Role of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 in Endotoxin-Induced Cerebral Hyperemia

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Background and Purpose—Bacterial lipopolysaccharide (LPS), an endotoxin, has been reported to induce the expression of inducible isoforms of both nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) in various cell types. LPS is also known to dilate systemic vasculature, including cerebral vessels. This study aimed to determine to what extent LPS induces iNOS and COX-2 expression in the brain and whether NO and/or cyclooxygenase metabolites derived from iNOS and/or COX-2 contribute to the LPS-induced cerebral hyperemia.

Methods—Regional cerebral blood flow (rCBF) was measured by laser-Doppler flowmetry in halothane-anesthetized, artificially ventilated rats for 4 hours after intracerebroventricular administration of LPS.

Results—LPS at doses of 0.01 mg/kg to 1 mg/kg caused dose-dependent, progressive increases in rCBF at 1 to 4 hours after administration. The increase in rCBF was attenuated by systemic administration of the selective iNOS inhibitor aminoguanidine (100 mg/kg IP) or the selective COX-2 inhibitor NS-398 (5 mg/kg IP), and it was abolished by preventing induction of these isoforms with dexamethasone (4 mg/kg IP). LPS significantly increased iNOS and COX-2 mRNA, iNOS protein, and iNOS and cyclooxygenase enzyme activity. The increases in iNOS and cyclooxygenase enzyme activity were eliminated by aminoguanidine and NS-398, respectively. Dexamethasone also prevented the increase in iNOS and cyclooxygenase activity.

Conclusions—These results indicate that induction of iNOS and COX-2 expression and the increased production of NO and vasodilator prostanoids in the brain contribute to the elevation in CBF after intracerebroventricular administration of LPS. (Stroke. 1998;29:1209-1218.)

Key Words: cerebral blood flow • endotoxins • lipopolysaccharides • nitric oxide synthase • prostaglandins • rats

Background
Bacterial LPS, an endotoxin, has been reported to induce expression of inducible isoforms of NOS (iNOS, type II NOS) in various cell types such as macrophages, vascular smooth muscle cells, astrocytes, and neurons. An elevated production of NO secondary to an increase in iNOS expression has been suggested to play an important role in the hyperemic effect of LPS in many vascular beds, including that of the brain. An isoform of prostaglandin synthase (cyclooxygenase), PGS-2 (COX-2), is also induced in fibroblasts, neurons, and astrocytes after administration of LPS, and studies using enzyme inhibitors have suggested that induction of both iNOS and COX-2 may contribute to LPS-induced increases in CBF. Most of the previous studies have utilized systemic administration of LPS. Some of these studies were unable to document elevated expression of iNOS and COX-2 mRNA in the brain because of a limited ability of LPS to cross the blood-brain barrier. Moreover, systemic administration of LPS produces hypotension, which complicates interpretation of the effects of LPS on CBF. Therefore, in the present study we sought to determine (1) whether direct intracerebroventricular administration of LPS increases rCBF and induces expression of iNOS and/or COX-2 in the brain and (2) whether the cerebral hyperemia produced by LPS is prevented by blocking the induction of iNOS and COX-2 in the brain with dexamethasone or by selective inhibitors of iNOS and COX-2, aminoguanidine and NS-398, respectively. To this end, we measured rCBF by laser-Doppler flowmetry after intracerebroventricular administration of LPS. Western blot analysis, RT-PCR, and enzyme assays were used to examine the expression of iNOS and COX-2 mRNA and protein levels in the brain.

Materials and Methods
All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care Committee of the Medical College of Wisconsin.

General Surgical Procedures
Experiments were performed in male Sprague-Dawley rats weighing between 265 and 350 g. Anesthesia was induced by intraperitoneal sodium pentobarbital (60 mg/kg, Sigma Chemical Co). The animals were tracheostomized, paralyzed with pancuronium bromide (1 mg/kg IP), and artificially ventilated (SAR-830, CWE) with 30% O₂ in N₂. After all surgery was completed, anesthesia was maintained by...
inhalation of 0.6% halothane (Anaquest Inc). Body temperature was maintained at 37 ± 1°C with the use of a water-circulated heating pad. One of the femoral arteries was cannulated to facilitate the measurement of arterial pressure and arterial blood gases. Arterial PaO2, PaCO2, and pH were measured with a blood gas/H2 analyzer (ABL-300, Radiometer). Arterial blood pressure, end-tidal carbon dioxide tension, inspired and expired oxygen, and halothane concentrations were continuously monitored (POET II, Criticare Systems, Inc) and recorded on an eight-channel polygraph recorder (Astro-Med, Inc). As previously reported, a 30-gauge stainless steel cannula (HTX-30, Small Parts) was placed into the left lateral ventricle for intracerebroventricular injection with the bregma chosen as the stereotaxic point (anteroposterior, −0.3 mm; lateral, +1.2 mm; dorsoventral, −4.5 mm). Intracerebroventricular infusions were performed at the rate of 1 μL/min with the use of a microinfusion pump (model 55-2222, Harvard Apparatus) with a microsyringe (25 μL, Hamilton) fitted with polyethylene tubing (PE-10). Preliminary studies demonstrated that Evan’s blue dye injected by this route was oxygenated, and pH was adjusted to 7.4. 14 Rats in group 2 (n = 1 rats) received intracerebroventricular LPS (Ecoli, 055:B5, Sigma) in doses of 2 μg/mg/kg. In group 4 (n = 6), the rats received a COX-2–selective inhibitor, NS-398 (5 mg/kg, BIOMOL), intraperitoneally 2 hours before and 2 hours after intracerebroventricular administration of LPS (1 mg/kg). In group 5 (n = 5), the rats received dexamethasone (4 mg/kg IP, Sigma) 4 hours before and immediately after intracerebroventricular administration of LPS (1 mg/kg) to prevent induction of iNOS and COX-2. In each experimental animal, rCBF was continuously monitored for 4 hours after intracerebroventricular injection of LPS. Amino guanidine, NS-398, and dexamethasone were dissolved in 1 mL of peanut oil with the aid of a sonicator. The doses of amino guanidine or NS-398 were the same as those used to inhibit iNOS or COX-2 selectively in previous studies without affecting constitutive enzyme activities,13,14 and we previously reported that the injection of a peanut oil alone has no effect on rCBF.14 Immediately after the experiments, the brains of rats were rapidly removed, the pial vessels were removed, and cerebral cortical tissue was frozen in liquid nitrogen and stored at −80°C until iNOS and COX-2 mRNA and protein levels and iNOS and cyclooxygenase activities were measured.

Expression of iNOS and COX-2 mRNAs (RT-PCR)
RNA was isolated from the cerebral cortex with the use of TRizol reagent (GIBCO BRL). The concentration of RNA in each sample was measured by a spectrophotometer at a wavelength of 260 nm (Gene quant, Pharmacia). The RNA underwent RT by incubation of 1 μg RNA for 40 minutes at 42°C with 2.5 U/μL MuLV reverse transcriptase with 2.5 μmol/L random hexamers, 1 mmol/L dNTP, 5 mmol/L MgCl2, and 1 U/L RNase inhibitor (GeneAmp, PerkinElmer) in a volume of 10 μL. The entire RT reaction was amplified by PCR in a 50-μL reaction containing 2 mmol/L MgCl2, 1.25 U Taq DNA polymerase, and 0.2 μmol/L of specific primers for iNOS, COX-2, or GAPDH, as a control for the RT reaction. The sequences of the primers used (Operon) have been reported previously17,18 and were as follows: iNOS forward, 5′-AACAAGTGGGAAACACCCAGGTGG-3′; iNOS reverse, 5′-ACAGTCCGGGCCATCGAA-GACC-3′; COX-2 forward, 5′-GAAGTTGGGGTTTGAAGACATC-3′; COX-2 reverse, 5′-CCTTTACTTCTTGGAATAAACA-3′; GAPDH forward, 5′-CACCAGGAAATCTGCAATGGCAC-3′; GAPDH reverse, 5′-GATTGTAGGGAGAGGTCTC-3′. The primers chosen amplify across several large interspersed introns to avoid the possibility of amplification of genomic DNA. The iNOS reactions were cycled at 96°C for 30 seconds, 65°C for 60 seconds, and 72°C for 90 seconds and yielded a single band corresponding to a 565-bp cDNA fragment. The COX-2 reactions were cycled at 96°C for 30 seconds, 60°C for 60 seconds, and 72°C for 90 seconds and yielded a single band corresponding to a 381-bp cDNA fragment. The GAPDH reactions were cycled under the same conditions as iNOS or COX-2 and produced a single band corresponding to a 970-bp cDNA fragment. RNA extracted from spleen of rats treated with LPS (10 mg/kg IP) was used as a positive control expression of iNOS or COX-2 mRNA. Twenty microliters of the RT-PCR reactions was electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining under UV light. The ratios of the intensities of iNOS or COX-2 to GAPDH bands were assessed by a fluororimeter (Vistra) and normalized with the intensity of GAPDH band, as previously reported19.

Experiments were also performed to verify that the RT-PCR reactions were linear under the present experimental conditions and could be used for semiquantitative comparisons of the amount of iNOS and COX-2 mRNA in the samples. In these experiments, various amounts of RNA (0.25 to 2 μg) extracted from the brain of an LPS-treated rat were added to RT-PCR reactions for iNOS, COX-2, and GAPDH. The products were separated on a 2% agarose gel stained with ethidium bromide, and the relative intensities of the bands were compared with the fluororimeter.

Cloning and Sequencing of Rat Brain iNOS and COX-2 PCR Products
The specificity of the RT-PCR reactions was verified by cloning and sequencing the 385-bp band amplified by the COX-2 primers and the
565-bp product amplified by the iNOS primers. PCR products were excised from the agarose gels and purified with the use of a dialysis membrane (Geno Technology). Purified PCR products were ligated into the pCRII vector (Invitrogen). Then 250 ng purified PCR product was added to a 10-μL ligation reaction containing 6 mmol/L Tris (pH 8.3), 5 mmol/L NaCl, 6 mmol/L MgCl₂, 5 mmol/L dNTPs, 0.1 mg/mL BSA, 7 mmol/L β-mercaptoethanol, 0.1 mmol/mL ATP, 2 μmol/L dithiothreitol, 1 mmol/L spermidine, 30 ng vector, and 4 U of T4 DNA ligase (Invitrogen). The reactions were incubated at 14°C for 16 hours. Escherichia coli strain TOP10F’ (50 μL) was transformed by heat shock with 2 μL ligation reaction. The cells were plated in 1 mL or 250 μL super optimal catablysate medium and incubated at 37°C for 1 hour, then plated on lauria broth agar plates with 50 mg/mL ampicillin. Colonies were grown in 10 mL lauria broth medium with 50 μg/mL ampicillin overnight at 37°C. Plasmid DNA was then extracted with the use of alkaline lysis and silica-gel membrane-based purification (Qiagen), resuspended in 10 mM Tris EDTA buffer (pH 7.4), and stored at 4°C.

Sequencing of plasmid DNA was performed by the dideoxy chain termination method with the use of an ABI model 377 sequencer. The plasmid DNA was digested with HindIII and EcoRI, and the fragments were analyzed by gel electrophoresis. The DNA was isolated from the gel, and the fragments were ligated into the pCRII vector (Invitrogen). Then 250 ng purified PCR product was added to a 10-μL ligation reaction containing 6 mmol/L Tris (pH 8.3), 5 mmol/L NaCl, 6 mmol/L MgCl₂, 5 mmol/L dNTPs, 0.1 mg/mL BSA, 7 mmol/L β-mercaptoethanol, 0.1 mmol/mL ATP, 2 μmol/L dithiothreitol, 1 mmol/L spermidine, 30 ng vector, and 4 U of T4 DNA ligase (Invitrogen). The reactions were incubated at 14°C for 16 hours. Escherichia coli strain TOP10F’ (50 μL) was transformed by heat shock with 2 μL ligation reaction. The cells were plated in 1 mL or 250 μL super optimal catablysate medium and incubated at 37°C for 1 hour, then plated on lauria broth agar plates with 50 mg/mL ampicillin. Colonies were grown in 10 mL lauria broth medium with 50 μg/mL ampicillin overnight at 37°C. Plasmid DNA was then extracted with the use of alkaline lysis and silica-gel membrane-based purification (Qiagen), resuspended in 10 mM Tris EDTA buffer (pH 7.4), and stored at 4°C.

Expression of iNOS and COX-2 Proteins

(Western Blot Analysis)

After careful removal of the pial vessels, the cerebral cortex was homogenized and centrifuged at 3000g for 5 minutes and 9000g for 5 minutes at 4°C. The concentration of protein was determined with the use of the Bio-Rad Protein Assay system (Bio-Rad Laboratories). An aliquot of protein (20 μg) was separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel (150 V for 100 minutes) and transferred to a nitrocellulose membrane (100 V for 60 minutes). After transfer, nonspecific binding was blocked by incubation in 10% nonfat dry milk in Tris-buffered saline solutions (50 mmol/L Tris HCl, 0.25 mol/L NaCl, 0.08% Tween 20, Sigma) followed by a 2-hour incubation at room temperature with monoclonal antibody for iNOS (1:2000 dilution, Transduction Laboratories) or polyclonal antibody for COX-2 (1:1000 dilution, Cayman Chemical Corp). The antibody for iNOS cross-reacts with nNOS. The bands at molecular weights of 131 and 155 kDa correspond to iNOS and nNOS, respectively. The membranes were incubated with a 1:1000 horseradish peroxidase–labeled secondary antibody (Bio-Rad). Immunoblots were detected by chemiluminescence (ECL, Amersham) on x-ray film, and optical density was scanned by a scanning laser densitometer (Vistra). LPS-stimulated murine macrophage lysate (Transduction Laboratories) was used as a positive control (39 kd) for equal loading, and the optical density ratio of iNOS and COX-2 bands to that of β-actin was used to compare steady state levels of the various proteins.

Measurement of the Brain Calcium-Independent (iNOS Activity)

Calcium-independent (iNOS) activity was measured by the conversion of [3H]l-arginine to [3H]l-citrulline by the high-performance liquid chromatography method originally described by Carlberg. Cerebral cortical tissue was homogenized in 20 mmol/L HEPES buffer (pH 7.4). After the homogenate was centrifuged twice at 9000g for 10 minutes at 4°C, aliquots of homogenate (150 μg protein) were incubated with [3H]l-arginine (0.2 μCi, 20 μmol/L, Amersham) in 100 μL of 20 mmol/L HEPES-calcium-free buffer containing 0.5 mmol/L EGTA, 1 mmol/L NADPH, 2.5 μmol/L flavin adenine dinucleotide, 1 μmol/L flavin mononucleotide, and 100 μM tetrahydrobipterin for 5 minutes at 37°C. The reactions were stopped by adding 50 μL of 20 mmol/L EDTA solution (pH 5.5) and frozen in liquid nitrogen. Products were separated by reverse-phase high-performance liquid chromatography on an LC-18 DB column (Supelco). Products were monitored with an on-line radioactive flow detector (A-100, Radiomatic Instruments). Results were expressed as picomoles citrulline produced per milligram protein per minute. All chemicals used in the iNOS assay except [3H]-arginine were purchased from Sigma.

In preliminary experiments, we compared the conversion of l-arginine to l-citrulline in cerebral homogenates prepared from a control rat in the presence or absence of 0.5 mmol/L EGTA in the reaction. Addition of 0.5 mmol/L EGTA to the reactions reduced the conversion rate by 50-fold to levels that were not significantly different from the blank samples. Therefore, this concentration of EGTA included in the reactions was sufficient to completely block calcium-dependent NOS catalytic activity in control brain homogenates and allowed for the selective measurement of calcium-independent conversion.

PGE₂ Levels by Enzyme Immunoassay

(Cyclooxygenase Activity)

Cyclooxygenase activity was assessed by measuring concentration of PGE₂ with the use of an enzyme immunoassay (Cayman Chemical).
Time Courses of MAP, Heart Rate, Arterial pH, and Blood Gases in Five Experimental Groups of Rats

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AG indicates aminoguanidine; DX, dexamethasone. Values are mean±SD. *P<0.05 vs 0-hour values; †P<0.05 vs ACSF-treated rats.

The time courses of changes in MAP, heart rate, arterial pH, PO2, and PCO2 in five experimental groups are presented in the Table (data from LPS group treated at 1 mg/kg are shown). There were no significant differences in baseline values (0 hours) among the treatment groups. MAP did not change significantly over the course of the study in groups 1, 3, 4, and 5, while a slight increase in MAP was seen in group 2 (LPS-treated rats) 4 hours after administration of LPS. Heart rate increased in groups 2 to 5 at 2 and 4 hours after administration of LPS compared with the respective values observed in the control group (group 1). Arterial pH, PO2, and PCO2 did not change significantly in any of the groups during the 4-hour experiments. Baseline values of rCBF and rCVC were similar in each treatment group.

The specificity of the RT-PCR reactions was verified by cloning and sequencing the 385-bp product amplified by the COX-2 primers and the 565-bp product amplified by the iNOS primers. RT-PCR of RNA extracted from the brain of an LPS-treated rat yielded single bands of the expected sizes of 565, 381, and 970 bp when amplified with the iNOS, COX-2, and GAPDH primers. The iNOS and COX-2 products were cloned into a PCRII vector (Invitrogen) and sequenced according to the fluorescent diodeoxynucleotide method in both directions. The results of these experiments indicate that the products exhibited 100% homology with published sequences.

The results of the experiments to verify that the PCR reactions were linear under the present experimental conditions are presented in Figure 2. We found that there was a linear relationship between the fluorescent intensity of the PCR products for iNOS, COX-2, and GAPDH and the amount of LPS-treated brain RNA added to the PCR reactions over the range of 0.25 to 2 μg.
A photograph of representative gels comparing the RT-PCR products for iNOS, COX-2 cDNA, and GAPDH when 1 μg of RNA from LPS- and vehicle-treated brains were amplified is presented in Figure 3. The intensity of bands corresponding to iNOS or COX-2 in cerebral cortices increased after intracerebroventricular administration of LPS compared with the degree of amplification seen when RNA was extracted from the brains of ACSF-treated control rats, whereas the intensity of GAPDH bands was not significantly different. A summary of the relative levels of iNOS and COX-2 mRNA is presented in Figure 4. In ACSF-treated control rats, the intensity ratio of the iNOS/GAPDH and COX-2/GAPDH bands increased to 0.15 ± 0.01 and 0.67 ± 0.2, respectively. These intensities of iNOS and COX-2 bands increased further 4 hours after administration of LPS compared with levels seen in control animals. Moreover, as expected, induction of iNOS and COX-2 mRNA was significantly attenuated in the animals treated by dexamethasone (Figure 4).

The effect of LPS on the levels of iNOS and COX-2 protein in the brain of rats is presented in Figure 5. After administration of LPS, the intensity ratio of the iNOS/GAPDH and COX-2/GAPDH bands increased to 0.15 ± 0.01 and 0.67 ± 0.2, respectively. These intensities of iNOS and COX-2 bands increased further 4 hours after administration of LPS compared with levels seen in control animals. Moreover, as expected, induction of iNOS and COX-2 mRNA was significantly attenuated in the animals treated by dexamethasone (Figure 4).

A photograph of representative gels comparing the RT-PCR products for iNOS, COX-2 cDNA, and GAPDH when 1 μg of RNA from LPS- and vehicle-treated brains were amplified is presented in Figure 3. The intensity of bands corresponding to iNOS or COX-2 in cerebral cortices increased after intracerebroventricular administration of LPS compared with the degree of amplification seen when RNA was extracted from the brains of ACSF-treated control rats, whereas the intensity of GAPDH bands was not significantly different. A summary of the relative levels of iNOS and COX-2 mRNA is presented in Figure 4. In ACSF-treated control rats, the intensity ratio of the iNOS/GAPDH and COX-2/GAPDH bands increased to 0.15 ± 0.01 and 0.67 ± 0.2, respectively. These intensities of iNOS and COX-2 bands increased further 4 hours after administration of LPS compared with levels seen in control animals. Moreover, as expected, induction of iNOS and COX-2 mRNA was significantly attenuated in the animals treated by dexamethasone (Figure 4).

Figure 2. Linearity of RT-PCR reactions. Known amounts of RNA (0.25 to 2.0 μg) extracted from the brain of an LPS-treated rat were amplified for 35 cycles with iNOS, COX-2, and GAPDH primers. The products were electrophoresed on a 1% agarose gel with ethidium bromide, and the intensities of PCR products corresponding to iNOS, COX-2, and GAPDH bands were linearly correlated with the initial amount of RNA added to the reaction.

Figure 3. Photograph showing RT-PCR products corresponding to iNOS (A), COX-2 (B), and GAPDH (C) mRNA in control and after administration of LPS. In lane 1 is a 100 bp-DNA ladder indicating the size of PCR products. Br indicates sample from the brain cortex; Sp, sample from spleen in rats treated with LPS (positive control). Note that the levels of iNOS and COX-2 mRNA in the brain are elevated in LPS-treated rats, while the GAPDH mRNA levels are similar in control and LPS-treated rats.
significantly different between the LPS-treated and the ACSF-treated groups (0.25 ± 0.04 versus 0.33 ± 0.1; P = 0.1).

As shown in Figure 6A, the calcium-independent iNOS activity in the brain was 11 times greater in rats treated with LPS than the levels seen in the brains of ACSF-treated control rats. Both the iNOS selective inhibitor aminoguanidine and dexamethasone attenuated the increase in calcium-independent iNOS activity in LPS-treated rats to levels that were not significantly different from those seen in the ACSF-treated control group. Aminoguanidine had no effect on constitutive NOS activity (measured as the difference in NOS activity in the presence and absence of calcium; n = 3; data not shown). Concentrations of PGE2 in the cerebral cortical tissue samples were significantly higher (by 21 ± 5%) in the LPS-treated rats than levels seen in the ACSF-treated control group. Both NS-398 and dexamethasone abolished the increase in PGE2 levels in the brain of LPS-treated rats (Figure 6B).

Figure 7A summarizes the effects of aminoguanidine, NS-398, and dexamethasone on LPS-induced cerebrocortical hyperemia compared with the effects of ACSF or LPS alone. The LPS-induced increases in rCBF were approximately 50% smaller in the rats treated with either aminoguanidine or NS-398 and were completely eliminated in the rats treated with dexamethasone. Similar effects of these inhibitors were observed when rCVC was used to represent the cerebrovascular effects of LPS (Figure 7B).

Discussion

In the present study we demonstrated that intracerebroventricular administration of LPS produces a progressive and dose-dependent increase in rCBF. This is associated with increases in the levels of iNOS and COX-2 mRNA, iNOS protein, iNOS activity, and PGE2 levels in the cerebral cortex of the rat. These effects were attenuated by the administration of either aminoguanidine or NS-398 and were completely abolished by pretreating the rats with dexamethasone. These findings suggest that the induction of both iNOS and cyclooxygenase activity and the subsequent increase in NO and cyclooxygenase metabolites of arachidonic acid contribute to the cerebral hyperemia produced by LPS.

The systemic physiological data presented in the Table demonstrate cardiovascular stability of the preparation achieved by using the intracerebroventricular endotoxin injection protocol at the dose of 1 mg/kg. The data also indicate that ventilation of the animals was well controlled, and there was no difference in blood pressure, Po2, pH, and PCO2 among the experimental groups. These results suggest that the cerebral hyperemia after intracerebroventricular administration of LPS was restricted to the brain and was not secondary to systemic effects.

The approximately twofold increase in CBF seen 4 hours after LPS treatment under normoxic, normocapnic conditions is clearly outside the normal physiological values. From a pathophysiologic point of view, such an increase in CBF would be expected to produce elevations in microvascular and intracranial pressures and increases in cerebrovascular permeability and intraparenchymal edema that would contribute to brain injury.
To date, three isoforms of NOS have been identified, i.e., neuronal NOS (nNOS or type I NOS), inducible NOS (iNOS or type II NOS), and endothelial NOS (eNOS or type III NOS), in the brain of rats. Among these isoforms, nNOS and eNOS are constitutively expressed. They produce NO in response to elevations of intracellular calcium concentration and mediate signal transduction in various organ systems. These enzymes play an important role in the maintenance of CBF. In contrast, iNOS is induced by inflammatory stimuli such as bacterial endotoxin, interferon-gamma, UV light, and brain ischemia. Subsequently, a large amount of NO can be produced from iNOS in many cell types, including macrophages, vascular smooth muscle and endothelial cells, astrocytes, microglia, and neurons. The overproduction of NO from iNOS has been thought to contribute to the pathogenesis of septic shock, host-defense response, cytotoxicity, and ischemia/reperfusion injury.

The bacterial endotoxin LPS is among the most important and well-documented stimuli for the induction of iNOS. It has been reported that LPS dilates rabbit cerebral arterioles after direct application through a cranial window. Since this vasodilation was accompanied by an increase in cGMP production and was attenuated by dexamethasone or amino-guanidine, these results suggested that LPS may have induced iNOS to dilate the cerebral vasculature. However, direct biochemical or molecular evidence that LPS actually increased the expression of iNOS in the brain after intracerebroventricular administration of LPS has yet to be provided in any study. We therefore designed and performed experiments to test this hypothesis and found that the levels of iNOS activity increased upon LPS treatment. These findings support the notion that iNOS is involved in the regulation of CBF.
mRNA and protein and calcium-independent NOS activity increased markedly after intracerebroventricular administration of LPS in the cerebral cortex. The time course of the changes in iNOS protein and enzyme activity correlated well with the changes in CBF. Moreover, we demonstrated that dexamethasone completely attenuated the increases in iNOS protein, iNOS, mRNA, and CBF. Similar effects were seen after administration of the iNOS selective inhibitor amino-guanidine, which blocked the increases in iNOS activity and the cerebral hyperemic response to LPS. These findings provide direct biochemical and molecular evidence to support the hypothesis that induction of iNOS expression and activity contributes to LPS-induced cerebral hyperemia after intracerebroventricular administration.

The present data showing enhanced levels of iNOS protein and mRNA in the cerebral cortex after intracerebroventricular administration of LPS contrast with previous reports that were unable to document increased levels of iNOS protein or RNA in the brain when given by an intravenous or intraperitoneal route. The difference is likely due to the limited ability of LPS to cross the blood-brain barrier.13,39 Recently, Minc-Golomb et al5 reported that direct injection of LPS into the cerebellum could increase iNOS mRNA or protein expression in cerebellar neurons. Therefore, it appears that LPS can increase iNOS levels when it is directly applied to the brain. The cellular mechanisms of cerebral hyperemia and the identity of vascular and/or parenchymal cells in the brain that increase iNOS expression after administration of LPS remain to be elucidated. The clinical significance of this experimental animal model is that direct administration of LPS could be used to investigate the mechanisms underlying changes in CBF during inflammation caused by bacterial meningitis, encephalitis, or ischemic injury.

In the present study iNOS mRNA was induced 2 hours after LPS. Such an early induction of iNOS seen in the present study is consistent with the recent findings of Bateson et al,46 who reported increased levels of iNOS mRNA in the heart as little as 30 minutes after systemic administration of LPS. This rapid induction of iNOS may explain the increase in rCBF in the early phase (1 to 2 hours) after the administration of LPS in our study. However, it has also been reported that activation of the production of NO41 and/or peroxynitrite42 by eNOS may also play role in the initial hyperemic response. Further studies will be necessary to clarify the mechanism of the initial rise in rCBF after administration of endotoxin. Nevertheless, it is clear from our findings that induction of iNOS does contribute importantly to the rise in rCBF seen 2 to 4 hours after administration of LPS.

Prostaglandins have also been reported to play a role in the regulation of CBF.43,44 During endotoxemia, an increased production of prostaglandins has been suggested to contribute to pathophysiological changes in brain, ie, fever,35 neuroendocrine changes,46 and cerebral hyperemia.4 Recently, inducible isomers of prostaglandin synthase (PGS-2) or cyclooxygenase (COX-2) have been identified, and expression of COX-2 mRNA and/or protein has been reported in many cell types, including fibroblasts,10 macrophages,19 endothelial and smooth muscle cells,48 heart,21 astrocytes,12 and neurons11 after induction by LPS.49 We hypothesized that in addition to iNOS, COX-2 might play a role in LPS-induced cerebral hyperemia. In the present study we demonstrated that the levels of COX-2 mRNA and PGE2 levels do increase in the brain increase after administration of LPS and that the rise in CBF was attenuated by NS-398. NS-398 has been reported to selectively reduce COX-2 (inducible) activity without affecting COX-1 (constitutive) activity at doses comparable to those used in our study.18,39,50 COX-2 catalyzes the formation of prostaglandins, thromboxanes, and prostacyclin,48 and PGE2 has been reported to be the major cyclooxygenase metabolite produced in the cerebral cortex.8,9,51 Therefore, we measured changes in cerebral cortical PGE2 levels as an index of total cyclooxygenase activity and found that they increased after administration of LPS. The rise in PGE2 levels was blocked by NS-398 or dexamethasone. These findings suggest that induction of COX-2 and increases in the production of vasodilator prostanooids may also contribute in LPSt-induced cerebral hyperemia.

Another interesting finding was the high level of expression of COX-2 protein in the brains of untreated rats. Although our finding of the constitutively expressed COX-2 in the brain is consistent with other reports,1,52 the physiological significance of constitutively expressed COX-2 in the brain remains to be determined. Since COX-2 has been reported to contribute to seizure-induced changes in synaptic activity,11,53 constitutively expressed COX-2 may also have some role in the regulation of synaptic signal transduction under certain physiological conditions. Further studies will be necessary to clarify the role of COX-2 in the regulation of CBF.

A possible cross-talk between the NO and prostaglandin systems was outside the scope of the present study, and therefore our results do not explain why COX-2 protein was not significantly altered while COX-2 activity was increased. Both stimulatory54 and inhibitory55 effects of NO on cyclooxygenase activity have been reported. We speculate that the high levels of NO after administration of LPS may have decreased COX-2 protein levels, perhaps by nitrosylating the enzyme and increasing protein degradation. Thus, the enhanced expression of COX-2 mRNA was uncoupled from the levels of COX-2 protein after LPS treatment.

The present results do not exclude the possibility that other vasodilator mediators may also contribute to the cerebrovascular dilatory response to LPS. For example, calcitonin gene–related peptide is known to be a potent dilator of cerebral blood vessels.56,57 It has been reported to contribute to endotoxin-induced cerebrovasodilation7 and interact with NO.58 Non–cyclooxygenase-derived eicosanoids, eg, cytochrome P-450–derived epoxyeicosatrienoic acid, have also been reported to play a role in maintaining CBF.59 and the production of these eicosanoids is known to be inhibited by NO.60 However, the question of to what extent these other mediators are involved in the LPS-induced cerebral hyperemia and to what extent they influence the iNOS and COX-2 pathways remains to be addressed in future studies.

In summary, we have demonstrated that intracerebroventricular administration of LPS increases the levels of iNOS and COX-2 mRNA, iNOS protein and enzyme activity, and PGE2 levels in the cerebral cortex of rats and that inhibitors of COX-2 and iNOS attenuate the increase in CBF produced by LPS. Our findings suggest that enhanced expression of both
iNOS and COX-2 followed by a rise in the production of NO and vasodilator prostanoids contribute to endotoxin-induced cerebral hyperemia.

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References

The inflammatory process is a very complicated cascade designed (a1) to defend against tissue injury and infection, (b2) to rid the body of injured or damaged tissues, and (c3) to subsequently regenerate the injured tissues.1–3 The process consists of two components: one is “tearing down” or destructive, and the other is “rebuilding” or regenerating. In an inflamed area, the destructive component often does not discriminate between invading pathogens, damaged cells, or healthy cells. In organs like the brain, the process can be particularly destructive since neurons may not regenerate in an orderly network of synaptic connections required for normal functioning. The inflammatory process in brain can be activated by such conditions as infections (bacterial endotoxin), damage produced by stroke or traumatic injury, and other pathological states.1–3 It is extremely important that we understand the inflammatory process in brain, with the ultimate goal of learning to therapeutically alter those processes that are undesirable and that ultimately exacerbate injury.

Okamoto and colleagues have presented a comprehensive study of one aspect of the inflammatory process involving iNOS and COX-2 and their effects on CBF. Using an endotoxin model and a combination of molecular tools with whole-animal physiological measurements, the authors clearly showed the involvement of iNOS and COX-2 in the inflammatory process and their respective roles in hyperperfusion in the brain. The authors were able to show that the major components, if not the exclusive components, of the increase in CBF after endotoxin administration was due to a combination of NO and COX-2 metabolites.

Although the maintenance of CBF is extremely important during pathological conditions, an excess flow in brain is associated with increases in intracranial pressure, altered blood-brain barrier permeability, and edema.1,3–5 Okamoto and colleagues have significantly added to our understanding of the inflammatory process in brain and have shown that pharmacological inhibition of the function or expression of these enzymes can reduce the hyperperfusion after endotoxin administration. This study is an important step toward the ultimate goal of therapeutically controlling the inflammatory process in the human during pathological states in brain.

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Role of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 in Endotoxin-Induced Cerebral Hyperemia

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