

# A Hypoxia-responsive Element Mediates a Novel Pathway of Activation of the Inducible Nitric Oxide Synthase Promoter

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## Summary

Picolinic acid, a catabolite of L-tryptophan, activates the transcription of the inducible nitric oxide synthase gene (iNOS) in IFN- $\gamma$ -treated murine macrophages. We performed functional studies on the 5' flanking region of the iNOS gene linked to a CAT reporter gene to identify the *cis*-acting element(s) responsible for the activation of iNOS transcription by picolinic acid. Transient transfection assays showed that the full-length iNOS promoter in the murine macrophage cell line ANA-1 was activated by the synergistic interaction between IFN- $\gamma$  and picolinic acid. Deletion or mutation of the iNOS promoter region from -227 to -209, containing a sequence homology to a hypoxia-responsive enhancer (iNOS-HRE), decreased picolinic acid- but not LPS-induced CAT activity by more than 70%. Functional studies using a tk promoter-CAT reporter gene plasmid demonstrated that the iNOS-HRE was sufficient to confer inducibility by picolinic acid but not by IFN- $\gamma$  or LPS. Electrophoretic mobility shift assays confirmed that picolinic acid alone induced a specific binding activity to the iNOS-HRE. Furthermore, we found that the iNOS-HRE activity was inducible by hypoxia and that hypoxia in combination with IFN- $\gamma$  activated the iNOS promoter in transient transfection assays and induced iNOS transcription and mRNA expression. These data establish that the iNOS-HRE is a novel regulatory element of the iNOS promoter activity in murine macrophages and provide the first evidence that iNOS is a hypoxia-inducible gene.

Nitric oxide (NO) is a pleiotropic molecule involved in neurotransmission, vascular homeostasis and effector functions of murine macrophages (1-3). NO is generated during the oxidation of L-arginine to L-citrulline by at least three different isoforms of the enzyme nitric oxide synthase (NOS). Neuronal and endothelial NOSs are constitutively expressed and their activity is dependent on the levels of Ca<sup>2+</sup> and calmodulin. Macrophages contain a transcriptionally inducible isoform of NOS (iNOS) (4, 5), whose activity is independent of elevated Ca<sup>2+</sup> or exogenous calmodulin (6). Maximal expression of iNOS mRNA in murine macrophages is achieved by stimulation with IFN- $\gamma$  plus a second signal, such as bacterial lipopolysaccharide (LPS) (4,

5). A 1.6-kb fragment of the 5'-flanking region of the mouse iNOS gene is sufficient to confer inducibility by IFN- $\gamma$  plus LPS (7, 8). Additionally, the consensus sequences for the binding of interferon regulatory factor-1 (IRF-1) (9, 10) and NF- $\kappa$ B (11) of the iNOS promoter are required for activation of transcription by IFN- $\gamma$  and LPS, respectively. No other regulatory elements important for the control of iNOS expression have been identified as yet.

The diversity of the pathological conditions in which macrophage NO is produced may be based on the existence of several signals inducing iNOS and implies the need for multiple regulatory elements to control its transcription (12). In an attempt to identify additional elements regulating iNOS expression, we studied the response of macrophages to activating agents structurally and biologically different from LPS. Picolinic acid is an end catabolite of L-tryptophan, whose metabolism is initiated by two inducible enzymes, tryptophan dioxygenase in the liver and indoleamine 2,3-dioxygenase in extra-hepatic tissue, that differ in the response to extracellular stimulation (13-15). The degradation of L-tryptophan occurs *in vitro* and *in vivo*, in

<sup>1</sup>Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; HIF-1, hypoxia inducible factor-1; HRE, hypoxia-responsive element; iNOS, inducible nitric oxide synthase; IRF-E, interferon regulatory factor binding site; NO, nitric oxide; NOS, nitric oxide synthase.

mice and humans (14–16), and has been implicated in some of the biological effects of IFN- $\gamma$  (17, 18). Elevated levels of tryptophan metabolites, such as kynurenine and quinolinic acid, have been detected *in vivo* in cerebrospinal fluid in pathological conditions (19, 20) as well as in cancer patients treated with IL-2 (21). Picolinic acid has been detected in body fluids (22, 23), and can activate murine macrophages *in vivo* (24–26). However, the cellular source of picolinic acid has not been defined yet and the pathophysiological role of picolinic acid remains to be fully determined. Picolinic acid is a potent costimulatory agent for the induction of tumoricidal activity in mouse peritoneal macrophages (27) as well as in the mouse macrophage cell lines ANA-1 and GG2EE (28, 29), the latter derived from the LPS-hyporesponsive C3H/HeJ strain of mouse. We recently demonstrated that picolinic acid is a costimulus with IFN- $\gamma$  for the induction of NO production (30) and iNOS mRNA transcription in murine macrophages (31). These observations suggest a possible connection between tryptophan and arginine metabolism, two oxidative amino acid metabolic pathways leading to biologically active molecules, and indicate that amino acid catabolites may play a role in the transcriptional control of gene expression in murine macrophages. However, the DNA regulatory element(s) required for picolinic acid-induced transcription of the iNOS gene are not known.

We have performed functional studies on the 5'-flanking region of the iNOS gene to characterize the *cis*-acting element(s) responsible for the activation of iNOS transcription by picolinic acid in ANA-1 murine macrophages. We have found that a 19-base pairs (bp) region of the iNOS promoter, containing a sequence homology to a hypoxia-inducible enhancer, is required for picolinic acid-, but not for LPS-induced, activation of iNOS promoter. The hypoxia-responsive element (HRE) present in the iNOS promoter responds also to hypoxia that in combination with IFN- $\gamma$  is a stimulus for the induction of iNOS transcription and mRNA expression. These data demonstrate the existence of a novel regulatory element of the iNOS promoter, that is active in the control of iNOS transcription in murine macrophages.

## Materials and Methods

**Cells.** 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 (32, 33). ANA-1 macrophages were cultured in DMEM (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (GIBCO BRL, Gaithersburg, MD) (complete medium). Cells were maintained at 37°C in a humidified incubator containing 20% O<sub>2</sub>, 5% CO<sub>2</sub> in air (referred to as normoxic conditions). In some experiments cells were cultured under hypoxic conditions in 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.

**Reagents.** Mouse rIFN- $\gamma$  (sp. act.  $\geq 10^7$  U/mg) was pur-

chased from GIBCO BRL. LPS (from *E. coli* serotype 0111:B4) was purchased from Sigma Chemical Company (St. Louis, MO). Picolinic acid was purchased from Sigma (purity  $\sim 99\%$ ) and was prepared as previously described (30). The content of endotoxin in all the reagents was below the detection limit of 6 pg/ml, as determined by assay with a chromogenic *Limulus* amoebocyte lysate test (Whittaker Bioproducts).

**Plasmids.** The 1,749-bp fragment of the 5'-flanking region of the murine iNOS gene inserted into pCAT-Basic (Promega Corp., Madison, WI) upstream of the promoterless chloramphenicol acetyltransferase (CAT) gene (p1-iNOS-CAT) was kindly provided by Q. Xie and C. Nathan (Cornell University Medical College, NY). Deletion mutants were obtained by digesting p1-iNOS-CAT with uniquely cleaving restriction enzymes and religating the linearized plasmid. Alternatively, oligonucleotide primers bearing a suitable restriction site at the 5' ends were synthesized in order to obtain the desired deletion or mutation; the PCR fragments were subsequently digested and inserted into linearized p1-iNOS-CAT plasmids. pBLCAT2 plasmid, containing the CAT reporter gene under the control of a HSV tk promoter fragment spanning from -105 to +51, was used for functional studies in the context of a heterologous promoter. Three tandem copies of the double-strand oligonucleotide depicted in Fig. 3A were subcloned in the HindIII/BamHI sites of pBLCAT2, 5' upstream of the HSV-tk promoter. The constructs were sequenced using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH).

**Transient Transfection of ANA-1 and CAT Assay.** ANA-1 macrophages were transfected by a modification of the DEAE-dextran method (34). After cells were washed twice with serum-free DMEM, 10  $\mu$ g of plasmid DNA was 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 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. The cells were washed, plated in 6-well plates at 1  $\times 10^6$ /ml in 5 ml of complete medium and incubated at 37°C in 5% CO<sub>2</sub>. 24 h later, the appropriate stimulus was added to each well and the cells were incubated for additional 18 h. The cells were then washed, resuspended in 0.25 M Tris-HCl (pH 7.5) and subjected to three cycles of rapid freezing and thawing. The lysates were centrifuged (11,000 g for 10 min), the supernatants were heated at 65°C for 10 min to inactivate CAT inhibitors and assayed for CAT activity by TLC method (35). Protein content was determined as described by Bradford, using the Bio-Rad Protein Assay (36) (Bio-Rad Labs., Richmond, CA). To control for differences in the uptake of transfected DNA, cells were cotransfected with 5  $\mu$ 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.

**Preparation of Nuclear Extracts.** Cells were incubated with appropriate stimuli, washed twice with cold Dulbecco's phosphate-buffered saline (PBS) and pelleted by centrifugation at 1,200 rpm for 5 min at 4°C. Nuclear extracts were prepared by modification of a standard protocol (37) as described (38), with buffers A and C containing 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 2  $\mu$ g/ml each of leupeptin, aprotinin and pepstatin. The cell pellet was washed with 5 packed cell volume of buffer A (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl), resuspended in 4 packed cell volume of buffer A, and then incubated on ice for 10 min. The cell suspension was homogenized with a glass Dounce homogenizer using a type B pestle. The nuclei were pelleted by centrifugation at 2,000 rpm for 5 min at 4°C and resuspended in 3 packed

nuclear volume of buffer C (0.42 M KCl, 20 mM Tris-HCl, pH 7.5, 20% glycerol, 1.5 mM MgCl<sub>2</sub>). 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). Dialysate was clarified by centrifugation at 15,000 rpm for 10 min at 4°C, and aliquots were frozen and stored at -70°C for subsequent analysis of DNA binding proteins.

**Electrophoretic Mobility Shift Assay (EMSA).** Probes were generated either by 5'-end labeling of the double strand oligonucleotide with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Corp., Arlington Heights, IL) and T4 polynucleotide kinase (GIBCO BRL) or by Klenow fragment of DNA polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP. Binding reaction was carried out in buffer D containing 5  $\mu$ g of nuclear extract and 0.4  $\mu$ g of denatured calf thymus DNA on ice. After incubation for 5 min, probe (1  $\times$  10<sup>4</sup> cpm; 0.1–0.2 ng) was added and the incubation was continued for an additional 15 min, after which the reaction mixtures were loaded onto 5% nondenaturing polyacrylamide gel. Electrophoresis was performed at 180 V in 0.3 $\times$  TBE (1 $\times$  TBE is 89 mM Tris-HCl, 89 mM boric acid, and 5 mM EDTA) at 4°C. Gels were vacuum dried and autoradiographed with intensifying screen at -70°C. Competitor DNAs were incubated with nuclear extracts for 5 min before addition of labeled probe.

**Northern Blot Analysis.** ANA-1 macrophages were cultured at 1  $\times$  10<sup>6</sup> cells/ml in 100-mm tissue culture plates and treated with appropriate reagents as detailed in Results. Total cellular RNA was harvested after 18 h of incubation and processed as previously described (31). Briefly, cells were solubilized with guanidine isothiocyanate, and the total cellular RNA was purified by centrifugation through a cushion of cesium chloride. 20  $\mu$ g 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 (Oncor Inc., Gaithersburg, MD). The cDNA probe that was specific for mouse macrophage NOS (39) 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<sup>6</sup> cpm/ml) during an overnight incubation, washed, and autoradiographed at -70°C on XAR-5 film (Eastman Kodak Co., San Diego, CA) with the use of Lightning Plus intensifying screens (DuPont NEN, Wilmington, DE). Equal loading of RNA was assessed by ethidium bromide staining of 28S and 18S rRNA.

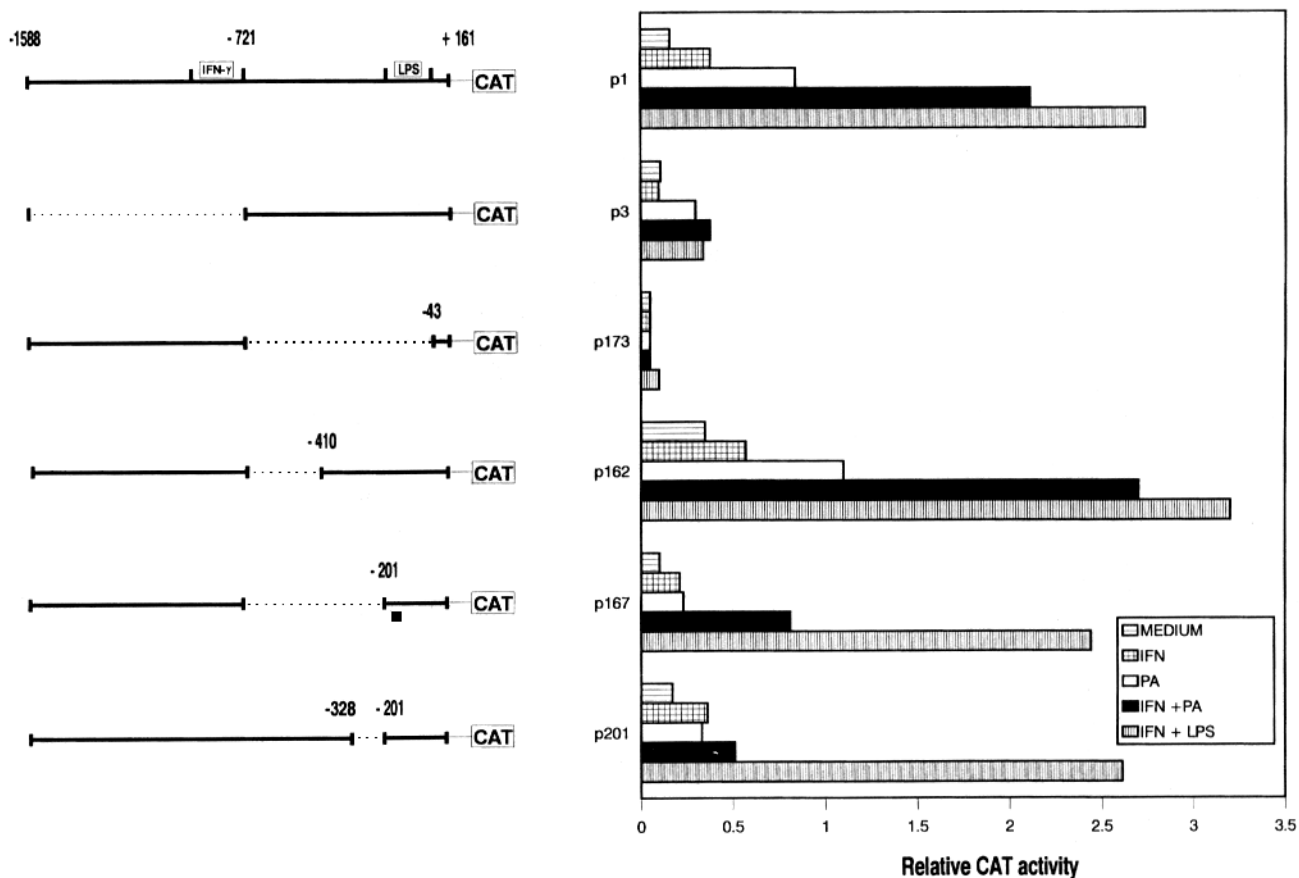
**Nuclear Run-on Experiment.** Nuclei were isolated as previously described (31) from 2.5  $\times$  10<sup>7</sup> ANA-1 cells treated as indicated for 12 h. Thawed nuclei were gently mixed with 150  $\mu$ l 2 $\times$  transcription mix (1 $\times$  = 100 mM Sucrose, 10% glycerol, 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl<sub>2</sub>, 2.5 mM DTT, 0.5 mM each of ATP, CTP, and GTP) and incubated with 100  $\mu$ Ci of [<sup>32</sup>P]UTP (800 Ci/mmol; Amersham Corp.) at 30°C for 30 min. 20  $\mu$ l of 100 mM CaCl<sub>2</sub> and 20 U 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 Trizol (GIBCO BRL) and the RNA was isolated according to the manufacturer's procedure. Approximately, 2  $\times$  10<sup>6</sup> cpm of RNA were used in hybridization for 48 h with 2.5  $\mu$ g of each slot-blotted denatured plasmid (pGEM-3Z vector alone, vector containing the 3.9-kb cDNA insert of macrophage iNOS (39), and vector containing a chicken  $\beta$ -actin cDNA insert). Filters were washed three times for 10 min at room temperature with 2 $\times$  SSC and 0.1% SDS and two times for 20 min at 60°C with 0.2 $\times$  SSC and 0.1% SDS. Fil-

ters were then autoradiographed as for the Northern blot experiment.

## Results

**Identification of a Putative Picolinic Acid-responsive Region in the iNOS Promoter.** To characterize the regulatory element(s) of the iNOS promoter responsive to picolinic acid, 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. The expression of the CAT gene was measured after stimulation of the cells with medium, IFN- $\gamma$  (100 U/ml) or picolinic acid (4 mM) alone or in combination. The CAT expression following stimulation of the cells with IFN- $\gamma$  plus LPS (10 ng/ml) was included as a positive control (7, 8). As shown in Fig. 1, ANA-1 cells transfected with the full-length construct (plasmid p1) expressed little or no CAT activity either constitutively or after treatment with IFN- $\gamma$  alone. Picolinic acid alone induced low but appreciable levels of CAT activity. However, high levels of transcriptional activation of iNOS promoter were induced by the synergistic interaction between IFN- $\gamma$  and picolinic acid, and were consistently observed in at least eight independent transfection experiments. IFN- $\gamma$  plus LPS induced levels of iNOS promoter activity similar to those induced by IFN- $\gamma$  plus picolinic acid. Deletion of the region from -1588 to -721 (plasmid p3), that contains an IFN- $\gamma$ -responsive region at position -923 to -913, inhibited the synergistic interaction of IFN- $\gamma$  with either picolinic acid or LPS. The CAT activity induced by picolinic acid alone or in combination with IFN- $\gamma$  was decreased by 64% and 82%, respectively, relative to that observed with the full-length iNOS promoter. In addition, deletion of the region from -1029 to -721 (EspI to SmaI site) also abrogated the synergistic induction of CAT expression by IFN- $\gamma$  plus picolinic acid (data not shown). These results demonstrated that the fragment -1588 to -721 is essential to achieve synergistic activation of iNOS promoter by IFN- $\gamma$  plus picolinic acid. Deletion of the region from -721 to -43 (plasmid p173) abolished CAT activity expressed either constitutively or after stimulation, suggesting that a putative element responsive to picolinic acid was present in this fragment.

The above results indicated that maximal activation of iNOS promoter required stimulation with IFN- $\gamma$  plus picolinic acid. To identify the regulatory sequence(s) targeted by picolinic acid in the synergistic interaction with IFN- $\gamma$  for the activation of iNOS transcription, we constructed mutants of the iNOS promoter in which the region containing IFN- $\gamma$ -responsive elements (from -1588 to -721) was conserved and the fragment from -721 to -43 was progressively deleted. As shown in Fig. 1, deletion of the region from -721 to -410 (plasmid p162) did not inhibit CAT expression induced by either IFN- $\gamma$  plus picolinic acid or IFN- $\gamma$  plus LPS. In contrast, deletion of the region from -410 to -201 (plasmid p167) strongly reduced CAT activity induced by picolinic acid alone or in combination



**Figure 1.** Identification of a picolinic acid responsive region of iNOS promoter. ANA-1 macrophages were transfected with the indicated CAT-constructs as described in Materials and Methods. 24 h later, cells were treated with medium, murine rIFN- $\gamma$  (100 U/ml) or picolinic acid (4 mM) alone or in combination, or IFN- $\gamma$  plus lipopolysaccharide (10 ng/ml) for an additional 18 h and CAT activity was assayed by TLC. The regions containing the element(s) responsive to IFN- $\gamma$  and LPS, previously described in other experimental systems, are indicated in plasmid p1. Results are expressed as relative CAT activity, obtained by dividing CAT activity (% acetylation) by relative light units for luciferase, and are from one representative experiment of at least four performed.

with IFN- $\gamma$  by 73 and 62%, respectively, without changing significantly the levels of CAT expression induced by IFN- $\gamma$  plus LPS. Furthermore, deletion of only 127 bp (from -328 to -201, plasmid p201) from the full-length iNOS promoter, caused a reduction greater than 70% of IFN- $\gamma$  plus picolinic acid-induced CAT expression, but did not affect the response to IFN- $\gamma$  plus LPS. These results demonstrate the existence of a novel regulatory region (-328 to -201) required for the synergistic activation of the iNOS promoter in response to picolinic acid but not to LPS.

**Functional Requirement of a Putative Picolinic Acid-responsive Element in the Activation of iNOS Promoter by IFN- $\gamma$  Plus Picolinic Acid.** Comparison of the sequence of the iNOS promoter region from -328 to -201 with the sequences reported in the GenBank was instrumental in pinpointing the putative element responsive to picolinic acid. We identified at position -226 to -212 of the iNOS promoter a sequence of 14 nucleotides identical to that present in the HRE, originally described in the 3' flanking region of the erythropoietin (Epo) gene, and comprising the consensus sequence for the binding of the hypoxia inducible factor-1 (HIF-1) transcription factor (Fig. 2 A) (38). To test whether

the picolinic acid-responsive element mapped in the region of homology with the HRE (referred to hereafter as iNOS-HRE) we transfected ANA-1 macrophages with two iNOS promoter-CAT reporter gene plasmids either deleted of 19 bp encompassing the putative binding site of HIF-1 (-227 to -209, plasmid p220) or mutated in two bases required for HIF-1 binding to DNA (-223 -222, plasmid p209). With either plasmid we observed a reduction of ~70% relative to the wild-type iNOS promoter (plasmid p1) in the expression of CAT reporter gene induced by IFN- $\gamma$  plus picolinic acid (Fig. 2 B). A reduction ranging from 65 to 80% was consistently observed in four independent experiments. The activation of iNOS promoter by treatment with picolinic acid alone was also decreased by 90% with both plasmids. In contrast, CAT activity induced by IFN- $\gamma$  plus LPS was not significantly affected. These data demonstrated that the iNOS-HRE is a functional regulatory element of the iNOS promoter required for the response to picolinic acid but not to LPS.

**iNOS-HRE Confers Picolinic Acid-inducibility in the Context of a Heterologous Promoter.** We then investigated the functional activity of the iNOS-HRE in ANA-1 macro-

A

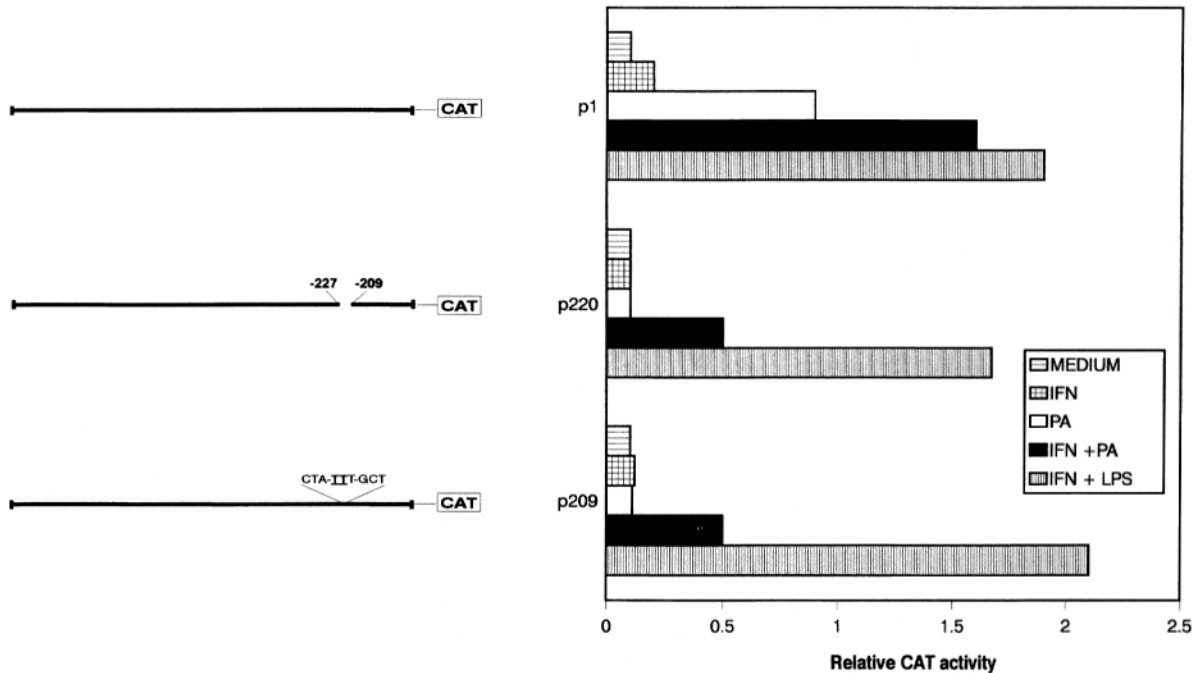
iNOS-HRE (-227 to -209)

5'-aCTACGTGCTGCCTaGggg-3'

Epo

5'-cCTACGTGCTGCCTcGcat-3'

B



**Figure 2.** A sequence homology to a hypoxia responsive element is required for induction of iNOS promoter activity by IFN- $\gamma$  plus PA. (A) The sequence homology between iNOS promoter and erythropoietin (*Epo*) gene is shown in uppercase. The putative binding site of HIF-1 is underlined. (B). ANA-1 macrophages were treated as described in Fig. 1. In plasmid p220, the sequence indicated in (A) was deleted. In plasmid p209 a 2-bp mutation (5'-TATTTGCT-3', underlined) was created at position -223 -222 internal to the putative binding site for HIF-1. Results are expressed as in Fig. 1 and are from one representative experiment of at least four performed.

phages and its responsiveness to picolinic acid alone. We subcloned three tandem copies of the iNOS-HRE (from -231 to -211), either wild type (pBL-iNOS.WT) or mutated at the putative site of HIF-1 binding (pBL-iNOS.Mu) (38) (Fig. 3 A), into the pBL-CAT2 plasmid that contains a HSV tk promoter linked to the CAT reporter gene. These constructs were transfected into ANA-1 macrophages to determine the enhancer activity of the iNOS-HRE on the expression of the CAT gene driven by the minimal tk promoter. The pBL-iNOS.WT construct was constitutively expressed at levels  $\sim 10$ -fold higher than the parental vector, and this value was taken as a reference to assess the effects of cell stimulation. Stimulation with picolinic acid alone caused a major increase in CAT activity of 4.7-fold over that of untreated cells, whereas IFN- $\gamma$  or LPS alone were not effective (Fig. 3 B). Similar results were obtained in five independent experiments. In contrast, the expression of the pBL-iNOS.Mu plasmid was decreased more than 10-fold, relative to that of the pBL-iNOS.WT in untreated cells, and was no longer inducible by picolinic acid.

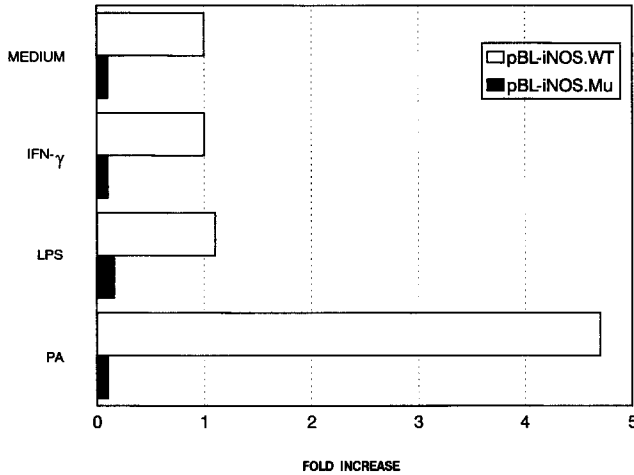
These results demonstrate that the iNOS-HRE is active in driving transcription of a heterologous promoter in murine macrophages and is sufficient to confer inducibility by picolinic acid.

*Picolinic Acid Induces DNA-binding Activity to the iNOS-HRE.* To establish whether picolinic acid induced DNA-binding activity to the iNOS-HRE, we performed EMSA with nuclear extracts prepared from ANA-1 macrophages either unstimulated or treated with picolinic acid alone and with a radiolabeled oligonucleotide of 19 bp (depicted in Table 1, AB.2) encompassing the iNOS-HRE. ANA-1 macrophages cultured in medium expressed a constitutive binding activity (Fig. 4). Treatment with picolinic acid increased the constitutive binding and caused the appearance of an inducible complex. Competition experiments demonstrated that the constitutive as well as the inducible binding complexes were specifically inhibited by excess unlabeled probe (AB.2), but not by an unrelated probe (AB.1). Because mutation of the HIF-1-binding site of the iNOS-HRE abolished its functional activity in transient transfection as-

A

pBL-iNOS.WT      5'-AGTGACT**ACGTGCTGCCTAGG**-3'  
 pBL-iNOS.Mu      5'-AGTGACTAAAAGCTGCCTAGG-3'

B



**Figure 3.** iNOS-HRE confers inducibility by picolinic acid in ANA-1 murine macrophages. (A) Sequence of the oligomers containing the iNOS-HRE from the mouse iNOS promoter that were subcloned in the pBLCAT2 vector as described in Materials and Methods. Bold type indicates the putative binding site of HIF-1, with the mutation underlined. (B) 24 h after the transfection with the indicated constructs, ANA-1 macrophages were treated with medium, IFN- $\gamma$  (100 U/ml), LPS (10 ng/ml), picolinic acid (4 mM) for additional 24 h and CAT activity was assayed. Results are presented as fold increase of CAT activity (% acetylation/luciferase) relative to that expressed by pBL-iNOS.WT in untreated cells (arbitrarily considered to be equal to 1) after normalization for the expression of the parental vector, and are from one representative experiment.

says (see Fig. 3 B), we tested whether the same mutation altered the ability of the probe to compete for the binding. As shown in Fig. 4, a probe mutated in three bases within the putative HIF-1-binding site (Table 1, Mu.AB2) did not compete for the picolinic acid-inducible binding complex, suggesting that the integrity of the HIF-1-binding site was required for the binding activity. In addition, the formation of the constitutive or picolinic acid-inducible binding complexes was competed for by an 18-bp probe containing the canonical HIF-1-binding site of Epo (Fig. 4), that differs from the AB.2 probe in five flanking bases (Table 1) (38). These results indicate that a specific DNA-binding factor, similar or closely related to HIF-1, is induced by picolinic acid in ANA-1 macrophages and binds to the iNOS-HRE.

*Hypoxia Activates iNOS-HRE and iNOS Promoter in Murine Macrophages.* Sequences similar to the iNOS-HRE drive

**Table 1.** Oligonucleotide Probe Used in EMSA

|                              |                                    |
|------------------------------|------------------------------------|
| AB.2 (iNOS,<br>-230 to -212) | 5'-GTGACT <b>ACGTGCTGCCTAG</b> -3' |
| Mu-AB.2                      | 5'-GTGACT <u>AAA</u> AGCTGCCTAG-3' |
| Epo                          | 5'-GCCCT <b>ACGTGCTGCCTCA</b> -3'  |
| AB.1                         | 5'-TGAGTCCCAGTTTTGAAGTG-3'         |

Bold characters indicate the consensus sequence for the binding of HIF-1; the mutated bases are underlined. Epo probe is from the 3' flanking region of the human Epo gene (from +3454 to +3471) (38). AB.1 is from an unrelated sequence of the iNOS promoter.

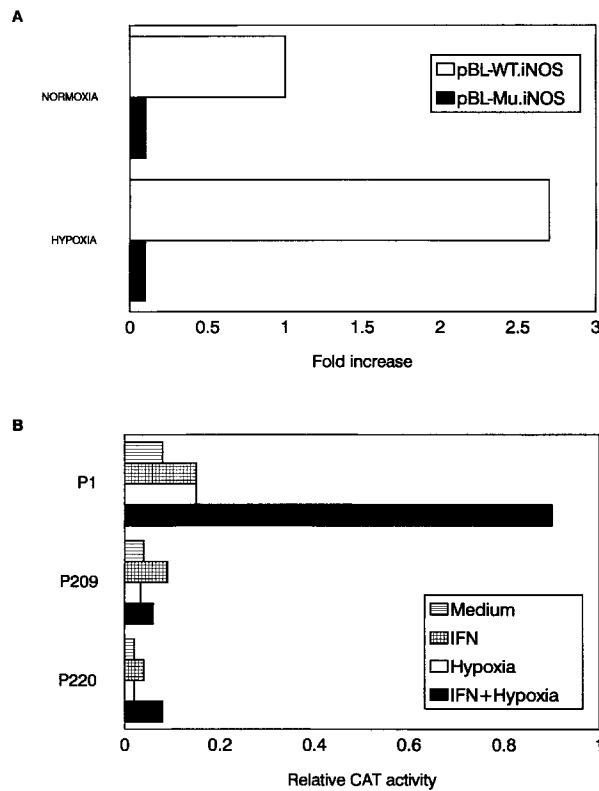


**Figure 4.** Picolinic acid induces a DNA-binding activity to the iNOS-HRE. Nuclear extracts from ANA-1 macrophages treated with medium or picolinic acid for 12 h were incubated with AB.2 probe, in the absence of competitor (0) or in the presence of 100-fold molar excess of the unlabeled AB.2, Mu-AB.2, AB.1, or Epo competitor oligonucleotides (described in Table 1) and analyzed by EMSA. Competitor DNAs were incubated with nuclear extracts for 5 min before addition of labeled probe. Binding activities are labeled as follows: C, constitutive; I, induced; Free, free labeled probe.

hypoxia-inducible transcriptional activation of reporter genes in other cell types (40, 41), raising the possibility that hypoxia was also a stimulus for iNOS-HRE activity in macrophages. We tested the response of the plasmids pBL-iNOS.WT and pBL-iNOS-Mu transfected in ANA-1 macrophages to normoxic (20% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions. We found that hypoxia induced the expression of pBL-iNOS.WT (2.7-fold), relative to control cells cultured under normoxic conditions (Fig. 5 A). In contrast, no induction of pBL-iNOS.Mu by hypoxia was observed. These data demonstrate that hypoxia is a stimulus for iNOS-HRE activity in murine macrophages. To establish whether hypoxia induced iNOS promoter activity, ANA-1 macrophages were transiently transfected with plasmid p1, containing the full-length 5'-flanking region of the iNOS gene. As shown in Fig. 5 B, hypoxia alone did not induce appreciable increase of CAT activity above the levels expressed by cells treated with IFN- $\gamma$  alone. However, a sig-

nificant induction of CAT expression was observed after treatment with IFN- $\gamma$  under hypoxic conditions, indicating that hypoxia is a costimulus for the induction of iNOS promoter activity. To investigate whether the iNOS-HRE was responsible for the activation of iNOS promoter by hypoxia, we tested the activity of plasmid p220 and plasmid p209, deleted or mutated in the iNOS-HRE, respectively. With both plasmids we observed a decrease of ~80% relative to the wild-type iNOS promoter in the induction of CAT activity following stimulation with IFN- $\gamma$  under hypoxic conditions (Fig. 5 B). These data establish that the iNOS-HRE is required for the induction of iNOS promoter activity by hypoxia.

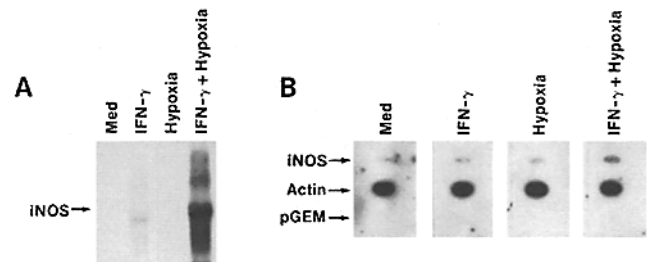
**Hypoxia Induces iNOS mRNA Expression and iNOS Transcription.** The inducibility of iNOS promoter by hypoxia raised the possibility that the expression of the endogenous iNOS gene was sensitive to a decrease in oxygen levels. To address this issue, ANA-1 macrophages were incubated under normoxic or hypoxic conditions, in medium alone or medium containing IFN- $\gamma$  and tested for iNOS mRNA expression (Fig. 6 A) and iNOS transcription (Fig. 6 B). The iNOS gene was not constitutively expressed either under normoxic or hypoxic conditions, and IFN- $\gamma$ , under normal oxygen tension, induced very low levels of iNOS mRNA expression. In contrast, under hypoxic conditions, IFN- $\gamma$  induced a major increase of iNOS mRNA expression and iNOS transcription, providing the first demonstration that iNOS is inducible by a decrease in oxygen tension.



**Figure 5.** Hypoxia induces iNOS-HRE and iNOS promoter activity. (A) 24 h after the transfection with the indicated constructs, described in the legend to Fig. 3 A, ANA-1 macrophages were cultured in medium either under normoxic (20% O<sub>2</sub>) or under hypoxic conditions (1% O<sub>2</sub>) for additional 24 h. Results are presented as fold increase of CAT activity (% acetylation/luciferase) relative to that expressed by cells cultured under normoxic conditions transfected with pBL-iNOS.WT (arbitrarily considered to be equal to 1) after normalization for the expression of the parental vector, and are from one representative experiment. (B) ANA-1 macrophages were transfected with the indicated constructs, described in the legend to Fig. 2. 24 h later the cells were treated with medium or IFN- $\gamma$  (100 U/ml) and cultured under either normoxic (20% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions, as described, for an additional 24 h. Results are expressed as in Fig. 1 and are from one representative experiment of at least three performed.

## Discussion

We have identified a novel regulatory element (iNOS-HRE, from -227 to -209) that is required to achieve full expression of iNOS promoter activity in response to IFN- $\gamma$



**Figure 6.** Hypoxia synergizes with IFN- $\gamma$  in the induction of iNOS mRNA expression and iNOS transcription. (A) Northern blot. ANA-1 macrophages were treated with medium or IFN- $\gamma$  (100 U/ml) and incubated for 18 h either in 20% O<sub>2</sub> or 1% O<sub>2</sub>, as described in Fig. 5. Total cellular RNA was examined for iNOS mRNA expression as described in Materials and Methods. Equal RNA loading was confirmed by ethidium bromide staining and monitoring of 28S and 18S rRNA (data not shown). Data shown are from one representative experiment of three performed. (B) Nuclear run-on. ANA-1 cells were treated as above and nuclei were isolated after 12 h. The rate of transcription of the iNOS gene was determined as described in Materials and Methods. The following plasmid DNA were used: pINOS, pGEM vector containing a 3.9-kb cDNA insert of mouse macrophage NOS; p-actin, vector containing a chicken  $\beta$ -actin cDNA insert; pGEM, vector lacking any cDNA insert. Data shown are from one representative experiment.

plus picolinic acid or IFN- $\gamma$  plus hypoxia. The iNOS-HRE is sufficient to drive the activation of a heterologous promoter following stimulation with picolinic acid or hypoxia alone. These data establish that a sequence homologous to a hypoxia-inducible enhancer regulates the iNOS promoter activity in murine macrophages and provide the first evidence of the existence of a regulatory element responsive to picolinic acid and of an inverse relationship between oxygen levels and iNOS expression.

We have previously reported that the simultaneous stimulation of ANA-1 macrophages with IFN- $\gamma$  plus picolinic acid caused a major induction of iNOS transcription and iNOS mRNA expression (31). These data are in agreement with the results of the transient transfection assays demonstrating that picolinic acid acted synergistically with IFN- $\gamma$  in inducing the expression of the full-length iNOS promoter. Deletion of the iNOS promoter fragment containing an IFN- $\gamma$ -responsive element abolished the cooperative interaction between IFN- $\gamma$  and picolinic acid in the activation of iNOS promoter, which is in agreement with the requirement for IFN- $\gamma$  to achieve maximal induction of iNOS transcription (5, 7, 8). Although picolinic acid alone did not induce detectable iNOS transcription, as assessed by nuclear run-on analysis (31), we found that picolinic acid alone induced low but consistent levels of expression of the iNOS promoter in transient transfection assays. This discrepancy might be explained by negative regulatory regions that control the expression of the endogenous iNOS gene or by differences in the sensitivity of the assays. A discrepancy between the extent of iNOS mRNA expression and the induction of transfected iNOS promoter-luciferase constructs by LPS was also observed in the RAW 264.7 macrophage cell line (8).

Deletion and site-directed mutagenesis experiments demonstrated that a 19 nt element containing a sequence homologous to a HRE was required for the transcriptional activation of the iNOS promoter by IFN- $\gamma$  plus picolinic acid. The HRE, originally identified in the 50 nt enhancer element of the 3' flanking region of the Epo gene (38), is highly conserved between human and mouse (42) and mediates hypoxia-inducible gene transcription in Epo producing cells (38). The hypoxia-inducible enhancer activity is also present in non-Epo producing cell lines, suggesting a general involvement of the HRE in the hypoxia-inducible gene expression (40, 41). The homology between the iNOS promoter and the HRE comprised the consensus sequence (5'-TACGTGCT-3') for the binding of a novel *trans*-activating factor (HIF-1) induced by stimulation of cells in low oxygen tension (38, 43). Deletion or mutation of the putative binding site of HIF-1 present in the iNOS-HRE consistently decreased CAT activity induced by picolinic acid in IFN- $\gamma$ -treated ANA-1 macrophages, demonstrating the functional role of the iNOS-HRE in the activation of iNOS promoter. Activation of the iNOS-HRE by picolinic acid was associated with a specific DNA-binding activity. Two different complexes were observed in ANA-1 macrophages: the first, constitutively present, was almost completely competed by excess cold probe but not by unrelated oligonu-

cleotide, suggesting that it was largely specific; the second complex was inducible by picolinic acid and was inhibited by excess specific probe. The binding pattern that we have observed in murine macrophages is strikingly similar to that reported in other cell types for HIF-1 transcription factor. HIF-1 binding activity has been originally described in nuclear extracts from hypoxia-treated Hep3B cells using as probe an 18-bp oligomer of the HRE present in the Epo gene (38, 43). Our results strongly suggest that HIF-1 or a closely related nuclear factor binds to the HIF-1 consensus sequence of the iNOS promoter. However, the biochemical characterization of the nuclear factor induced by picolinic acid in ANA-1 macrophages requires additional studies.

The presence of a functional HRE in the iNOS promoter suggested that hypoxia was a stimulus for its transcriptional activation. Indeed, we found that hypoxia consistently induced iNOS promoter activity in cooperation with IFN- $\gamma$ . The iNOS-HRE was the target of hypoxia because deletion or mutation of the iNOS-HRE almost completely abrogated the cooperative interaction between hypoxia and IFN- $\gamma$ . These data provided the first indication that iNOS could be a hypoxia-responsive gene. This hypothesis was supported by the finding that hypoxia caused a major increase of iNOS transcription and mRNA expression in IFN- $\gamma$ -treated ANA-1 macrophages. So far, hypoxia responsive sequences have been demonstrated to play a functional role only in the hypoxia-inducible transcriptional activation of genes encoding Epo (38, 42, 44) and glycolytic enzymes (45, 46), whose functions are to counteract the adverse effects of decreased oxygen levels. Our data demonstrating that a hypoxia-inducible enhancer controls iNOS transcriptional activity suggest that iNOS might be the prototype of a different class of oxygen-sensitive genes.

The studies of the functional activity of the iNOS-HRE in the context of a heterologous promoter established that this sequence responded to picolinic acid or to hypoxia alone. Three copies of the wild-type iNOS-HRE inserted 5' to a tk promoter-CAT reporter gene mediated picolinic acid- or hypoxia- but not IFN- $\gamma$ - or LPS-inducibility in ANA-1 macrophages, indicating that either picolinic acid or hypoxia can independently activate the iNOS-HRE. These results are consistent with the possibility that the synergistic activation of the iNOS promoter by IFN- $\gamma$  and picolinic acid or hypoxia may be achieved through stimulation of interferon regulatory factor binding site (IRF-E) and iNOS-HRE, respectively, suggesting a cooperative interaction between hypoxia- and IFN- $\gamma$ -regulated sequences. A three-bp mutation of the HIF-1-binding site abolished the constitutive activity of the iNOS-HRE and eliminated the picolinic acid- or the hypoxia-inducibility, indicating that the integrity of the consensus sequence for the binding of HIF-1 was required for the functional activity of the iNOS-HRE. In agreement with these results, the same mutation was shown to inhibit the HIF-1 binding and eliminate the hypoxia-inducible enhancer activity of the element present in the Epo gene (38, 41, 43).



Two regulatory sequences have been identified so far in the 5'-flanking region of the iNOS gene and they are involved in the activation of iNOS transcription induced by IFN- $\gamma$  plus LPS. An upstream region (from -923 to -913), containing an IRF-E, binds IRF-1 and is required for the synergistic enhancement of iNOS expression by IFN- $\gamma$ , as demonstrated in vivo in IRF-1 knock-out mice (9), and in vitro by functional studies with the murine macrophage-like cell line RAW 264.7 (10). A downstream region containing a NF- $\kappa$ B consensus sequence (at position -85 to -76) is necessary for LPS inducibility of the iNOS promoter (11). The pathway of iNOS transcriptional activation induced by IFN- $\gamma$  plus LPS is particularly relevant for NO production during the course of microbial infections. However, the pleiotropic expression of iNOS and the complex structure of its promoter suggest that different signals and multiple regulatory elements control iNOS transcription. The finding that picolinic acid or hypoxia induce iNOS transcription through a novel regulatory sequence provides evidence of the existence of a distinct pathway of iNOS transcriptional activation that may be important dur-

ing a microbial-independent activation of the immune response. L-tryptophan catabolites have been detected in vivo in patients treated with IFN- $\gamma$  (15) or IL-2 (21) or during the rejection of transplanted tumors (47, 48), and in localized compartments, such as the brain, during inflammatory conditions (19, 20). However the presence of picolinic acid in pathological conditions or during therapy with cytokines remains to be established.

The demonstration that iNOS responds to low oxygen tension is important in macrophage biology because macrophages can be exposed to broad variations of oxygen tension ranging from ambient in the lung to hypoxic in inflammatory tissues. Hypoxia may occur in acute and chronic inflammation (49-51), in the rejection of transplanted organs (52-54) and in necrotic areas of neoplastic lesions (55, 56) where the involvement of NO has been demonstrated. The requirement for IFN- $\gamma$  costimulation in iNOS induction by hypoxia may be important to prevent iNOS expression in noninflammatory hypoxic conditions, such as anemia.

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