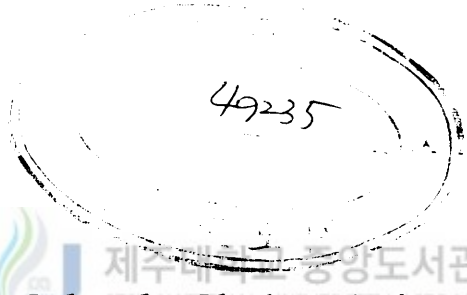
A Thesis

For The Degree of Master of Science

Upregulation of Constitutive Nitric Oxide Synthase in the Spinal Cord of Rats with Experimental Autoimmune Encephalomyelitis



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Abstract

Upregulation of Constitutive Nitric Oxide Synthase in the Spinal Cord of Rats with Experimental Autoimmune Encephalomyelitis

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To elucidate the role of nitric oxide (NO) in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), we examined the expression of three isoforms of nitric oxide synthase (NOS), an enzyme of NO production, in the spinal cords of rats with EAE using Western blot analysis and immunohistochemistry. We also examined the expression of inducible NOS (iNOS) in EAE lesions with treatment using aminoguanidine, a selective iNOS inhibitor, to see whether iNOS in the target organ spinal cords is associated with the exacerbation of EAE.

Western blot analysis showed that three isoforms of NOS, constitutive neuronal, endothelial, and inducible NOS, increased in the spinal cords of rats at the peak stage of EAE and persisted through the recovery stage, while minimal amounts of these same enzymes are identified in normal rat spinal cords. Using immunohistochemistry, some inflammatory cells as well as some neurons were positive for constitutive neuronal NOS during the peak and recovery stages of EAE, while it was only localized in a few neurons in the normal spinal cord. Endothelial NOS was localized mainly in endothelial cells and astrocytes in the spinal cords with EAE. Inducible NOS was found mainly in the inflammatory macrophages in the perivascular EAE lesions.

Aminoguanidine (AG), an iNOS inhibitor, significantly reduced the clinical severity of EAE paralysis when it was administered during the induction of EAE. According to Western blot analysis, the amelioration of EAE was closely associated with the reduced expression of inducible NOS in the target organ spinal cords.

Combined, these findings suggest that the 3 isoforms of NOS are upregulated in inflammatory autoimmune central nervous system lesions, and the sequential production of NO via constitutive and inducible NOS plays an important role in the induction of autoimmune diseases including EAE.

Key words: nitric oxide synthase, autoimmune disease, astrocytes, neurons.

CONTENTS

1. Introduction

Nitric oxide (NO) is a readily diffusible apolar gas synthesized from L-arginine via nitric oxide synthase (NOS) (Xie and Nathan, 1994). The enzyme responsible for NO formation exists in two forms: (1) a constitutive, Ca2+-dependent form (cNOS), which is rapidly activated by agonists that elevate intracellular free Ca2+ (Moncada et al., 1991) and includes neuronal NOS (nNOS) and endothelial NOS (eNOS); and (2) a Ca2+-independent inducible form (iNOS), which is induced after several hours of immunological stimulation (Moncada et al., 1991; Xie et al., 1992). In the central nervous system (CNS) tissues, constitutive NOS is thought to synthesize NO, which is important for intracellular signaling, neurotransmission, and vasoregulation (Bredt and Snyder, 1992). Unless activated, iNOS is not expressed in brain cells (Lee et al, 1993, Murphy et al, 1993). In the CNS, the local generation of toxic concentrations of NO via cNOS and iNOS has been implicated in mediating excitotoxic neuronal injury (cNOS; Dawson et al., 1991, 1993), hypoxic-ischemic brain damage (cNOS; Buisson et al., 1992; Nowiki et al., 1992; Huang et al., 1994; iNOS, Iadecola et al., 1995a, b), inflammatory demyelinating disorders (iNOS, MacMicking et al., 1992; Lin et al., 1993; Hooper et al., 1995).

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS (Raine, 1976; Bernard et al., 1983) used to study human demyelinating diseases such as multiple

sclerosis (Raine, 1984). The clinical course of EAE is characterized by weight loss, ascending progressive paralysis, and spontaneous recovery (Raine, 1984). This coincides with an inflammatory response in the CNS that is characterized by infiltration of T cells and macrophages and activation of microglia and astrocytes (Shin et al., 1995).

Although several studies have shown that iNOS is an important mediator of CNS inflammation through the generation of NO, little is known about the expression of constitutive NOS isoenzymes, including neuronal and endothelial NOS. These forms of NOS, as well as iNOS, are also possible enzymes for NO production *in vivo* and *in vitro* (Tran et al, 1997).

In this study, we studied the expression of three isoforms of NOS (nNOS, eNOS, and iNOS) in the course of rat EAE using Western blot analysis and immunohistochemistry to examine the functional role of NOS in autoimmune CNS disease models. We also examined the role of iNOS in the induction of EAE using aminoguanidine (AG), an iNOS inhibitor.

2. Materials and Methods

2.1. Animals

Lewis rats were obtained from the Korean Research Institute of Bioscience and Biotechnology, KIST (Taejeon, Korea) and bred in our animal facility.

2.2. EAE induction

EAE was induced in 7-12 week old Lewis rats as described previously (Ohmori et al, 1992). Briefly, each rat was injected in the hind foot pads bilaterally with an emulsion containing an equal part of fresh rat spinal cord homogenates in phosphate buffer (g/mL) and complete Freunds adjuvant (CFA; *Mycobacterium tuberculosis* H37Ra, 5 mg/mL; Difco Lab, Detroit, MI). After immunization, some rats were given *Bordetella pertussis* toxin (Sigma Chemical Co., St. Louis, MO) intraperitoneally. Immunized rats were observed daily for clinical signs of EAE. The progress of EAE was divided into seven clinical stages (Grade (G) 0, no signs; G1, floppy tail; G2, mild paraparesis; G3, severe paraparesis; G4, tetraparesis; G5, moribund condition or death; R0, recovery stage) (Ohmori et al, 1992). Control rats were immunized with CFA only. Five rats were killed under ether anesthesia at various stages of the EAE.

2.3. *Tissue sampling*

Tissue sampling was performed on days 12-14 and 21-25 post-immunization (PI), during the peak and recovery stages of EAE, respectively. For the Western blot analysis and histology, experimental rats were sacrificed under ether anesthesia, and the spinal cords were removed and frozen in a deep freeze (-70°C) for protein analysis. Pieces of the spinal cords were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. A segment of spinal cord was further fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer and processed for EPON 812 embedding. Thin sections were stained with uranyl acetate and lead citrate, and studied by transmission electron microscopy (JEOL EX II 1200).

2.4. *Western blot antalysis*

Frozen spinal cords with EAE were thawed at room temperature, minced, weighed, placed in PBS (1:4 w/v), and homogenized with a Tissue-Tearor (Biospec, USA). The homogenate was sonicated three times (5 sec at room temperature) and centrifuged at 12,000g for 10 min. The supernatant was diluted with electrophoretic sample buffer to obtain a protein concentration of 3g/L, and heated at 100°C for 5 min. Samples were electrophoresed under denaturing conditions in sodium

dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using a discontinuous procedure (Laemmli, 1970). Stacking gels were 4.5% polyacrylamide and separating gels were 7.5% polyacrylamide. Paired mini-gels (Mini-protein II cell, Bio-Rad Laboratories, U.S.A.) were loaded with 30g protein per well. The protein concentration was estimated using the method of Bradford (1970). Samples containing iNOS (130 kDa), eNOS (140 kDa), or standard markers (Transduction laboratories, Lexington, KY) were run at 100V/gel slab. After electrophoresis, one mini-gel was routinely stained by the Coomassie blue-staining method and the other was equilibrated in a transfer buffer (25mM Tris, 192mM glycine and 20% v/v methanol at pH 7.3). The proteins were then electrotransferred in the transfer buffer to a PROTRAN® nitrocellulose transfer membrane (Schleicher and Schuell, Keene N. H., USA) overnight at 4℃ and 30 Volts. The transferred proteins were visualized by staining the membrane for 10 min with Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO). Incubating the nitrocellulose membrane in TBS-5% BSA (50mM Tris/HCl, 20nM NaCl, pH 7.4 containing 5% bovine serum albumin) for 2 hours at room temperature blocked non-specific sites. The blot was then rinsed with TBS-T (TBS with 0.1% Triton X-100). The iNOS and eNOS binding was detected by incubating the membrane in a moist chamber overnight at 4°C, with the primary antibody (rabbit anti-iNOS, rabbit anti-eNOS, or rabbit anti-nNOS (Transduction Laboratories, Lexington, KY) and rabbit anti-nitrotyrosine (Upstate Biotechnology, NY) diluted with TBS-T, 1:1000). The finding of NT indicates that peroxynitrite was generated

nearby, either in the past or recently, and that one of its potentially damaging effects (nitration) on protein occurred (Beckman et al., 1994). After washing in TBS-T, the membrane was incubated with the second antibody (anti-rabbit IgG and anti-mouse IgG peroxidase conjugate diluted in TBS 1:3000, Sigma Chemical Co., St. Louis, MO) for 3h at room temperature. Visualization was achieved using 1% 3,3'-diaminobenzidine-HCl in 0.1% TBS.

2.5. *Immunohistochemistry*

Five-micron sections of paraffin-embedded spinal cords were deparaffinized and treated with 0.3% hydrogen peroxide in methyl alcohol for 30 minutes to block endogenous peroxidase. After three washes with PBS, the sections were exposed with 10% normal goat serum, and then incubated with primary antisera including rabbit anti-nNOS, rabbit anti-eNOS or rabbit anti-iNOS antisera (1:200 dilution) for 1 hour at room temperature. After three washes, the appropriate biotinylated second antibody and the avidin-biotin peroxidase complex *Elite* kit (Vector, Burlingame, CA) were added sequentially. Peroxidase was developed with diaminobenzidine-hydrogen peroxidase solution (0.001% 3,3'-diaminbenzidine and 0.01% hydrogen peroxidase in 0.05M Tris buffer). Before being mounted, the sections were counterstained with hematoxylin.

2.6. *Treatment of EAE rats with aminoguanidine (AG), an iNOS inhibitor*

To evaluate the effect of NO in the induction of acute EAE, rats were treated with AG, a selective iNOS inhibitor, from day 0 to 14 PI or from day 0 to 7 PI. The rats were administered a daily dose of 200 mg/kg of AG dissolved in PBS intraperitoneally. A third group of animals received 200 mg/kg of AG from day 7 to 14 PI. Control rats were treated with only PBS in the same way.

2.7. *Statistical analysis*

Statistical calculations were evaluated by ANOVA to examine the variation in the course of EAE. Differences with a p-value < 0.05 were considered as significant.

3. Results

3.1. Clinical observation of EAE

The clinical course of EAE is shown in Fig. 1. EAE rats immunized with spinal cord homogenates developed floppy tail (G1) on days 9-10 PI, and showed severe paresis (G3) on days 11-15 PI. All the rats recovered subsequently (Fig. 1).

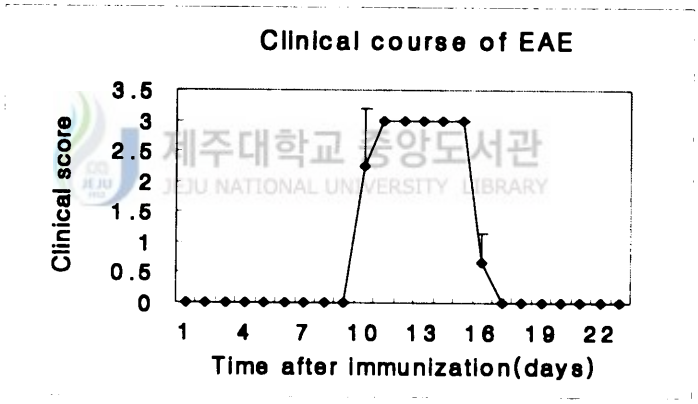

Figure 1. The clinical course of rat spinal cord homogenat- induced experimental autoimmune encephalomyelitis (EAE) in Lewis rats. Each rat was injected in the hind foot pads bilaterally with an emulsion containing raw rat spinal cord in complete Freunds adjuvant supplemented with Mycobacterium tuberculosis H37Ra (5 mg/mL). Rats were given Bordetella pertussis toxin intraperitoneally. The clinical course was classified into seven categories: G0. No clinical signs; G1. Floppy tail; G2. Mild paraparesis; G3. Severe paraparesis; G4. Tetraparesis; G5. Moribund condition or death; R0, recovery stage.

3.2. *Histological findings of EAE*

A large number of inflammatory cells infiltrated the perivascular lesions and parenchyma of the spinal cord of rats with EAE at the peak stage (Fig. 2B). In the normal spinal cord, no infiltrating cells were detected in the spinal cord parenchyma (Fig. 2A). Using transmission electron microscopy, focal demyelination was recognized in the dorsal root entry zone of the spinal cord of rats with EAE (Fig. 2C).

3.3. *Western blot analysis of three isoforms of NOS in EAE*

The expression of both nNOS (Fig. 3, left column), eNOS (Fig. 3, right column), and iNOS (Fig. 4) was assessed semiquantitatively using densitometry.

Intense immunoreactivity of both nNOS and eNOS was identified at the peak stage (G3) of EAE (Fig. 3, lane 2/ea), and remained until the recovery stage of EAE (day 21 PI, R0) (Fig. 3, lane 3/ea), although little nNOS and eNOS was identified in normal spinal cords (Fig. 3, lane 1/ea). Increased expression of nNOS and eNOS compared with normal rat spinal cords ($p<0.01$) was evident by densitometric semiquantitative analysis (Fig. 2, graphs)($P<0.01$).

Unlike the expression of cNOS in the spinal cords, iNOS was

barely identified in normal spinal cords (Fig. 4, lane 1). iNOS immunoreacted during the peak and recovery stages of EAE (Fig. 4, lanes 2 and lane 3). Using densitometric semiquantitative analysis (Fig. 4, graph), iNOS immunoreactivity in the spinal cord of EAE-affected rats increased significantly compared with that in normal and 5CFA treated rat spinal cords ($p<0.05$). Increased expression of iNOS ($p<0.05$) persisted through the EAE recovery stage (day 21 PI).

The amounts of cNOS and iNOS increased in the 5CFA treated rat spinal cords, as compared with normal controls (data not shown).

These data indicate that the induction of EAE upregulates both constitutive nNOS and eNOS in addition to iNOS.

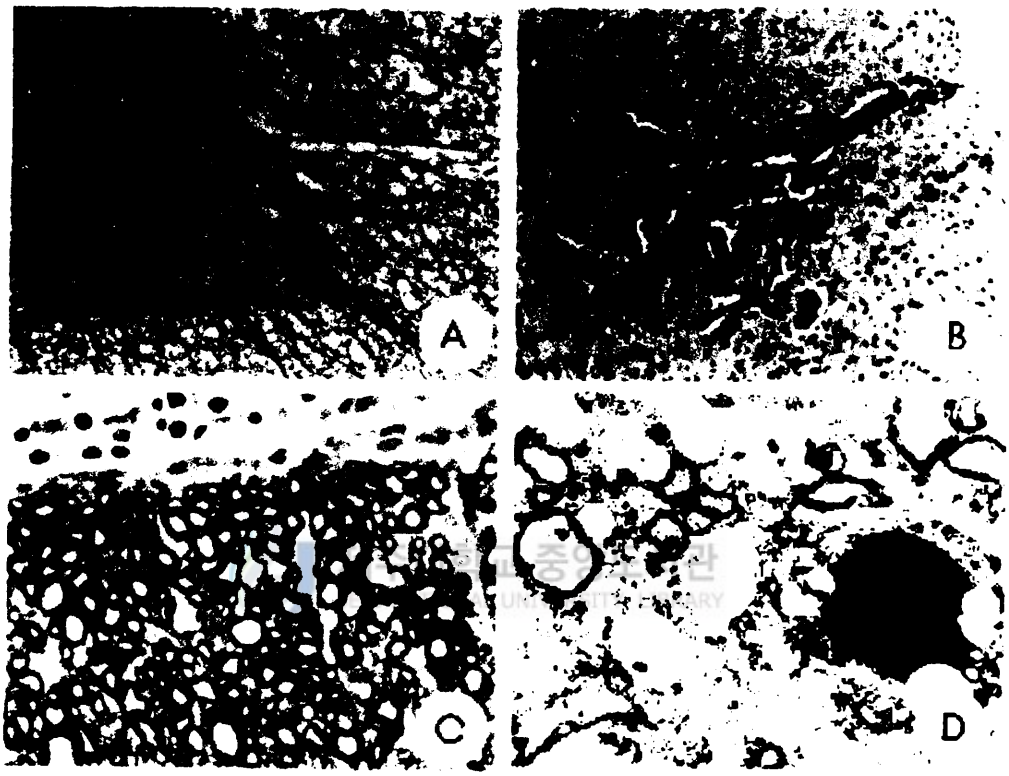

Figure. 2. Photomicrographs of the spinal cord from a normal (A) and an EAE-induced rat (B, C) and transmission electron micrograph of EAE-affected spinal cord (D). A: Cross section of a normal spinal cord. B: Infiltration of inflammatory cells in the parenchyma and perivascular cuffing is evident in the EAE spinal cord during the peak stage. C, D: Myelination and demyelination plaque are seen in the spinal cord parenchyma of EAE affected rat. A, normal spinal cord; B, C and D, EAE (G3, day 13 PI). A, B: H-E staining; C: Toluidine blue staining; D: transmission electron micrograph. Original magnification: A and B, x100; C, x220; D, x5000.

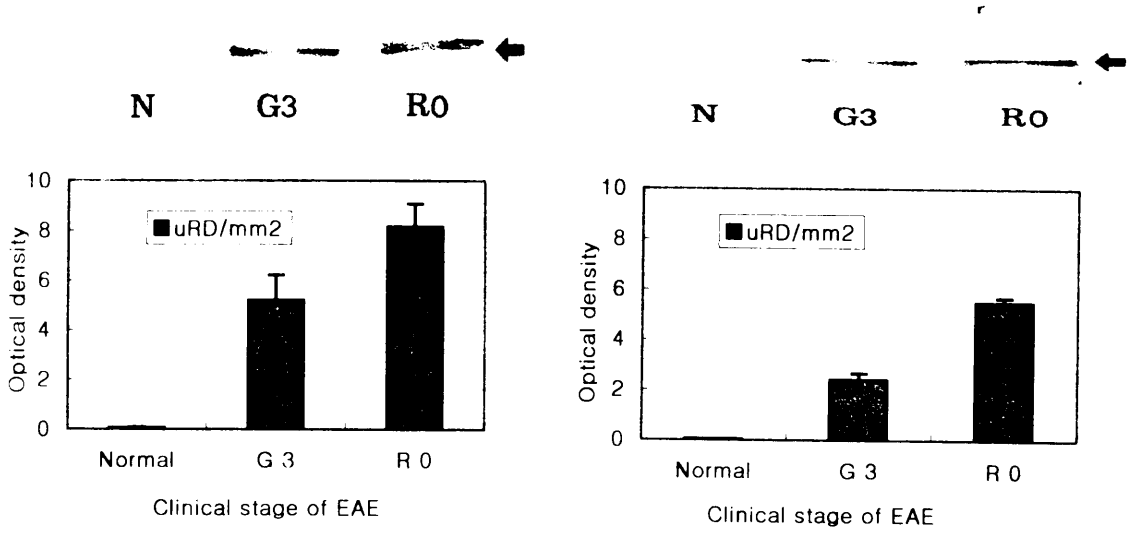

Figure 3. Western blot analysis of constitutive neuronal NOS (nNOS) (left) and endothelial NOS (eNOS) (right) in the spinal cords of rats with normal (N), 5CFA (data not shown), peak (G.3) and recovery stage(R.0) of EAE. Minimal amounts of nNOS and eNOS were identified in normal spinal cords (lane 1 per each blot) but its expression increased in spinal cord of 5CFA treated rats (data not shown), as compared with normal control. In spinal cords with EAE, both nNOS and eNOS were upregulated in the spinal cord of a rat with grade 3 paralysis (G3) (lane 2, day 10 PI) and its expression persisted during the EAE recovery stage (R0) (lane 3, day 15 PI). The molecular weight of nNOS (155 kDa) and eNOS (140 kDa) was indicated respectively. *Graphs,* A semiquantitative analysis of nNOS and eNOS and at different clinical states (normal, peak stage, recovery stage) was made using optical density (OD) measurements on Western blot signals (uRD/mm2). (uRD uncalibrated reflective density.) The data are the mean SEM (n=3 per clinical score) and represent significant changes in the EAE-induced spinal cord versus the normal spinal cord ($p<0.01$).

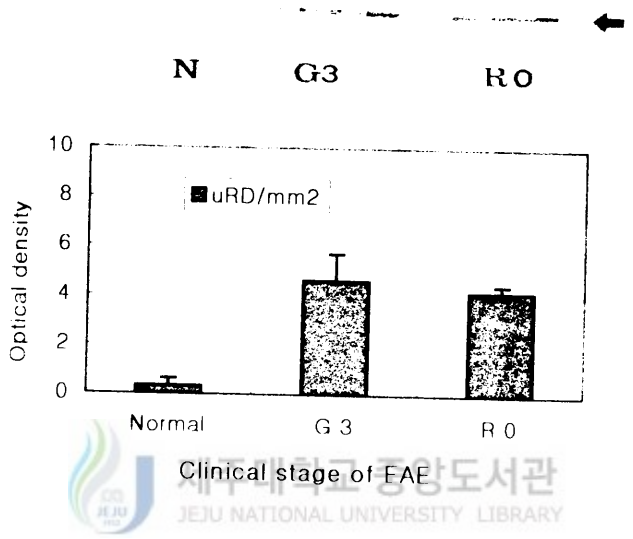

Figure 4. Western blot analysis of iNOS in the spinal cords of rats with EAE. Minimal amounts of iNOS were identified in normal spinal cords (lane 1) but, in the 5CFA treated rat spinal cord, iNOS expression increased (data not shown), as compared with normal control. In the EAE spinal cord, iNOS was upregulated in the spinal cord of a rat with grade 3 paralysis (G3) (lane 2, day 10 PI) and its expression persisted during the EAE recovery stage (R0) (lane 3, day 15 PI). The molecular weight of inducible NOS (130 kDa) was indicated. *Graph*, A semiquantitative analysis of iNOS at different clinical states (normal, peak stage, recovery stage) represents significant changes in the EAE-induced spinal cord versus the normal spinal cord ($p<0.05$). The data are the mean SEM (n=3) at the same conditions.

3.4. *Cell phenotypes of bNOS, eNOS, and iNOS in EAE*

The spinal cords of normal and EAE-affected rats were used for immunohistochemical examination. In EAE-affected rats, nNOS was strongly expressed in neurons and some inflammatory cells in the EAE lesions of the spinal cord (Fig. 5B). In the same lesions, eNOS immunoreactivity was detected in the endothelial cells of blood vessels and some astrocytes (Fig. 5D). This shows that brain cells, including neurons and astrocytes, are prone to express cNOS upon stimulation by invading cells. iNOS immunoreactivity was found predominantly in infiltrating ED1+ macrophages in the EAE lesions (Fig. 5F), but not in other brain cells. In normal rats, nNOS, eNOS and, iNOS were rarely identified in the parenchyma of normal spinal cords (Fig. 5A, C, E).

3.5. *Evidence for NO and peroxynitrite production by Western blot analysis of nitrotyrosine (NT)*

Nitrotyrosine immunoreactivity was recognized during the peak (Fig. 6, lane 2) and recovery stages (Fig. 6, lane 3) of EAE, but not in normal or CFA immunized spinal cords (lane 1). The increased expression of NT during the peak stage of EAE suggests that peroxynitrite or NO is generated in the autoimmune spinal cord lesions.

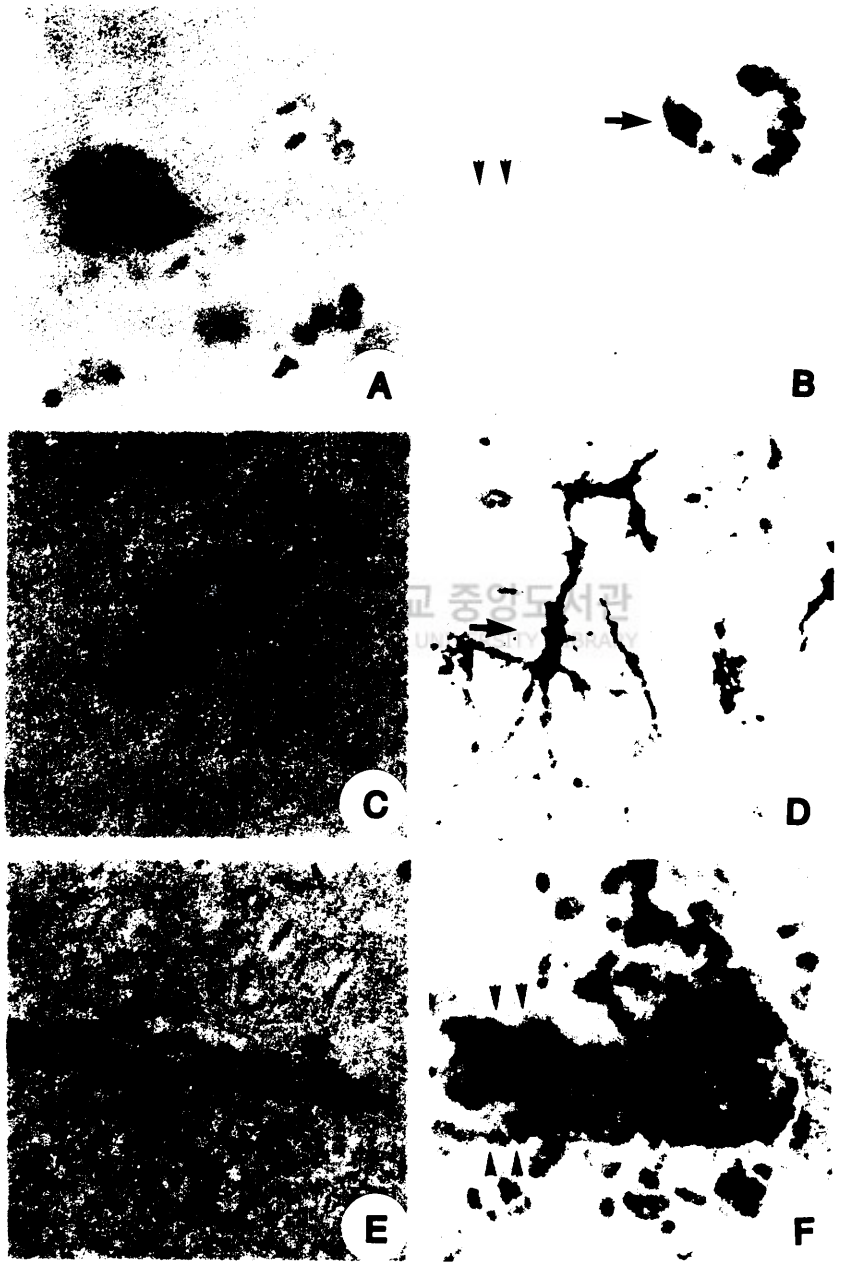

Figure 5. Immunostaining three isoforms of NOS in the spinal cords of normal (A, C, E) and EAE-affected rats (B, D, F). In normal rats, minimal

amounts of all three types of NOS (iNOS, eNOS, and nNOS) were identified in the spinal cord parenchyma. At the peak stage of EAE, nNOS-positive cells were seen in neuronal cell bodies (arrowhead) in the gray matter and some inflammatory cells (arrow) in the parenchyma of spinal cord (B). eNOS-positive cells were found in vessels and some astrocytes (arrow) (D). Oval type iNOS-positive cells (arrowhead) were found mainly in perivascular lesions (F). Counterstained with hematoxylin. A, C and E, normal spinal cord; B, D and E, EAE (G3, days 12-14 PI). Original magnification: x200. A and B, Rb anti-nNOS, C and D, Rb anti-eNOS, E and F, Rb anti-iNOS antibody.

3.6. Aminoguanidine (AG), a selective iNOS-inhibitor, delayed the onset and reduced the duration of clinical EAE

In AG-treated EAE rats, the onset was delayed in comparison with control EAE rats ($p<0.0006$, Table 1). A significant reduction in the duration of the illness was also observed in AG-treated versus control EAE rats ($p<0.02$, Table 1). These data indicate that daily intraperitoneal injection of AG at a dose of 200 mg/kg body weight significantly reduced the duration of paralysis in the acute EAE model. To visualize the effect of AG on iNOS expression in AG treated EAE rats, we compared iNOS expression in the spinal cord of EAE rats in AG- versus placebo-treated groups. At day 10 PI, no iNOS was

detected in the spinal cord of EAE rats treated with AG and there was no clinical EAE paralysis, while iNOS expression was significantly increased in placebo treated EAE rats at the same time. However, all of the immunized rats treated with either placebo or AG subsequently developed EAE paralysis. At this stage (day 15 PI), equal amounts of iNOS were found in the spinal cords of both placebo- and AG-treated EAE rats (Fig 7), although in reduced amounts.

Figure 6. Immunoblotting analysis of nitrotyrosine in the spinal cord of the complete Freunds adjuvant (CFA)-treated group (lane 1) and the peak (lane 2) and recovery (lane 3) stages of EAE-affected rats. These spinal cord extracts were resolved by electrophoresis, transferred to nitrocellulose and probed with rabbit anti-nitrotyrosine (2 g/mL). Immunoblot analysis of nitrotyrosine (2 g/mL) detected nitrotyrosine molecular weight standards at 14, 31, 66, 116, and 200 kDa. Nitration in the ortho position of tyrosine residues in proteins is a widespread observation in diverse pathological conditions. Nitrotyrosine immunoreactivity was seen in the CFA-treated group (lane 1), and peak (lane 2) and recovery (lane 3) stages of EAE, but not in the normal group.

Table 1. The effect of aminoguanidine on the clinical parameters of EAE.

Parameter[a]	Control	Aminoguanidine-treated group		
		EXP I (D0-D7pi)	EXP II (D7-D14pi)	EXP III (D0-D14Pi)
Incidence of clinical EAE	5/5	4/5	5/5	4/4
First onset.	9.2±0.8[b]	11.8±0.25[b]	11±0.0[b]	11.7±0.3[b]
Maximam clinical severity (Scale from 0 to 5)	3±0.0	3±0.0	3.8±0.84	3±0.0
Duration of illness(days)	6.3±0.6[c]	4±0.6[c]	4.7±0.3[d]	4±0.0[c]

All rats in the treated group received a daily intraperitoneal injection with 200 mg/kg body weight of aminoguanidine (AG).

a Data are expressed as mean ± S.E. except incidence of clinical EAE, in which the second number indicates the number of rats studied. In the AG-treated group, only those rats with clinical EAE were included to evaluate the maximum clinical severity.

b First onset differences between the control and AG-treated groups are statistically significant ($p<0.0006$), but are not significant between the AG-treated groups.

c, d The duration of paralysis between the two groups was considered significant ($p<0.02$), except in the EXP-II group (d).

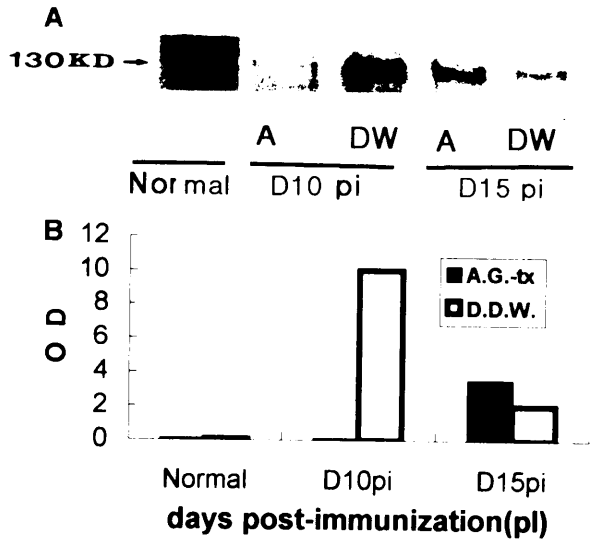

Figure 7. A: Western blot analysis of iNOS in the spinal cords of rat EAE with AG (lane 2: day 10 PI, lane 4: day 15 PI) and placebo treatment (lane 3: day 10 PI, lane 5: day 15 PI). No iNOS immunoreactivity was detected in normal spinal cords (lane 1). In the placebo treated EAE control spinal cord, iNOS was upregulated in the spinal cord of a rat with grade 3 paralysis (G3) (lane 3, day 10 PI), but its expression decreased during the recovery stage of EAE (R0) (lane 5, day 15 PI). Arrows indicate the location of 130 kDa iNOS molecular weight markers. In AG-treated rats, iNOS immunoreactivity was not detected at day 10 PI (lane 2). During the recovery stage (lane 4: day 15 PI), a low level of iNOS immunoreactivity was generated.

B: Semiquantitative analysis of iNOS for different clinical scores (normal, peak stage, recovery stage), determined by optical density (OD) measurements of Western blot signals. 1, normal stage; 2 and 3, day 10 PI; 4 and 5, day 15 PI. uRD/mm2: uncalibrated reflective density.

4. Discussion

In this study, semiquantitative analysis showed that the immunoreactivity of constitutive NOS, including nNOS and eNOS, was significantly increased during the peak and recovery stages of EAE.

Constitutive neuronal and endothelial NOS are Ca2+-dependent and act as a neurotransmitter in the central nervous system (Bredt and Synder, 1992), and regulate vasorelaxation (Moncada et al., 1991), respectively. Although both nNOS and eNOS are known physiological mediators, increased expression of these molecules in the CNS is associated with extension of the injury (Sharma et al, 1995) and exacerbation of neuronal injury in viral infections of the CNS (Barna et al., 1996). Furthermore nNOS is readily inducible in some neurons by stimulation with formalin (Lam et al., 1996), and in spinal cord injury (Sharma et al., 1996). Also, eNOS is upregulated in ischemia (Samdani et al., 1997) and autoimmune disorders such as experimental anti-myeloperoxidase associated crescentic glomerulonephritis (Heeringa et al., 1998).

Our findings in the rat model of autoimmune encephalomyelitis are consistent with previous studies of viral infection in the brain (Barna et al, 1996). This suggests that cNOS has the potential to be upregulated after immunological stimulation (this study), as well as after neurotropic viral infections (Barna et al, 1995) and in the focal ischemia model (Samdani et al., 1997).

The functional role of constitutive NOS in CNS diseases is not well understood because the cNOS-containing cells generate picomole level amounts of NO and peroxynitrite. However, a recent study suggests that eNOS also has the capacity to induce apoptosis or programmed cell death (Zini et al., 1996). If this is the case, the low level of NO produced by nNOS and eNOS in autoimmune CNS disease is effective in inducing apoptosis of inflammatory cells combined with the large amounts of NO generated by inflammatory macrophages containing iNOS in EAE. This finding supports the hypothesis that a low level of NO is associated with the inhibition of T cell proliferation in the spinal cord in EAE. Indeed, autoimmune T cells do not proliferate vigorously in the target organ spinal cords (Ohmori et al., 1992).

In this study, we identified the diffuse expression of both cNOS and iNOS in the parenchyma of spinal cords. Since iNOS is expressed in astrocytes in mouse EAE (Tran et al., 1997) and constitutive endothelial isoforms of NOS are expressed by brain cells and inflammatory cells in rat EAE (this study), the generation of NO by both cNOS and iNOS may play an important role in either the exacerbation or recovery of EAE. The precise role of constitutive NOS in EAE should be studied further. Because T cells from PVG rat treated with cNOS inhibitor induce EAE suggesting that cNOS play a role in downregulation of CNS inflammation (Cowden et al., 1998).

NO has two contradictory effects on EAE, it exacerbates EAE (Zhao et al, 1996) and has a host protective effect through apoptosis, which is associated with EAE recovery (Okuda et al., 1997). However, little is

known on the expression of iNOS in the autoimmune target organ when a selective iNOS inhibitor is administered during the induction of EAE. In the iNOS inhibition experiment using the iNOS inhibitor aminoguanidine, we found that the administration of AG (200 mg/kg body weight) tended to shorten the duration of EAE paralysis. This finding supports the fact that iNOS is involved in the induction of autoimmune paralysis via the generation of NO.

iNOS has been well studied in host tissue defense in response to viral infections, and has been identified in stimulated astrocytes and microglia by cytokines. These cells are the major brain cell types that express iNOS *in vitro* (Lee et al, 1993) and *in vivo* in the mouse EAE model (Tran et al, 1997), although iNOS is not normally expressed in brain cells (Koprowski et al., 1993) or in rat EAE (this study). The lack of iNOS staining of the astrocytes in rat EAE tissues might result from differences in the antisera or the immunostaining methods used in this study. This negative finding does not mean that iNOS is not involved in the apoptosis of inflammatory cells in the EAE lesions, because the excess production of NO in cultured macrophages *in vitro* (Sarih et al., 1993), and the intense expression of iNOS in EAE lesions (Okuda et al, 1997) are both associated with apoptosis in either an autocrine or a paracrine manner. We are currently investigating the structural interaction between apoptosis and NOS-expressing cells in autoimmune disease models.

Our study showed that both cNOS and iNOS are upregulated in the initiation of EAE, and that NOS (including nNOS, eNOS, and iNOS)

has a detrimental effect during the induction of EAE. The sequential generation of NO from both nNOS and eNOS in brain cells, including astrocytes, plays a defensive role in inhibiting the proliferation of invading cells with a low level of NO. Ultimately, the stasis of T cell proliferation induces apoptosis through multiple cascades.

We conclude that a low level of NO, produced from both nNOS and eNOS, has a beneficial role by removing inflammatory cells through apoptosis in autoimmune encephalomyelitis, but that a higher level of NO from exogenous iNOS in perivascular lesions has a detrimental effect during the induction of EAE.

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초 록

자기면역성 뇌척수염에서 Constitutive Nitric Oxide Synthase의 발현 증가

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자기면역성 뇌척수염 (experimental autoimmune encephalomyelitis, EAE) 발병에 nitric oxide (NO)의 관여여부를 규명하기 위하여, Western blot과 면역조직화학적 방법을 이용하여 NO를 생산하는 효소인 nitric oxide synthase (NOS)의 발현을 자기면역성 뇌척수염 랫트의 척수에서 조사하였다. 또한 NO의 역할을 조사하기 위하여 Inducible NOS (iNOS) 억제제인 aminoguanidine를 EAE 랫트에 투여한 후 다각적으로 마비 정도를 검사하였다.

Western blot 결과 constitutive neuronal NOS (nNOS)와 endothelial NOS (eNOS) 그리고 iNOS는 심한 마비기에서 강하게 발현하였고, 그 발

현정도는 회복기 까지 지속되었다. 면역염색 결과, nNOS는 심한 마비기와 회복기에서 일부 neuron및 침윤된 일부 염증세포에서 양성반응을 보였지만, 정상 상태의 척수에서는 거의 확인되지 않았다. eNOS는 주로 혈관 내피세포와 일부 astrocytes에서 강하게 발현되었다. iNOS는 주로 척수 혈관 주위에 침윤한 염증세포에서 양성반응을 보였다.

iNOS가 심급성 자기면역성 마비에 미치는 영향을 확인하기 위하여 iNOS 억제제인 aminoguanidine을 면역일 부터 계속하여 투여한 결과, 마비기간이 대조군보다 유의성 있게 감소되고, 그 질병의 유발 시점도 2-3일 지연되었다.

이상의 결과, 뇌조직에서 상존하는 형태의 NOS인 nNOS 및 eNOS 그리고 iNOS는 자기면역성 중추신경계 질병 부위에서 강하게 발현하고, 이러한 효소에 의해 생성된 NO는 자기면역성 질병에서 중요한 역할을 할 것으로 생각된다.

주요어 : nitric oxide synthase, autoimmune disease, astrocytes, neurons.

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