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Biochemistry

Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme

(Kupffer cells/endotoxin/interferon/long-term potentiation)

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ABSTRACT **Nitric oxide (NO) is a messenger molecule of macrophages, endothelial cells in blood vessels, and neurons. A neuronal form of NO synthase (NOS) has been previously cloned. We now report the molecular cloning of macrophage NOS. The macrophage enzyme displays 50% sequence identity to the neuronal enzyme. Like neuronal NOS, macrophage NOS has recognition sites for FAD, FMN, and NADPH and also has a consensus calmodulin binding site. Macrophage NOS mRNA is strikingly inducible; it is absent in quiescent macrophages or spleen but is prominent 2–6 hr after endotoxin treatment.**

Nitric oxide (NO) is a recently identified messenger molecule with diverse functions throughout the body. In the brain and peripheral nervous system, NO displays many properties of a neurotransmitter; it is implicated in neurotoxicity associated with stroke and neurodegenerative diseases (1–4), neural regulation of smooth muscle including peristalsis (5–7), and penile erection (8, 9). NO is responsible for endothelial-derived relaxing factor activity regulating blood pressure (reviewed in refs. 10 and 11). In macrophages NO mediates tumoricidal and bactericidal actions, because inhibitors of NO synthase (NOS) block these effects (reviewed in ref. 12).

At least two distinct isoforms of NOS exist. Neuronal NOS (n-NOS) and macrophage NOS (mac-NOS) differ in several biochemical properties. Both n-NOS and mac-NOS are unusual among oxidative enzymes in requiring several electron donors, FAD, FMN, NADPH, and tetrahydrobiopterin (13–18). n-NOS is absolutely dependent upon Ca^{2+} and calmodulin, neither of which are required by mac-NOS (19–21). n-NOS activity is constitutive, whereas mac-NOS activity can be induced by interferon γ and lipopolysaccharide (LPS) (22–25). Endothelial NOS requires the same oxidative cofactors as the other two forms of the enzyme and, like n-NOS, is dependent upon Ca^{2+} (10). Whether endothelial NOS is a distinct gene product is not clear.

Molecular cloning and expression of n-NOS has revealed specific recognition sites on the protein for FAD, FMN, NADPH, and calmodulin (26). Additionally, n-NOS has consensus sequences for phosphorylation, consistent with phosphorylation of n-NOS that has been demonstrated for cAMP-dependent protein kinase, protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinase (26). The only mammalian protein with close sequence similarity to n-NOS is cytochrome P450 reductase (CPR). The mac-NOS protein has recently been purified (27–29). To clarify functions of mac-NOS, we have cloned its cDNA‡ and expressed the enzyme in 293 human kidney cells. The mac-NOS cDNA, like that of n-NOS, encodes recognition sites for FMN, FAD, and NADPH, but not for calmodulin. It also contains a cAMP-dependent phosphorylation site. The mRNA for mac-NOS is dramatically enhanced in macrophages treated with LPS and interferon γ and is present in peripheral tissues of rats treated with these agents.

MATERIALS AND METHODS

Molecular Biology. To obtain the initial mac-NOS DNA probe, RNA was harvested from LPS- and interferon γ-stimulated macrophages (30, 31) and was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (BRL) using the following degenerate antisense primer: GCCGGAATTC-RTCNCCRCANACRTADATRTG (corresponding to the consensus NADPH binding region His-Ile-Tyr-Val-Cys-Gly-Asp). Amplification of this cDNA pool by PCR was performed (32) with the following degenerate sense orientation primer: GGGGGATCC-GTNGGNCCNGGNACNGG (corresponding to the consensus NADPH binding region Val-Gly-Pro-Cys-Thr-Gly). Three cDNA libraries were made with kits according to the manufacturers' instructions (Invitrogen, San Diego; Stratagene) from mRNA harvested from LPS-stimulated RAW 264.7 macrophages (30, 31). The vectors were screened (33) with a DNA probe labeled with random hexamers (34). Isolated cDNA inserts were ligated into a unique *Not* I site in the BlueScript KS vector (Stratagene), sequenced by the dideoxy chain-termination method with radioactive chain terminators (35), and then resequenced with fluorescent chain terminators (Applied Biosystems; according to the manufacturer's instructions). Northern blot analysis of mRNA was performed according to standard techniques using the mac-NOS cDNA as a probe (36, 37). Total RNA was also extracted from tissues harvested from BALB/c mice that were stimulated 6 hr previously with an i.p. injection of LPS (from *Escherichia coli*; GIBCO; 4 mg/kg) in 0.9% NaCl. *In situ* hybridization was performed as described (38, 39) on tissues from BALB/c mice harvested 6 hr after stimulation with an i.p. injection of LPS (from *E. coli*; GIBCO; 4 mg/kg). The assay for NOS activity was performed as described (20).

Tissue Culture. Macrophage tissue culture was performed as described (40). Briefly, RAW 264.7 cells, a monocyte–macrophage cell line from BALB/c mice (American Type Culture Collection), were stimulated with *E. coli* LPS (GIBCO; 3.5 ng/ml) and recombinant murine interferon γ (Upstate Biotechnology Inc., Lake Placid, NY; 5 units/ml) for 0–24 hr. The mac-NOS cDNA was inserted into the pCIS expression vector (Genentech), which includes a cytomegalovirus enhancer–promoter, a cytomegalovirus intron, a multiple cloning site, a simian virus 40 polyadenylylation site, and a simian virus 40 origin. Human embryonic kidney 293 cells (American Type Culture Collection) were transfected by the transient Ca^{2+} phosphate method (41, 42).

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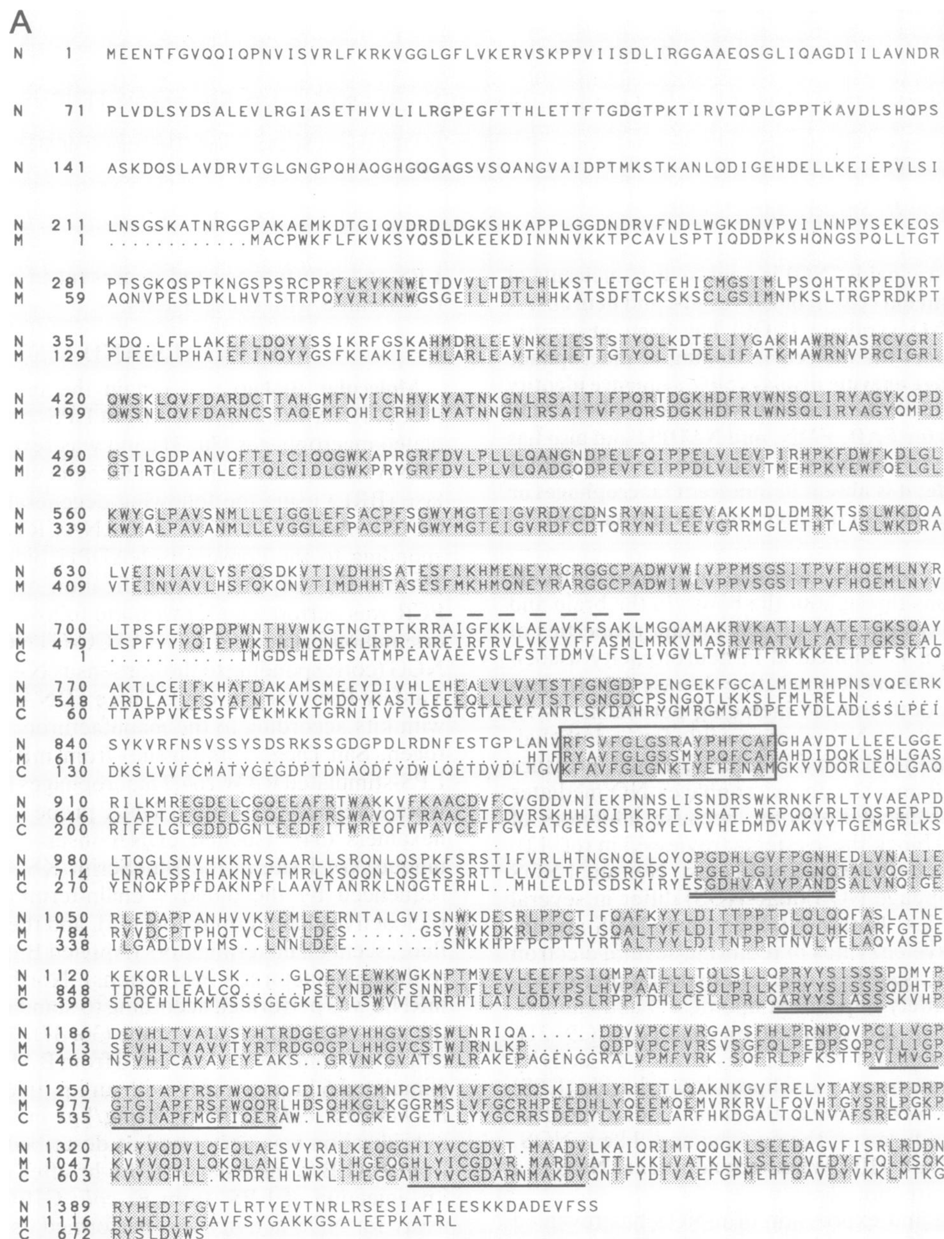
Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; LPS, lipopolysaccharide; n-NOS, neuronal NOS; mac-NOS, macrophage NOS; CPR, cytochrome P450 reductase.
‡The nucleotide sequence of the mac-NOS cDNA has been deposited in the GenBank data base (accession no. M92649).

RESULTS

Cloning Strategy. We designed degenerate PCR primers based on NADPH consensus binding sites in n-NOS, which presumably would be conserved in mac-NOS. The 360-base-pair product obtained by PCR of reverse-transcribed macrophage RNA was used as a probe to screen a cDNA library constructed from RAW 264.7 macrophage cells stimulated



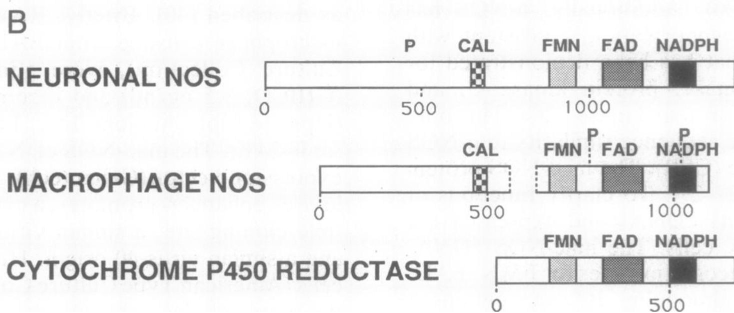


FIG. 1. mac-NOS is closely related to n-NOS and CPR. (*A*) Homologous regions between the mac-NOS (M), n-NOS (N), and CPR (C) deduced amino acid sequences are shaded. Consensus binding sites for FMN are boxed, those for FAD are doubly underlined, and those for NADPH are singly underlined. The putative NOS calmodulin sites are overlined with a dashed line. (*B*) Schematic alignment of mac-NOS, n-NOS, and CPR. Compared to n-NOS, mac-NOS contains a 40-amino acid residue internal deletion and is 200-amino acid residues shorter at the amino terminus and 15 residues shorter at the carboxyl terminus. Consensus binding sites are shown: CAL, calmodulin; P, potential site for phosphorylation by cAMP-dependent protein kinase.

for 6 hr with LPS and interferon γ. Screening with the NOS probe revealed one cDNA clone (CL-3), which was employed to rescreen the library, resulting in the identification of a second clone (CL-44). These two clones were ligated at a unique *Afl* III site and subcloned, and the cDNA was sequenced.

mac-NOS Sequence Contrasted to n-NOS. The 4110-base-pair cDNA sequence of mac-NOS contains a 3432-base-pair open reading frame encoding a polypeptide of 1144 amino acids with a calculated molecular mass of 130 kDa (Fig. 1*A*). An in-frame stop sequence TAG precedes the start site by 60 bases. The start site contains a Kozak consensus sequence (CAGACATGG) (43).

The mac-NOS sequence is shorter than n-NOS. Alignment of the two sequences shows that, compared to mac-NOS, n-NOS is extended at the amino terminus by an additional 200 amino acids and is 15 amino acid residues longer at the carboxyl terminus (Fig. 1*A*). Additionally, mac-NOS manifests a deletion of 40 amino acids internally.

Like n-NOS, mac-NOS possesses consensus recognition sites for the oxidative cofactors FAD, FMN, and NADPH in the carboxyl half of the molecule (Fig. 1*B*). These sites are virtually identical in the two enzymes. In addition, mac-NOS has a consensus calmodulin recognition site although its activity is calcium independent (44).

mac-NOS displays a consensus site for phosphorylation by cAMP-dependent protein kinase located at a site different for the comparable sequence in n-NOS. In both n-NOS and mac-NOS this is a weak phosphorylation sequence, Lys-Arg-Xaa-Xaa-Ser (45).

The amino acid sequence of mac-NOS displays an overall 50% identity to n-NOS, with 65% homology. However, in certain portions of the molecule similarity is particularly high. The sites for binding of oxidative cofactors in the carboxyl-terminal half are virtually identical in the two enzymes. Quite strikingly, in the amino-terminal half of the molecule, a sequence of 300 amino acid residues from position 180 to 480 displays 67% identity and 80% homology to n-NOS. It is possible that this area of similarity incorporates the recognition sites for arginine and/or tetrahydrobiopterin. There is no generally recognized consensus sequence for tetrahydrobiopterin binding. To evaluate a possible arginine consensus site, we compared the mac-NOS sequence to the sequences of the following arginine binding enzymes: mouse argininosuccinate synthase (46), rat liver arginase (47), and rat argininosuccinate synthase (48), but we were unable to locate appropriately homologous regions.

Like n-NOS, mac-NOS displays substantial similarity to CPR in the carboxyl-terminal half of the molecule (49). In this region, at amino acids 750–1120, there is 36% identity and 43% homology between mac-NOS and CPR, about the same level of similarity as occurs between n-NOS and CPR (26).

Transfection Establishes the Identity of the Cloned cDNA as mac-NOS. The cDNA for mac-NOS was ligated into the expression vector pCIS and transfected into 293 embryonic human kidney cells, which were assayed for NOS catalytic activity (Fig. 2). Control cells as well as cells transfected with antisense mac-NOS displayed negligible levels of NOS activity. In contrast, cells transfected with sense mac-NOS displayed substantial enzymatic activity in the absence of NADPH, with an increase in enzymatic activity upon the addition of NADPH. The failure of EDTA to reduce NOS activity establishes that the mac-NOS is Ca^{2+} independent.

A kinetic analysis of NOS activity in transfected cells demonstrates saturable activity, with a K_m for arginine of 19 μM, which is similar to published values for purified mac-NOS (27–29).

mac-NOS mRNA Is Inducible. One of the hallmarks of mac-NOS catalytic activity is its inducibility following treatment with LPS and interferon γ (22–25). To assess if this

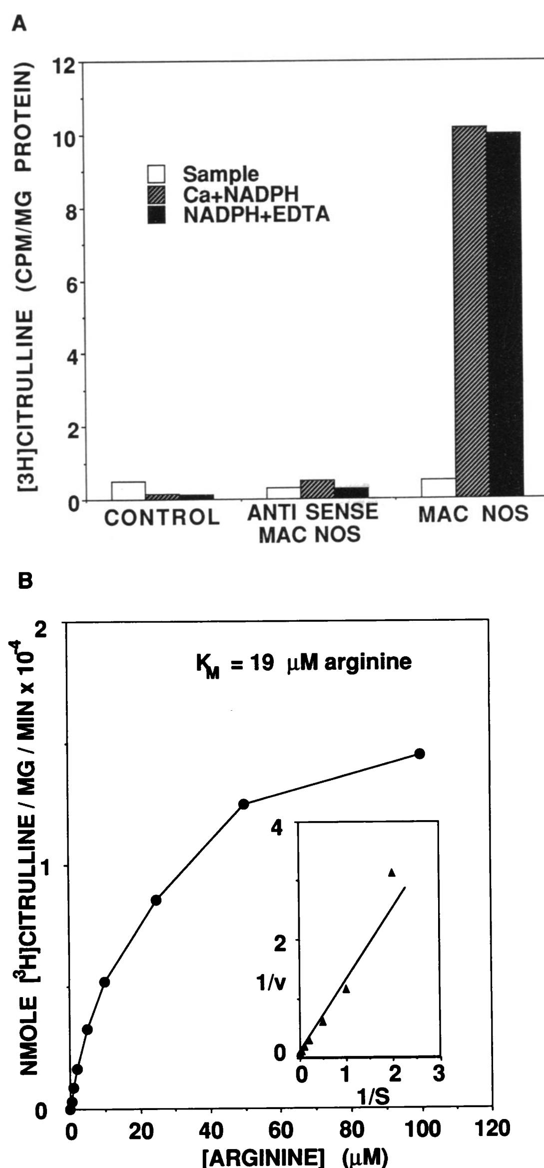


FIG. 2. The putative mac-NOS cDNA encodes a protein with NOS catalytic activity. (*A*) Human embryonic kidney 293 cells contain NOS catalytic activity only after transfection with mac-NOS cDNA, but not after transfection with antisense mac-NOS DNA or vector alone. mac-NOS sense or antisense cDNA was inserted into the pCIS expression vector and transiently transfected into 293 cells, which were assayed for NOS activity after 48 hr. (*B*) Human embryonic 293 cells transfected with mac-NOS cDNA (as described above) were assayed for NOS catalytic activity with increasing concentrations of arginine. The NOS catalytic activity is saturable by arginine with a K_m = 19 μM.

inducibility reflects gene transcription, we measured both catalytic NOS activity and mac-NOS mRNA levels in macrophages treated with LPS and interferon γ (Fig. 3). This treatment provoked a marked enhancement of mac-NOS mRNA analyzed by Northern blot probed with the mac-NOS cDNA. A 4-kilobase band corresponding to the mac-NOS mRNA was first detectable at 2 hr, peaked at 6 hr, and remained elevated at 24 hr. mac-NOS catalytic activity in the same cells was absent at 0 and 2 hr, increased slightly at 4 hr, and reached peak values at 6 hr, which were maintained at 24 hr. Ca^{2+} failed to augment enzyme activity, which was also unaffected by EDTA. NADPH treatment enhanced enzyme activity, most notably at 24 hr, though in some experiments NADPH provided varying degrees of enhancement at earlier

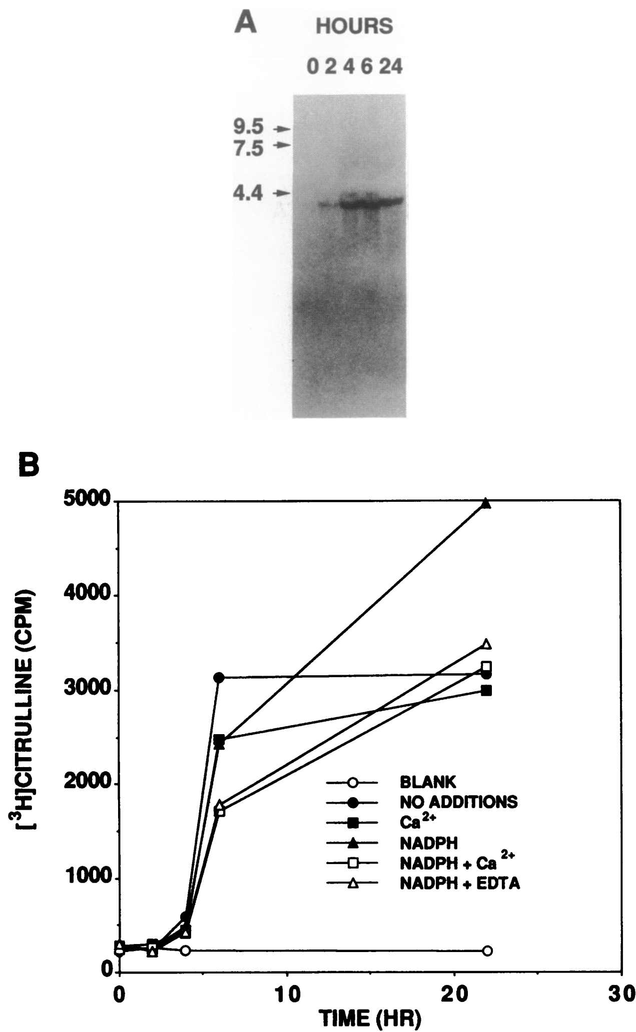


FIG. 3. mac-NOS mRNA and NOS catalytic activity are induced in macrophages by LPS and peak 6 hr after induction. (*A*) RAW 264.7 macrophages were stimulated with LPS and interferon γ for 0–24 hr as shown. Total RNA (20 μg) was isolated from the macrophages, electrophoresed on an agarose/formaldehyde gel, and hybridized with labeled mac-NOS cDNA. Size markers (in kilobases) are given at left. (*B*) RAW 264.7 macrophages were stimulated with LPS and interferon γ. Macrophage homogenates were assayed for catalytic NOS activity as described.

time points (data not shown). Interestingly, Ca^{2+}-treated samples consistently displayed some reduction of NOS activity compared to samples treated with NADPH in the absence of Ca^{2+}. Conceivably this reflects the influence of Ca^{2+}-dependent proteases.

To explore mac-NOS regulation in peripheral tissues, we treated mice with LPS and monitored NOS catalytic activity. Six hours after LPS administration, mac-NOS activity was markedly enhanced in liver and spleen in a calcium-independent fashion, moderately induced in brain, but not in lung, heart, and kidney (data not shown). To visualize mac-NOS mRNA we conducted *in situ* hybridization. In resting RAW macrophage cells *in vitro*, we detected no mac-NOS signal. However, 6 hr after LPS and interferon γ treatment, extremely high levels of mRNA were evident (data not shown). Uninduced spleen possesses negligible mac-NOS mRNA, but after LPS treatment of mice, the mRNA signal was pronounced (Fig. 4). The mRNA levels were most abundant in the red pulp. However, substantial levels were also evident in the central area of the white pulp as well. Red pulp contains the highest levels of macrophages in the spleen

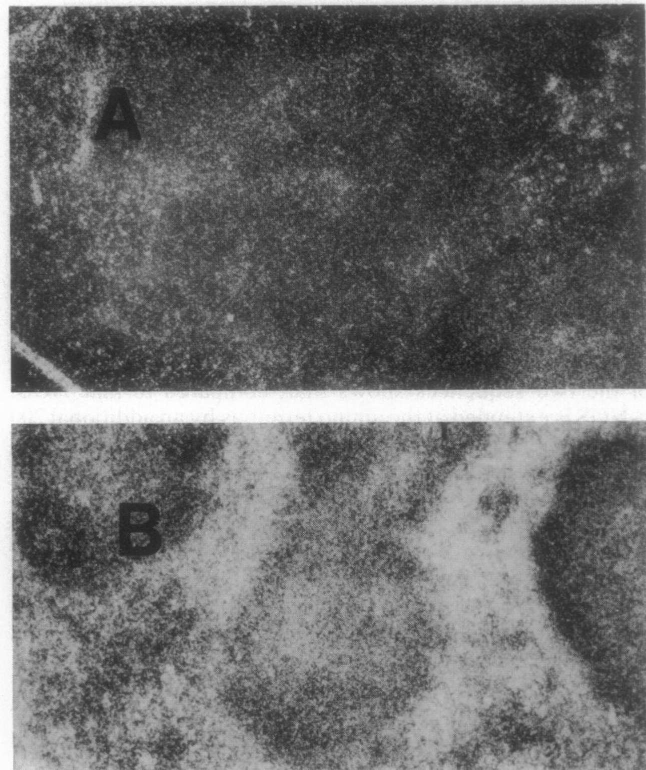


FIG. 4. mac-NOS mRNA is induced in spleens of LPS-activated mice. *In situ* hybridization with antisense mac-NOS RNA probes was performed on sections of spleens harvested from control (*A*) and LPS-activated (*B*) BALB/c mice. (Darkfield photographs; ×10.)

(50, 51). In the absence of LPS induction, macrophages are restricted to the red pulp and outer margin of the white pulp (52). However, following stimulation of the immune system, macrophages migrate to the central portion of the white pulp to present antigen to the T cells, which are localized in this zone of white pulp (53). Thus, the localization of mac-NOS mRNA to the central portion of the white pulp and the red pulp fits with the known distribution of macrophages in stimulated spleen.

DISCUSSION

Cloned and expressed mac-NOS displays both similarities and differences from n-NOS. Both enzymes contain recognition sites for FAD, FMN, and NADPH with closely similar sequences. Both of them resemble CPR. Both also possess a consensus site for phosphorylation by cAMP-dependent protein kinase. Moreover, mac-NOS contains a calmodulin consensus binding site as does n-NOS, although its activity is Ca^{2+} independent. mac-NOS is shorter than n-NOS, due primarily to the lack of about 200 amino acids at the amino terminus, 15 amino acids at the carboxyl terminus, and a 40-amino acid deletion in the interior of the molecule.

A striking difference between n-NOS and mac-NOS has to do with the tissue expression of mRNA for the two enzymes. n-NOS mRNA is abundantly expressed in the brain; negligible levels are detected by Northern blot analysis in numerous peripheral tissues (data not shown). By contrast, we fail to detect mac-NOS mRNA in brain tissue. The inducible character of mac-NOS is evident in the pronounced enhancement of mRNA in liver and spleen following LPS treatment of mice. Interestingly, inducible, calcium-independent NOS activity has been demonstrated in endothelial cells and hepatocytes, indicating that mac-NOS may not be restricted to macrophages (54, 55). Whether mac-NOS mRNA that we

detect in liver occurs in Kupffer cells, the liver equivalent of macrophages, or in hepatocytes awaits *in situ* hybridization studies. Strikingly, we observe an enhancement of EDTA-resistant NOS catalytic activity in brain following LPS treatment. Our failure to detect mac-NOS mRNA in the brain by Northern analysis may reflect the anticipated lower levels compared to those of peripheral tissues. In the brain, microglia functionally resemble macrophages and conceivably might be the source of mac-NOS activity stimulated by LPS. By implication, NO production in the brain increases following certain bacterial infections and possibly other inflammatory stimuli, so that the brain sequelae play a role in the pathophysiology of these conditions.

Our *in situ* hybridization experiments revealed a selective enrichment of mac-NOS mRNA in the center of splenic white pulp in endotoxin-treated mice. This presumably reflects a migration of macrophages associated with immune stimulation. Monitoring mac-NOS mRNA localizations may facilitate delineation of the physiology of macrophages and related cell types throughout the body. For instance, in the brain during disease states such as human immunodeficiency virus infection, microglia may function as macrophages, or peripheral macrophages may penetrate the blood–brain barrier (56, 57). Studies of mac-NOS gene expression may clarify the role of microglia–macrophages in this and other conditions.

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