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Regulation of Nitric-oxide Synthase mRNA Expression by Interferon-γ and Picolinic Acid*

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Giovanni Melillo‡§, George W. Cox‡, Arya Biragyn¶, Lynn A. Sheffler‡, and Luigi Varesio‡

From the ‡Macrophage Cell Biology Section, Laboratory of Experimental Immunology, and the ¶Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland 21702-1201

Picolinic acid, a catabolite of L-tryptophan, is a potent co-stimulatory agent for the induction of tumoricidal activity and the production of L-arginine-dependent reactive nitrogen intermediates (RNI) in murine macrophages. We studied whether picolinic acid could affect nitric-oxide synthase (NOS) expression at the gene level in the macrophage cell line ANA-1. NOS mRNA was neither constitutively expressed nor induced by treatment with picolinic acid alone. However, low levels of NOS mRNA were induced by interferon (IFN)-γ alone. In contrast, a major increase of NOS mRNA expression was observed after treatment with IFN-γ plus picolinic acid. The synergism was already detectable after 5–6 h and increased up to 20 h of treatment. The ability of picolinic acid to augment IFN-γ-dependent NOS mRNA expression was associated with a parallel increase in transcription, as demonstrated by nuclear run-on experiments. Protein synthesis was required for the induction of NOS mRNA because addition of cycloheximide dramatically reduced IFN-γ plus picolinic acid-induced NOS mRNA expression. Finally, interleukin-4 significantly decreased IFN-γ plus picolinic acid-induced NOS mRNA expression and NOS transcription. These data provide evidence of a molecular event connecting arginine and tryptophan metabolic pathways in the generation of RNI, and they indicate that picolinic acid can induce transcriptional activation of gene expression.

Nitric oxide (·NO) has been identified as a major effector molecule, involved in the expression of tumoricidal and microbicidal activities exerted by activated murine macrophages (1). The production of ·NO, from the L-arginine metabolic pathway, is controlled in murine macrophages by an inducible enzyme, nitric-oxide synthase (NOS).[1] At least three different forms of NOS have been isolated, characterized, and cloned from brain tissue, endothelial cells, and macrophages (2–8) and, comparing across species, they have approximately 50–60% homology at the amino acid level (2–8). NOS is constitutively expressed in endothelial cells and in the brain, and its activity is dependent on exogenous Ca^{2+} and calmodulin. In contrast, the macrophage NOS is independent of exogenous Ca^{2+} and calmodulin and is inducible by IFN-γ alone or IFN-γ in combination with several agents, including LPS (9), TNF (10), and IL-2 (11). Although much information has been accumulated regarding the role of NOS in the production of ·NO by activated murine macrophages, the molecular mechanism(s) underlying this process are still poorly understood.

The metabolism of L-tryptophan is also associated with the expression of effector functions by mononuclear phagocytes (12, 13). The catabolic pathway of L-tryptophan is controlled in monocytes/macrophages by indoleamine 2,3-dioxygenase, whose activity is induced by IFN-γ (14–16). The degradation of L-tryptophan has been implicated in some of the biological effects of IFN-γ (17, 18), and it has been demonstrated, *in vitro* and *in vivo*, in mice and humans (14–16, 19). Tryptophan metabolites were found in mice treated with LPS (19) and patients treated with IFN-γ (16). Picolinic acid, a terminal metabolite of L-tryptophan, is a potent co-stimulatory agent for the induction of tumoricidal activity in mouse peritoneal macrophages (20) as well as in the mouse macrophage cell lines ANA-1 and GG2EE (21, 22), the latter derived from the LPS-hyporesponsive C3H/HeJ strain of mouse. Picolinic acid has been detected in body fluids (23, 24), and its ability to activate macrophages *in vivo* has been reported in mice (25–27). However, the type(s) of cell that produces it has not been defined yet. We recently demonstrated that picolinic acid is a co-stimulus for the induction of L-arginine-dependent RNI production in murine macrophages (28). These data provided the first evidence of a possible connection between tryptophan and arginine metabolism and suggested that amino acid catabolites may be important for the activation and expression of effector functions by murine macrophages. However, little is known about the mechanism(s) by which tryptophan degradation products interact with the ·NO synthesis pathway during the process of murine macrophage activation.

To investigate the molecular basis of picolinic acid-dependent induction of RNI production, we studied the expression of NOS mRNA in the macrophage cell line ANA-1. We report now that IFN-γ plus picolinic acid synergistically induced the expression of NOS mRNA. The accumulation of NOS mRNA was dependent upon *de novo* protein synthesis and was associated with an increase in the rate of transcription of the NOS gene. Furthermore, IL-4 significantly decreased IFN-γ- plus picolinic acid-induced NOS transcription and mRNA expression. These data indicate that the interaction between arginine and tryptophan metabolic pathways in the generation of RNI can be explained by induction of NOS mRNA expression, and they provide the first evidence that picolinic acid can induce transcriptional activation of gene expression.

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§ To whom correspondence and reprint requests should be addressed: Laboratory of Experimental Immunology, BRMP, Bldg. 560, Rm. 31–52, NCI-FCRDC, NIH, Frederick, MD 21702-1201. Tel.: 301-846-1547; Fax: 301-846-1673.

[1] The abbreviations used are: NOS, nitric-oxide synthase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; RNI, reactive nitrogen intermediate; CHX, cycloheximide.

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 (29). ANA-1 macrophages were cultured in Dulbecco's modified Eagle's medium (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) (complete medium). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 in air. The content of endotoxin, as determined by assay with a chromogenic *Limulus* amebocyte lysate test (QCL-1000, Bio-Whittaker, Walkersville, MD), was below the detection limit of 6 pg/ml in all the reagents used.

Reagents—Mouse rIFN-γ (specific activity ≥ 10^7 units/mg) was purchased from Amgen Biologicals. Mouse rIL-4 was a gift from Immunex Corp. (Seattle, WA). LPS (from *Escherichia coli* serotype 0111:B4) and actinomycin D were purchased from Sigma. Picolinic acid was purchased from Sigma (purity approximately 99%). During the course of the experiments, several batches of picolinic acid were used and all of them gave consistent and reproducible results. The biological activity of picolinic acid was fully dialyzable, and we routinely tested for potential endotoxin contamination. The content of endotoxin, as determined by assay with a chromogenic *Limulus* amebocyte lysate test (Bio-Whittaker), was below the detection limit of 6 pg/ml in all the preparations used. Picolinic acid was dissolved in Hanks' balanced salt solution, and the pH was adjusted to 7.4. The stock solution was then passed through a 0.2-μm filter, aliquoted, and stored at −20 °C.

Northern Blot Analysis—Cells were solubilized with guanidine isothiocyanate, and the total cellular RNA was purified by centrifugation through a cushion of cesium chloride according to the method of Chirgwin *et al.* (30). In some experiments, phenol-chloroform extraction was used to purify total RNA. Twenty μg of RNA were size-fractioned in a 1.2% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the gel was rinsed with water, treated briefly with 50 mM NaOH, and blotted onto a Nytran membrane (Schleicher & Schuell). The RNA were cross-linked to the membrane by using ultraviolet irradiation and incubated overnight at 42 °C in Hybrisol I hybridization solution (Oncor, Gaithersburg, MD). The cDNA probes that were specific for mouse macrophage NOS (7) or for human glyceraldehyde-3-phosphate dehydrogenase (CLONTECH Laboratories Inc.) were radiolabeled with [^{32}P]dCTP (Amersham Corp.) by using a random priming (31) kit (Boehringer Mannheim). The blot was hybridized individually with the radiolabeled probes (1 to 2 × 10^6 cpm/ml) during an overnight incubation at 42 °C in Hybrisol I (Oncor). After hybridization, the blot was washed three times in 2 × SSC/0.1% SDS for 5 min at room temperature and then washed twice in 0.1 × SSC/0.1% SDS for 15 min at 60 °C. The blot was autoradiographed at −70 °C on XAR-5 film (Eastman Kodak) with the use of Lightning Plus intensifying screens (DuPont NEN). For total cellular RNA extraction, 1 × 10^6 cells/ml were cultured in 100-mm tissue culture plates and treated with appropriate reagents as detailed under "Results."

Nuclear Run-on Experiments—Nuclear run-on assays were performed as described (32), with modification. Briefly, nuclei were isolated from 2.5 × 10^7 ANA-1 cells treated as indicated for 5 h. Cells were lysed in 10 mM Tris-HCl, pH 7.4, buffer containing 0.5% Nonidet P-40, 2 mM $MgCl_2$, 3 mM $CaCl_2$ for 5 min on ice. Nuclei were pelleted by centrifugation (700 × *g*, 5 min) and resuspended in lysis buffer without Nonidet P-40. The mixture was centrifuged as described above, and nuclei were resuspended in 100 μl of freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and stored in liquid nitrogen.

Nuclear run-on assays were performed for 30 min at 30 °C. The thawed nuclei were mixed with 100 μl of reaction buffer (10 mM Tris-HCl, pH 8.0, 260 mM KCl, 5 mM dithiothreitol, 0.2 mM EDTA, 5 mM $MgCl_2$, and 0.8 mM concentration each of ATP, GTP, and CTP) and 150 μCi of [^{32}P]UTP (800 Ci/mmol; Amersham Corp.). The nuclei were lysed in 400 μl of 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol, mixed with 85 μl of 2 M NaAc, pH 4.0, and extracted with 400 μl of water-saturated phenol and 80 μl of chloroform. RNAs were precipitated twice with an equal volume of isopropyl alcohol and resuspended in 200 μl of ETS buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS), cooled on ice for 5 min, and mixed with 1 N NaOH for 10 min. The reaction was stopped by mixing with 100 μl of acidic HEPES and 35 μl of NaAc. RNAs were precipitated with 3 volumes of 96% ethanol and resuspended in 200 μl of ETS buffer. About 10^6 cpm of hot RNAs were used in hybridization for 48 h with 5 μg each of slot-blotted denaturated plasmid DNA (pGEM vector alone; vector containing a 3.9-kilobase cDNA insert of mouse macrophage NOS (7); and vector containing a mouse β-actin cDNA insert). Filters were washed twice at room temperature for 30 min in 2 × SSC/0.1% SDS and 3 times at 56 °C for 30 min each in 0.1 × SSC/0.1% SDS. Filters were then autoradiographed at −70 °C on XAR-5 film (Eastman Kodak) with the use of Lightning Plus intensifying screens (DuPont NEN). Filters were scanned using a PhosphorImager (Ambis Systems Inc.), and data were normalized for the content of β-actin present in each sample.

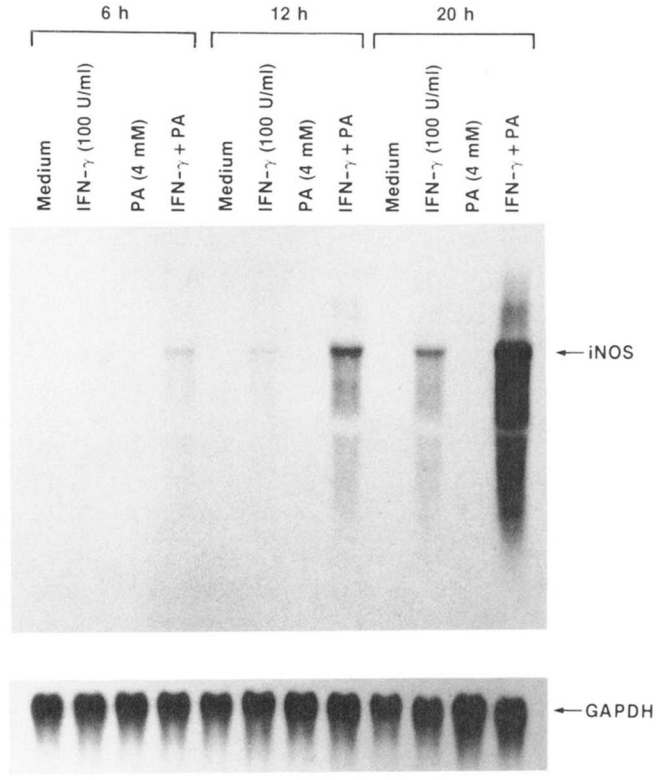

FIG. 1. **Picolinic acid synergizes with IFN-γ for induction of NOS mRNA expression in ANA-1 macrophages.** ANA-1 macrophages (1 × 10^6 cells/ml) were incubated with complete medium, 100 units/ml IFN-γ, 4 mM picolinic acid (*PA*), or a combination of these reagents for the indicated times. Total cellular RNA was examined for NOS mRNA expression as described under "Materials and Methods." Data shown are from one representative experiment of four performed. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Picolinic Acid and IFN-γ Synergistically Induce NOS mRNA Expression in ANA-1 Macrophages—We investigated the ability of picolinic acid to influence the expression of NOS mRNA. ANA-1 Mϕ were incubated with medium, IFN-γ (100 units/ml), and picolinic acid (4 mM) alone or in combination with IFN-γ, and total RNA was harvested after 6, 12, and 20 h. 4 mM picolinic acid was optimal for the synergistic interaction with IFN-γ in the induction of cytotoxic activity and RNI production. ANA-1 Mϕ did not express NOS mRNA either constitutively or after treatment with picolinic acid alone. However, low levels of NOS mRNA were detectable following stimulation of Mϕ with IFN-γ alone (Fig. 1); the IFN-γ-dependent induction was barely evident after 6 h and increased after 18 h of treatment. In contrast, addition of picolinic acid caused a major increase of IFN-γ-induced NOS mRNA expression that was already detectable after 5–6 h of treatment. After 12 h of incubation, picolinic acid caused a 5-fold augmentation of NOS mRNA expression over that induced by IFN-γ alone, and, after 20 h, the increase was 10–15-fold greater. The extent of picolinic acid-dependent NOS mRNA induction was consistently observed in four independent experiments performed.

To determine the dose dependence of picolinic acid effects, ANA-1 Mϕ were incubated with increasing concentrations of

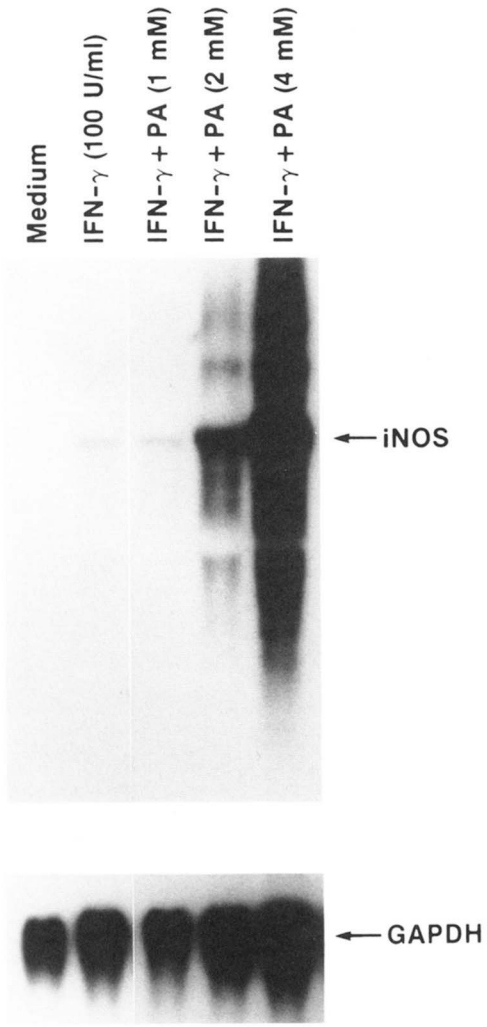

FIG. 2. **Dose-dependent induction of NOS mRNA expression by IFN-γ plus picolinic acid.** ANA-1 macrophages (1×10^6 cells/ml) were incubated with complete medium, IFN-γ (100 units/ml) alone or in combination with the indicated concentrations of picolinic acid for 18 h, and total cellular RNA was examined for NOS mRNA expression as described. Data are from one of three similar experiments.

picolinic acid in the presence or absence of IFN-γ (100 units/ml) for 18 h. Picolinic acid alone, at doses ranging from 0.5 mM to 4 mM, failed to induce NOS mRNA (data not shown). The combination of IFN-γ and picolinic acid increased the expression of NOS mRNA (4–5-fold greater than IFN-γ alone) at a dose of 2 mM picolinic acid; the synergistic interaction between IFN-γ and picolinic acid in the induction of NOS mRNA reached its maximum at a dose of 4 mM picolinic acid (Fig. 2). Results from experiments in which ANA-1 Mϕ were preincubated with picolinic acid (4 mM) for either 4 or 12 h and then treated with IFN-γ (100 units/ml) for an additional 6 h indicated that the pretreatment with picolinic acid did not induce higher levels of NOS mRNA expression than those observed when IFN-γ and picolinic acid were added simultaneously (data not shown). Furthermore, the levels of NOS, as measured by NO_2^- production, induced by sequential treatment with picolinic acid followed by IFN-γ (3.5 μM) were similar to those induced by IFN-γ alone (4.5 μM) and negligible in cells treated with picolinic acid alone. Lack of augmenting activity by sequential treatment was also observed when ANA-1 Mϕ were exposed to IFN-γ followed by picolinic acid (data not shown). These data demonstrate that picolinic acid acted synergistically with IFN-γ in a dose-dependent fashion for the induction of NOS mRNA expression in murine macrophages.

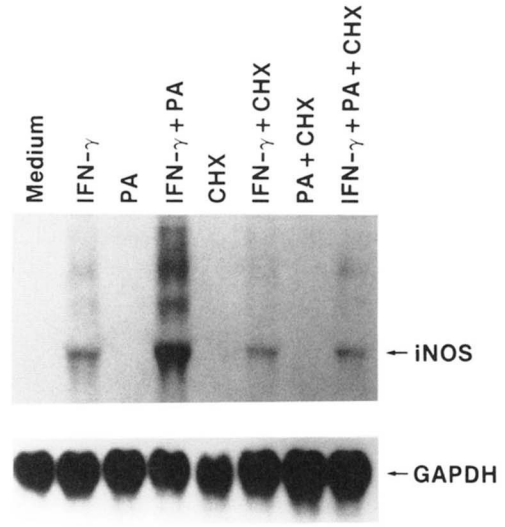

FIG. 3. **Protein synthesis is required for IFN-γ plus picolinic acid induction of NOS mRNA expression.** ANA-1 macrophages (1×10^6 cells/ml) were incubated with medium, IFN-γ (100 units/ml), picolinic acid (4 mM) alone or in combination with IFN-γ in the absence or presence of 7.5 μg/ml CHX. Total cellular RNA was harvested 5 h later and examined for NOS mRNA expression as described. Data are from one of two similar experiments.

Protein Synthesis Is Required for NOS mRNA Induction by IFN-γ Plus Picolinic Acid—To establish whether picolinic acid-induced NOS mRNA expression was dependent upon *de novo* protein synthesis, ANA-1 Mϕ were incubated with medium, IFN-γ, picolinic acid alone, or in combination with IFN-γ, in the presence or absence of CHX (7.5 μg/ml) for 5 h. Under our experimental conditions, CHX inhibited protein synthesis by more than 90%. ANA-1 Mϕ treated with either medium or picolinic acid alone did not express NOS mRNA in the absence or presence of CHX (Fig. 3). Moreover, the addition of CHX did not significantly affect induction of NOS mRNA by IFN-γ alone. In contrast, the induction by IFN-γ plus picolinic acid was almost completely abolished by CHX. We conclude that *de novo* protein synthesis was required for NOS mRNA induction by IFN-γ plus picolinic acid.

IFN-γ Plus Picolinic Acid Induces Transcriptional Activation of Nitric-oxide Synthase—To investigate the mechanism(s) by which picolinic acid increased NOS mRNA expression, nuclear run-on experiments were performed. ANA-1 Mϕ were incubated with medium, IFN-γ, picolinic acid, or IFN-γ plus picolinic acid, and the nuclei were isolated after 5 h of treatment. As shown in Fig. 4, the NOS gene was neither transcribed constitutively nor after treatment with picolinic acid. IFN-γ alone induced very low levels of transcription at the limit of detection after 5 h. In contrast, the combination of picolinic acid plus IFN-γ caused a significant increase in the rate of transcription of the NOS gene. A similar pattern of results was observed when the cells were treated with IFN-γ alone or in combination with picolinic acid for 16 h (data not shown).

Next, experiments were performed to investigate whether picolinic acid influenced the stability of NOS mRNA. Inasmuch as NOS mRNA is not constitutively present in ANA-1 Mϕ and it is marginally expressed in IFN-γ-treated cells, we compared the half-life of IFN-γ plus picolinic acid-induced NOS mRNA with that of cells treated with IFN-γ plus LPS (10 ng/ml), in order to utilize conditions inducing similar levels of NOS mRNA (33). ANA-1 macrophages were treated for either 12 or 18 h before addition of actinomycin D (5 μg/ml), and total RNA was harvested at different time points. The half-life of NOS mRNA in ANA-1 Mϕ treated with IFN-γ plus picolinic acid was approximately 3.5–4.0 h, and it was not significantly different

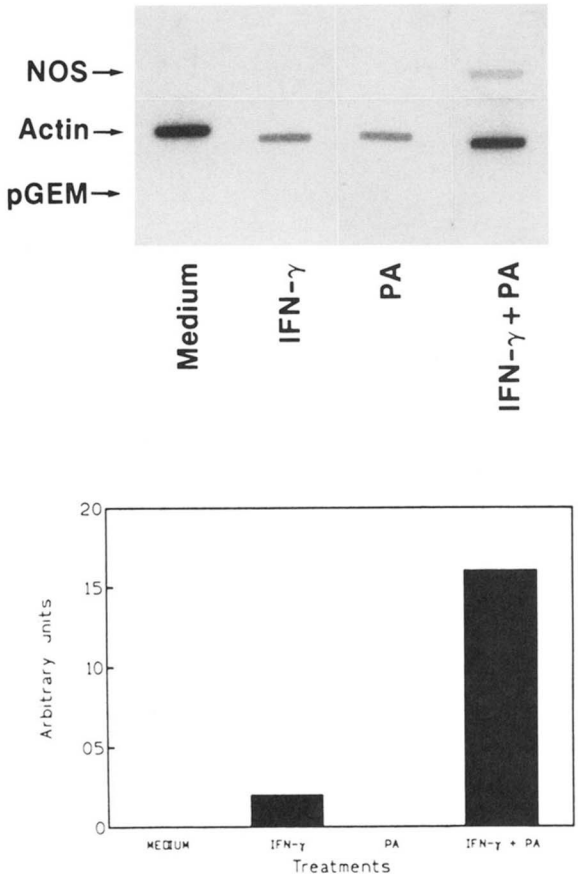

FIG. 4. **Effect of picolinic acid plus IFN-γ on NOS gene transcription.** ANA-1 macrophages (2.5×10^7) were treated with medium alone, IFN-γ (100 units/ml), picolinic acid (4 mM) alone or in combination with IFN-γ. Nuclei were isolated after 5 h, and the rate of transcription of the NOS gene was then determined by nuclear run-on analysis as described under "Materials and Methods." pGEM denotes pGEM vector lacking any cDNA insert. Results of scanning PhosphorImager analysis of the filters are presented as a bar graph. The relative level of *de novo* transcription for each sample was determined after normalization to the respective amount of β-actin. Data presented are from one of four similar experiments.

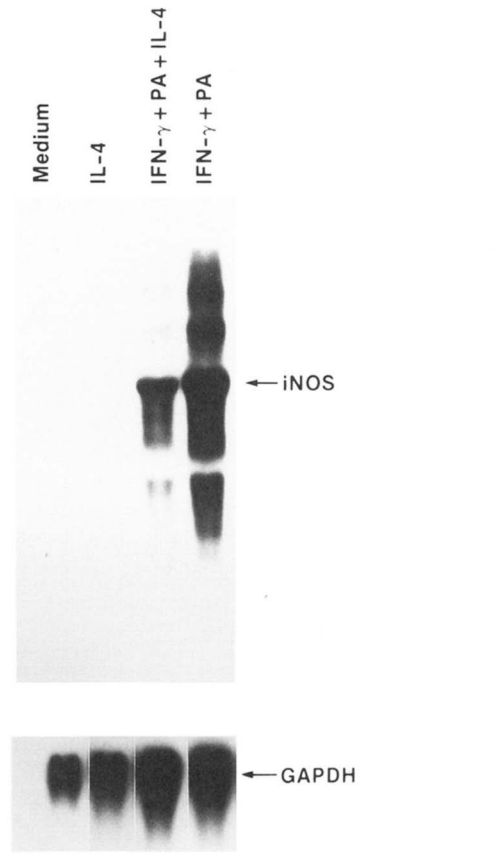

FIG. 5. **IL-4 inhibits NOS mRNA expression induced by IFN-γ plus picolinic acid.** ANA-1 macrophages (1×10^6) were stimulated with medium, IL-4 (400 units/ml), or IFN-γ (100 units/ml) plus picolinic acid (4 mM) in the absence or presence of IL-4. Total cellular RNA was isolated after 18 h and examined for NOS mRNA expression as described. Data shown are from one representative experiment of three performed.

from that of IFN-γ- plus LPS-treated cells (data not shown). Overall, these data suggest that the major mechanism by which picolinic acid augments NOS mRNA expression is the induction of *de novo* transcription of the NOS gene.

IL-4 Decreases NOS mRNA Expression Induced by IFN-γ Plus Picolinic Acid—IL-4 inhibits the co-stimulatory activity of picolinic acid in the induction of RNI production in ANA-1 macrophages (28). Therefore, we examined whether IL-4 influenced IFN-γ plus picolinic acid-induced NOS mRNA expression. ANA-1 macrophages were treated with medium, IFN-γ, picolinic acid, or IFN-γ plus picolinic acid in the presence or absence of IL-4 (400 units/ml), and total RNA was harvested after 18 h. IL-4 did not induce NOS mRNA either by itself or with picolinic acid alone and almost completely suppressed IFN-γ-induced NOS mRNA expression (data not shown). Furthermore, IL-4 caused a major decrease of NOS mRNA induced by IFN-γ plus picolinic acid (Fig. 5). The inhibitory effect of IL-4 was observed in three independent experiments, and the extent of the decrease was ≥50%. Nuclear run-on experiments were performed to establish whether IL-4 inhibited the transcription of the NOS gene. ANA-1 macrophages were incubated with medium, IFN-γ alone or in combination with picolinic acid, in the presence or absence of IL-4, and the nuclei were isolated after 5 h. No basal transcription was observed in the presence of IL-4. However, IL-4 significantly reduced the rate of transcription induced by IFN-γ plus picolinic acid (Fig. 6). IL-4 consistently inhibited the rate of transcription by approximately ≥50% in three independent experiments. To establish whether IL-4 acted also at the post-transcriptional level, ANA-1 Mϕ were treated with IFN-γ plus picolinic acid in the presence or absence of IL-4 for 18 h. Then, actinomycin D (5 μg/ml) was added to the culture, total RNA was harvested at different time points, and the half-life of the NOS mRNA was estimated. We did not observe any significant difference in the half-life of NOS mRNA, regardless of the presence or absence of IL-4 (Fig. 7). Therefore, these data indicate that IL-4 exerts its inhibitory effects on IFN-γ plus picolinic acid-induced NOS mRNA expression at the transcriptional level.

DISCUSSION

We have studied the ability of picolinic acid to influence the expression of macrophage NOS mRNA. We demonstrate that picolinic acid, although ineffective by itself, acted synergistically with IFN-γ in the transcriptional activation of the NOS gene. Furthermore, we show that IL-4 exerted a negative regulation of IFN-γ- plus picolinic acid-induced NOS mRNA expression by inhibiting the transcription of the gene. These results provide evidence of the molecular basis of the synergistic interaction between IFN-γ and picolinic acid in the generation of RNI production, and they suggest that amino acid catabolites may play a role in the transcriptional control of gene expression in murine macrophages.

Murine macrophages respond to IFN-γ with low levels of NO_2^- production (34). These data are consistent with our ob-

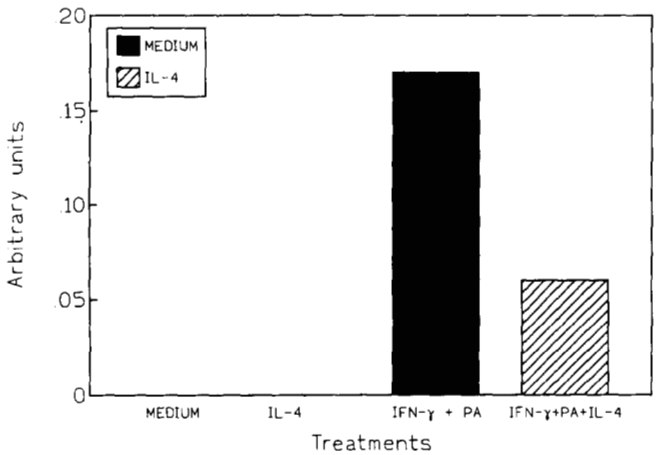

FIG. 6. **Effect of IL-4 on NOS gene transcription.** ANA-1 macrophages were treated with medium alone, IL-4 (400 units/ml), or IFN-γ (100 units/ml) plus picolinic acid (4 mM) in the absence or presence of IL-4. Nuclei were isolated after 5 h, and the rate of transcription of the NOS gene was then determined by nuclear run-on analysis as described. Results are presented as a bar graph after scanning PhosphorImager analysis of the filters performed as described under "Materials and Methods." The relative level of *de novo* transcription for each sample was determined after normalization to the respective amount of β-actin. Data shown are the average of three independent experiments.

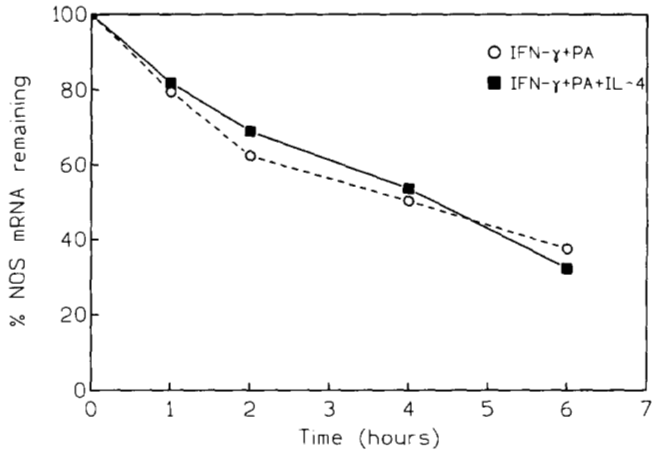

FIG. 7. **Effects of IL-4 on the NOS mRNA stability.** ANA-1 macrophages were treated with IFN-γ (100 units/ml) plus picolinic acid (4 mM) in the absence or presence of IL-4 (400 units/ml) for 18 h. Then, actinomycin D (5 μg/ml) was added to the culture, and total RNA was harvested after 1, 2, 4, and 6 h, and Northern blot analysis was performed. Data are presented as the relative amount of NOS mRNA remaining after addition of actinomycin D and normalization to the respective amount of glyceraldehyde-3-phosphate dehydrogenase. Scanning PhosphorImager analysis of the filters was performed as described under "Materials and Methods."

servation that incubation with IFN-γ alone induced, although at a very low level, the expression of NOS mRNA which was not constitutively present in ANA-1 Mϕ. ANA-1 Mϕ behave like murine peritoneal macrophages in terms of RNI production, and this is further supported by the evidence that IFN-γ alone induced NOS mRNA expression in thioglycollate-elicited peritoneal macrophages from C57BL/6 mice (data not shown), to a degree similar to that observed in ANA-1 macrophages.

Picolinic acid can influence gene expression in murine macrophages either by exerting inhibitory effects on 28 S rRNA accumulation (20) and retroviral mRNA expression (22) or by providing a positive signal for augmentation of IFN-γ-dependent TNF-α mRNA expression (28). We report now that picolinic acid caused a major increase of IFN-γ-dependent NOS mRNA expression. The synergistic interaction between IFN-γ and picolinic acid was an early event, as demonstrated by a significant accumulation of NOS mRNA after 5–6 h, and the effect was sustained for at least 20 h, the latest time point that we have studied. The magnitude of picolinic acid-dependent NOS mRNA increase, over that induced by IFN-γ alone, ranged from 2–3-fold after 6 h up to 10–15-fold after 20 h. This effect was not limited to the ANA-1 macrophages, in that a similar induction of NOS mRNA expression by IFN-γ plus picolinic acid was observed in thioglycollate-elicited peritoneal macrophages from C57BL/6 mice (data not shown). The increase of NOS mRNA was dose-dependent, with 3–4 mM picolinic acid being the most effective doses. These data are consistent with our previous observation that the co-stimulatory effects of picolinic acid *in vitro* required concentrations of about 3 to 4 mM. The augmented expression of NOS mRNA by IFN-γ alone or IFN-γ plus picolinic acid was associated with a parallel induction of NOS enzymatic activity in ANA-1 Mϕ, as measured by accumulation of NO_2^- in culture supernatants as previously reported (28). Nuclear run-on experiments demonstrated that picolinic acid in combination with IFN-γ increased the rate of transcription of the NOS gene, providing the first evidence that picolinic acid can exert transcriptional control of gene expression. Activation of NOS gene expression by IFN-γ plus LPS in the mouse macrophage cell line RAW 264.7 also occurred at the transcriptional level (6, 33), and we confirmed in ANA-1 macrophages that LPS either alone or in combination with IFN-γ is a potent activator of NOS gene transcription.[2] Other mechanism(s) might have been involved in the induction of NOS mRNA expression by IFN-γ plus picolinic acid. However, we were not able to demonstrate any post-transcriptional effect of picolinic acid on NOS mRNA stability that could have accounted, at least in part, for the accumulation of mRNA. This objective was hampered by the absence of constitutive NOS mRNA in medium-treated cells and by the extremely low levels obtained with stimulation by IFN-γ alone. However, no differences in the half-life of NOS mRNA were reported in RAW 264.7 cells treated with LPS or LPS and IFN-γ (33). A direct rather than a cytokine-mediated effect of picolinic acid on NOS gene is suggested by the observation that IFN-γ plus picolinic acid induced activation of NOS transcription after 4–5 h of treatment. Furthermore, we previously reported that TNF-α was only partially involved in picolinic acid-dependent induction of RNI production and that picolinic acid augmented IFN-γ-dependent TNF-α mRNA expression after 10 h, but not after 4 h of treatment, and did not affect the secretion of TNF-α protein (28). However, it cannot be ruled out from our data that, after prolonged incubation, TNF-α or other cytokines may be involved in an amplification loop that enhances the picolinic acid-dependent induction of NOS mRNA expression.

A body of literature has been accumulating during the past several years on the involvement of ·NO in the generation of tumoricidal activity by murine macrophages (1, 11, 35–37). Our previous work indicated that under the same experimental conditions in which IFN-γ plus picolinic acid induced RNI production in ANA-1 macrophages, a parallel increase of tumoricidal activity was also observed. Indeed, under conditions in which the L-arginine metabolism was inhibited, ANA-1 macrophages treated with IFN-γ plus picolinic acid did not express cytotoxic activity against TNF-resistant P815 tumor target cells.[2] These findings, together with data presented here, suggest that at least one molecular mechanism of the synergistic induction of tumoricidal activity by IFN-γ plus picolinic acid is activation of NOS gene expression.

Inhibition as well as superinduction of gene expression has

[2] G. Melillo, G. W. Cox, A. Biragyn, L. A. Sheffler, and L. Varesio, unpublished observation.

been reported in mononuclear phagocytes treated with different stimuli in the presence of CHX (38, 39). We found that the picolinic acid-dependent increase of NOS mRNA expression was almost completely abolished by addition of CHX, demonstrating a requirement for *de novo* protein synthesis. In contrast, CHX had little or no effect on the induction of NOS mRNA by IFN-γ alone. However, protein synthesis was also needed for NOS mRNA induction by LPS alone or LPS plus IFN-γ in the mouse macrophage cell line RAW 264.7 (33), suggesting the existence of a common regulatory mechanism in the activation of NOS gene expression. Whether the differential requirements for protein synthesis between IFN-γ and IFN-γ in combination with picolinic acid reflects involvement of different pathways of activation of NOS gene expression remains to be established.

IL-4 has long been known as a cytokine which inhibits the expression of several genes (40, 41) and functions (42–44) in murine macrophages. We report now that IL-4 down-regulated, although did not completely abolish, IFN-γ- plus picolinic acid-induced NOS mRNA expression. The extent of IL-4 inhibition was ≥50%; under the same conditions, IL-4 caused a reduction, to a similar degree, of IFN-γ- plus picolinic acid-induced NO_2^- production in ANA-1 macrophages (28). In addition to IL-4 (45), other cytokines, such as transforming growth factor-β and macrophage deactivating factor (46), have been described to exert a negative influence on NO_2^- production by murine macrophages. Our data provide the first evidence for a molecular basis of a negative regulation of NOS activity. Nuclear run-on experiments demonstrated that IL-4 affected the transcription of the NOS gene. Transcriptional inhibition of gene expression by IL-4 has been previously reported (47). Furthermore, recent evidence suggests that IL-4 can specifically influence the activity of transcription factors (48) and can block the transcription of IFN-inducible genes in human monocytes (49). Although there is no information available regarding the transcription factors involved in the regulation of NOS gene expression, we speculate that IL-4 might interfere with DNA binding proteins involved in IFN-γ- plus picolinic acid-dependent induction of the NOS gene.

Picolinic acid is a natural molecule and could be the physiologic mediator of the interaction between arginine and tryptophan metabolism. Elevated levels of tryptophan metabolites, such as kynurenine and quinolinic acid, have been detected *in vivo* in cerebrospinal fluid in pathologic conditions (50) as well as in cancer patients treated with IL-2 (51). Although there is only one report of RNI production *in vitro* by human monocyte-derived macrophages (52), elevation of nitrate levels in urine and plasma of IL-2-treated patients has also been demonstrated (53). Therefore, in pathologic conditions or following therapy with cytokines, an increased production of IFN-γ might be responsible for the concomitant activation of tryptophan and arginine metabolic pathways. Under the same conditions, activation of indoleamine 2,3-dioxygenase activity by IFN-γ might lead to accumulation of picolinic acid, which in localized compartments could feedback on macrophages amplifying the production of RNI.

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