Regulation of the mouse inducible-type nitric oxide synthase gene promoter by interferon- γ , bacterial lipopolysaccharide and N^G -monomethyl-L-arginine

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Cytokines and bacterial lipopolysaccharides (LPSs) stimulate nitric oxide production in macrophages by inducing transcription of the gene coding for the inducible isoform of nitric oxide synthase (iNOS). We have cloned the mouse iNOS gene promoter and analysed its structural features and its response to interferon- γ (IFN- γ) and *Escherichia coli* LPS in RAW 264.7 mouse macrophage-like cells. Transcription of a recombinant reporter gene including the promoter and 4 kb of its 5'-flanking DNA, linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene, is stimulated by IFN- γ and, more efficiently, by LPS upon transient transfection in RAW 264.7 cells. Two upstream DNA regions are required for maximal promoter activation by LPS: the first maps between positions –1541 and

–775 and the other between –420 and –47, with respect to the major transcriptional start site of the iNOS gene. The upstream-most region also mediates promoter *trans*-activation by IFN- γ . As reported earlier for transcription of the endogenous iNOS gene, combined stimulation of RAW 264.7 cells with IFN- γ and LPS results in lower activation of the transfected promoter, when compared with LPS alone. N^G -Monomethyl-L-arginine, a competitive inhibitor of nitric oxide synthase activity, enhances iNOS gene mRNA induction and promoter activation by IFN- γ and LPS, indicating that nitric oxide can influence negatively the responsiveness of this gene to inducers. These results suggest the possibility of a negative regulatory feedback exerted by iNOS on the transcriptional activation of its own gene.

INTRODUCTION

Nitric oxide, a highly bioactive but potentially toxic molecule, is synthesized in multiple cell types by a set of specific enzymes, nitric oxide synthases (NOSs), that act in response to a wide array of stimuli [1]. Nitric oxide is thought to be involved in several physiological functions [2], as well as to be implicated in the onset and maintenance of certain pathological conditions [3]. The beneficial effects of this compound, and its toxicity, are due to its reactivity towards a variety of molecular targets. Chemistry of nitric oxide involves, in fact, various redox forms that include NO (commonly designated nitric oxide, but most properly identified as nitrogen monoxide), NO[•] (nitric oxide), NO⁺ and NO[–] (nitrosonium and nitroxyl anion, respectively), and the related oxides, anhydrides and acids [4,5]. The high reactivity and potency of these molecules induce the existence in the cells of control mechanisms overseeing excessive synthesis of their common precursor, NO. Indeed, in the case of the 'constitutive' NOS isoforms, which require calcium as their main activator, the intracellular concentration of free calcium ions undergoes rapid fluctuations, allowing readily reversible enzyme activation. In contrast, fine regulation of NO production by the 'inducible' NOS (iNOS), characterized by stimulus-independent catalytic activity, is rather different. Inducers and modulators, in fact, determine fluctuations of its activity in the cell by affecting expression of the iNOS gene [6]. As a consequence, induction of enzyme activity is less readily reversible in this case when compared with that involving constitutive NOS isoforms, since the main controls for NO production reside here on trans-

criptional regulation of this gene. The pathways regulating NO production by iNOS are thus likely to be endowed with feedback controls acting on transcription of this gene that avoid NO, and its redox derivatives, reaching toxic concentrations in the cell.

One of the best-characterized responses of the murine iNOS gene occurs during activation of macrophages by interferon γ (IFN- γ) or bacterial lipopolysaccharide (LPS). In this case, exposure of quiescent cells to the inducer is followed by transcriptional activation of this gene, with subsequent accumulation of the corresponding mRNA and active enzyme [1]. Transcription requires on-going protein synthesis, indicating that it is a delayed cellular response to the stimulus mediated, at least in part, by the products of 'primary-response' genes [7,8]. While both IFN- γ or LPS are effective on their own, they do not seem to co-operate efficiently with each other on enhancement of iNOS gene transcription, since full stimulation of the cells with both inducers is not followed by higher transcriptional activity of this gene [7]. Some authors reported, however, that IFN- γ is not always effective on its own, requiring LPS to achieve full transcriptional stimulation [9,10], implying that the two inducers can, in certain circumstances, co-operate in inducing iNOS gene expression.

The basic mechanisms for transcriptional regulation of the iNOS gene are currently being actively investigated, and certain *cis*-regulatory elements involved in this regulation have been identified [11]. For example, gene 'knock-out' experiments involving interferon regulatory factor 1 (IRF-1) and nuclear factor interleukin 6 (NF-IL6), as well as IFN- γ , are consistent with the previous assumption that IRF-1, in conjunction with IFN- γ , is critical for transcriptional regulation of the iNOS gene,

Abbreviations used: AMV, avian myeloblastosis virus; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; dNTP, deoxyribonucleotide triphosphate; IFN- γ , interferon γ ; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; IRF, IFN regulatory factor; ISREs/IREs, IFN-stimulated response elements/IFN response elements; L-NMMA, N^G -monomethyl-L-arginine; LPS, lipopolysaccharide; NF-IL6, nuclear factor interleukin 6; NF- κ B, nuclear factor κ B; NO, nitric oxide; NOS, NO synthase; Oct, octamer factors; ssDNA, single-stranded DNA; SV40, simian virus 40; TBE, Tris-borate EDTA buffer; TNF- α , tumour necrosis factor- α .

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whereas NF-IL6 does not seem to be essential [12–14]. There are, however, still many aspects of the regulation of this gene in different cells and under different conditions that remain to be solved. In particular, it is clear that transcriptional activation of the iNOS gene results from the balanced effects of both positively and negatively acting factors, which combine under different conditions to yield various degrees of gene activation.

Because NO is a potentially toxic agent, much attention has been paid to understanding its cytotoxic effects, especially in immune responses. The ever increasing amount of information on the effects of NO on gene expression, however, clearly indicates that NO may be an important mediator of gene regulation [15]. A quite interesting model for NO action through cellular iron has been proposed recently [16], in addition to its well-accepted action through cyclic GMP. In this respect, possible effects of NO on iNOS gene expression have been reported [17,18]. These experiments suggest that NO can be a regulator of the iNOS gene, although defined mechanism(s) remain to be determined.

To further our understanding of the mechanisms that control iNOS activity, we have cloned the mouse iNOS gene promoter and analysed its response to both IFN- γ and LPS by transfecting it in mouse macrophage-like RAW 264.7 cells [7]. In this study we performed a genetic analysis of the regulatory regions endowed in its 5'-flanking DNA. This allowed the identification of two main regulatory regions: one, centred around the transcriptional start site of the gene, that includes *cis*-acting element(s) responsive to LPS, and the second, located far upstream, endowed with element(s) responsive to both IFN- γ and LPS. Furthermore, by using a competitive inhibitor of NOS, we could identify in the activity of iNOS itself a factor negatively affecting transcription of this gene.

MATERIALS AND METHODS

Reagents and cell culture

The mouse macrophage-like cell line RAW 264.7 (A.T.C.C. TIB71) was obtained from the American Type Culture Collection (U.S.A.). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, U.S.A.), supplemented with 2 mM L-glutamine, antibiotics (50 units/ml penicillin-G, 50 mg/ml streptomycin and 125 ng/ml amphotericin B; Sigma, U.S.A.) and 10% (v/v) fetal bovine serum (MultiSer; Cytosystems, Australia). Bacterial LPS (from *E. coli* serotype 0127:B8) was from Sigma, recombinant mouse IFN- γ was a generous gift of Shionogi Pharmaceuticals Company (Japan). RNase ONE, RNasin ribonuclease inhibitor, T3 and T7 RNA polymerases, ribonucleotides, deoxyribonucleotide triphosphates (dNTPs), RQ1 DNase and T4 polynucleotide kinase were from Promega (Madison, WI, U.S.A.); [α - 32 P]UTP (800 Ci/mmol), [α - 35 S]dATP (400 Ci/mmol) and [γ - 32 P]ATP (7000 Ci/mmol) were from Amersham International; avian myeloblastosis virus (AMV) reverse transcriptase was from Seikagaku Co. (Japan). All other chemicals were from Sigma (St. Louis, MO, U.S.A.) or Wako Ltd. (Japan).

Mapping of the transcriptional start site by RNase protection

RNase protection experiments were carried out using labelled antisense and sense (negative control) RNA probes synthesized according to standard procedures [19].

The DNA fragments containing the presumptive 5'-end of the iNOS gene were subcloned from the λ clones to the *Sma*I or *Xba*I sites of the phagemid Bluescript II SK+. After digestion with the appropriate restriction endonucleases (*Acc*I, *Hinc*II or *Sac*I),

RNA probes (riboprobes) were synthesized and treated with RQ1 DNase to remove the plasmid template according to standard protocols [19]. Following phenol/chloroform extraction, the labelled probes were purified by electrophoresis on 5% polyacrylamide/7 M urea gels, followed by elution from the matrix in 2 M ammonium acetate, 1% SDS and 25 mg/ml tRNA, at 37 °C for 4 h. Purified riboprobes were recovered by ethanol precipitation and dissolved in hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 0.4 M sodium acetate, 1 mM EDTA). Total RNA (10 μ g), purified from RAW 264.7 cells as described previously [7], was precipitated and carefully redissolved in 30 μ l of hybridization buffer containing 1.5×10^5 c.p.m. labelled RNA probe. Samples were then heated to 85 °C for 10 min, followed by hybridization at 45 °C for 12–16 h. Non-hybridized riboprobe and excess cellular RNA were removed by RNase ONE digestion (in 300 μ l of 10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 200 mM sodium acetate containing 0.1 unit of RNase ONE) according to standard protocols [19]. Following inactivation of the ribonuclease by addition of 0.1% SDS, 10 μ g of tRNA was added as carrier to each sample, and the RNase-resistant fragments were concentrated by ethanol precipitation in 4 ml of 80% formamide/10 mM EDTA/0.1% Bromophenol Blue/0.1% xylene cyanol/0.1% SDS and resolved on an 8% polyacrylamide/7 M urea sequencing gel. The labelled products were detected by autoradiography at -90 °C using Kodak XAR-5 films.

Molecular-mass markers were labelled with DNA fragments obtained in a sequencing reaction carried out using a single-stranded DNA (ssDNA) template of known sequence. Sizes of RNA fragments were calculated considering the differences in electrophoretic mobility between ssDNA and RNA [19].

Determination of iNOS mRNA capsite by primer extension

Three different antisense oligonucleotide primers, derived from the known sequence of the murine iNOS cDNA [20], were used: m0 (5'-AAC CAG TGA CAC TGT GTC CCG TGG; from position 29 to position 6 of the cDNA: shown in Figure 1), m1 (5'-ACA GCT CAG TCC CTT CAC CAA; from position 68 to position 48 of the cDNA), and m2b (5'-GTG AAC AAG ACC CAA GCG TGA GG; from position 110 to position 88 of the cDNA). Primers were 5'-end-labelled with [γ - 32 P]ATP using polynucleotide kinase and then purified by electrophoresis on 15% polyacrylamide gels in TBE buffer (Tris-borate EDTA buffer), according to standard protocols [19]. Labelled oligonucleotide (1×10^5 c.p.m.) was hybridized to 10 μ g of total RAW 264.7 cells RNA in 15 ml of hybridization buffer (150 mM KCl, 10 mM Tris, pH 8.3, 1 mM EDTA) for 90 min at 65 °C, followed by slow cooling of the samples to room temperature. Primer was then elongated at 42 °C for 1 h using 20 units of AMV reverse transcriptase in 45 μ l of 20 mM Tris, pH 8.3, containing 10 mM MgCl₂, 6 mM DL-dithiothreitol, 0.6 mM dNTP and 150 μ g/ml actinomycin D. Sonicated DNA (70 μ g/ml) was then added to each sample and RNA was removed by digestion with RNase A (14 μ g/ml) for 15 min at 37 °C. The samples were extracted once with phenol/chloroform and concentrated by ethanol precipitation in 5 ml of 80% formamide/10 mM EDTA/0.1% Bromophenol Blue/0.1% xylene cyanol/0.1% SDS and resolved on an 8% polyacrylamide/7 M urea sequencing gel.

The same primers were used also for sequencing genomic DNA clones collinear with the 5'-end of the cDNA. In each case, the sequencing reactions were run side by side with the corresponding samples and used as molecular markers to determine the exact size of each reverse transcription product.

Construction of iNOS-CAT reporter recombinants

Cloning of the mouse iNOS gene promoter region and construction of the reporter gene piNOS CAT1 has been described previously [7]. All deletion mutants of piNOS CAT1 were constructed by restriction enzyme digestion and re-ligation, according to standard protocols [19].

Transient transfection and chloramphenicol acetyltransferase (CAT) assays

For transient transfections, RAW 264.7 cells were plated on Day 1 at about 10–20% confluency in DMEM/10% (v/v) fetal calf serum culture medium. On Day 2, fresh medium was added and cells were transfected, using the calcium phosphate–DNA coprecipitation method [21] with 5 µg of piNOS CAT1 DNA (or equivalent amounts of DNA of each reporter), 3 µg of the β -galactosidase expression vector pnlS-LAC-Z [containing the bacterial LAC-Z open reading frame fused to the nuclear localization signal of simian virus 40 (SV40) large T antigen], as an internal control for transfection efficiency, and carrier DNA (Bluescribe M13+) up to 15 µg of total DNA/cell culture dish. After 18–20 h, cells were washed once for 30 min with PBS, before addition of fresh medium and incubation for an additional 24 h. Where indicated, 10^2 units/ml of IFN- γ and/or 5 µg/ml LPS were added to the cell culture media starting from Day 2.

CAT enzyme assays were performed on whole-cell extracts as described previously [22], after normalization for β -galactosidase activity and under assay conditions where substrate conversion was within a linear range with respect to the concentration of cell extract used. CAT activity was calculated as c.p.m. of acetylated chloramphenicol formed/h per unit of β -galactosidase.

Northern-blot analysis

Cells were seeded on 150-mm-diam. dishes as described above and 24 h later were treated with 5 µg/ml LPS or 10^2 units/ml IFN- γ , in the absence or presence of 1 mM *N*^G-monomethyl-L-arginine (L-NMMA), for an additional 24 h. Total cellular RNA was then extracted with 4 M guanidinium isothiocyanate and iNOS and β -actin mRNAs were detected by Northern-blot hybridization, after 1% agarose gel electrophoresis, as described previously [7,8].

RESULTS AND DISCUSSION

Isolation of genomic clones including the 5'-end of the mouse iNOS gene has been described previously [7]. A detailed characterization of the promoter region of this gene was carried out by both 'RNase mapping' and 'primer extension', and the results are summarized in Figure 1. Challenge by the RNase protection assay of various genomic DNA subclones with RNA extracted from RAW 264.7 cells allowed us to map the transcriptional start site of the gene to within a *Pst*I-*Hinc*II region, including a region of sequence identity with the 5'-end of the iNOS cDNA (Figure 1A, top and results not shown). Analysis of the first 4 kb of 5'-flanking DNA by RNase mapping failed to show any evidence of other transcribed regions upstream of the *Pst*I restriction site reported in Figure 1 (results not shown), confirming the location of the iNOS gene promoter as indicated in the Figure. Furthermore, as shown in Figure 1(A), no iNOS mRNA was detectable in samples extracted from non-stimulated cells (Figure 1, lane 2), confirming that basal expression of this gene is very low in quiescent RAW 264.7 cells. Upon exposure of the cells for 5 h to either IFN- γ (10^2 units/ml), LPS (5 µg/ml) or both, iNOS mRNA becomes easily detectable (Figure 1A: lanes

Figure 1 Mapping of the mouse iNOS gene promoter and determination of iNOS mRNA capsite in RAW 264.7 macrophages

Top: Schematic representation of the genomic organization of the mouse iNOS gene promoter region, including the first, non-coding exon of the gene (boxed) and the transcriptional start site (+1). The riboprobes used in the 'RNase mapping' experiments, with the expected protected fragment, are also shown. (A) Analysis by 'RNase mapping' of the 5'-end of the mouse iNOS mRNA. ³²P-labelled riboprobes, reproducing the antisense strand of the *Hinc*II-*Sac*I genomic DNA region, shown at the top of the Figure and including the putative promoter region of the iNOS gene, were hybridized to 10 µg of tRNA (control: lane 1) or total RNA isolated from semi-confluent cultures of RAW 264.7, either non stimulated (lane 2), or incubated for 5 h with 100 units/ml IFN- γ (lane 3), 5 µg/ml LPS (lane 4) or IFN- γ and LPS combined (lane 5). Following RNase digestion, the protected RNA fragments were resolved by electrophoresis on sequencing gels and visualized by autoradiography, as described in the text. Arrows shows the specific protected fragments obtained, which are about 155 to 165 bases long. (B) Identification of iNOS mRNA capsite by primer extension. The antisense oligonucleotide primer used is shown in italic, below the sequence around the transcriptional start site of the gene; its sequence is complementary to bases 29 to 6 of the mouse macrophage iNOS cDNA [20]. Total RNA (10 µg) was from non-stimulated (lane 1) or from IFN- γ (lane 2), LPS (lane 3) or IFN- γ plus LPS-stimulated RAW 264.7 cells. The sequence ladder shown on the autoradiogram was obtained by 'di-deoxy' DNA sequencing of a mouse genomic DNA subclone collinear with the 5'-end of the iNOS cDNA, using the same oligonucleotide as sequencing primer.

Figure 2 Identification of IFN- γ - and LPS-responsive regions in the 5'-flanking region of the mouse *iNOS* gene promoter

Top: Schematic representation of the genomic organization of the mouse *iNOS* gene promoter region and 5'-flanking DNA and of the *iNOS*-CAT reporter plasmids used. Bottom: Effects of IFN- γ and LPS on expression of the *iNOS*-CAT reporters in RAW 264.7 cells. The *iNOS*-CAT DNAs (5 μ g of piNOS CAT1 and an equivalent molar amount of each of the deletion mutants) were transfected in semi-confluent cultures by the calcium phosphate-DNA co-precipitation method. CAT activity was measured in whole-cell extracts from cells treated without (—) or with (+) the indicated inducer for 24 h before harvesting. The data reported are representative of multiple, independent transfection experiments, corrected for changes in transfection efficiency as described in the Materials and methods section.

3, 4 and 5, respectively). In all cases, the 5'-end of the mRNA is identical and corresponds essentially to that described previously both in subclones of RAW 264.7 cells unresponsive to IFN- γ alone [9,10] and in primary mouse macrophages [9]. RNase mapping results were confirmed by primer extension analysis of *iNOS* mRNA in RAW 264.7 cells (Figure 1B, lane 4).

A 5 kb *Bgl*III-*Bgl*III fragment, including about 4 kb of 5'-flanking DNA, the first non-coding exon and 340 bp of the first

intron of the *iNOS* gene, was subcloned upstream of the CAT coding sequence in pBLCAT5, to constitute the reporter piNOS CAT1. We have shown previously that this recombinant gene is expressed upon transient transfection in RAW 264.7 cells and it is inducible by either IFN- γ or LPS [7,8]. The transfected *iNOS* gene promoter was found to be active in different mouse as well as human cell lines, including Balb/3T3 fibroblasts, Hep G2 hepatoma cells, MCF-7 breast cancer cells, U 251 MG and A-172 glioblastoma cells, confirming the notion that this gene is expressed in tissues of different embryonal origin (results not shown). Moreover, transcription of this reporter could be activated by IFN- γ and LPS in RAW 264.7 and Balb/3T3 cells (about 10–15-fold and 2–3-fold respectively), but not by LPS in all other cell types investigated here (results not shown). Since these cell lines are responsive to various different inducers, they will be useful to identify within the *iNOS* gene promoter the elements responsive to multiple signal transduction pathways. Indeed, in A-172 cells, piNOS CAT1 was found to be responsive to a combined stimulation of the cells with IFN- γ , tumour necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β) [23].

A series of deletion mutants of piNOS CAT1 were then constructed and tested for their responses to IFN- γ and LPS in RAW 264.7 cells. The results, normalized for variations in transfection efficiency by using β -galactosidase as an internal control, are reported in Figure 2 (as actual autoradiograms of the TLC plates from representative transfection experiments) and in Table 1 (as the average results obtained in multiple, independent transfection experiments by quantifying CAT activity in cell extracts). piNOS CAT1, 2, 3 and 4 were all equally expressed and responsive to either LPS or IFN- γ stimulation, indicating that elements within the first 1541 bp upstream of the *iNOS* gene are sufficient to confer both basal activity and inducibility to this promoter. As reported previously [7], while each inducer alone was efficient in enhancing transcription of these reporters, co-stimulation of the cells with IFN- γ and LPS was not followed by a synergistic effect on the promoter (compare, for example, lanes 2 and 3 with lane 4, or lanes 14 and 15 with lane 16, and see the corresponding data in Table 1). The possible significance of these data is discussed below. Deletion of *iNOS* DNA comprised between the *Sph*I (–1541) and either the *Acc*I (–775) or the *Xba*I (–420) restriction sites, as in piNOS CAT5 and 6, is followed by a complete loss of the response to IFN- γ , while that to LPS, although strongly reduced, is still detectable (Figure 2, compare lane 17 with lanes 18 and 19, or lane 21 with lanes 22 and 23). The residual responsiveness to LPS is lost when deletion is extended to the *Pst*I site located at –47, as in piNOS CAT7 (Figure 2, lanes 29 to 32). These results indicate that *cis*-acting elements mediating stimulation of the *iNOS* gene promoter by LPS in RAW 264.7 macrophages are multiple and included within two separate DNA regions, located at a distance from each other: one between positions –1541 and –775 and the other between positions –420 and –47. In contrast, the element(s) mediating the IFN- γ response are confined in the upstream-most region, since cloning of this DNA upstream of the *iNOS* gene promoter, as in piNOS CAT47, restores the ability of IFN- γ to activate the reporter gene (compare lane 25 with lane 26 in Figure 2 and see Table 1).

Both of these DNA regions comprise multiple sequences homologous to genetic elements known to mediate the genomic effects of either of these inducers, including activator protein 1 (AP-1), nuclear factor κ B (NF- κ B), NF-IL6 and octamer factor (Oct) sites, IFN-stimulated response elements/IFN response elements (ISREs/IREs) and γ -activated factor binding sequences (GASs) [9,10] and our results not shown), that in most cases are fairly well conserved, in structure and localization, in the human

Table 1 Identification of IFN- γ - and bacterial LPS-responsive regions upstream of the mouse iNOS gene

The data represent the average ($\pm$ S.D.) of results obtained in multiple (n) transfection experiments, performed in RAW 264.7 cells as described in the text.

	Transcriptional activity (c.p.m./unit of β -galactosidase per h)				<i>n</i>
	–	IFN- γ	LPS	IFN- γ + LPS	
piNOS CAT1	1.48 ($\pm$ 0.36)	10.91 ($\pm$ 0.92)	25.96 ($\pm$ 1.18)	14.55 ($\pm$ 1.10)	9
piNOS CAT2	1.22 ($\pm$ 0.31)	6.18 ($\pm$ 1.03)	19.24 ($\pm$ 2.01)	12.39 ($\pm$ 1.31)	3
piNOS CAT3	2.03 ($\pm$ 0.58)	11.20 ($\pm$ 1.58)	21.96 ($\pm$ 1.11)	18.04 ($\pm$ 0.95)	3
piNOS CAT4	1.07 ($\pm$ 0.55)	6.56 ($\pm$ 0.15)	24.18 ($\pm$ 1.10)	15.4 ($\pm$ 0.99)	5
piNOS CAT5	1.15 ($\pm$ 0.60)	1.05 ($\pm$ 0.47)	5.52 ($\pm$ 0.96)	1.88 ($\pm$ 0.18)	5
piNOS CAT6	1.63 ($\pm$ 0.80)	1.18 ($\pm$ 0.54)	9.45 ($\pm$ 0.28)	3.67 ($\pm$ 0.70)	5
piNOS CAT47	0.95 ($\pm$ 0.37)	3.61 ($\pm$ 0.44)	2.21 ($\pm$ 0.10)	3.04 ($\pm$ 0.18)	3
+1 mM L-NMMA					
piNOS CAT1	1.28 ($\pm$ 0.51)	20.66 ($\pm$ 1.12)	39.00 ($\pm$ 2.07)	45.99 ($\pm$ 5.00)	5
piNOS CAT2	1.75 ($\pm$ 0.32)	20.05 ($\pm$ 3.08)	30.85 ($\pm$ 2.19)	49.52 ($\pm$ 2.04)	4
piNOS CAT4	0.98 ($\pm$ 0.61)	15.46 ($\pm$ 2.32)	29.78 ($\pm$ 1.75)	40.98 ($\pm$ 3.07)	3
piNOS CAT6	0.85 ($\pm$ 0.21)	1.09 ($\pm$ 0.32)	5.10 ($\pm$ 0.86)	5.86 ($\pm$ 0.55)	4
piNOS CAT47	0.86 ($\pm$ 0.53)	5.95 ($\pm$ 0.89)	3.18 ($\pm$ 0.30)	6.02 ($\pm$ 0.41)	3

iNOS gene promoter also [24]. There are several lines of evidence indicating that some of these elements mediate, at least in part, the effects of LPS and IFN- γ on transcription of the iNOS gene. Kamijio et al. [12], using IRF-1-deficient mice, demonstrated that this transcription factor is essential for induction of NOS by IFN- γ in macrophages. Two adjacent IREs (IRF-response elements) are located within the upstream DNA region shown here to mediate IFN- γ activation. In addition, since the IRF-1 gene is generally induced by treatment with IFNs, as well as TNF- α and IL-1 [25], the known requirement for *de novo* protein synthesis in induction of the iNOS gene by cytokines [7] could well be explained by the need for neosynthesis of such a transcription enhancer factor. It is worth mentioning here that the iNOS gene is not always readily responsive to IFN- γ in macrophages, since several reports indicate that co-stimulation of the cells with LPS is often required to achieve detectable activation of this gene by the lymphokine [9,10]. IRF-1, and possibly other factors synthesized in response to IFN, could in certain conditions require post-translational modifications, mediated by the signal transduction pathways activated by LPS, to be fully active. Alternatively, since both IRF-1 and IRF-2 genes are responsive to the NF- κ B transcription factor [25], known to be activated by LPS in macrophages [26], activation of an LPS-responsive pathway leading to NF- κ B could be required to boost IRF expression when this is too low to unchain the IFN- γ effects. At the same time, IFN-responsive and LPS-responsive factors could co-operate with each other in stimulating transcription of the iNOS gene: an NF- κ B site, for example, is located in close proximity to the two IRF-1 sites within the –1541 to –775 region of the gene, so that DNA-bound factors could functionally interact with each other in this case.

Concerning the elements responsive to LPS, multiple putative target sequences, in particular potential AP-1, octamer proteins, NF-IL6 and NF- κ B binding sites, are located in both responsive regions. An NF- κ B-like element is located in each of the two LPS-responsive regions identified here, the first at position –970 and the other at –80. It is possible that these two sites co-operate with each other, and possibly with other *cis*-acting elements, in achieving full stimulation of the iNOS gene promoter by LPS (Table 1). In fact, NF- κ B mediates multiple effects of LPS on transcription in macrophages, and low concentrations of

cycloheximide, a condition known to induce NF- κ B activity [19], have been shown to super-induce iNOS mRNA and to stimulate transcription of piNOS CAT1 in RAW 264.7 cells [7,8]. More convincingly, Xie et al. [11] showed recently that the NF- κ B site located at about position –80 is critical in the stimulation of iNOS transcription by LPS in RAW 264.7 cells and that pyrrolidine dithiocarbamate, an antioxidant that acts as an inhibitor of NF- κ B activation, can block NO production by LPS-treated RAW 264.7 cells. Recently, mice lacking the NF-IL6 gene were described by Tanaka et al. [14] in which the iNOS gene is fully induced by LPS. This suggests that the putative NF-IL6 site is not strictly required for full iNOS induction in this case. Alternatively, however, this sequence could mediate the action of NF-IL6-like factors that are responsive to LPS stimulation.

Analysis of iNOS gene transcription in IFN- γ - and LPS-stimulated RAW 264.7 cells shows that while the two inducers are active on their own they do not seem to co-operate efficiently with each other in achieving a synergistic effect on transcription of the iNOS gene [7]. They do co-operate, however, at the post-transcriptional level, so that the final result is a relatively modest co-operation between the two stimuli in inducing NO production by these macrophages [7]. Interestingly, this is also true for the cloned gene promoter, when this is re-introduced in RAW 264.7 cells (Table 1). Comparison of the response of piNOS CAT1–4 to IFN- γ , LPS or both inducers combined shows how this is, paradoxically, bipartite: strong when the cells are stimulated with only one inducer and moderate when the two stimuli are combined (Table 1). This is apparently independent of the nature of the DNA elements involved, since it occurs also when the reporter gene includes only the promoter-near region, responsive only to LPS (compare, in Table 1, expression of piNOS CAT5 or 6 in cells treated with LPS versus cells exposed to IFN- γ + LPS). It seems that the signal transduction pathways involved, i.e. IFN-responsive and LPS-responsive, and/or their effectors, in this case are not fully efficient when activated together. Alternatively, the two stimuli combined could induce in the macrophage a condition that prevents excessive activation of the iNOS gene, that would in turn result in overproduction of NO with possible toxic effects for the cell. NO itself is a possible candidate for a central role in regulation of its synthesis via

Figure 3 Northern-blot analysis of iNOS mRNA in the RAW264.7 cells treated with LPS and IFN- γ in the presence or absence of L-NMMA

Cells were exposed to various compounds for 24 h, before lysis followed by RNA extraction and analysis by Northern-blot hybridization, as described in the text. Total RNA (10 μ g) was from non-stimulated cells (lane 1) or from cells stimulated with 5 μ g/ml LPS (lane 2), 10² units/ml IFN- γ (lane 3), 5 μ g/ml LPS and 1 mM L-NMMA (lane 4), 10² units/ml IFN- γ and 1 mM L-NMMA (lane 5), 5 μ g/ml LPS plus 10² units/ml IFN- γ (lane 6).

iNOS, since certain effects of this molecule, and its redox derivatives, on gene transcription have been documented [27]. Furthermore, an increase of NO concentration is bound to modify the oxidoreductive potential within the cell, as a consequence of its high reactivity [4]. The activity of certain transcription factors, including NF- κ B [28], is influenced by changes in the redox balance within the cells. To test this last possibility, i.e. if NO can affect expression of the iNOS gene, we have used L-NMMA, a competitive inhibitor of NO synthases, to prevent NO production by iNOS synthesized in RAW 264.7 cells in response to IFN- γ and LPS. L-NMMA (1 mM) was added to the culture medium prior to exposure of the cells to either IFN- γ , LPS or both inducers combined. As shown in Figure 3, L-NMMA can stimulate significantly iNOS mRNA accumulation in cells treated either with LPS or with IFN- γ (2- to 3-fold increase on average, compared with that achieved by each inducer alone). The effects of the inhibitor were also tested on the cloned iNOS promoter. The results, reported in Figure 4 and in Table 1, show clearly that inhibition of NO synthesis in macrophages with L-NMMA potentiates iNOS gene promoter activation by IFN- γ and LPS, and more so when both inducers are used in combination. The permissive effects of the inhibitor are more evident on piNOS CAT1, 2 or 4 reporters, which include both the regulatory regions described above (compare lane 4 with lane 8 and lane 12 with lane 16 in Figure 4 and see the data relative to piNOS CAT 1, 2 and 4 expression in Table 1). No changes in expression of pSV2-CAT or pRSV-CAT, two constitutive CAT reporters based on the SV40 early promoter and the Rous sarcoma virus long terminal repeat (LTR), could be detected in the presence either of the inducers or of the inhibitor (results not shown), suggesting that L-NMMA does not have a generalized effect on transcription or CAT enzyme activity itself, but instead that its action is likely to be specific for the iNOS gene, mediated by inhibition of NO synthase activity. PTIO (4,4,5,5-tetramethyl-2-phenyl-imidazoline-1-oxyl 3-oxide), an

Figure 4 Inhibition of NO synthesis by L-NMMA enhances the inducible expression of iNOS-CAT reporter genes transfected in RAW 264.7 macrophages

Aliquots (5 μ g) of piNOS CAT1 or 2 DNA were transfected by the calcium phosphate–DNA coprecipitation method. The transfected cells were then incubated for 24 h in medium alone, or in medium supplemented with IFN- γ and/or LPS, in the presence or absence of 1 mM L-NMMA as indicated. CAT activity was measured in whole-cell extracts, corrected for differences in transfection efficiency by normalizing the amount of extract used in each case to its content in β -galactosidase internal control, as described in the Materials and methods section. The data reported are representative of two, independent transfection experiments.

imidazolinoyl *N*-oxide that traps NO released from the cells [29], did not have any effect on transcription of the iNOS-CAT reporters, either in the presence or absence of IFN- γ and LPS (results not shown), suggesting that the putative activity of NO and/or its derivatives on the iNOS gene promoter is very rapid and occurs predominantly through an intracellular mechanism. In preliminary experiments, overexpression of NOS in RAW 264.7 cells by transfection of an expression vector encoding the rat iNOS cDNA results in a dramatic reduction of the piNOS CAT1 response to either IFN- γ or LPS, which is relieved by pretreatment of the cells with L-NMMA (A. Weisz, H. Adachi and H. Esumi, unpublished work). Preliminary results show that sodium nitroprusside, an NO-generating compound, did not show an inhibitory effect on the iNOS reporter genes in these cells (results not shown), suggesting that only certain redox derivatives of NO could be involved in this putative autoregulatory loop. Further studies, now in progress, are required to clarify the full role of NO and its derivatives on the fine tuning of iNOS gene transcriptional activation by cytokines and other inducers.

The possibility that NO derivatives influence negatively gene expression in NOS-expressing cells, and possibly also in NO target cells, reveals for the first time wholly new possible scenarios that, with the identification of the target molecules mediating these effects, are likely to expand our knowledge on the full range of biological activities of these molecules.

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