result, at least in part, of the cooperative interaction between the iNOS-HRE and IFN- γ -responsive sequences. Deletion or mutation of the HIF-1 binding site of the iNOS promoter demonstrated that the DFX-dependent iNOS transcriptional activation required the integrity of the HIF-1 binding site, providing further evidence of the involvement of HIF-1 in the regulation of iNOS promoter activity.

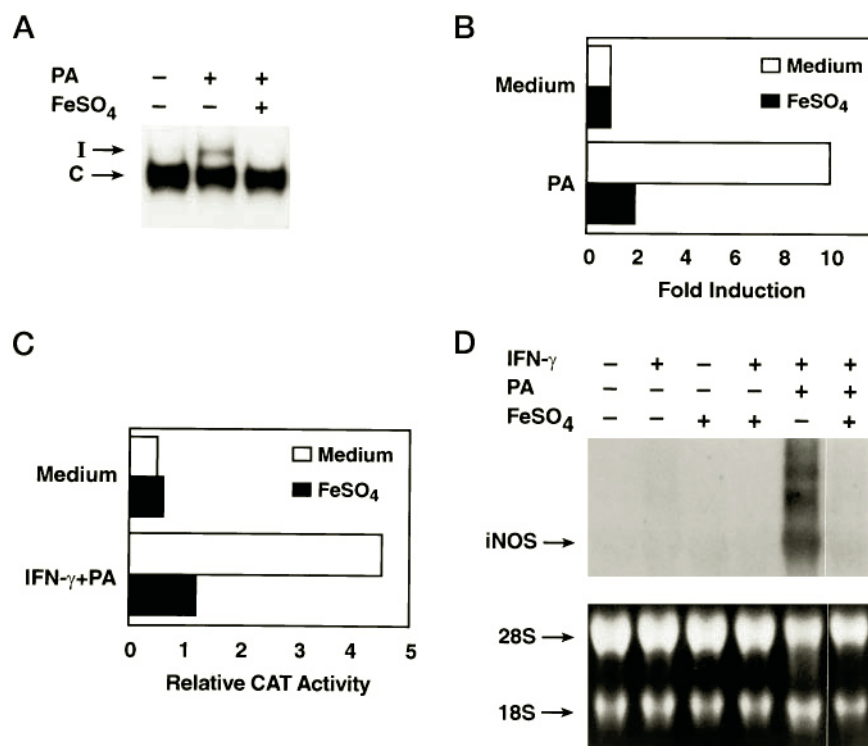


FIG. 8. **Ferrous sulfate inhibits PA-dependent induction of iNOS-HRE activity and iNOS expression.** A, electrophoretic mobility shift assay. ANA-1 macrophages were treated with medium or PA (4 mM) in the absence or presence of FeSO₄ (300 μM) for 12 h. Nuclei were isolated, and EMSA was performed with radiolabeled AB.2 probe as described. B, iNOS-HRE activity. ANA-1 macrophages, transfected with plasmid pBL-WT.iNOS, were treated with medium or PA (4 mM) in the absence or presence of FeSO₄ (300 μM) for 18 h, and CAT activity was assayed as described. C, iNOS promoter activity. ANA-1 cells, transfected with plasmid pI, were treated with medium, IFN-γ plus PA (4 mM) in the presence or absence of FeSO₄ (300 μM) for 18 h, and CAT activity was assayed. D, Northern blot analysis. ANA-1 macrophages were treated with medium, IFN-γ, FeSO₄ (300 μM) alone or in combination with IFN-γ, IFN-γ plus PA (4 mM) in the presence or absence of FeSO₄ (300 μM) for 18 h, and total cellular RNA was examined for iNOS mRNA expression as described.

The DFX-dependent induction of HIF-1 binding and iNOS promoter activity was associated with increased expression of the endogenous iNOS gene in ANA-1 macrophages. DFX induced iNOS mRNA expression and increased the rate of transcription of the iNOS gene in combination with IFN-γ. The synergistic interaction between IFN-γ and DFX in the induction of iNOS mRNA was dose-dependent and required *de novo* protein synthesis, as demonstrated by inhibition of DFX-induced iNOS mRNA expression in the presence of CHX. HIF-1 binding activity observed in hypoxia- or DFX-treated Hep3B cells was also abolished by addition of CHX (7, 25). This finding is consistent with the involvement of interferon regulatory factor 1 and HIF-1, both of which require ongoing protein synthesis, in the induction of iNOS transcriptional activity induced by IFN-γ plus DFX (7, 20). The augmented expression of iNOS mRNA induced by IFN-γ plus DFX in ANA-1 macrophages was also associated with increased levels of NO production and with the expression of tumoricidal activity against the tumor necrosis factor-α-resistant tumor target cell line P815.² These data demonstrate that DFX functions as a macrophage co-stimulator in combination with IFN-γ.

Iron chelation appeared to be the mechanism by which DFX induced HIF-1 activity in Hep3B cells (25). Cellular iron has been involved in the control of iNOS transcriptional activity induced by IFN-γ plus LPS in the J774 macrophage-like cell line (24). Data presented here suggest that the augmenting effect of DFX on IFN-γ plus LPS-dependent iNOS transcription can be accounted for by induction of HIF-1 binding and activation of the iNOS-HRE and that DFX and LPS activate iNOS

transcription through distinct pathways. However, a possible effect of DFX on LPS activation cannot be ruled out at the present. Addition of iron sulfate completely abolished the DFX- or PA-dependent induction of HIF-1 binding and iNOS-HRE expression in ANA-1 macrophages. The abrogation of these activities was paralleled by lack of iNOS promoter activation and iNOS mRNA expression following stimulation with IFN-γ plus either DFX or PA. These data indicate that the iNOS-HRE may be activated by iron chelation, and they provide evidence of a molecular mechanism by which iron metabolism may affect iNOS promoter activation in murine macrophages. Furthermore these data provide the first indication of the mechanism by which PA acts on murine macrophages to induce the expression of the iNOS gene.

The data presented here, together with previously published observations (23), demonstrate that the involvement of HIF-1 binding and iNOS-HRE expression is the common feature of the induction of iNOS promoter activity by hypoxia, picolinic acid, and DFX, and they suggest the existence of a common pathway of activation of iNOS transcription. This pathway of iNOS expression appears to be clearly distinct from that induced by LPS, which is largely dependent on NF-κB activation (22). Hypoxia and DFX are nonclassical macrophage activating agents because their signals are delivered through an intracellular sensor rather than through cell surface receptors. Our results are consistent with the possibility that hypoxia, picolinic acid, and DFX use a common sensor in the signal transduction pathway. The putative hemoprotein, which would function as intracellular O₂ sensor, may be responsive to changes in O₂ tension as well as to chelation of iron (36). Picolinic acid shares with DFX the property of acting as a co-stimulus with IFN-γ for the induction of tumoricidal activity (37), NO produc-

² G. Melillo, L. S. Taylor, A. Brooks, T. Musso, G. W. Cox, and L. Varesio, unpublished observations.

tion (38), and iNOS mRNA expression (28) in murine macrophages. In contrast, IFN- γ plus hypoxia induce significant levels of iNOS mRNA and iNOS protein but not of NO production, which can be achieved only by subsequent reoxygenation (39). Therefore, the biological implications of the induction of iNOS expression by hypoxia, picolinic acid, or DFX are quite different. In fact, hypoxia may occur in inflammatory and neoplastic lesions (14, 40, 41), whereas picolinic acid might be induced in those conditions in which elevated levels of tryptophan metabolites, such as kynurenine and quinolinic acid, have been detected (42–44). In contrast, DFX is a pharmacological agent used for the treatment of several pathological conditions, including iron overload (45), cancer (46–48), and Alzheimer's disease (49, 50). The notion that, in the presence of IFN- γ , DFX might be responsible for the induction of iNOS expression is important for the potential role of nitric oxide in mediating undesired side effects or beneficial therapeutic activities.

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