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Central Role of Transcription Factor NF-IL6 for Cytokine and Iron-Mediated Regulation of Murine Inducible Nitric Oxide Synthase Expression

Margit Dlaska and Günter Weiss

We have previously shown that iron regulates the transcription of inducible nitric oxide synthase (iNOS). To elucidate the underlying mechanisms we performed a series of transient transfections of murine fibroblast (NIH-3T3) and macrophage-like cells (J774.A1) with reporter plasmids containing the iNOS promoter and deletions thereof. By means of this and subsequent DNase I footprinting analysis we identified a regulatory region between −153 and −142 bp upstream of the transcriptional start site of the iNOS promoter that was sensitive to regulation by iron perturbation. Gel shift and supershift assays revealed that the responsible protein for this observation is NF-IL6, a member of the CCAAT/enhancer binding protein family of transcription factors. Binding of NF-IL6 to its consensus motif within the iNOS promoter was inducible by IFN-γ and/or LPS, was reduced by iron, and was enhanced by the iron chelator desferrioxamine. Introduction of a double mutation into the NF-IL6 binding site of this NO exerts cytostatic effects toward target cells by direct controlling the expression of those proteins at the translational/post-transcriptional level (10, 11). This reaction is catalyzed by the enzyme NO synthase (NOS), of which two constitutively expressed (NOSI and NOSIII) and one cytokine-inducible form (NOSII or iNOS) have been identified to date (1–6). The latter enzyme is responsible for high output formation of NO by macrophages in response to cytokines such as IFN-γ, TNF-α, IL-1β, and/or LPS. NO formation is a major effector mechanism of macrophages against invading microorganisms and tumor cells, and many of the cytotoxic actions of NO can be referred to its high affinity to iron (7). As an example of this NO exerts cytostatic effects toward target cells by direct interference with the catalytic iron-containing centers of enzymes in the citric acid cycle, mitochondrial respiration, or DNA synthesis such as aconitase, succinate oxidoreductase, or ribonucleotide reductase (8, 9). Moreover, NO directly affects the regulation of cellular iron homeostasis via its stimulatory effect on the binding activity of iron regulatory proteins to cis-acting RNA stem loop structures, the so-called iron-responsive elements, within untranslated regions of ferritin and transferrin receptor mRNA, thus providing evidence for the existence of an autoregulatory feedback loop in macrophages that links maintenance of iron homeostasis with optimal formation of NO for host defense (13). However, the underlying mechanism by which iron exerts transcriptional regulation of iNOS has remained elusive to date, although recent data suggested that activation of hypoxia inducible factor-1 (HIF-1) may be involved (14).

The murine iNOS promoter contains numerous consensus sequences for known transcription factors. These are located in two clusters: one called region I (ranging from +10 to −300 bp upstream of the TATA box), and the other called region II (−1100 to −800 bp) (15, 16). Region II was supposed to be primarily important for IFN-γ-mediated induction of iNOS because it contains binding sites for IFN regulatory factor-1 (IRF-1), STAT1α, and NF-κB (17–19). On the other hand, region I has been shown to be the principal target region for LPS-mediated iNOS induction since it contains three NF-IL6 binding sites, one TNF response element, one NF-κB binding site, and one octamer binding site. A number of groups have demonstrated the importance of this proximal promoter region for LPS-mediated induction of murine iNOS transcription (15, 16, 20).

This study was initiated to identify the regulatory regions and transcription factors responsible for iron-mediated regulation of iNOS in cytokine-stimulated macrophage-like cells.

Materials and Methods

Cell culture techniques

The J774.A1 murine macrophage cell line and the murine fibroblast cell line NIH3T3 were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% heat-inactivated FCS (very low endotoxin, Biochrom-Seromed, Berlin, Germany), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were then supplemented with 100 μM ferric iron (Fe(3+) );
applied as FeCl₃ (6H₂O) or 200 μM desferrioxamine (all from Sigma, Munich, Germany) before stimulation with 100 μM murine rIFN-γ (sp. act., 10⁷ U/mg; Life Technologies, Vienna, Austria) and/or with up to 10 μM dCTP instead of [3²P]dCTP (Amersham, Aylesbury, U.K.) and the three other competitors preincubated with 15 μM of double-stranded poly(dI-dC). CAT activity was determined using a CAT-ELISA (Boehringer Mannheim, Mannheim, Germany) before stimulation with 100 U/ml murine rIFN-γ, with a site-specific mutation in the IRF-1 binding site at position −975 bp (p1del Nhel-764, p1del BsrXI-206, and p1del PstI-47) are deletion constructs of p1NOS-CAT (for details, see Materials and Methods). Numbers indicating the position in the promoter sequence relative to the transcriptional start site.

**EMSA**

For performing EMSAs different oligonucleotide probes, representing standard consensus sequences of known transcription factors or specific sequences within the murine iNOS promoter, were used: NF-IL6 153/162 sense, 5′-CCACAGGTAGTGAATCCA-3′; antisense, 5′-GTGTGCTT GATTTGCTAATTCTTGTC-3′; NF-IL6 153/142 sense, 5′-CCACAGGT GAAATAATCA-3′; antisense, 5′-CTCTCGGA AAGTCCCCTCTG-3′; NF-IL6 135/153/142 sense, 5′-AAGTCGCAAAGGGGACCTCCGAGAGG-3′; antisense, 5′-TCGAC CTCTCGGA AAGTCCCCTCTG-3′. For preparation of double-stranded probes oligonucleotides were annealed, and overhanging ends were filled with TdT before addition of 2 ng of double-stranded poly(dI-dC) (Pharmacia Biotech) on ice for 10 min after addition of 10 μM of competitor double-stranded poly(dI-dC). The DNA binding reactions were conducted in the presence of 200 μM HEPES (pH 7.8), 10 mM EDTA, and 10 mM DTT for 30 min at room temperature (final volume, 10 μl). After addition of 50 ng of heparin to the binding reaction and subsequent incubation at room temperature for 10 min, 1.7 μl of 87% glycerol was added, and samples were analyzed on a 6% nondenaturating polyacrylamide gel (22).

For competition studies a 30- to 50-fold molar excess of unlabeled competitors dNTPs (from Pharmacia Biotech, Piscataway, NJ) using the Klenow fragment of DNA polymerase I. For preparation of unlabeled competitor dNTPs instead of [α-³²P]dCTP was used. Nuclear proteins (10 μl) were preincubated with 2 μg of competitor double-stranded poly(dI-dC)poly(dl-dC) (Pharmacia Biotech) on ice for 10 min before addition of 2 ng of the radiolabeled oligonucleotide probe (sp. act., 50,000 cpm/ng). The DNA binding reactions were conducted in the presence of 200 mM HEPES (pH 7.8), 10 mM EDTA, and 10 mM DTT for 30 min at room temperature (final volume, 10 μl). After addition of 50 ng of heparin to the binding reaction and subsequent incubation at room temperature for 10 min, 1.7 μl of 87% glycerol was added, and samples were analyzed on a 6% nondenaturating polyacrylamide gel (22).

**Relative CAT-activity**

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<td>p1del BsrXI−206</td>
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<td>p1del PstI−47</td>
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**FIGURE 1.** Transient transfection of NIH-3T3 cells with murine iNOS wild-type promoter plasmid constructs and mutants thereof. NIH-3T3 cells were transiently transfected with the plasmid constructs described below and then stimulated with IFN-γ (50 U/ml) and LPS (10 μg/ml) for 18 h after pretreatment with ferric chloride (50 μM) or DFO (100 μM). CAT activity was related to the control value (C; untreated cells), which was set at 100%. Results are the mean ± SD for three to eight experiments with each construct. Plasmid constructs are shown schematically. The p1iNOS-CAT contains the full-length iNOS promoter (−1588 to +161). Construct p1(IRF⁻¹) contains the full-length promoter with a mutation in the IRF-1 binding site at position −975 bp (p1del Nhel−764, p1del BsrXI−206, and p1del PstI−47) are deletion constructs of p1NOS-CAT (for details, see Materials and Methods). Numbers indicating the position in the promoter sequence relative to the transcriptional start site.

**Plasmid construction and transient transfection assays**

A 1749-bp HindII fragment, corresponding to the 5′-flanking region of murine iNOS fused to a promoterless chloramphenicol acetyltransferase (CAT) reporter gene (p1NOS-CAT) (5) and mutants thereof, p10.5iNOS-CAT, with a deletion of the 5′ region up to −975 bp, and p1(IRF⁻¹), with a site-specific mutation in the IRF-1 binding site (at position −920 bp) (17) were provided by Drs. E. Martin, Q.-w. Xie, and C. Nathan (Cornell University, Ithaca, NY). Further deletion mutants were constructed by stepwise digestion of the 5′ region of the iNOS promoter using restriction endonucleases as indicated below (Fig. 1). Progressive deletions of the 5′ region were obtained using the restriction enzyme NhIi up to position −764 bp (p1del Nhel−764, BsrXI up to position −206 bp (p1del BsrXI−206), and PstI up to position −47 bp (p1del PstI−47), respectively. Moreover, the 1.6-kb full-length promoter fragment (−1588 to +15 bp) and further deletion mutants were generated by PCR and subcloned into the pGL3 promoterless firefly luciferase reporter gene vector (pGL3-basic, Promega, Madison, WI). Point mutations of the transcription factor consensus sequences sequences NF-IL6 153/142 and the HRE 224/216 were introduced by site-directed mutagenesis via overlap extension using PCR (23) and then subcloned into the pGL3-basic vector. All plasmids were checked for accuracy by sequencing.

Cells were transiently transfected by a lipofection method using the liposomal transfection reagent DOTAP (Boehringer Mannheim, Mannheim, Germany). Plasmid (2.5 μg) was preincubated with 15 μg of DOTAP for 10 min at room temperature was added to 2 × 10⁶ cells/ml serum-free medium in six-well plates and incubated at 37°C for 8 h. Before stimulation cells were washed with fresh medium supplemented with 10% FCS. After 16 h of treatment with the various stimuli described above, cells were washed once with PBS and subjected to determination of CAT or luciferase activity. CAT activity was determined using a CAT-ELISA (Boehringer Mannheim, Mannheim, Germany). CAT activities were normalized by cotransfection with a pGL3 luciferase reporter vector, (pGL3-Control, Promega) and were reported as relative CAT activity. Luciferase activity was determined by the dual luciferase system from Promega. Firefly luciferase activity was corrected upon cotransfection of cells with a renilla luciferase vector pKL-SV40 (Promega). The dual luciferase assay was conducted according to the manufacturer’s instruction.

**DNase I footprinting**

DNase I footprinting was conducted as described by Leblanc and Moss (24). Nuclear extracts were prepared as outlined above. Probes for DNase I footprinting representing position −220 to +14 bp (relative to the transcription start site) of the murine iNOS promoter were generated by PCR. The binding reaction was performed in a total volume of 50 μl containing 2–3 ng of end-labeled DNA fragment (15,000 cpm), 10 μg of nuclear extracts, and 2 μg of competitor double-stranded poly(dI-dC)poly(dl-dC). DNase I (from bovine pancreas, Sigma) digestion was conducted with 0.005 and 0.02 Kunitz units for naked DNA and 0.08 and 0.16 Kunitz units for DNA and proteins at room temperature for 2 min. Probes were then analyzed on a sequencing gel (6% PAGE, 7 M urea, and 1× TBE). To localize the position of the footprint, G and G+A chemical sequencing
Results

Promoter activity of the full-length 1749-bp fragment and its 5'-deleted constructs

In cells transiently transfected with a plasmid containing the whole murine iNOS promoter (p1iNOS-CAT) CAT activity is inducible upon treatment with IFN-γ/LPS. This 2.5-fold induction compared with that in the untreated cells was reduced after the treatment of cells with iron, but was significantly enhanced by addition of the iron chelator desferrioxamine (DFO; p < 0.01). These data are in agreement with our previous results, showing transcriptional regulation of iNOS expression by iron perturbation (12). We then tried to identify the region within the iNOS promoter that is responsible for iron-mediated regulation of the iNOS promoter. Recent reports suggested that IRF-1 may be the central player in cytokine-mediated iNOS expression (17, 18, 26). Since knockout of IRF-1 resulted in dramatic reduction of iNOS expression without any effect on MHC class II expression (18), an observation consistent with the findings observed after iron perturbations (12), we investigated an iNOS promoter construct bearing a mutation within the IRF-1 binding region (p1IRFΔm). Although, inducibility by IFN-γ/LPS was reduced compared with that of the parent plasmid (p1iNOS-CAT), regulation of promoter activity by iron perturbation was still present (Fig. 1), thus excluding IRF-1 as a potential candidate for iron-mediated regulation. Therefore, we performed a series of further deletions of the 5’ region of the iNOS promoter. Interestingly, deletions of the region of the iNOS gene down to position −206 bp (p1del BstXI −206) still showed inducibility by IFN-γ/LPS and regulation by iron and DFO, although to a lesser extent than with the parent plasmid (p1iNOS-CAT, Fig. 1). Further deletion of the iNOS promoter down to position −47 bp (p1del PstI −47) resulted in loss of both IFN-γ/LPS inducibility and iron-mediated regulation of CAT activity (Fig. 1).

Structural and functional analysis of the 5’-flanking region −220 to +15 bp of the murine iNOS gene by DNase I footprinting

Since our transient transfection experiments indicated that the region between −206 and −47 bp upstream from the transcriptional start site of the iNOS promoter may bear the regulatory motif that mediates regulation of iNOS expression by cytokines and iron, we conducted DNase I footprinting analysis to identify a transcription factor responsible for this. DNase I footprinting analysis of this region with nuclear extracts from J774A.1 cells after treatment with IFN-γ/LPS revealed an inducible protection between position −160 to −140 bp of the iNOS promoter (Fig. 2, compare lane 4 with lane 3). This protection peaked after 4 and 8 h of cytokine stimulation, but could not be observed after treatment of cells for shorter periods (2 h; data not shown). A protection in the DNase I footprint assay is indicative for an interaction of a transcription factor with its DNA target sequence. The interaction of this protein with the iNOS promoter was weakened upon addition of iron and enhanced by DFO (Fig. 2, compare lanes 4, 5, and 6).

Characterization of the DNA binding protein by EMSA and supershift assay

To investigate whether the observed protection in the footprint analysis can indeed be referred to inducible binding of a transcription factor we next performed EMSA with labeled oligonucleotides representing the protected region (−140 to −160 bp) within the iNOS promoter. Using the same nuclear extracts as that for footprint analysis we identified IFN-γ- and LPS-inducible binding of a protein to this region (Fig. 3A). Treatment with IFN-γ or LPS resulted in induction of protein binding to the labeled DNA oligonucleotide compared with the untreated control, and both stimuli when applied together were synergistic in this respect. Nevertheless, perturbation of cells with iron decreased binding of this protein to its DNA sequence, while DFO even enhanced protein/DNA interaction. Notably, this effect was less pronounced when cells were treated with IFN-γ and LPS at the same time, indicating that a strong stimulus may weaken regulation by iron perturbation (Fig. 3A). Finally, this protein/DNA interaction could be completely abolished by competition with the cold oligonucleotide construct of the same region, pointing to the specificity of the observed protein/DNA interaction (Fig. 3A).

To further identify this transcription factor we performed a series of supershift assays. Previous studies of other groups (15, 16) indicated that at least three known transcription factors, namely NF-IL6, STAT-1α, and NF-κB, could potentially bind to this region.

Upon EMSA supershift analysis only an anti-NF-IL6 (C/EBPβ) Ab produced a supershift (Fig. 3B), while no altered migration of protein/DNA complexes was observed upon incubation of nuclear extracts with anti-NF-κB-p50, anti-NF-κB-p65, anti-RelC, or anti-STAT1α Abs (data not shown). The complex supershifted with anti-NF-IL6 Ab was completely abolished by competition with a 30-fold excess of the appropriate unlabeled duplex oligonucleotide, now called NF-IL6 −153/−144 (Fig. 3B, compare lanes 3 and 4). The specificity of this interaction was further sustained by the observation that an excess of cold oligonucleotide with a double mutation within the NF-IL6 binding site, called NF-IL6 −153/−144m, was not able to compete the supershift (Fig. 3B, lane 5).

To further confirm the specific involvement of the transcription factor NF-IL6 we performed EMSAs using nuclear extracts from
and from cells activated with IFN-γ oligonucleotides NF-IL6 was confirmed by cold competition with a 30-fold excess of the unlabeled and lanes 3–5 Ab (IFN-γ stimulated with IFN-γ (50 U/ml; lanes 5–7).

Modulation of protein binding to iNOS promoter fragments

Functional analysis of the NF-IL6−153−142 promoter element for IFN-γ and/or LPS inducibility and its regulation by iron

To determine whether iron-mediated regulation of NF-IL6 binding to its consensus sequence NF-IL6−153−142 accounts for altered expression of iNOS we constructed a series of new plasmids and performed transient transfections. When transfecting cells with a plasmid containing the region from −230 to +15 bp (piNOS-230) of the iNOS promoter, we found inducibility by IFN-γ/LPS, which was in agreement with our previous data (Fig. 4). However, iNOS promoter activity in response to IFN-γ/LPS was reduced by about 30% upon deletion of the 5’ end between −230 and −1588 bp compared with the parent plasmid (piNOS-LUC) and to a lesser extend with IFN-γ and LPS when applied alone (Fig. 4). Beside the NF-IL6 motif at position −153 to −142 bp, the plasmid piNOS-230 also contains a hypoxia-responsive element (position −224 to −216 bp; HRE−224/−216) that binds HIF-1. The latter has been shown previously to participate in iron- and IFN-γ-mediated regulation of iNOS in ANA-1 macrophages (14). To investigate the impact of these two transcription factors on the induction of iNOS in response to IFN-γ and/or LPS on iNOS promoter activity was observed compared with that in cells transfected with the plasmid without the mutation. When we introduced a mutation into the NF-IL6 site while HRE remained unaffected, we observed an almost complete loss of IFN-γ/LPS inducibility of the promoter (Fig. 4). The same was observed by further deletion of the promoter down to position −140 bp, thus eliminating the NF-IL6 site between −153 to −142 bp (Fig. 4). This indicated that NF-IL6 is essential for IFN-γ/LPS-mediated induction of iNOS.

We then investigated the impact of HRE and NF-IL6 on iron-mediated regulation of iNOS in response to IFN-γ/LPS. Using the piNOS-230 plasmid for transfection of NIH-3T3 cells, which were then stimulated with IFN-γ and/or LPS, we found hyperinducibility of promoter activity upon treatment with DFO by a factor between 2.1 and 3.3 (Fig. 5, A–C). However, in this setting iron had no effect on IFN-γ/LPS-treated cells, which could probably be due to the high iron content of the medium supplemented with FCS (Fig. 5). When cells were transfected with a plasmid bearing a double mutation within the HRE region (piNOS-230HREim), we

J774.A1 cells and a synthetic oligonucleotide bearing the NF-IL6 consensus sequence (NF-IL6cons.; Fig. 3B, lanes 6–11). As with the NF-IL6 sequence within the iNOS promoter (NF-IL6−153−142), stimulation of cells with IFN-γ and/or LPS induced binding of the protein to the NF-IL6 consensus DNA motif (Fig. 3B, lanes 6 and 7). Addition of a polyclonal Ab against NF-IL6 was able to supershift this protein/DNA complex (Fig. 3B, lane 8). DNA binding of supershifted NF-IL6 was completely abolished upon competition with a 30-fold excess of the appropriate unlabeled consensus NF-IL6 oligonucleotide (NF-IL6cons.) and with a 30-fold excess of cold NF-IL6−153−142 but not with the mutated NF-IL6−153−142m oligonucleotide (Fig. 3B, lanes 9–11).

Our data obtained to date indicated that the protein binding to the iNOS promoter as shown by in vitro footprints and EMSAs, which is both cytokine inducible and iron regulated, is NF-IL6. However, since the NF-IL6 Ab was not sufficient to supershift all protein/DNA binding complexes with the NF-IL6−153−142 oligonucleotide (Fig. 3B, lane 3), it is possible that other unrecognized transcription factors may also bind to this region, although to a much lesser extent than NF-IL6.

FIGURE 3. Modulation of protein binding to iNOS promoter fragments and the NF-IL6 transcription factor consensus sequence by cytokine and iron perturbation. The binding reaction was performed with nuclear extracts from cells treated for 8 h with or without the indicated stimuli. A, Gel shift analysis of the iNOS promoter region −158 to −132 bp (NF-IL6−153−142) with nuclear extracts from untreated J774A.1 cells (C) and from cells stimulated with IFN-γ (50 U/ml)/LPS (10 μg/ml; lanes 2–4) or with IFN-γ (50 U/ml; lanes 5–7) or with LPS (10 μg/ml; lanes 8–10) after previous addition of ferric chloride (1, 100 μM; lanes 3, 6, and 9) or DFO (D, 200 μM; lanes 4, 7, and 10). The specificity of binding was confirmed by cold competition with a 30-fold excess of the same unlabeled oligonucleotide (lane 11). B, Gel supershift analysis of the iNOS promoter region −158 to −132 (NF-IL6−153−142) and the NF-IL6 consensus sequence (NF-IL6cons.) with nuclear extracts from untreated J774A.1 cells (C; lanes 1 and 6) and from cells activated with IFN-γ (50 U/ml)/LPS (10 μg/ml) and incubated without (lanes 2 and 7) or with a polyclonal anti-NF-IL6 (C/EBPβ) Ab (lanes 3–5 and lanes 8–11). Nuclear extracts were preincubated with polyclonal anti-NF-IL6 (C/EBPβ) on ice for 1 h. The specificity of binding was confirmed by cold competition with a 30-fold excess of the unlabeled oligonucleotides NF-IL6−153−142 (lanes 4 and 10), NF-IL6cons. (lane 9), or NF-IL6−153−142m the latter bearing a double mutation within the NF-IL6 binding site (lanes 5 and 11).
observed a reduction in the stimulatory potential of DFO on luciferase activity by about 30–40% compared with that of the parent plasmid (piNOS-230). However, the difference in luciferase activity between IFN-γ/LPS and IFN-γ/LPS plus DFO in cells transfected with piNOS-230lHREm was still significant (p < 0.05). A similar observation was made when cells were transfected with the plasmid piNOS-170 (Fig. 5). Transfection of cells with the NF-IL6 mutated construct (piNOS-230NF-IL6m) reduced inducibility by cytokines by >90% as shown above (Fig. 4). However, at this very low level, treatment of piNOS-230NF-IL6m-transfected cells with IFN-γ/LPS plus DFO increased the albeit little promoter activity by 2.5-fold compared with stimulation with IFN-γ/LPS alone (Fig. 5A). This phenomenon should be due to activation of HRE binding affinity by DFO (14), since a double-knockout deletion of HRE and NF-IL6 by using the plasmid piNOS-170NF-IL6m failed to show even this little of an effect of the drug (Fig. 5). Regulation of promoter activity by DFO was similar in cells treated with IFN-γ or LPS alone and in those treated with a combination of both (Fig. 5).

In summary, binding of NF-IL6 to the iNOS promoter appears to be of central importance for the induction of iNOS by LPS and/or IFN-γ and along with other transcription factors, such as HIF-1 and NF-IL6, mediates iron regulation of the iNOS gene (Figs. 4 and 5).

Discussion

In the present study we investigated the underlying mechanism responsible for the previously observed transcriptional regulation of iNOS by iron perturbation (12). By means of transient transfections, deletion, and mutational analysis and in vitro DNase I footprint, gel shift, and supershift assays, we could identify a trans-activating factor that targets to a NF-IL6 consensus motif within the murine iNOS promoter and whose DNA binding affinity is modulated by iron.

Even more importantly, NF-IL6 appears to be essential for cytokine/LPS-mediated induction of iNOS, since mutation of the NF-IL6-153/142 binding motif almost fully abolished inducibility of iNOS promoter by IFN-γ and/or LPS.

NF-IL6, a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, belongs to a class of DNA binding proteins called basic leucine zipper protein family, which includes C/EBPα, C/EBPβ (NF-IL6), C/EBPγ, and C/EBPδ (NF-IL6β) (29). These proteins are all characterized by a leucine zipper domain and a DNA-binding basic region located in the C-terminus of the proteins. Members of the C/EBP family can associate through the leucine zipper domain to form homo- and heterodimers with each other and bind with similar affinity to various C/EBP binding sites (27–29).

The binding affinity of NF-IL6 to its DNA consensus sequence is induced in various tissues after stimulation with LPS or proinflammatory cytokines such as IL-1, TNF-α, and IL-6 (30, 31). NF-IL6 expression is also induced during macrophage differentiation. NF-IL6 consensus binding motifs are found in the functional regulatory regions of genes that are induced in activated macrophages, such as IL-6, IL-1α, IL-8, TNF-α, G-CSF, lysozyme genes, and iNOS (15, 32–34).

Interestingly, the data presented here demonstrate that NF-IL6 binding to the iNOS promoter is induced by LPS and IFN-γ; however, the latter stimulus is not able to sufficiently stimulate the induction of iNOS at least in transiently transfected NIH3T3 or J774A.1 cells, which is in accordance with previously published data (35). This implies that although NF-IL6 appears to be an essential component for iNOS expression as shown by our mutational analysis, other transcription factors must be activated in addition to gain full induction of iNOS expression. This is not surprising, since numerous consensus binding sequences for known transcription factors have been identified within the iNOS promoter. While the essential transcription factor for induction of iNOS by IFN-γ is IRF-1 (19), numerous trans-activating factors have been identified to transduce LPS-mediated stimulation of iNOS (15, 16, 20, 36). Beside binding of transcription factor NF-κB/Rel to promoter elements (37) at position −85 to −76 bp (15, 16, 20) or position −971 to −962 bp (15, 16), other LPS-responsive elements within the iNOS promoter, e.g., at position −62 to −56, and a binding site for an octamer-like binding protein in an as yet unidentified complex with other factors have been identified (20, 36).

Although NF-κB/Rel was suggested to be essential for the LPS-mediated induction of iNOS, it is not sufficient (37, 38). It has
between NF-IL6 and NF-κB has been provided for a functional and physical interaction between these transcription factors, and it has been shown that C/EBP and Rel proteins activate the IL-12/p40 promoter in a synergistic manner, as specific mutations in either recognize critical sequences have been shown to be inducible by hypoxia and DFO (14), and it has been suggested to be a major player in iron-mediated regulation of iNOS, at least in ANA-1 cells stimulated with IFN-γ alone (14). However, knockout of HRE still showed significant regulation of iNOS by iron perturbation, which is mainly due to modulation of NF-κB binding to its consensus sequence upon iron depletion (49). After stimulation of cells with LPS the binding affinity of NF-κB p50/p65 and p50/c-Rel to the iNOS promoter peaks at 30 min after treatment and is then decreased (37); however, synthesis of iNOS mRNA continues for >24 h (2, 50). In contrast, induction of NF-IL6 binding to the iNOS promoter, as shown by our DNase I footprint analysis, occurs at later time points, with maximum binding affinities 8 h after cytokine/LPS stimulation of J774.A1 cells. This would fit into the hypothesis that NF-κB acts as an inducer of iNOS gene transcription, and NF-IL6 maintains the active transcription state.

Recent work has demonstrated that macrophages from NF-IL6−/− mice produce similar amounts of NO after stimulation with IFN-γ/LPS as those from NF-IL6+/− mice (51). However, this does not automatically exclude the potential importance of NF-IL6 for iNOS transcription in vivo, since, as suggested by these authors, other members of the C/EBP transcription factor family, such as NF-IL6β (52), could overcome a lack of NF-IL6, thus leading to similar transcriptional activities toward target genes such as iNOS.

Moreover, NF-IL6 participates in iron-mediated regulation of iNOS as shown herein and may cooperate with other transcription factors, such as HIF-1, in this respect. The binding of HIF-1 to target sequences has been shown to be inducible by hypoxia and DFO (14), and it has been suggested to be a major player in iron-mediated regulation of iNOS, at least in ANA-1 cells stimulated with IFN-γ alone (14). This results were also confirmed by us; however, knockout of HRE still showed significant regulation of iNOS by iron perturbation, which is mainly due to modulation of NF-IL6 binding to its consensus sequence upon iron depletion (Fig. 5). In contrast, the binding affinity of NF-κB to the iNOS promoter appears to be unaffected by iron perturbations (data not shown).

However, DFO when combined with IFN-γ was able to induce iNOS promoter activity even when IFN-γ alone was quite ineffective. Nevertheless, DFO when applied alone did not have a direct effect on transcriptional iNOS expression as shown by nuclear run-off analysis (12, 14), iNOS mRNA stability (12), or iNOS enzymatic activity (12). Moreover, all these experiments provided evidence that the modulating effects of DFO on iNOS mRNA expression are exclusively due to the transcriptional mechanism investigated in this paper and are not due to modulation of iNOS mRNA half-life by the drug (12). Thus, DFO may not be a direct inducer of gene transcription in the absence of inflammatory stimuli. More likely, DFO acts as a costimulus able to modulate or strengthen a signal induced by a cytokine or LPS and thus contributes to gene expression via its stimulatory effect on binding of transcription factors. The latter effect could be due in part to the potential of DFO to inhibit iron-catalyzed formation of radicals (14, 53), since radicals are also involved in activation/deactivation of transcription factors (54). This is in accordance with the data showing that DFO induces HIF-1 binding activity and could thus be a costimulus for the activation of iNOS expression in IFN-γ-treated macrophages along the hypoxia pathway.

Therefore, NF-IL6 may cooperate with NFκB to maintain full iNOS induction. As suggested by other authors, NF-IL6 could maintain a high transcription rate of iNOS rather than triggering the initial induction (48). This is in agreement with the observation that after cytokine/LPS stimulation of cells the activation of NF-IL6 occurs later than that of NF-κB. Therefore, NF-κB could act as a first inducer of iNOS transcription, and this stimulus is preserved by the action of NF-IL6. This is in agreement with the induction kinetics of the acute phase genes (α1-acid glycoprotein and serum amyloid A and P) by LPS, where STAT3 functions as an inducer of transcription, and NF-IL6 then maintains a high rate of transcription (49). After stimulation of cells with LPS the binding affinity of NF-κB p50/p65 and p50/c-Rel to the iNOS promoter peaks at 30 min after treatment and is then decreased (37); however, synthesis of iNOS mRNA continues for >24 h (2, 50). In contrast, induction of NF-IL6 binding to the iNOS promoter, as shown by our DNase I footprint analysis, occurs at later time points, with maximum binding affinities 8 h after cytokine/LPS stimulation of J774.A1 cells. This would fit into the hypothesis that NF-κB acts as an inducer of iNOS gene transcription, and NF-IL6 maintains the active transcription state.

Recent work has demonstrated that macrophages from NF-IL6−/− mice produce similar amounts of NO after stimulation with IFN-γ/LPS as those from NF-IL6+/− mice (51). However, this does not automatically exclude the potential importance of NF-IL6 for iNOS transcription in vivo, since, as suggested by these authors, other members of the C/EBP transcription factor family, such as NF-IL6β (52), could overcome a lack of NF-IL6, thus leading to similar transcriptional activities toward target genes such as iNOS.

Moreover, NF-IL6 participates in iron-mediated regulation of iNOS as shown herein and may cooperate with other transcription factors, such as HIF-1, in this respect. The binding of HIF-1 to target sequences has been shown to be inducible by hypoxia and DFO (14), and it has been suggested to be a major player in iron-mediated regulation of iNOS, at least in ANA-1 cells stimulated with IFN-γ alone (14). This results were also confirmed by us; however, knockout of HRE still showed significant regulation of iNOS by iron perturbation, which is mainly due to modulation of NF-IL6 binding to its consensus sequence upon iron depletion (Fig. 5). In contrast, the binding affinity of NF-κB to the iNOS promoter appears to be unaffected by iron perturbations (data not shown).

However, DFO when combined with IFN-γ was able to induce iNOS promoter activity even when IFN-γ alone was quite ineffective. Nevertheless, DFO when applied alone did not have a direct effect on transcriptional iNOS expression as shown by nuclear run-off analysis (12, 14), iNOS mRNA stability (12), or iNOS enzymatic activity (12). Moreover, all these experiments provided evidence that the modulating effects of DFO on iNOS mRNA expression are exclusively due to the transcriptional mechanism investigated in this paper and are not due to modulation of iNOS mRNA half-life by the drug (12). Thus, DFO may not be a direct inducer of gene transcription in the absence of inflammatory stimuli. More likely, DFO acts as a costimulus able to modulate or strengthen a signal induced by a cytokine or LPS and thus contributes to gene expression via its stimulatory effect on binding of transcription factors. The latter effect could be due in part to the potential of DFO to inhibit iron-catalyzed formation of radicals (14, 53), since radicals are also involved in activation/deactivation of transcription factors (54). This is in accordance with the data showing that DFO induces HIF-1 binding activity and could thus be a costimulus for the activation of iNOS expression in IFN-γ-treated macrophages along the hypoxia pathway.
In summary, our data demonstrate that the NF-IL6-153/-142 element in the iNOS promoter plays a central role for the induction of iNOS gene expression in response to cytokine stimulation, and that it is centrally involved in iron-mediated regulation of the iNOS promoter.

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