Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures
(t-arginine/astrocytes/endotoxin/cytokines/microglia)

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ABSTRACT Exposure of primary cultures of neonatal rat cortical astrocytes to bacterial lipopolysaccharide (LPS) results in the appearance of nitric oxide synthase (NOS) activity. The induction of NOS, which is blocked by actinomycin D, is directly related to the duration of exposure and dose of LPS, and a 2-hr pulse can induce enzyme activity. Cytosol from LPS-treated astrocyte cultures, but not from control cultures, produces a Ca2+-independent conversion of L-arginine to L-citrulline that can be completely blocked by the specific NOS inhibitor N2-monomethyl-L-arginine. The induced NOS activity exhibits an apparent K_m of 16.5 μM for L-arginine and is dependent on NADPH, FAD, and tetrahydrobiopterin. LPS also induces NOS in C6 glioma cells and microglial cultures but not in cultured cortical neurons. The expression of NOS in astrocytes and microglial cells has been confirmed by immunocytochemical staining using an antibody to the inducible NOS of mouse macrophages and by histochemical staining for NADPH diaphorase activity. We conclude that glial cells of the central nervous system can express an inducible form of NOS similar to the inducible NOS of macrophages. Inducible NOS in glia may, by generating nitric oxide, contribute to the neuronal damage associated with cerebral ischemia and/or demyelinating diseases.

The enzyme nitric oxide synthase (NOS), which catalyzes the biosynthesis of nitric oxide from L-arginine, exists in two principal isoforms differing with respect to Ca2+-dependency, tissue distribution, and regulation of expression. The constitutive form of NOS is a Ca2+-calmodulin-dependent enzyme expressed in numerous tissues (1). The Ca2+-dependence of this isotype allows it to be rapidly activated by biological signals which increase intracellular Ca2+ (2). The inducible form of NOS is not regulated by Ca2+ and is synthesized de novo in cells closely allied to the immune-cellular defense systems, including macrophages (3, 4) and neutrophils (5, 6). NO generated by inducible NOS appears to mediate some bactericidal and tumoricidal actions of activated cells (7).

In brain NO is synthesized by a constitutive form of NOS that is localized primarily to neurons (8-10) and possibly to astrocytes (11, 12). Astrocytes share several properties with macrophages, including the capacity to synthesize class II major histocompatibility antigens (13) and cytokines (14) and to respond to treatment with bacterial lipopolysaccharide (LPS) (15). Microglial cells present in brain exhibit macrophage-like properties (16) and hence are also cells in which LPS might elicit a response. These considerations raise the question of whether astrocytes and/or microglia can express inducible NOS. We report here that exposure of primary cultures of rat cortical astroglial cells to LPS will stimulate the appearance of NOS in astrocytes as well as in brain-derived microglial cells.

MATERIALS AND METHODS

Materials. Recombinant human tumor necrosis factor α (TNF-α, 2 × 107 units/mg) was from Genzyme. Calmodulin, LPS (Salmonella typhimurium), biotin-labeled Ricinus communis RCA-1 lectin (RCA120), and N2-monomethyl-L-arginine were from Sigma. Rhodamine-labeled RCA-1 lectin was from Vector Laboratories. Rabbit anti-bovine glial fibrillary acidic protein (GFAP) was from Dako. LPS was from E. coli 055:B5 (Sigma) or the lipopolysaccharide of E. coli 0111:B4 (Difco).

Cell Culture. Primary astrocyte cultures were prepared from the cerebral cortex of 1-day-old neonatal rats (17). At confluence the cells were shaken at 200 rpm for 16 hr at 37°C to dislodge cells adhering to the astrocyte layer, mainly oligodendrocytes (18). The medium was replaced, and the cells were allowed to recover for 1 day before experiments. In some experiments, the shaking procedure was omitted to allow the microglial content to increase. Secondary astrocyte cultures were obtained by mild trypsinization (0.1% trypsin for 5 min at 37°C) of primary cells, with replating at 5 × 105 cells per 35-mm plate. These cells were used for experiments after 3–4 days, at which point they had reached confluency.

Microglia cultures were prepared by continuously shaking 10-day-old primary astrocyte cultures overnight in serum-free medium, collecting detached cells, and replating them into 6-well plates at a density of 50,000 cells per well. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum until use.

Primary rat cortical neuronal cultures were prepared as described (19). The rat RT4-D6 cell line, an ethynilnitrosourea-induced schwannoma, was a gift of Noburo Sueoka (20). The rat C6 glioma cell line and PC12 pheochromocytoma cell line were obtained from the American Type Culture Collection.

For all experiments, the cell culture medium was removed, and then test drugs (or controls) were added in fresh DMEM for the desired incubation times.

Preparation of Cytosolic and Membrane Fractions. After a 24-hr incubation in the presence or absence of LPS (500 ng/ml), cells were harvested and homogenized in 50 mM Tris-HCl, pH 7.8/320 mM sucrose/1 mM EDTA/1 mM 3-mercaptopropanol/0.2 mM benzamidine/10 μM pepstatin A containing chymostatin (10 μg/ml) and phenylmethylsulfonyl fluoride (10 μg/ml) (buffer A). The protein content of this homogenate was determined as total cell Da protein. The homogenate was centrifuged at 100,000 × g for 30 min at 4°C, and the supernatant was dialyzed (cutoff of 12 kDa) 2 hr against 1 liter of 50 mM Tris-HCl, pH 7.8/1 mM 3-mercaptopropanol/0.2 mM benzamidine (buffer B); the buffer was replaced once during dialysis. To obtain a membrane fraction, the pellet from the first centrifugation was resuspended in buffer A and centrifuged a second time at 100,000 × g for

Abbreviations: NOS, nitric oxide synthase; LPS, lipopolysaccharide; GFAP, glial fibrillary acidic protein; TNF, tumor necrosis factor.

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30 min at 4°C. Pelleted membranes were then suspended in buffer B. Sample aliquots were stored at −80°C.

Measurement of NOS Activity. Accumulated nitrate levels were determined by mixing 100 μl of cell culture medium with 100 μl of Griess reagent (21), incubating the mixture at room temperature for 15 min, and then determining the absorbance at 570 nm in a microplate reader (MR6000, Dynatech). Fresh culture medium served as the blank, and solutions of NaNO2 diluted in culture medium were used as standards.

NOS activity was also measured by production of L-[14C]citrulline from L-[14C]arginine (22). In these experiments, 50 μl of sample, corresponding to ~100 μg of total cellular protein, was incubated at 37°C in the presence, unless otherwise indicated, of 50 mM Tris-HCl (pH 7.8), 0.5 mM NADPH, 5 μM FAD, 5 μM tetrahydrobiopterin, 20 μM L-arginine, and 50 nCi of L-[14C]arginine (305 mCi/mmol; Amersham; 1 Ci = 37 GBq) in a total volume of 100 μl. The reactions were halted by the addition of 1.25 ml of ice-cold 20 mM Hepes (pH 5.5), and the total volume was applied to a Dowex-50W column pre-equilibrated with the same buffer. L-[14C]Arginine was retained on the column, whereas L-[14C]citrulline was recovered in the effluent and its concentration was determined by liquid scintillation counting.

Immunostaining. Primary astrocyte cultures on glass slides were incubated for 24 hr with or without LPS (500 ng/ml). The cells were washed with phosphate-buffered saline (PBS), fixed 1 hr at room temperature in 4% paraformaldehyde, washed with PBS, and incubated with primary antibodies (1:1000 dilution in the presence of 3% goat serum) for 16 hr at 4°C. To detect astrocytes, rabbit anti-bovine GFAP polyclonal serum was used (23), and for NOS, a rabbit antimacrophage NOS serum (a gift of Q.-W. Xie, H. J. Cho, and C. Nathan) that specifically detects the inducible form of NOS (24). Microglia were visualized by incubating fixed cells with rhodamine- or biotin-labeled RCA-1 (10 μg/ml) for 1 hr at room temperature (25). After removal of the primary antibody or biotin-labeled lectin, cells were visualized by the peroxidase-antiperoxidase method using diaminobenzidine as chromogen (Vector Laboratories). Slides were counterstained with thionin. For each treatment cells were counted and scored in 7–10 different fields of view at ×40 magnification.

RESULTS
Effects of LPS on NOS Activity in Primary Glial Cultures. Incubation of primary cultures of neonatal rat cortical astrocytes with LPS (500 ng/ml) led to the accumulation of nitrates in the culture medium (Fig. 1A). Nitrite was first detected between 6 and 8 hr of continuous exposure to LPS and continued to increase linearly up to 48 hr, the longest period tested. Accumulation was 9-fold and 15-fold greater than basal levels at 24 and 48 hr, respectively.

The response to LPS was dose-dependent, with 50% of maximal nitrite accumulation occurring at a LPS dose of ~50

**Table 1. Characteristics of LPS-induced NOS activity from primary cultures**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (μM)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>$K_m$ (L-arginine)</td>
<td>16.5 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>$V_{max}$ (pmol of citrulline per min per mg)</td>
<td>210.0 ± 5.0</td>
<td>4</td>
</tr>
<tr>
<td>IC$_50$ (L-NMMA), μM</td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td>Relative activity, %</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Complete reaction mix</td>
<td>0.6 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>-NADPH</td>
<td>59.3 ± 1.5</td>
<td>4</td>
</tr>
<tr>
<td>-FAD</td>
<td>59.1 ± 1.8</td>
<td>4</td>
</tr>
</tbody>
</table>

Data are means ± SEM from n separate experiments, except for IC$_{50}$, which is the mean from two experiments, each done in duplicate. Activities were obtained by measuring the conversion of radiolabeled l-arginine to l-citrulline by a dialyzed cytosolic preparation of astrocyte cultures treated for 24 hr with LPS (500 ng/ml). Reactions were carried out for 15 min at 37°C.

*IC$_{50}$ for N$^\text{O}$-monomethyl-L-arginine (L-NMMA) was determined in the presence of 20 μM L-arginine.
Table 2. Cytological specificity of NOS activity

<table>
<thead>
<tr>
<th>Assay</th>
<th>Activity, nmol/mg of total cell protein</th>
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<tbody>
<tr>
<td>L-Citrulline production</td>
<td></td>
</tr>
<tr>
<td>LPS-treated astrocyte cytosol</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>Control astrocyte cytosol</td>
<td>nd</td>
</tr>
<tr>
<td>Control astrocyte membrane</td>
<td>nd</td>
</tr>
<tr>
<td>Nitrite accumulation</td>
<td></td>
</tr>
<tr>
<td>Primary astrocyte culture</td>
<td>88.2 ± 15.0</td>
</tr>
<tr>
<td>Secondary astrocyte culture</td>
<td>nd</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>nd</td>
</tr>
<tr>
<td>RT4-D6 schwannoma</td>
<td>nd</td>
</tr>
<tr>
<td>Primary neuronal culture</td>
<td>nd</td>
</tr>
<tr>
<td>C6 glioma</td>
<td>nd</td>
</tr>
<tr>
<td>C6 glioma plus TNF-α*</td>
<td>30.6 ± 1.4</td>
</tr>
</tbody>
</table>

NOS activity was assessed as the conversion of L-arginine to L-citrulline assayed in standard reaction mixture for 30 min in the presence of Ca²⁺ (10 μM) and calmodulin (10 μg/ml), or as the accumulation of nitrites in the cell culture medium measured after 24 hr of incubation with LPS (500 ng/ml). Data are expressed as the mean ± SEM. All experiments were done in triplicate, nd, Not detected. Nitrite accumulation was assayed at cell protein concentrations of 0.5–1.0 mg/ml for tumor cell lines and 100–150 μg/ml for others. Limits of detection were 1 pmol of L-citrulline and 1 μM nitrite.

*Recombinant human TNF-α was present at 0.1 μg/ml during the entire 24 hr. TNF-α alone had no effect.

ng/ml (when measured after 24 hr of incubation) (Fig. 1B). Maximal accumulation occurred at LPS concentrations of >500 ng/ml.

Characteristics of NOS Induced in Glial Cells After Exposure to LPS. The kinetics and substrate requirements of NOS induced in glial cells by LPS treatment were determined by measuring the conversion of L-[14C]arginine to L-[14C]citrulline by a dialyzed cytosolic fraction. The accumulation of L-[14C]citrulline was linear for up to 30 min, completely blocked by addition of the specific NOS inhibitor N⁶G-monomethyl-L-arginine, and not affected by the addition of 10 μM CaCl₂ (Fig. 2). Enzyme activity was slightly reduced by the presence of 1 mM EGTA (86 ± 2.7% versus no EGTA, n = 3). The enzyme activity had an apparent Kₘ for L-arginine of 16.5 μM and a Vₘₐₓ of 210 pmol per min per mg of protein, was dependent upon the presence of NADPH, and was enhanced by the addition of tetrahydrobiopterin or FAD (Table 1). N⁶G-Monomethyl-L-arginine was a potent inhibitor of NOS, having an IC₅₀ value of 1.6 μM when assayed in 20 μM L-arginine (Table 1).

NOS activity was not detectable in cytosol of untreated cells even though Ca²⁺ (10 μM) and calmodulin (10 μg/ml) were added to the reaction mixture (Table 2). Nor could enzyme activity be detected in membranes prepared from untreated cells, ruling out the presence of a particulate form of constitutive NOS in these cells (27). Thus LPS induces a form of NOS comparable to the Ca²⁺-independent inducible form of the enzyme characteristic of activated macrophages (24).

Cytological Specificity of the Response. Cellular localization. In both LPS-treated and untreated primary cultures, >95% of the cells could be identified as astrocytes as defined by GFAP staining (Fig 3 a and c), and 3–5% of the cells were identified as microglia by staining with rhodamine- or bioin-labeled lectin RCA-1 (25). Microglial content was not increased by LPS treatment. Immunostaining with an antibody to the inducible NOS of mouse macrophages (24) revealed that after 24 hr of exposure to LPS (500 ng/ml), the majority of cells were weakly stained and only 15–20% of the cells stained intensely (Fig. 3d). Control cells did not show any staining for NOS (Fig. 3b). Since microglia account for up to 5% of the cells, these results demonstrate that a subpopulation of astrocytes can be induced to express NOS.

Expression of NOS in glial cells was also detected by staining primary cultures for NADPH diaphorase activity (28). In untreated cultures, an occasional cell exhibited diaphorase staining. After LPS treatment, many cells contained the blue reaction product, including cells with typical astrocyte appearance, as well as numerous microglia (Fig. 4A). Subsequent immunostaining for GFAP resulted in conversion of the blue-colored formanzan product to black in cells

![Fig. 3.](image-url) Immunochemical localization of NOS in astrocyte cultures. Primary astrocyte cultures were plated directly onto glass slides and maintained for 10 days. The cells were then incubated for 24 hr in 1 ml of medium alone (a and b) or containing LPS (500 ng/ml) (c and d). The cells were then processed for immunostaining using either rabbit anti-GFAP serum (a and c) or rabbit anti-NOS serum (b and d). (x 100.)
having astrocyte morphology (Fig. 4B). Other cells (presumably microglia) were observed which had stained strongly for diaphorase activity (Fig. 4C) but were not stained for GFAP, and thus the blue formazan color was preserved. Thus, cytochemical studies indicate that LPS induces NOS expression in both astrocytes and microglia.

Relative contribution of microglial NOS activity. To estimate the relative contributions of microglial and astrocytic induced NOS to the total observed activity, we examined the effects of LPS on microglial cultures, and on primary astrocyte cultures in which the microglia content was 10-fold that of normal levels.

Following exposure to LPS (500 ng/ml, 24 hr), nitrite levels in microglial cultures reached 240 nmol/mg of protein, 3-5 times greater than that determined for primary astrocyte cultures (50-80 nmol/mg of protein). This suggests that in primary astrocyte cultures, microglial NOS activity contributes significantly (perhaps 25%) but not entirely to the total observed activity. In astrocyte cultures containing 31% microglial content (achieved by omission of the overnight shaking step) rather than the typical 3-5%, treatment with LPS (500 ng/ml, 24 hr) resulted in ~2-fold greater NOS activity compared with that found in normally prepared astrocyte cultures (100 ± 12 vs. 52 ± 1, nmol of nitrite per mg of protein, mean ± SD, n = 3). Thus, a relatively large increase in microglial content led to a much smaller increase in NOS activity, supporting the premise that both astrocytic and microglial NOS activities contribute significantly to the total observed activity.

Neurons and Related Cell Lines. In contrast to primary astrocyte cultures, primary cultures of cortical neurons did not display LPS-inducible NOS activity as measured by nitrite accumulation (Table 2). Furthermore, secondary astrocyte cultures and three different clonal cell lines were also found to be unresponsive to LPS treatment. However, in C6 glioma cells simultaneous exposure to LPS and TNF-α resulted in nitrite accumulation that was about one-third of that obtained with primary astrocyte cultures (Table 2). Thus, cells of an astrocyte-derived tumor line are also capable of expressing an inducible form of NOS, in agreement with previous findings (29).

Effects of Actinomycin D on LPS Induction of NOS Activity. To assess whether the increased accumulation of NOS depended upon de novo transcription, cultures were incubated with LPS in the presence of the transcriptional inhibitor actinomycin D. Over 92% of the nitrite accumulation elicited by LPS exposure (500 ng/ml, 24 hr) was blocked when actinomycin D (1 μg/ml) was present throughout the incubation period (96.8 ± 4.7 vs. 9.0 ± 0.9 nmol of nitrite per mg of protein, mean ± SEM, n = 3). A short pulse of LPS (2 hr, followed by 22 hr in the absence of LPS) resulted in nitrite accumulation (33.9 ± 3.8 nmol/mg of protein) that was 35% of that obtained by 24 hr of continuous exposure. When actinomycin was present during the 2-hr pulse, there was no subsequent detectable nitrite accumulation. These results indicate that LPS induction of NOS activity occurs at the level of gene transcription and that a brief exposure to LPS is sufficient to initiate NOS expression.

**DISCUSSION**

In the present study we have demonstrated that exposure of primary cultures of glial cells obtained from the cerebral cortex of neonatal rats to LPS increases the activity of NOS. Enzyme activity is detectable after a latency of 6-8 hr and is directly dependent upon the dose and duration of exposure to the stimulus. Glial NOS shares many of the properties of the inducible form of the enzyme in macrophages (24): both are cytoplasmic; are dependent upon NADPH, FAD, and tetrahydrobiopterin for full activity; are competitively inhibited by N-monomethyl-L-arginine; and do not depend upon Ca2+ for activity. The fact that the LPS-stimulated, but not control,
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Note Added in Proof. After acceptance of this manuscript for publication, a report also describing induction of NOS-like activity in primary astrocyte cultures appeared (33).