Molecular Cloning and Functional Expression of an Inducible Nitric Oxide Synthase from a Murine Macrophage Cell Line*

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Macrophages activated by exposure to cytokines and/or to endotoxin produce nitric oxide (NO), a free radical that is a mediator of the host response to infection. Activation induces the expression of nitric oxide synthase, the enzyme that catalyzes formation of NO from L-arginine and molecular oxygen. We report the cloning of a cDNA encoding the inducible nitric oxide synthase from a murine macrophage cell line, RAW264.7, exposed to interferon-γ and lipopolysaccharide. Oocytes injected with mRNA transcribed from this cDNA demonstrate arginine-dependent production of nitrite, a stable metabolite of NO. Nitrite production is blocked by the enzyme inhibitor, Nω-monomethylarginine, and is independent of calcium/calmodulin. RAW264.7 cells demonstrate rapid accumulation of the nitric oxide synthase-encoding mRNAs upon activation. Comparison of the deduced amino acid sequence to the calcium/calmodulin-dependent nitric oxide synthase previously purified (Bredt, D. S., and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 682-685) and cloned (Bredt, D. S., Hwang, P. M., Giatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714-718) from rat brain identifies shared binding sites for the cofactors NADPH and flavins in the C-terminal half of both proteins, and an additional conserved region near the N terminus that may recognize L-arginine and/or contribute to the active site.

Diverse roles for nitric oxide (NO) in different tissues have been identified. In macrophages, nitric oxide is an important mediator of tumoricidal (10, 11) and microbicidal activity, particularly against Cryptococcus neoformans (4) and intracellular organisms such as Toxoplasma gondii (12) and Leishmania major (13). Production of NO by endothelial cells is an important regulator of vascular resistance and blood pressure (5, 14). Indeed, excess production of NO by macrophages and other cells exposed to endotoxin and interleukin-1 may contribute to the hemodynamic changes observed in septic shock (15, 16). In particular, a sepsis-related decrease in blood pressure may result from cGMP-mediated loss of vascular tone that is secondary to activation of smooth muscle guanylyl cyclase by NO (17). Nitric oxide is also an important messenger molecule in the brain (18, 19) and neurotransmitter in the peripheral nervous system (9).

Regulation of NO production in different tissues is mediated by control of nitric oxide synthase activity. In endothelial cells and neurons, NOS may be activated by phosphorylation (9) and requires Ca2+ and calmodulin (8). Purification (8) and cDNA cloning (9) have demonstrated that brain NOS is a 160,000-kDa protein that is related to cytochrome P450 reductase.

A second nitric oxide synthase (Mac-NOS) that is distinct from the brain/endothelium enzyme has been identified in macrophages activated by cytokines (1-3). Unlike brain NOS, activation of Mac-NOS requires protein synthesis and is independent of calcium/calmodulin. Recently, Mac-NOS has been purified (20-22). Although it is smaller (125-130 kDa) than brain NOS, both enzymes require FAD, FMN, and NADPH and demonstrate a similar Ks for arginine (2-5 μM) and sensitivity to inhibition by arginine analogues such as Nω-monomethylarginine (L-NMMA).

In this report, we describe the cloning and functional expression of a cDNA encoding an inducible nitric oxide synthase from a macrophage-derived mouse cell line, RAW264.7, that has been activated by exposure to interferon-γ and lipopolysaccharide (LPS). Analysis of the deduced amino acid sequence demonstrates that Mac-NOS is related to brain NOS.

**EXPERIMENTAL PROCEDURES**

Cells and Reagents—RAW264.7 were obtained from American Type Tissue Collection and grown at 37°C, 5% CO2 in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum. In experiments which required nitrite measurements, phenol red was omitted from the Dulbecco's modified Eagle's medium. Interferon-γ was purchased from Genzyme (Cambridge, MA). All other reagents were obtained from Sigma. The serotype of the LPS was Escherichia coli 0128:B12.

Nitric Oxide Synthase Assay—NOS activity was measured as nitrite production (6, 7) in injected oocytes. Stage 5 and 6 oocytes were removed from female Xenopus laevis and separated by incubation with collagenase as described (23). Each oocyte was injected with 50 nl of RNA (0.5-1.0 ng/μl) and incubated at 19°C for 72 h in 50 μl Leibovitz L-15 medium (GIBCO) without phenol red. Unless otherwise indicated, the incubation medium contained 2 mM arginine and 0.67 mM calcium. Each assay was performed in triplicate using 5 oocytes per well. Nitrite was measured by mixing 50 μl of the oocyte supernatant with an equal volume of Griess reagent (1 part 0.1% naphthylethenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) (24). The absorbance at 550 nm was measured.

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The abbreviations used are: NOS, nitric oxide synthase; L-NMMA, Nω-monomethylarginine; LPS, lipopolysaccharide.
and nitrite concentration determined using a curve calibrated from sodium nitrite standards. In the depletion studies, the oocytes were incubated in arginine- or calcium-free medium for 4 days prior to injection.

cDNA Cloning—RNA was prepared from RAW264.7 cells 6 h after exposure to interferon-γ (50 units/ml) and LPS (20 ng/ml) by a guanidine isothiocyanate/CsCl procedure (25). Polyadenylated RNA (poly(A) RNA) was isolated by oligo(dT)-cellulose column chromatography and size-fractionated by density gradient centrifugation on 15–30% (w/v) sucrose gradients containing methylmercury hydroxide (26). After centrifugation at 4 °C for 16 h at 76,800 × g, 0.3-ml fractions were collected and assayed for nitric oxide synthase activity by injection into Xenopus oocytes. RNA from the positive fractions was combined, concentrated by ethanol precipitation, and used as a template to synthesize a cDNA library that was ligated into the phage vector, lambda ZAP II (Stratagene). DNA was prepared from amplified pools of 10^8 phage and used as template in a polymerase chain reaction (40 cycles, 4 °C annealing temperature) using primers from

![Image of nitrite synthesis by frog oocytes injected with size-fractionated poly(A) RNA from activated RAW264.7 cells. Poly(A) RNA from activated RAW264.7 cells was fractionated on 15–30% sucrose gradients, and aliquots were assayed for nitric oxide synthase-encoding activity by measuring nitrite production in injected oocytes. Fractions containing the greatest activity (10 and 11) were used as the template to construct the cDNA library.](image1)

![Image of Sucrose gradient fractions.](image2)

![Image of NOS activity in oocytes injected with RNA transcribed from pMac-NOS. NOS production was measured in oocytes injected with RNA transcribed from pMacNOS as described under "Experimental Procedures." Each data point is a mean obtained from triplicate samples. Incubation medium was modified to assess the dependence of Mac-NOS activity on L-arginine and on Ca^2+ and calmodulin. The standard deviation of absorbance values among triplicate samples ranged from 0.018 to 0.036. Background absorbance values ranged from 0.063 to 0.080 (0.002–0.003 nmol of nitrite). Concentration of L-NMMA was 75 μM and trifluoperazine (TFP), 200 μM.](image3)

![Image of Expression of Mac-NOS RNA in activated RAW264.7 cells. A Northern blot was prepared using poly(A) RNA from RAW264.7 cells activated by γ-INF (50 units/ml) and LPS (20 ng/ml) and from control cells. The migration positions of marker 28 and 18 S RNAs are indicated. An additional hybridization using a mouse actin cDNA as probe demonstrated a comparable amount of the actin transcript in each lane (data not shown).](image4)

suspected cofactor binding sites. Deoxyinosine was inserted at ambiguous codon positions (27). The sense oligonucleotide, AAATATTCTCTTATATATACGC, was derived from KYLDITTP, an amino acid sequence conserved in enzymes that bind NADPH and FAD (9, 28). The antisense oligonucleotide, AAAGGGCGTCCCGCTTCCGCGCG, encodes GPGTGIAPF, an amino acid sequence conserved in enzymes that bind NADPH and FAD (9, 28). The antisense oligonucleotide, AAAGGGCGTCCCGCTTCCGCGCG, encodes GPGTGIAPF, an amino acid sequence conserved in enzymes that bind NADPH and FAD (9, 28). The antisense oligonucleotide, AAAGGGCGTCCCGCTTCCGCGCG, encodes GPGTGIAPF, an amino acid sequence conserved in enzymes that bind NADPH and FAD (9, 28). The antisense oligonucleotide, AAAGGGCGTCCCGCTTCCGCGCG, encodes GPGTGIAPF, an amino acid sequence conserved in enzymes that bind NADPH and FAD (9, 28).

Functional Expression of cDNA Clone—pMac-NOS was digested with Kpnl, blunt-end with T4 polymerase, and used as a template to prepare RNA by in vitro transcription using SP6 RNA polymerase (Stratagene). Each oocyte was then injected with 50 nl (0.5 ng/ml).

Northern Blot Analysis—Poly(A) RNA was prepared as above from activated and control RAW264.7 cells. Five μg was separated on an agarose-formaldehyde gel, transferred onto a nitrocellulose filter, and hybridized to a ^32P-labeled insert from pMac-NOS (29). The blot was washed (0.2 x SSC, 0.1% sodium dodecyl sulfate 65 °C) and autoradiographed for 1 h.

Sequence Analysis—All programs used for sequence analysis were from the University of Wisconsin Genetics Computer Group (31).

RESULTS AND DISCUSSION

Previous studies demonstrated that nitrite production by RAW264.7 cells activated by exposure to γ-INF and LPS correlated with induction of NOS activity (1–3). We observed an arginine-dependent increase in nitrite production by frog oocytes injected with poly(A) RNA from activated, but not from resting, RAW264.7 cells. Incubation of injected oocytes in the presence of L-NMMA, an arginine analogue that inhibits NOS (14), blocked nitrite production (data not shown).
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Fig. 4. Comparison of the macrophage and brain nitric oxide synthases. a, nucleotide sequence of the pMac-NOS insert. The sequence is numbered with respect to the first potential initiator methionine residue. The deduced amino acid sequence is shown using the single letter code. Two methionine-encoding sequences near the 5' end of the long open reading frame have neighboring nucleotide sequences consistent with translational start sites (33). The first methionine is encoded within TCAGACTAGTG. b, alignment of deduced amino acid sequence of Mac-NOS and brain-NOS. Gaps introduced to optimize the alignment are marked as dots. Identical amino acids are connected with vertical lines. The two proteins demonstrate 55% identity assuming translation initiation from the first methionine. Cofactor binding regions are framed. An NADPH binding region is found between residues 936 and 1238. The binding sites for the ribose and adenine rings are found at residues 923-990 and 1070-1085, respectively (9). The region between residues 157 and 476 of Mac-NOS demonstrates 71% identity to brain NOS. The residues used to design the polymerase chain reaction primers are underlined. c, schematic diagram displaying the relationship between Mac-NOS and brain NOS. The positions of the NADPH and flavin binding sites are labeled, and the conserved N-terminal region is darkened. The predicted recognition regions for calmodulin (C) and protein kinase A phosphorylation (P) previously reported (9) are included in brain-NOS.
addition, sucrose gradient sedimentation of this RNA identified a 5–6 kilobase fraction that was enriched for nitrite-producing activity (Fig. 1). This RNA is large enough to encode the 130 kDa NOS previously purified from these cells (20–22).

A cDNA library was constructed from the RNA in the fractions enriched for this activity. Our screening strategy took advantage of previous reports of conserved amino acid residues in the cofactor binding regions of several NADPH/flavin-dependent proteins (28), including brain-NOS (9). We synthesized oligonucleotides that encoded these conserved residues and used them as primers in a polymerase chain reaction on phage DNA obtained from the library. A single 450-base pair product was generated and used as a hybridization probe to screen the library. Analysis of the nucleotide sequence obtained from several overlapping clones that hybridized to this probe revealed a single long open reading frame that encoded a protein of greater than 130 kDa.

To test if this open reading frame encoded NOS, a full-length cDNA was constructed from two overlapping clones and inserted into the pGEM plasmid vector (Promega). RNA transcribed from this clone (pMac-NOS) was injected into oocytes and evaluated for NOS activity. These oocytes demonstrated arginine-dependent nitric oxide production that was inhibited by L-NMMA (75 μM), and this inhibition was reversed by excess arginine (Fig. 2). Nitrite production did not require the presence of Ca²⁺ in the incubation medium and was not inhibited by trifluoperazine (200 μM), an inhibitor of brain NOS (9) and calmodulin-mediated reactions in frog oocytes (32). Hybridization of pMac-NOS insert to poly(A) RNA identified three transcripts in activated, but not in resting, macrophages.

The nucleotide sequence of the pNOS insert is shown in Fig. 4b. Examination of the deduced amino acid sequence identifies two potential translation start sites (33) that encode proteins of 130,574 and 118,067 kDa. Translation from both potential initiator methionine residues could, in part, explain the binding sites in the C-terminal portion of both proteins, the reported migration of purified NOS as multiple bands identified and may represent use of alternative polyadenylation sites in the C-terminal portion of both proteins, the reported migration of purified NOS as multiple bands identified and may represent use of alternative polyadenylation sites.

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