Dexamethasone and Nitric Oxide Synthase Gene Expression in Brain

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Systemic administration of lipopolysaccharide (LPS), which causes endotoxemia and systemic inflammation, has been reported to induce expression of the gene for type II inducible nitric oxide synthase (iNOS) in peripheral organs. This study was carried out to examine whether intraperitoneally injected LPS elicits the expression of iNOS messenger ribonucleic acid (mRNA) in the rat brain. We also investigated whether intraperitoneal treatment with dexamethasone (DEX) prevents this induction. To determine levels of iNOS mRNA, a quantitative reverse transcription–polymerase chain reaction (RT-PCR) method was employed. Treatment with LPS induced the expression of iNOS mRNA in various brain regions, accounting for approximately 1×10^5 to 4×10^5 molecules per μg of poly A^+ RNA, and these inductions were markedly suppressed by DEX. The results suggest that, during systemic inflammation, iNOS mRNA induction occurs in brain through a DEX-sensitive mechanism.

Key Words: brain, dexamethasone, inducible nitric oxide synthase, lipopolysaccharide, messenger ribonucleic acid, reverse transcription–polymerase chain reaction

INTRODUCTION

The production of nitric oxide ($NO^\bullet$) is catalyzed by the enzyme $NO^\bullet$ synthase (NOS). Type I NOS (nNOS), which is localized in neurons, is constitutively expressed and is regulated by the influx of calcium (Ca^{2+}) and the activation of calmodulin (Bredt and others 1991). By contrast, type II NOS (iNOS) is a Ca^{2+}-independent, transcriptionally regulated enzyme (Xie and others 1992; Park and others 1994).

Peripheral treatment with an endotoxin, LPS, causes endotoxemia and systemic inflammation, which are often fatal. Recently, it has been shown that null mutant iNOS mice ($iNOS^{-/-}$) are much more resistant to peripheral administration of LPS than wild type mice (MacMicking and others 1995; Wei and others 1995), suggesting that iNOS plays an important role in the pathophysiology of endotoxemia and systemic inflammation. With regard to cultured glial cells, many groups have reported that LPS induces iNOS mRNA (Galea and others 1992; Simmons and Murphy 1992; Murphy and others 1993; Sparrow 1995), but whether peripheral administration of LPS induces iNOS in glial cells in vivo remains an unsettled question. The 1st purpose of this study was to examine whether iNOS gene expression is induced in the rat brain by peripheral administration of LPS.

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Table 1

Effects of LPS on iNOS mRNA levels ($\times 10^5$ molecules per μg of total poly A^+ RNA)

Brain region	Control	LPS	DEX + LPS
Hypothalamus	ND	1.8 ± 0.9	0.17 ± 0.08
Hippocampus	ND	1.0 ± 0.4	0.13 ± 0.10
Frontal cortex	ND	1.1 ± 8.0	0.29 ± 0.05
Brain stem	ND	1.9 ± 1.2	0.27 ± 0.09
Cerebellum	ND	2.5 ± 1.0	0.25 ± 0.13

Data are presented as the mean ± standard error of 4 rats.
Six hours after intraperitoneal injection of 15 mg/kg of LPS, rats were decapitated under light ether anesthesia. DEX (1.0 mg/kg) was systemically administered at 30 min before LPS injection (DEX + LPS). Control rats were injected with saline alone.
Methods for quantification of mRNA are described in the legend of Figure 2.
ND = not detectable.

It has been suggested that NO• is involved in the pathogenesis of neurologic impairment (Moncada and others 1991; Sparrow 1995). For example, iNOS has been implicated in neurotoxicity in experimental viral encephalitis (Koprowski and others 1993). The pathophysiology of encephalopathy arising during systemic inflammation, including weakness, malaise, listlessness, hypersomnia, and anorexia, has not been well clarified. Induction of iNOS mRNA, if it occurs, may provide additional evidence for this mechanism.

Dexamethasone (DEX), a nonspecific suppressor of inflammation, has been reported to prevent LPS-induced iNOS gene expression in peripheral organs, and this suppression has been suggested to contribute to survival after LPS challenge (Liu and others 1993). Our 2nd objective was to test whether this cerebral induction can be inhibited by DEX.

To measure the levels of mRNA, the quantitative RT-PCR was employed with primers selected from the specific complementary deoxyribonucleic acid (cDNA) sequence of astrocyte iNOS (Galea and others 1994). To enhance the reliability of quantification, an original synthetic ribonucleic acid (RNA), which could be coamplified with the iNOS mRNA using the same primers, was used as an internal standard.

METHODS

Animals and treatments

Seven-week-old male Sprague-Dawley rats (Sankyo Laboservice, Tokyo, Japan) were housed in a temperature-controlled room (23 to 25 °C) under a 12-h reversed light–dark schedule for 1 week before experimentation. Food and water were freely provided at all times. Rats were intraperitoneally injected with the endotoxin LPS from *Escherichia coli* (Sigma Chemical Company, St Louis, USA, 15.0 mg/kg), dissolved in saline with a final volume of 1 mL per rat. Controls received the same volume of saline alone. Rats in the DEX plus LPS group were intraperitoneally injected with DEX (Sigma Chemical Company, St Louis, USA) 1.0 mg/kg dispersed in saline with a final volume of 1 mL at 30 min before administration of LPS. After 6 h, the rats were decapitated under light ether anesthesia, and the brains were quickly removed and dissected into the hypothalamus, hippocampus, frontal cortex, brain stem, and cerebellum. To examine the time course of LPS effect, a whole brain was removed at 1, 3, 6, 9, 12, and 24 h after LPS injection.

RT-PCR

Total RNA was extracted according to a previously described method (MacDonald and others 1987). Poly A^+ RNA was purified with Oligotex-dt30 super (Takara, Kyoto, Japan) and was reverse transcribed into cDNA. A 20-μL quantity of reaction mixture consisted of 1 μg of mRNA, 1 pg of modified complementary RNA (cRNA) as the internal standard (described below), 20 U Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, USA), 1 × reverse transcription buffer (20 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 100 mg/mL bovine serum albumin), 5 mM deoxyribonucleoside 5′-triphosphate (dNTP) (Takara, Kyoto, Japan), 10 mM dithiothreitol, 20 U ribonuclease inhibitor (TOYOBO, Osaka, Japan), and 100 pmol of DNA random hexamers (Takara, Kyoto, Japan). The reaction mixture was incubated at 41.5 °C for 45 min, heated to 75 °C for 10 min, and then quick-chilled on ice to stop the reverse transcription and the denaturation of the products.

One microliter of each reverse-transcription product was used as a template of PCR. PCR was performed at a final concentration of 1 × PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$), 50 mM dNTPs, 0.1 mM each 5′and 3′ primer, 1×10^6 counts per minute of ^{32}P-end-labeled primer, 1 unit of *Thermus aquaticus* DNA polymerase (Pharmacia Biotech, Milwaukee, USA) in a final volume of 50 μL.

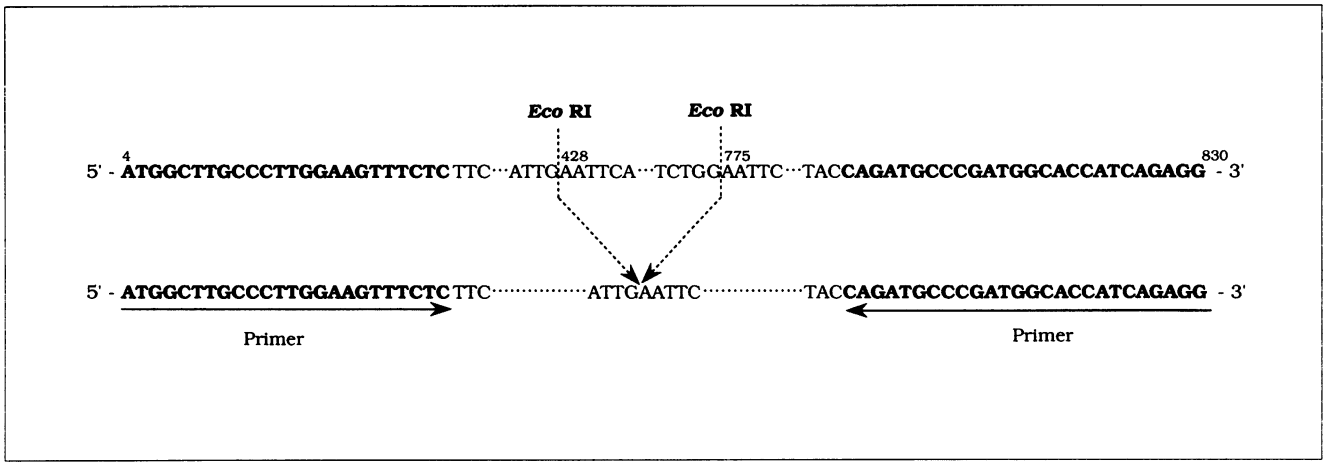

Figure 1. Preparation of internal standard iNOS cDNA: PCR products from the 827-bp original iNOS cDNA (upper) and from the 348-bp deletion construct (lower) serving as an internal standard. Arrows represent an iNOS primer pair selected from the astrocyte iNOS cDNA sequence. Broken arrows represent the *Eco* RI site. Standard cRNA was synthesized using this standard iNOS cDNA as a template.

An iNOS primer pair was selected from the rat astrocyte iNOS sequence (Galea and others 1994) (Figure 1). Reverse primer was labeled with [γ-^{32}P]ATP with a Kination kit (TOYOBO, Osaka, Japan) according to the protocol of TOYOBO, and unincorporated nucleotides were removed on a Puresil C18 column (Nihon Waters Ltd, Tokyo, Japan). The amplification profile involved denaturation at 92 °C for 20 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s with 35 cycles. PCR products were inserted into M13 plasmids for sequencing.

Internal standard

To devise an internal standard, PCR was performed with the 5′ and 3′ primers, respectively, each of which had been added—*Nco* I site or *Sal* I site—to the primer described above (see Figure 1) at the 5′ side. After digestion with *Nco* I and *Sal* I (Takara, Kyoto, Japan), the PCR products were purified using a Millipore ultra free C3LTK filter (Nihon Millipore Ltd, Yonezawa, Japan) and then inserted into pGEM-5Zf(+) (Pharmacia Biotech, Milwaukee, USA), which contains the T7 polymerase promoter. Next, the inserted plasmid was cut by *Eco* RI and, after purification by agarose gel electrophoresis, again ligated with a DNA ligation kit (Takara, Kyoto, Japan). As shown in Figure 1, the inserted DNA consists of an iNOS DNA fragment of 827 base pair (bp) (bases 4 to 830), having a 347-bp deletion (bases 428 to 774), thereby allowing amplification with the same primer pair for the original cDNA and electrophoretic separation of the original cDNA from the constructed PCR products. This plasmid was used as a template for transcription by T7 polymerase (Takara, Kyoto, Japan) according to the protocol of Takara. The template was digested with ribonuclease-free deoxyribonuclease I (Takara, Kyoto, Japan) after transcription at 37 °C for 20 min and heated to 90 °C for 10 min to stop the digestion. The resulting cRNA was used as the internal standard.

Quantification of PCR products

Quantification of mRNA was based on the method of Wang and others (1989). Serial 1:3 dilutions of 1-μL aliquots of the reverse transcription product were used as the PCR template. After the reaction, the remaining primers were removed with a Millipore ultra free C3LTK filter, the product was resolved by 2% agarose gel electrophoresis, and appropriate bands were cut from the gel. The PCR product amounts were determined by Cerenkov counting of bands.

RESULTS

The sequences of PCR-amplified iNOS cDNA from all of the brain regions examined corresponded exactly with the cloned rat astrocyte iNOS cDNA (Galea and others 1994).

Figure 2 shows an example of quantitative analysis. In this example, it was confirmed that the PCR efficiencies of iNOS mRNA and standard cRNA amplification were identical and that it is possible to calculate the amount of iNOS mRNA from the standard data. We elaborated 1 standard curve in each sample and used only the data that gave parallel lines with a slope of approximately 1. For example, the counts per minute of iNOS cDNA amplified from a nondiluted mixture (10^0 dilution) was equal to that of modified iNOS cDNA from one-tenth diluted mixture (10^{-1} dilution), indicating that 1 μg of mRNA from a brain sample and 0.1 pg of internal standard cRNA produced the same amount of PCR products. The detection limit of iNOS poly A$^+$ RNA was $< 1 \times 10^3$ molecules (data not shown).

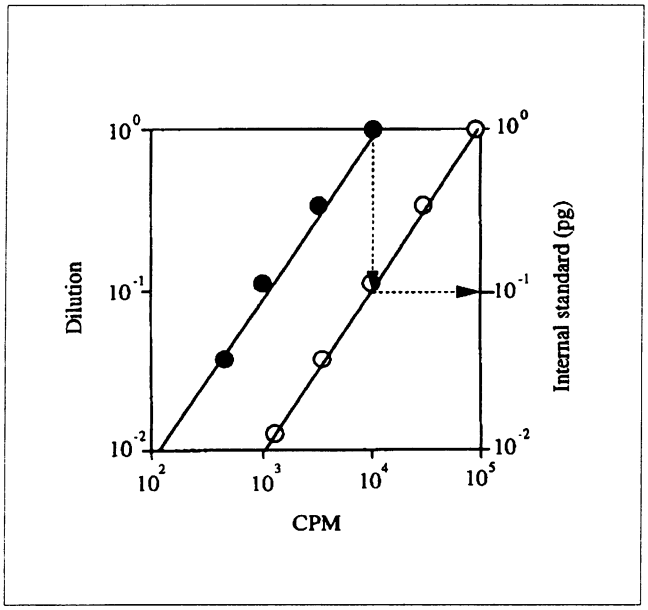

Figure 2. An example of quantitative analysis of iNOS mRNA levels. Standard cRNA (1 pg) (see Figure 1 legend) was added to 1 μg poly A^+ RNA prepared from rat brain. The mixture was subjected to reverse transcription. The mixture was serially diluted by the factor 3 and then was amplified by PCR with ^{32}P-end-labeled primer. After unreacted primers had been removed, the products were electrophoresed and appropriate bands were cut out. The amounts were determined by Cerenkov counts per min (CPM). The data from the internal standard DNA (open circles) and iNOS cDNA derived from a brain sample (closed circles) fall on parallel lines with slopes of approximately 1. All data shown in this figure were within exponential range.

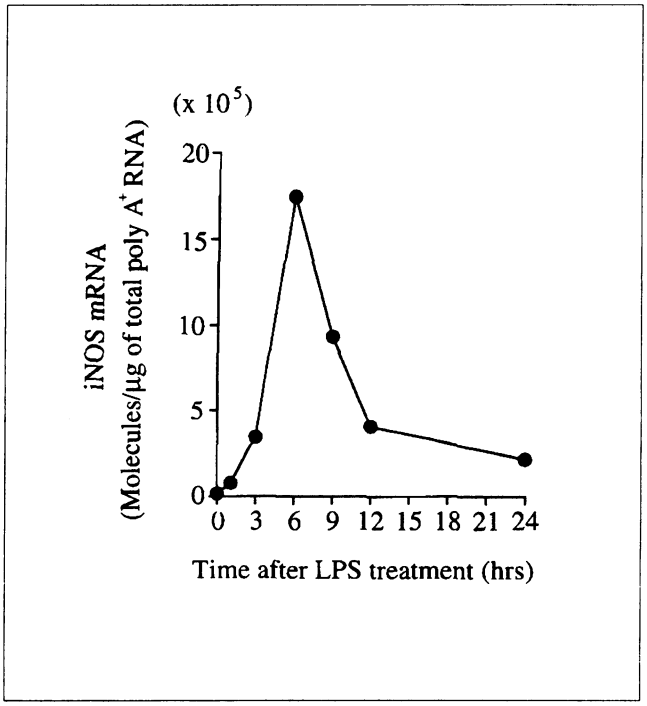

Figure 3. Time course of the appearance of iNOS mRNA in the rat brain. At various times after the intraperitoneal injection of bacterial LPS (endotoxin), whole brains were removed for analysis. Quantification was performed as described in the legend of Figure 2.

Figure 3 shows the time course of LPS-induced iNOS mRNA expression in a whole brain. The PCR products were detectable at 1 h after the LPS injection and peaked at approximately 6 h.

As shown in Table 1, iNOS mRNA was undetectable in all brain regions of the control group. In the brain regions of LPS-treated rats, amounts of iNOS mRNA accounted for approximately 1×10^5 to 4×10^5 molecules per μg of total brain poly A^+ RNA. The induction of iNOS mRNA was markedly inhibited by pretreatment with DEX.

DISCUSSION

This study yielded 2 major findings. First, intraperitoneally administered LPS induced an iNOS mRNA expression in the brain. Second, this induction was prevented by systemic pretreatment with DEX.

In a preliminary report using the RT-PCR method, Liu and others (1993) found that iNOS mRNA was detectable in only 3 of the 6 rat brains treated by intraperitoneal LPS administration, whereas the mRNA was markedly induced in all peripheral organs. We (Suzuki and others 1995) also reported the slight induction of iNOS mRNA in the brain. The primers used in the previous studies, however, were taken from the sequence of macrophage (Xie and others 1992) or vascular smooth muscle iNOS (Nunokawa and others 1993), which is not identical with that of rat astrocyte iNOS (Galea and others 1994). So it was considered that the nonspecific primer pair may lower the PCR efficiency. In this study, we chose a primer pair from rat astrocyte iNOS sequence and performed the quantitative RT-PCR. The level of iNOS mRNA was undetectable ($< 1 \times 10^3$ molecules) under normal conditions. Intraperitoneal administration of LPS induced a more than 50-fold increase in iNOS gene expression in the brain regions examined (see Table 1). When we completed our experiments, a new report in the literature demonstrated, using an in situ hybridization method with a rat astrocyte iNOS probe, that systemic treatment with LPS induces the expression of iNOS mRNA (Wong and others 1996).

In contrast to the investigation of gene expression, neither iNOS activity nor protein has ever been detected in the brain after systemic administration of LPS (Salter and others 1991; Oguchi and others 1992; Cook and others 1994; Sato and

others 1995; Van Dam and others 1995). In agreement with these studies, we were unable to detect the induction of iNOS by Western blot analysis (data not shown). Considering the fact that iNOS mRNA was increased, however, it is possible that the large increase in mRNA dose did not lead to iNOS protein synthesis. Nevertheless, Wong and others (1996) were able to show that nitrite, a metabolite of NO, was indeed increased in brain parenchyma and cerebrospinal fluid by systemic administration of LPS. Other groups also suggested that iNOS is transcriptionally regulated, and its activity depends on newly induced mRNA (Xie and others 1992; Park and others 1994). Taken together, these facts lead us to postulate that the RT-PCR method, using the primer pair chosen from rat astrocyte iNOS sequence, is a sensitive method in iNOS detection in brain.

Pretreatment with DEX suppressed the LPS-induced expression of iNOS mRNA in the brain. This finding suggests a clinical usefulness of DEX for treatment of neurologic symptoms, since iNOS is postulated to be involved in several pathogenic processes in the brain. The mechanism underlying this suppression remains to be elucidated.

In summary, we have shown that iNOS mRNA expression is induced by intraperitoneally administered LPS in various regions of the rat brain and that this induction is suppressed by DEX pretreatment. The results suggest that systemic inflammation induces the iNOS mRNA expression in the brain by means of a DEX-sensitive mechanism. Further studies aimed at determining whether iNOS is involved in neurologic symptoms during endotoxemia or systemic inflammation are warranted.

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