

# Regulation of Inducible Nitric Oxide Synthase Expression in IFN- $\gamma$ -Treated Murine Macrophages Cultured Under Hypoxic Conditions<sup>1</sup>

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We recently reported that a hypoxia-responsive element mediates a novel pathway of transcriptional activation of the inducible nitric oxide synthase (iNOS) promoter in murine macrophages treated with IFN- $\gamma$  plus hypoxia (1% O<sub>2</sub>). In this study, we investigated the expression of iNOS activity and the regulation of iNOS induction in IFN- $\gamma$ -treated ANA-1 murine macrophages or thioglycollate-elicited peritoneal macrophages cultured under hypoxic conditions. We found that murine macrophages stimulated with IFN- $\gamma$  plus hypoxia, despite a significant accumulation of iNOS mRNA, did not release nitrite into culture supernatant. However, cytosol from macrophages treated with IFN- $\gamma$  plus hypoxia contained significant levels of iNOS protein and enzymatic activity. Experiments in which cells were treated with IFN- $\gamma$  plus hypoxia and then cultured in normoxic conditions (20% O<sub>2</sub>) demonstrated that reoxygenation was required to achieve detectable accumulation of nitrite in the culture supernatant. Furthermore, we demonstrated that IL-4 inhibited IFN- $\gamma$  plus hypoxia-dependent induction of iNOS mRNA expression, iNOS protein, and enzymatic activity. Experiments in which ANA-1 macrophages were transfected transiently with the full-length iNOS promoter linked to a chloramphenicol acetyltransferase reporter gene demonstrated that IL-4 also down-regulated the IFN- $\gamma$  plus hypoxia-induced activation of the iNOS promoter. These data establish that hypoxia is a costimulus with IFN- $\gamma$  for the induction of iNOS activity in ANA-1 macrophages as well as in murine peritoneal macrophages, and they provide the first evidence that IL-4 inhibits hypoxia-inducible gene expression. In addition, our results suggest that hypoxia, which occurs in many pathologic conditions, may play an important role in the activation of murine macrophages. *The Journal of Immunology*, 1996, 157: 2638–2644.

Nitric oxide (NO)<sup>4</sup> is a free radical gas produced by at least three different isoforms of the enzyme nitric oxide synthase (NOS). NO has been implicated in a variety of biologic functions, including neurotransmission, vascular homeostasis, and antimicrobial and antitumor activities (1–3). Murine macrophages contain a transcriptionally inducible isoform of NOS (iNOS), whose expression can be induced by different pathways. The pathway of iNOS expression following stimulation with IFN- $\gamma$  plus LPS has been investigated extensively (4, 5). At least two regulatory sequences of the 5' flanking region of the iNOS gene mediate the activation of transcription by IFN- $\gamma$  and LPS (6,

7), through the binding of the transcription factors IRF-1 (8, 9) and nuclear factor- $\kappa$ B (10), respectively, to the corresponding responsive elements.

We have recently provided evidence of the existence of a novel pathway of iNOS transcriptional activation in murine macrophages (11) on the basis of functional studies, in which deletion mutants of the iNOS promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene were transfected transiently in the murine macrophage cell line ANA-1. We demonstrated that a 19-bp element containing a sequence homology to a hypoxia-responsive enhancer (iNOS-HRE) was required for the synergistic induction of iNOS promoter activity by either hypoxic conditions (1% O<sub>2</sub>) or picolinic acid, a catabolite of L-tryptophan, in combination with IFN- $\gamma$  (11). Furthermore, transfection experiments using a tk promoter-CAT reporter gene construct demonstrated that the iNOS-HRE was sufficient to confer inducibility by hypoxia or picolinic acid alone (11). The HRE, originally identified in the 3' flanking region of the erythropoietin (Epo) gene (12), mediates hypoxia-inducible gene transcription in Epo-producing and non-Epo-producing cell lines, suggesting its general involvement in the induction of gene expression by hypoxia (13, 14). The iNOS-HRE contains the consensus sequence for the binding of the transcription factor hypoxia-inducible factor 1 (12, 15, 16). Site-directed mutagenesis experiments demonstrated that the hypoxia-inducible factor 1 binding site of the iNOS-HRE was essential for the hypoxia- or picolinic acid-dependent transcriptional activation of the iNOS promoter (11). Picolinic acid is known to act as a costimulus with IFN- $\gamma$  for the induction of tumoricidal activity (17), nitrite (NO<sub>2</sub><sup>-</sup>) production (18), and iNOS transcription (19) in murine macrophages. In contrast, the activation of the iNOS promoter by hypoxia suggested for the first time that iNOS expression could be

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<sup>4</sup> Abbreviations used in this paper: NO, nitric oxide; CAT, chloramphenicol acetyltransferase; Epo, erythropoietin; HRE, hypoxia-responsive enhancer; iNOS, inducible nitric oxide synthase; NOS, nitric oxide synthase.

sensitive to a decrease in oxygen tension. Indeed, we found that hypoxia caused a major increase of iNOS transcription and iNOS mRNA expression in IFN- $\gamma$ -treated macrophages (11). These results provided the first evidence that iNOS is a hypoxia-inducible gene, and demonstrated that multiple cis-acting elements control iNOS transcriptional activation in response to different pathways of activation. However, the generation of NO from L-arginine is an oxidative process, and the relationship between induction of iNOS mRNA by hypoxia and iNOS enzymatic activity remains to be elucidated. Furthermore, the mechanisms that may down-regulate the IFN- $\gamma$  plus hypoxia-dependent pathway are not defined.

We studied the expression of iNOS in ANA-1 macrophages and in thioglycollate-elicited peritoneal macrophages stimulated with IFN- $\gamma$  under low oxygen tension. We found that the cytosolic fraction of macrophages treated with IFN- $\gamma$  plus hypoxia contained substantial levels of iNOS protein and iNOS enzymatic activity. However, reoxygenation had to follow hypoxia to yield NO production by IFN- $\gamma$ -treated macrophages. Furthermore, we demonstrated that IL-4 almost completely abolished the IFN- $\gamma$  plus hypoxia-mediated increase in iNOS promoter activity and iNOS protein levels. These data establish that hypoxia enhances the IFN- $\gamma$ -dependent induction of iNOS activity, and they provide the first evidence that IL-4 inhibits the expression of a hypoxia-inducible gene.

## 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-*raft/v-myc*) recombinant retrovirus (20, 21). ANA-1 macrophages were cultured in DMEM (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Life Technologies, Grand Island, NY) (complete medium). Normal peritoneal macrophages were obtained from C57BL/6 mice that were injected i.p. with 1 ml of 3% thioglycollate broth. After 4 days, the peritoneal exudate cells were collected by lavage of the peritoneal cavity with 10 ml of sterile HBSS. Cells were washed, resuspended, and plated in RPMI 1640 supplemented as described above. Macrophages were isolated by adherence to plastic tissue culture dishes. Cells were maintained at 37°C in a humidified incubator containing 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub> (referred to as normoxic conditions). Cells were cultured under low oxygen tension by using a modular incubator flushed with a mixture of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub> at 37°C in a humidified atmosphere (referred to as hypoxic conditions). Mouse rIFN- $\gamma$  (sp. act.  $\geq 10^7$  U/mg) was purchased from Life Technologies. LPS (from *Escherichia coli* serotype 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). Mouse rIL-4 was a gift from Immunex Corporation (Seattle, WA).

### Nitrite assay

The accumulation of NO<sub>2</sub><sup>-</sup>, a stable end product of NO formation, in macrophage culture supernatants was used as a relative measurement of NO production. Fifty microliters of cell-free supernatant were incubated with 50  $\mu$ l of Griess reagent (22) for 10 min at room temperature, and the absorbance at 550 nm was measured in a Dynatech MR5000 microplate reader (Dynatech Labs., Chantilly, VA). The concentration of NO<sub>2</sub><sup>-</sup> was determined from a least squares linear regression analysis of a sodium nitrite standard that was generated with each experiment. The limit of sensitivity of the NO<sub>2</sub><sup>-</sup> assay was 1.5  $\mu$ M.

### Preparation of cytosols

Cytosols were prepared as described previously (23), with modifications. ANA-1 macrophages (15–20  $\times 10^6$ ), treated as described, were gently scraped from tissue culture plates, centrifuged, and washed twice with PBS. The cell pellet was resuspended in 300  $\mu$ l water containing 1 mM PMSF, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin. The cells were lysed by three rapid freeze-thaw cycles, and the lysates were spun at approximately 100,000  $\times g$  in an ultracentrifuge at 4°C for 1 h. The resulting supernatant was used as the iNOS-containing cytosol and was routinely stored at -70°C. The protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

### Cytosolic iNOS activity

The iNOS activity was measured by the capacity of the cytosol to generate NO<sub>2</sub><sup>-</sup> in the presence of cofactors and substrate, according to modifications of a previously described procedure (23). Samples containing 225  $\mu$ g of cytosol were incubated with iNOS buffer (1 mM nicotinamide adenine dinucleotide phosphate, 4 mM L-arginine, 4 mM flavin adenine dinucleotide, 5  $\mu$ M glutathione, 5  $\mu$ M tetrahydrobiopterin, and 40 mM Tris, pH 8) at 37°C for 6 h. The levels of NO<sub>2</sub><sup>-</sup> were then measured in 50  $\mu$ l of sample, as described above.

### Western blot analysis

Ten micrograms of cytosolic protein were subjected to 12% SDS-PAGE under reducing conditions. The western blot was performed as described previously (24). Briefly, the proteins were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) by electroblotting at approximately 300 mA for 4 to 5 h at 4°C. The membrane was incubated with 1% BSA in wash buffer (blocking buffer: 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. The membrane was subsequently incubated with anti-macNOS mAb (Transduction Laboratories, Lexington, KY) (diluted 1/500 in blocking buffer) for 16 to 18 h at 4°C with gentle shaking. The membrane was washed six times with wash buffer at room temperature for 5 min and then incubated with horseradish peroxidase-conjugated sheep anti-mouse Ig F(ab')<sub>2</sub> fragment (Amersham Corp., Arlington Heights, IL) (diluted 1/300 in 5% nonfat milk in wash buffer) for 1 h at room temperature. The membrane was washed as described previously, and the level of iNOS protein expression was determined using the enhanced chemiluminescence assay kit (Amersham Corp.).

### Northern blot analysis

Total cellular RNA was purified from ANA-1 macrophages using the TriZOL RNA isolation reagent (Life Technologies, Gaithersburg, MD). Thioglycollate-elicited peritoneal macrophages were solubilized with guanidine isothiocyanate, and the total cellular RNA was purified by centrifugation through a cushion of cesium chloride. Twenty micrograms (from ANA-1 macrophages) or five micrograms (from thioglycollate-elicited peritoneal macrophages) of RNA were size-fractionated in a 1.2% agarose gel, blotted onto a Nytran membrane (Schleicher & Schuell, Keene, NH), and incubated overnight at 42°C in Hybrisol I hybridization solution (Onco, Gaithersburg, MD). The cDNA probe that was specific for mouse macrophage NOS (25) was radiolabeled with [<sup>32</sup>P]dCTP (Amersham Corp.) by using a random priming kit (Boehringer Mannheim Corp., Indianapolis, IN). The blot was hybridized with the radiolabeled probe (1  $\times 10^6$  cpm/ml) during an overnight incubation, washed, and autoradiographed, as described previously (19). Equal loading of RNA was assessed by ethidium bromide staining of 28S and 18S rRNA.

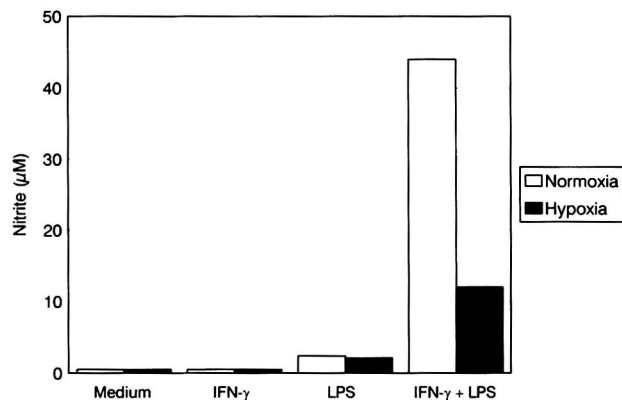
### Transient transfection of ANA-1 and CAT assay

The 1749-bp fragment of the 5' flanking region of the murine iNOS gene inserted into pCAT-Basic (Promega Corp., Madison, WI) upstream of the promoterless CAT gene (p1-iNOS-CAT) was kindly provided by Q. Xie and C. Nathan (Cornell University Medical College, New York, NY) (6). ANA-1 macrophages were transfected by a modification of the DEAE-dextran method, as described previously (11). Ten micrograms of plasmid DNA were added to 10<sup>7</sup> cells in 1 ml of DMEM without serum, prewarmed to 37°C and containing 250  $\mu$ g/ml of DEAE-dextran and 50 mM of Tris-HCl (pH 7.5). The cells were incubated at 37°C for 1 h, followed by a 2-min shock with 10% DMSO at room temperature. Replicate transfectants were pooled and cells were plated in six-well plates at 1  $\times 10^6$ /ml in 2.5 ml of complete medium. Following the appropriate treatment for 18 h, the cells were 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 (26). Protein content was determined using the Bio-Rad protein assay kit.

## Results

### NO production is inhibited under hypoxic conditions

Initial experiments were performed to investigate the influence of low oxygen tension on the accumulation of NO<sub>2</sub><sup>-</sup> in culture supernatants of stimulated ANA-1 macrophages. ANA-1 cells were treated with medium, IFN- $\gamma$ , or LPS alone or in combination with IFN- $\gamma$ , and cultured for 24 h under either normoxic or hypoxic conditions. As shown in Figure 1, no NO<sub>2</sub><sup>-</sup> production was observed in cells untreated or stimulated with either IFN- $\gamma$  alone or

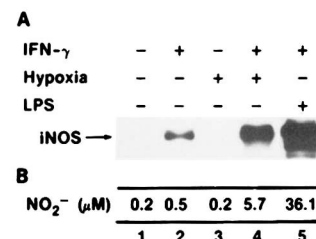


**FIGURE 1.** Inhibition of  $\text{NO}_2^-$  accumulation in ANA-1 macrophages stimulated under hypoxic conditions. ANA-1 macrophages ( $1 \times 10^6$  cells/ml) were plated in duplicate wells of a 24-well plate in 500  $\mu\text{l}$  of complete medium. Cells were treated with medium, IFN- $\gamma$  (100 U/ml), or LPS (10 ng/ml) alone or in combination with IFN- $\gamma$ , and cultured under either normoxic (20%  $\text{O}_2$ ) or hypoxic (1%  $\text{O}_2$ ) conditions for 24 h, as described in *Materials and Methods*. Cell-free culture supernatants were assayed for the presence of  $\text{NO}_2^-$ , as described. Data shown are from one representative experiment of four performed.

IFN- $\gamma$  plus hypoxia. Very low levels of  $\text{NO}_2^-$  were detected in ANA-1 macrophages treated with LPS alone and cultured under either normoxic or hypoxic conditions. As previously reported (22), high levels of  $\text{NO}_2^-$  were induced in ANA-1 cells by stimulation with IFN- $\gamma$  plus LPS in the presence of oxygen. In contrast, a 70% reduction of  $\text{NO}_2^-$  accumulation was observed in the culture supernatant of IFN- $\gamma$  plus LPS-treated cells under hypoxic conditions. Cell viability was not affected significantly by culture in hypoxia for 24 h, as assessed by trypan blue dye exclusion (data not shown). These results indicated that culture under low oxygen levels prevented  $\text{NO}_2^-$  accumulation in the supernatant of stimulated ANA-1 cells.

#### Hypoxia augments IFN- $\gamma$ -dependent expression of iNOS protein and enzymatic activity

Despite the fact that ANA-1 cells treated with IFN- $\gamma$  plus hypoxia expressed high levels of iNOS mRNA (11), no  $\text{NO}_2^-$  accumulation was detected in the culture supernatant under the same experimental conditions. Therefore, we measured the levels of iNOS protein in lysates from cells stimulated with IFN- $\gamma$  and incubated for 18 h under hypoxia or normoxia. As a positive control, we tested the levels of iNOS protein in lysates from ANA-1 macrophages treated with LPS alone or in combination with IFN- $\gamma$ . As shown in Figure 2A, immunoblot analysis did not reveal iNOS protein in untreated cells under either normoxic (lane 1) or hypoxic (lane 3) conditions. ANA-1 cells treated with IFN- $\gamma$  alone expressed low but detectable levels of iNOS protein (lane 2). In contrast, cells treated with IFN- $\gamma$  plus hypoxia showed a major increase in iNOS protein levels (lane 4), demonstrating that hypoxia is a costimulus for the induction of iNOS protein expression. Very low levels of iNOS protein were induced by LPS alone (data not shown), whereas a dramatic synergistic induction of iNOS protein was observed in ANA-1 macrophages treated with IFN- $\gamma$  plus LPS (lane 5). Next, we measured the levels of iNOS activity present in the cytosolic fraction of ANA-1 macrophages treated with medium or IFN- $\gamma$  for 18 h under normoxic or hypoxic conditions. No detectable enzymatic activity, measured by the conversion of L-arginine to  $\text{NO}_2^-$  in the presence of cofactors and substrate, was observed in cytosol isolated from untreated cells or



**FIGURE 2.** Hypoxia synergizes with IFN- $\gamma$  in the induction of iNOS protein and enzymatic activity. ANA-1 macrophages ( $1\text{--}1.5 \times 10^6$  cells/ml) were treated with medium, IFN- $\gamma$  (100 U/ml), hypoxia, IFN- $\gamma$  plus hypoxia, or IFN- $\gamma$  plus LPS (10 ng/ml) for 18 h. Cytosols were then isolated and assayed for the presence of iNOS protein (A) or iNOS activity (B), as described in *Materials and Methods*. Results shown are from one representative experiment of three performed.

Table I. Reoxygenation restores IFN- $\gamma$  plus hypoxia-dependent production of  $\text{NO}_2^-$  in the culture supernatant<sup>a</sup>

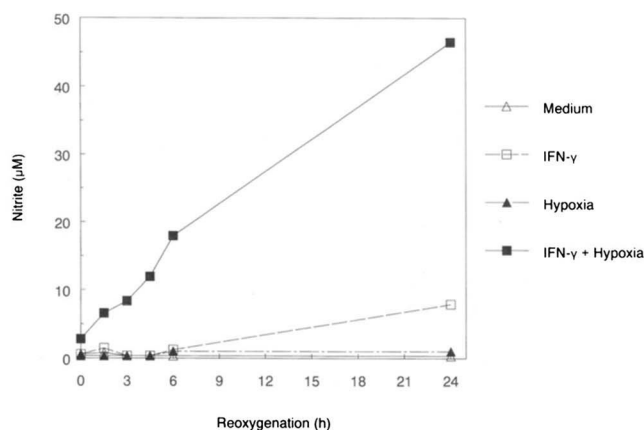
Treatments	Culture Conditions			$\text{NO}_2^-$ (μM)	
	24 h	⇒	24 h	24 h	48 h
Medium	Normoxia	⇒	Normoxia	0.5	0.6
Medium	Hypoxia	⇒	Normoxia	0.4	0.7
IFN- $\gamma$	Normoxia	⇒	Normoxia	0.6	1.5
IFN- $\gamma$	Hypoxia	⇒	Normoxia	0.4	8.5

<sup>a</sup> ANA-1 macrophages ( $1 \times 10^6$  cells/ml) were treated with medium or IFN- $\gamma$  (100 U/ml) and cultured under either normoxic conditions for 48 h or hypoxic conditions for 24 h and then normoxic conditions for an additional 24 h. Cell-free culture supernatants were harvested at 24 h and 48 h and assayed for the presence of  $\text{NO}_2^-$  as described in *Materials and Methods*. Data shown are from one representative experiment of four performed.

cells stimulated with IFN- $\gamma$  and cultured under normoxic conditions (Fig. 2B, lanes 1 and 2). Hypoxia alone also failed to induce measurable levels of iNOS activity (lane 3), whereas a significant conversion of L-arginine to  $\text{NO}_2^-$  was detected in lysates from ANA-1 macrophages treated with IFN- $\gamma$  plus hypoxia (lane 4). High levels of  $\text{NO}_2^-$  were measured in lysates from cells treated with IFN- $\gamma$  plus LPS (lane 5). These data demonstrated that hypoxia, although ineffective by itself, augments the IFN- $\gamma$ -dependent induction of enzymatically active iNOS protein in murine macrophages.

#### Reoxygenation restores the production of NO following stimulation with IFN- $\gamma$ plus hypoxia

Because IFN- $\gamma$  plus hypoxia-treated ANA-1 macrophages expressed elevated levels of functional iNOS protein, the failure to produce NO could be due to insufficient oxygen tension and consequent impairment of the oxidation of L-arginine. Therefore, it was possible that reoxygenation following stimulation with IFN- $\gamma$  plus hypoxia was needed to allow NO production by macrophages. To test this possibility, ANA-1 cells were treated with medium or IFN- $\gamma$  and cultured either under hypoxia for 24 h, followed by additional 24 h in normoxia (reoxygenation), or under normoxic conditions for the entire length of the experiment (48 h). No detectable  $\text{NO}_2^-$  production was observed in untreated cells or in cells cultured under hypoxia for 24 h and then in normoxia (Table I). Very low levels of  $\text{NO}_2^-$  were measured in supernatants of cells treated with IFN- $\gamma$  alone for 48 h. In contrast, appreciable levels of  $\text{NO}_2^-$  production were detected in the supernatant of cells treated with IFN- $\gamma$  plus hypoxia for the first 24 h and then cultured in the presence of normal oxygen levels for additional 24 h. To investigate whether hypoxia was a costimulus for NO

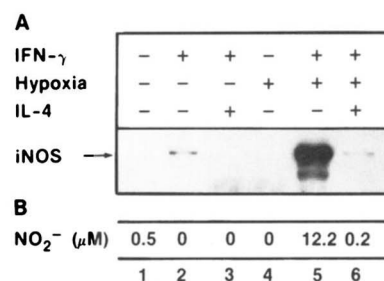


**FIGURE 3.**  $\text{NO}_2^-$  accumulation in IFN- $\gamma$ -treated murine peritoneal macrophages cultured under hypoxia-reoxygenation conditions. Thioglycollate-elicited peritoneal macrophages ( $2 \times 10^5$  cells) were treated with medium or IFN- $\gamma$  (100 U/ml) and cultured under hypoxic or normoxic conditions for 24 h. Then, cells were cultured for additional 24 h under normoxic conditions. Cell-free culture supernatants were harvested after the first 24 h of incubation (time 0) and at the indicated times thereafter. The levels of  $\text{NO}_2^-$  were measured as described in *Materials and Methods*. Data are from one of two similar experiments.

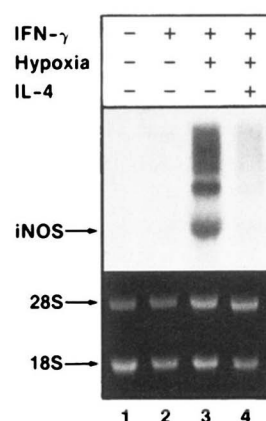
production in primary murine macrophages, thioglycollate-elicited peritoneal macrophages were treated with medium or IFN- $\gamma$  and cultured under either normoxic or hypoxic conditions for 24 h, and then in normoxic conditions for up to an additional 24 h. Cell-free culture supernatants were harvested after 24 h (time 0) and 1.5, 3, 4.5, 6, and 24 h later (Fig. 3). No  $\text{NO}_2^-$  production was detected in untreated peritoneal macrophages under either normoxic or hypoxic conditions. Significant levels of  $\text{NO}_2^-$  were produced by macrophages stimulated with IFN- $\gamma$  under normal oxygen levels for 48 h. However, a major synergistic induction of  $\text{NO}_2^-$  production was observed in peritoneal macrophages treated with IFN- $\gamma$  plus hypoxia for 24 h and then cultured under normal oxygen levels (Fig. 3). A significant accumulation of  $\text{NO}_2^-$  was already detected after 1 to 1.5 h of reoxygenation, and  $\text{NO}_2^-$  levels steadily increased over a 24-h period. These data demonstrate that reoxygenation is required for the production of NO by murine macrophages stimulated with IFN- $\gamma$  plus hypoxia.

#### IL-4 inhibits iNOS expression induced by IFN- $\gamma$ and hypoxia

We have reported previously that IL-4 inhibits  $\text{NO}_2^-$  production (18) and iNOS mRNA expression (19) induced by IFN- $\gamma$  plus picolinic acid in ANA-1 macrophages. Because hypoxia and picolinic acid activate a common element of the iNOS promoter (11), we investigated whether IL-4 inhibited iNOS expression induced by IFN- $\gamma$  plus hypoxia. ANA-1 macrophages were treated with medium, or IFN- $\gamma$  alone or plus hypoxia in the presence or absence of IL-4, and the cell lysates were tested for the content of iNOS protein (Fig. 4A) and iNOS activity (Fig. 4B). ANA-1 cells treated with IFN- $\gamma$  alone expressed very low levels of iNOS protein that were inhibited completely by the addition of IL-4 (lanes 2 and 3). As shown above, cells treated with IFN- $\gamma$  plus hypoxia expressed significantly higher levels of iNOS protein (lane 5). However, IL-4 almost completely abolished the induction of iNOS protein by IFN- $\gamma$  plus hypoxia (lane 6). Consistent with these data, the expression of iNOS activity observed in cytosol isolated from ANA-1 macrophages treated with IFN- $\gamma$  plus hypoxia (Fig. 4B, lane 5) was abrogated completely in the presence of IL-4 (lane 6). To investigate whether IL-4 also inhibited IFN- $\gamma$  plus hypoxia-



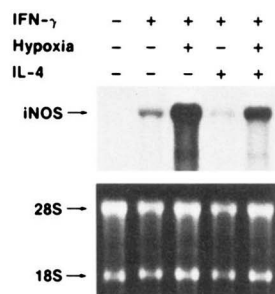
**FIGURE 4.** IL-4 inhibits the expression of iNOS protein and iNOS activity induced by IFN- $\gamma$  plus hypoxia. ANA-1 macrophages ( $1\text{--}1.5 \times 10^6$  cells/ml) were treated with medium or IFN- $\gamma$  (100 U/ml) in the presence or absence of IL-4, and were cultured under either normoxic or hypoxic conditions for 18 h. Cytosols were then isolated and assayed for the presence of iNOS protein by western blot (A) or iNOS activity (B), as described. Results shown are from one experiment of two performed.



**FIGURE 5.** IL-4 inhibits iNOS mRNA expression induced by IFN- $\gamma$  plus hypoxia in ANA-1 macrophages. ANA-1 macrophages ( $1 \times 10^6$  cells/ml) were plated in 100-mm tissue culture dishes and treated with medium, IFN- $\gamma$  (100 U/ml) alone, or IFN- $\gamma$  plus hypoxia in the presence or absence of IL-4 (400 U/ml) for 18 h. Total cellular RNA was examined for iNOS mRNA expression, as described. Equal RNA loading was confirmed by ethidium bromide staining and monitoring of 28S and 18S rRNA. Data shown are from one representative experiment of three performed.

induced iNOS mRNA expression, ANA-1 macrophages were treated with medium or IFN- $\gamma$  in the presence or absence of IL-4, and cultured under either hypoxic or normoxic conditions for 18 h. No expression of iNOS mRNA was observed in untreated ANA-1 macrophages (Fig. 5, lane 1) or in cells treated with hypoxia alone, regardless of the presence of IL-4 (data not shown). IFN- $\gamma$  alone induced very low levels of iNOS mRNA expression (lane 2). As previously reported (11), a major increase of iNOS mRNA expression was induced in cells treated with IFN- $\gamma$  plus hypoxia (lane 3). The addition of IL-4 caused a dramatic inhibition of IFN- $\gamma$  plus hypoxia-dependent induction of iNOS mRNA expression (lane 4). The inhibitory effects of IL-4 were always greater than 80% in three experiments performed. To establish whether IL-4 was an inhibitory signal for IFN- $\gamma$  plus hypoxia-induced iNOS mRNA expression in primary macrophages, thioglycollate-elicited peritoneal macrophages were treated with medium or IFN- $\gamma$  in the presence or absence of IL-4, and cultured under either normoxic or hypoxic conditions for 18 h. Peritoneal macrophages did not express iNOS mRNA either constitutively (Fig. 6) or following stimulation with hypoxia alone, in the absence or presence of IL-4





**FIGURE 6.** IL-4 inhibits the IFN- $\gamma$  plus hypoxia-dependent synergistic induction of iNOS mRNA expression in murine peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages ( $1.5 \times 10^7$  cells) were treated with medium and IFN- $\gamma$  (100 U/ml) in the presence or absence of IL-4 (400 U/ml), and were cultured in normoxic or hypoxic conditions for 18 h. Total cellular RNA was examined for iNOS mRNA expression. Data shown are from one of two similar experiments.

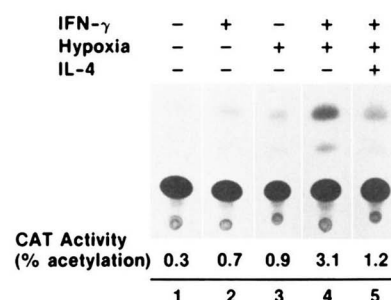
(data not shown). Cells treated with IFN- $\gamma$  alone expressed low but significant levels of iNOS mRNA that were reduced by the addition of IL-4. A major increase in iNOS mRNA expression was observed in peritoneal macrophages treated with IFN- $\gamma$  and cultured under hypoxic conditions (Fig. 6). However, the addition of IL-4 caused a significant inhibition of IFN- $\gamma$  plus hypoxia-induced iNOS mRNA expression. IL-4 also inhibited the expression of iNOS protein induced in peritoneal macrophages by treatment with IFN- $\gamma$  plus hypoxia (data not shown). In conclusion, these data demonstrate that IL-4 inhibits the synergistic interaction between IFN- $\gamma$  and hypoxia in the induction of iNOS expression both in the ANA-1 macrophage cell line and in thioglycollate-elicited peritoneal macrophages.

#### *IL-4 inhibits the activation of the iNOS promoter induced by IFN- $\gamma$ plus hypoxia*

IFN- $\gamma$  plus hypoxia induce the transcriptional activation of the iNOS gene and the expression of iNOS promoter-CAT reporter gene constructs transiently transfected in ANA-1 macrophages (11). To establish whether IL-4 inhibited the IFN- $\gamma$  plus hypoxia-dependent transcriptional activation of the iNOS promoter, ANA-1 macrophages were transfected transiently with a plasmid containing the full-length 1.6-kb 5' flanking region of the iNOS gene linked to a CAT reporter gene and stimulated with medium or IFN- $\gamma$  under normoxic or hypoxic conditions, in the presence or absence of IL-4. ANA-1 cells expressed little or no CAT activity either constitutively or after treatment with IFN- $\gamma$  or hypoxia alone (Fig. 7). IL-4 did not induce CAT expression, either under normoxic or hypoxic conditions (data not shown). As previously reported (11), a synergistic induction of CAT activity was observed in ANA-1 cells treated with IFN- $\gamma$  under hypoxic conditions (Fig. 7, lane 4). However, a significant decrease (>50%) of the IFN- $\gamma$  plus hypoxia-dependent induction of CAT reporter gene expression was caused by the addition of IL-4, indicating that IL-4 inhibited the transcriptional activation of the iNOS promoter induced by IFN- $\gamma$  under low oxygen tension.

## Discussion

We have demonstrated that hypoxia acted synergistically with IFN- $\gamma$  in inducing the expression of iNOS protein and cytosolic iNOS enzymatic activity in murine macrophages. However, production of NO required that reoxygenation followed hypoxia in IFN- $\gamma$ -treated macrophages. The induction of iNOS mRNA and protein expression through the IFN- $\gamma$  plus hypoxia-dependent



**FIGURE 7.** IL-4 inhibits the activation of iNOS promoter induced by IFN- $\gamma$  plus hypoxia. ANA-1 macrophages were transfected with the p1-iNOS-CAT construct, as described in *Materials and Methods*. Twenty-four hours after the transfection, cells were treated with medium or IFN- $\gamma$  (100 U/ml) in the presence or absence of IL-4 (400 U/ml), and cultured in either normoxic or hypoxic conditions for an additional 18 h. Cells were lysed and CAT activity was assayed by TLC, as described. Data shown are from one representative experiment of four performed.

pathway was susceptible to inhibition by IL-4, which acted on the transcriptional activation of the iNOS promoter. These data elucidate the role of hypoxia in the production of NO in murine macrophages, and they provide evidence of the molecular mechanism by which IL-4 inhibits the cooperative interaction between IFN- $\gamma$  and hypoxia in the activation of iNOS gene expression.

Hypoxia is a condition of decreased O<sub>2</sub> availability that elicits the expression of several genes that favor the adaption of the organism to the increased demand of O<sub>2</sub> (27). A systemic compensatory mechanism to hypoxia is the induction of erythropoietin, a glycoprotein hormone that stimulates erythropoiesis in bone marrow and increases O<sub>2</sub> delivery in the tissue (28). Other genes that are part of the local and intracellular adaptive response to hypoxia include vascular endothelial growth factor (29), a potent angiogenic factor leading to increased vascularization of the affected tissue, and glycolytic enzymes (30, 31), which activate an anaerobic metabolic pathway for ATP production. The molecular mechanism of hypoxia-inducible gene expression has been the subject of intensive investigation in the past several years. Cis-acting DNA sequences responsive to hypoxia have been demonstrated to play a functional role in the hypoxia-inducible transcriptional activation of genes encoding Epo (12, 32, 33) and glycolytic enzymes (30, 31). The identification of a functional hypoxia-responsive sequence in the iNOS promoter indicated that iNOS is a hypoxia-inducible gene (11). However, the expression of iNOS induced by low oxygen tension is different from that of classical hypoxia-inducible genes in that it requires a second signal (IFN- $\gamma$ ) to be effective and hypoxia alone is not sufficient.

The relationship between iNOS and oxygen levels is intriguing because hypoxia induces iNOS expression, but oxygen is required as a co-substrate for its enzymatic activity (34). The effect of low oxygen tension on the activity of NOS enzyme is still controversial. Hypoxia inhibited neural cNOS in penile corpus cavernosum (35), but stimulated NO release in the heart (36). We found that murine macrophages stimulated with IFN- $\gamma$  plus hypoxia, despite a significant induction of iNOS mRNA expression, did not release NO in the culture supernatant and that cells treated with IFN- $\gamma$  plus LPS under hypoxia produced much less NO than under normoxic conditions. Thus, macrophage iNOS activity is inhibited by low oxygen tension. However, ANA-1 macrophages treated with IFN- $\gamma$  plus hypoxia contained increased levels of iNOS protein that paralleled the expression of iNOS mRNA. In addition, the amount of iNOS protein observed in ANA-1 macrophages treated

with IFN- $\gamma$  plus picolinic acid, which also induce iNOS mRNA expression, was similar in the presence or absence of oxygen (data not shown). These data indicate that hypoxia did not inhibit iNOS mRNA translation. Accordingly, significant levels of iNOS activity were detected in the cytosol isolated from ANA-1 macrophages treated with IFN- $\gamma$  plus hypoxia, demonstrating that hypoxia is a costimulus for the induction of iNOS activity in the presence of cofactors and substrate. Experiments in which either ANA-1 cells or murine peritoneal macrophages were stimulated with IFN- $\gamma$  plus hypoxia and then cultured in the presence of oxygen demonstrated that reoxygenation was sufficient to restore iNOS activity in the intact cells and to achieve significant accumulation of NO<sub>2</sub><sup>-</sup> in the culture supernatant. These results provide the first evidence that hypoxia is a costimulus with IFN- $\gamma$  for the induction of NO production in murine macrophages. Under the same experimental conditions, no significant difference in the content of iNOS protein was observed between IFN- $\gamma$ -treated cells cultured under either hypoxia or hypoxia reoxygenation, ruling out the possibility that the reoxygenation increased the levels of iNOS protein (data not shown). Experiments in which the levels of NO<sub>2</sub><sup>-</sup> were measured at different time intervals after reoxygenation in IFN- $\gamma$  plus hypoxia-treated peritoneal macrophages indicated that NO<sub>2</sub><sup>-</sup> accumulation followed a steady kinetic over a 24-h period. The rise in the rate of iNOS activity after reoxygenation supports the notion that oxygen is required as a co-substrate in NO production. However, many cofactors, in addition to molecular oxygen, are required for iNOS enzymatic activity. Therefore, reoxygenation might also control the production of such cofactors, allowing the enzymatic reaction to occur. Whether oxygen is needed only as a substrate or also as an inducer of other cofactors remains to be fully established.

The biologic implications of the finding that hypoxia induces the expression of the iNOS gene but inhibits its enzymatic activity are quite interesting. It appears that the activation of iNOS may be a two-step process involving first the augmentation of the levels of iNOS protein under hypoxic conditions, followed by the expression of the enzymatic activity in the reoxygenated tissue. The transient nature of tissue hypoxia supports the hypothesis of a biologic role of the hypoxia-reoxygenation sequence in NO production. Hypoxia may occur in many pathologic conditions in which the involvement of NO has been demonstrated, including acute and chronic inflammation (37–39), rejection of transplanted organs (40–42), and necrotic areas of neoplastic lesions (43, 44). The hypoxia inducibility of iNOS expression suggests that tissue hypoxia, besides its effects on cellular metabolism, may be an important local environmental factor in the activation and expression of effector functions by macrophages. This is particularly relevant to macrophage biology because macrophages can be exposed to broad variations of oxygen tension in different tissues and pathologic conditions. In this scenario, exposure of macrophages to low oxygen tension may cause, in the presence of IFN- $\gamma$ , increased expression of iNOS mRNA and iNOS protein, whose full biologic activities are achieved by restoration of sufficient oxygen concentration. It is also tempting to speculate that NO production itself might be part of the adaptive response to decreased oxygen levels. A role for NO in the angiogenic process that is induced in hypoxic conditions has been suggested by the requirement of an L-arginine/iNOS-dependent mechanism in the production of angiogenic activity by human monocytes (45), and by the increased vascularization and enhanced growth in nude mice of a human colon adenocarcinoma cell line engineered to generate NO continuously (46).

The expression of iNOS is regulated tightly and is controlled by inhibitory mechanisms aimed at preventing the detrimental effects of prolonged or excessive NO production (47). Several cytokines,

including TGF- $\beta$  (48), macrophage-deactivating factor (49), macrophage-stimulating protein (50), and IL-4 (22, 51), inhibit iNOS expression. IL-4 has long been known as a cytokine that inhibits the expression of several genes (52) and functions (53) in murine macrophages. IL-4 inhibited the costimulatory activity of IL-2 or picolinic acid, but not of LPS on IFN- $\gamma$ -treated macrophages (53). Furthermore, we have demonstrated previously that IL-4 inhibited IFN- $\gamma$  plus picolinic acid-dependent induction of NO<sub>2</sub><sup>-</sup> production (18), iNOS mRNA expression, and iNOS transcription, as assessed by nuclear run-on experiments (19). Consistent with the finding that picolinic acid and hypoxia activate a common pathway of iNOS promoter transcriptional activation, we report that IL-4 down-regulated iNOS mRNA expression induced by IFN- $\gamma$  plus hypoxia both in murine peritoneal macrophages and in the ANA-1 macrophage cell line. Therefore, IL-4 exerts a negative control on the expression of a hypoxia-inducible gene. The IL-4-dependent inhibition of iNOS mRNA expression caused a parallel inhibition of iNOS protein and enzymatic activity. The physiologic regulator of hypoxia-inducible genes is the oxygen tension itself, which once restored to normal levels readily inhibits hypoxia-induced gene expression (16). The peculiarity of the hypoxia-dependent induction of iNOS expression, which is under the immunologic control exerted by IFN- $\gamma$ , suggests that additional cytokine-dependent mechanisms may be required to control its expression.

IL-4 also down-regulated the expression of iNOS promoter activity induced by IFN- $\gamma$  plus picolinic acid in ANA-1 macrophages (unpublished observation), consistent with previous results obtained by nuclear run-on analysis. We report now that IL-4 down-regulated IFN- $\gamma$  plus hypoxia-dependent transcriptional activation of iNOS promoter. The IL-4-dependent inhibition was observed consistently, always greater than 50% and in agreement with the inhibition of iNOS mRNA expression. These data suggest that the IL-4 inhibition of IFN- $\gamma$  plus hypoxia-dependent induction of iNOS mRNA expression may be accounted for, at least in part, by inhibition of iNOS transcriptional activation. Recent evidence suggests that IL-4 activates members of the signal transducers and activators of transcription (STAT) gene family that bind to IFN- $\gamma$  activation sites (GAS) originally found in the promoters of IFN- $\gamma$ -inducible genes (54–56). Interestingly, the iNOS promoter contains several putative GAS elements that may be potential targets of IL-4-induced transcription factors. Whether any of these sequences plays a functional role in the control of iNOS expression remains to be established. The 5' flanking region of the iNOS gene contains large stretches of DNA whose functional role is still unknown, and the issue of negative regulatory elements is a question to be addressed.

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