Increased expression of neuronal nitric oxide synthase in astrocytes and macrophages in the spinal cord of Lewis rats with autoimmune encephalomyelitis

Taekyun Shin*

Department of Veterinary Medicine, Cheju National University, Jeju 690-756, Republic of Korea

Abstract

Neuronal nitric oxide synthase (nNOS) is constitutively expressed in neurons of the central nervous system, where it plays a physiological role in neurotransmission. In this study, we examined the functional role of nNOS in experimental autoimmune encephalomyelitis (EAE). The effects of the specific nNOS inhibitor 7-nitroindazole on normal and EAE rats were studied by immunohistochemistry and Western blot analysis. We found that nNOS is constitutively expressed in the spinal cords of normal rats, whilst in the spinal cords of EAE rats, nNOS expression slightly increased, concomitant with the infiltration of T cells and macrophages. Immunohistochemical studies showed that nNOS expression in macrophages and astrocytes increased at the peak stage of EAE and declined thereafter. Treatment with 7-nitroindazole (30 mg/kg) significantly delayed the onset of EAE paralysis, but had no effect on either the incidence or the severity of the paralysis. These findings suggest that nNOS inhibition has a limited role in the induction of rat EAE, and that constitutive nNOS in the spinal cord functions as a novel neurotransmitter, rather than a pro-inflammatory agent.

Key word: neuronal nitric oxide synthase, autoimmune encephalomyelitis, macrophages

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the central nervous system (CNS) that is used as a model for human demyelinating diseases, such as multiple sclerosis. The clinical course of EAE is characterized by weight loss, ascending progressive paralysis, and spontaneous recovery. These symptoms coincide with an inflammatory response that is characterized by the infiltration of T cells and macrophages into the CNS, the activation of microglia, and reactive astrogliosis [15].

Nitric oxide (NO) is an important mediator of CNS inflammation during EAE induction, when it is mainly generated by inducible NO synthase (iNOS) [14, 18]. However, despite the pathogenic effect of NO during the induction stage of EAE, NO also plays a critical role in the recovery of Lewis rats from EAE and in the maintenance of resistance to re-induction [10].

iNOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are the three isoforms of Ca++-dependent NOS that generate NO from L-arginine [3, 8, 9]. Constitutive NOS is thought to synthesize NO in CNS neurons, and is important for intracellular signaling and neurotransmission [3, 13]. The NOS isoform is also found in astrocytes and other brain cells [4, 7]. Previous studies have linked NOS to neural damage in response to ischemic brain injury [1, 2, 5, 16], and spinal cord NOS levels increase following the induction of adjuvant-induced arthritis [11, 17]. Many brain cells express constitutive NOS and eNOS under both normal and pathologic conditions, which include the ischemia and traumatic brain injury caused by remote activation after peripheral inflammation. However, little is known about the functional role of NOS and its correlation with cell phenotype in the pathogenesis of EAE. In this study, we demonstrate that the expression of NOS is increased slightly in the neurons and reactive astrocytes of EAE lesions, and that the specific inhibition of NOS has no effect on the incidence or severity on EAE paralysis.

*Corresponding author
Phone: +82-64-754-3363; Fax: +82-64-756-3354
E-mail: shint@cheju.cheju.ac.kr
MATERIALS AND METHODS

Animals

Lewis rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN, U.S.A.) and bred at our animal facility. Male rats of 8-12 weeks of age and weighing 160-200 grams were used throughout these experiments.

Induction of EAE

The rear footpads of each rat were injected bilaterally with an emulsion containing equal parts of fresh rat spinal cord homogenate (1mg/ml) in phosphate-buffered saline, pH 7.4 (PBS) and complete Freund's adjuvant (CFA) (1 mg/ml) (Mycobacterium tuberculosis H37Ra, Difco). At the time of immunization, rats received an intraperitoneal injection of 500 ng of pertussis toxin (Sigma, St. Louis, MO). Immunized rats were observed daily for clinical signs of EAE. Disease progress was divided into seven clinical stages (Grade 0, no signs; G1, floppy tail; G2, mild paraparesis; G3, severe paraparesis; G4, tetraparesis; G5, moribund condition or death; R0, recovery stage). Control rats were immunized with CFA only.

Treatment with 7-nitroindazole

Daily dosing with 7-nitroindazole (7-NI) has previously been shown to reduce NO production in gerbils [1]. In this study, immunized rats were treated intraperitoneally with an equivalent daily dose of 7-NI (30 mg/kg), either from day 0 through day 7 post-immunization (PI) or from day 7 through day 13 PI. Control animals received sham treatment with the 7-NI vehicle only.

Tissue sampling

In this study, tissue sampling was performed on days 13 and 21 PI, during the peak and recovery stages of EAE, respectively. Five rats in each group were sacrificed under ether anesthesia. The spinal cords were removed and stored at -70°C for later analysis. Pieces of the spinal cords were fixed in paraformaldehyde (4%) in PBS and embedded in paraffin.

Immunohistochemistry

Sections (5 μm) of paraffin-embedded spinal cords were deparaffinized and treated with 0.3% hydrogen peroxide in distilled water for 30 min to block endogenous peroxidase. After three washes with PBS, the sections were exposed to 10% normal goat serum and incubated with mouse monoclonal anti-nNOS antibody (1:200 dilution) (Transduction Laboratories, Lexington, KY, U.S.A.) for 1 h at room temperature. Rabbit anti-glial fibrillary acidic protein (GFAP) (Sigma Chemical Co., St. Louis, MO, U.S.A.) and ED1 (Serotec, London, U.K.) antibodies were then applied to identify astrocytes and macrophages, respectively. After three washes, the appropriate biotinylated secondary antibodies and avidin-biotin peroxidase complex reagents (Elite kit, Vector, Burlingame, CA, U.S.A.) were added sequentially. Color was developed with a diaminobenzidine substrate (Vector), and the sections were counterstained with hematoxylin before mounting.

RESULTS

Western blot analysis of nNOS in EAE rat spinal cords

Western blot analysis showed that nNOS was constitutively expressed in untreated and adjuvant-immunized rat spinal cords. In EAE spinal cords, nNOS expression was expressed at a slightly higher level, but this was not significantly different from that of the adjuvant-immunized spinal cords (Fig. 1). In contrast, iNOS expression was observed to have increased significantly at the peak stage of EAE, and to have declined thereafter. These findings suggest that nNOS does not increase significantly as a result of autoimmune inflammation of the CNS.

Figure 1. Western blot analysis of neuronal NOS in the rat spinal cord. Untreated (A, lane 1), CFA-immunized (lane 2), paralytic stage of EAE (G.3) (lane 3), recovery stage of EAE (lane 3). Neuronal NOS was detected in untreated rat spinal cords (lane 1) and was insignificantly increased in the spinal cords of rats with EAE (lanes 3 and 4). Spinal cord tissues were obtained on day 13 (lanes 2 and 3) and at day 21 (lane 4) post-immunization. The molecular mass of neuronal NOS is 150 kDa.

Immunohistochemistry of nNOS in EAE rat spinal cords

In spinal cords from control rats, nNOS was constitutively expressed in ependymal cells, and some neurons (Fig. 2, A) and glial cells (Fig. 2, B). In EAE
Increased expression of neuronal nitric oxide synthase in astrocytes and macrophages in the spinal cord of Lewis rats with autoimmune encephalomyelitis

lesions, neurons showed increased immunoreactivity of nNOS (Fig. 3, A). In addition, nNOS immunoreaction (Fig. 3, C and E) was also recognized in glial cells, which were either GFAP- positive (Fig. 3, D), or ED1-positive macrophages (Fig. 3, F).

**Figure 2.** Immunohistochemical staining of neuronal NOS in the spinal cords of normal rats. Neuronal NOS was weakly expressed in some neurons (A) and astrocytes (B) in normal rat spinal cords. Counterstaining was performed with hematoxylin. Scale bar = 50 μm.

**Figure 3.** Immunohistochemical staining of neuronal NOS in the spinal cords of EAE rats. Neurons showed increased nNOS immunoreactivity (A). nNOS immunoreaction (C and E) was also recognized in GFAP- positive glial cells (Fig. 3, D) and in ED1-positive macrophages (Fig. 3, F). Arrows indicate matched cell groups in each mirror section. B is a negative control for immunostaining. Counterstaining was performed with hematoxylin. Scale bar = 50 μm.

We questioned whether the slight increase of nNOS observed in EAE spinal cords had an effect on EAE paralysis. Accordingly, the nNOS inhibitor 7-NI was used to block nNOS activity. As shown in Table 1, 7-NI treatment (30 mg/kg) significantly delayed the onset of paralysis, but it had little effect on either the incidence or the severity of EAE paralysis compared with vehicle-treated animals. Treatment with 7-NI at either the induction stage (days 0-7 PI) or the effector stage (days 7-13 PI) had no effect upon the clinical parameters. Both 7-NI- and vehicle-treated rats that were previously immunized with spinal cord homogenates developed floppy tails and paralysis (G3) at days 11-15 PI, and both groups of rats subsequently recovered. Histopathologically, EAE spinal cords from rats with and without 7-NI treatment showed typical perivascular cuffing. Moreover, the two groups were similar in terms of nNOS immunoreactivity. In general, the application of 7-NI appeared to have little effect on the pathogenesis of EAE.

**Table 1.** Effect of 7-nitroindazole (30 mg/kg) on clinical symptoms during EAE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Clinical First onset of</th>
<th>Mean peak clinical score*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>score ? 2 floppy tail</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8 / 8</td>
<td>3 / 8</td>
<td>11.5 ± 0.29</td>
</tr>
<tr>
<td>7-NI</td>
<td>7 / 7</td>
<td>3 / 7</td>
<td>12.75 ± 0.25**</td>
</tr>
<tr>
<td>(Days 1-7 PI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-NI</td>
<td>7 / 7</td>
<td>2 / 7</td>
<td>13.5 ± 0.96</td>
</tr>
<tr>
<td>(Days 7-14 PI)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data are expressed as the mean ± S.E.M.  
** p < 0.05, 7-NI-treated compared with vehicle-treated, Student’s unpaired, two-tailed t-test.

**DISCUSSION**

We examined the functional role of nNOS in EAE by Western blot and immunohistochemical analyses, and found that nNOS inhibition by the administration of the specific nNOS inhibitor 7-NI delayed the onset of rat EAE paralysis. However, the quantitative analysis demonstrated that nNOS levels do not increase significantly in EAE. These results suggest that nNOS is not actively involved in the induction stage of EAE, and are quite different from results obtained in previous studies of iNOS [14, 18], in which the inhibition of hematogenous macrophage iNOS was found to ameliorate or delay the onset of EAE paralysis. In fact, the results of the present study indicate that nNOS in EAE-affected spinal cords plays only a minor role in the induction stage of EAE, if any, and does not generate detrimental levels of NO.

Although the toxic effect of nNOS has been well documented in ischemic injury, little is known about the
role of nNOS in autoimmune diseases. In recent studies of the effects of 7-NI on the pathogenesis of adjuvant-induced arthritis, an inflammatory role was indicated for nNOS, but not for iNOS [17]. However, these NOS isoforms appear to have different involvements in the pathogenesis of CNS autoimmune disease, including rat EAE, than they do in adjuvant-induced arthritis. In another report [12], nNOS mRNA was detected in cultured human macrophages, and nNOS immunoreactivity was detected in some rat macrophages after EAE induction by the administration of pertussis toxin [6]. Since nNOS was detected in ED1-positive macrophages in the present study, we postulate that pertussis toxin may specifically induce or activate nNOS as well as iNOS in inflammatory macrophages during EAE.

There is a general agreement on the phenotype of nNOS expression in rat spinal cord neurons and ependymal cells [13]. In addition, nNOS is known to immunostain in reactive astrocytes of transgenic mice expressing a human Cu/Zn SOD mutation [4], and in astrocytes and Bergmann glia in normal spinal cords [7]. We postulate that brain cells, including neurons and astrocytes, are possible sources of nNOS in EAE, and that constitutive neuronal NOS plays a role in normal spinal cord physiology. We conclude that, in CNS autoimmune disease models, brain cell derived nNOS probably plays a physiological role in neurotransmission rather than in tissue injury, while macrophage associated nNOS has a detrimental effect in animal models of CNS neuroimmunological disorders.

ACKNOWLEDGEMENTS

This work was supported by a Korean Research Foundation Grant (KRF-2000-041-G00118). The author wishes to express his gratitude to a graduate school student, M. Ahn, for the technical assistance.

REFERENCES


