

Enhanced expression of constitutive and inducible forms of nitric oxide synthase in autoimmune encephalomyelitis

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To elucidate the role of nitric oxide synthase (NOS) in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), we analyzed the expression of constitutive neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) in the spinal cords of rats with EAE. We further examined the structural interaction between apoptotic cells and spinal cord cells including neurons and astrocytes, which are potent cell types of nitric oxide (NO) production in the brain. Western blot analysis showed that three forms of NOS significantly increased in the spinal cords of rats at the peak stage of EAE, while small amounts of these enzymes were identified in the spinal cords of rats without EAE. Immunohistochemical study showed that the expression of either nNOS or eNOS increased in the brain cells including neurons and astrocytes during the peak and recovery stages of EAE, while the expression of iNOS was found mainly in the inflammatory macrophages in the perivascular EAE lesions. Double labeling showed that apoptotic cells had intimate contacts with either neurons or astrocytes, which are major cell types to express nNOS and eNOS constitutively. Our results suggest that the three NOS may play an important role in the recovery of EAE.

Key words: nitric oxide synthase, microglia, astrocytes, autoimmune encephalomyelitis

Introduction

Nitric oxide (NO) is a readily diffusible apolar gas synthesized from L-arginine via nitric oxide synthase (NOS) [49]. The enzyme exists in two forms: (1) a Ca²⁺-dependent and constitutive NOS and (2) a Ca²⁺-in-

dependent inducible NOS (iNOS) [31, 48]. The constitutive NOS includes neuronal NOS (nNOS) and endothelial NOS (eNOS) which are rapidly activated by agonists that elevate intracellular free Ca²⁺. The inducible NOS is induced several hours after an immunological stimulation [31, 48]. In the central nervous system (CNS), the constitutive NOS synthesizes NO, which is known to play an important role in intracellular signaling, neurotransmission, and vasoregulation [6, 32]. However, iNOS is not expressed in the brain cells unless activated [26, 32, 44]. In the CNS, the local generation of NO by nNOS and/or iNOS has also been implicated in toxic injuries including excitotoxic neuronal injury (nNOS) [12, 13], hypoxic-ischemic brain damage (nNOS, iNOS) [8, 20-22, 33], traumatic brain injury (nNOS) [41], and autoimmune disorders (iNOS) [19, 27-29, 43].

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS, which is designed to study human demyelinating diseases such as multiple sclerosis [37]. The clinical course of EAE is characterized by weight loss, ascending progressive paralysis, and spontaneous recovery. 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 at the peak stage of EAE [34, 42], and apoptotic elimination of inflammatory cells leading to recovery [1, 23].

Several studies have shown that iNOS is an important mediator of CNS inflammation through the generation of NO in the course of EAE [7, 11, 28, 35, 45, 50] as well as in human multiple sclerosis lesions [14]. Contrary to these previous findings, NO and its relevant enzymes including iNOS have been shown to play a beneficial role in the course of EAE because iNOS inhibition aggravated EAE progression depending on the stage of inflammation [10, 17, 36, 38] and because EAE was exacerbated in mice lacking the NOS2 gene [15]. Furthermore, animals with EAE at high levels of NO and iNOS recover from

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paralysis [35], suggesting that iNOS may have a capacity to prevent immunologically privileged CNS from invading inflammatory cells in EAE. Recently, Gonzalez-Hernandez and Rustioni [18] reported that the three isoforms of NOS including nNOS, eNOS and iNOS, exert a beneficial effect on peripheral nerve regeneration.

In the course of acute EAE in mice, we examined the quantitative changes of the three isoforms of NOS by Western blot analysis and the structural interaction between apoptotic cells and brain cells by immunohistochemistry.

Materials and Methods

Animals

Lewis male rats (7-12 weeks old) were obtained from the Korea Research Institute of Bioscience and Biotechnology, KIST (Taejon, Korea) and bred in our animal facility. The animals weighing 160-200 g were used throughout the experiments.

EAE induction

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 Freund's adjuvant (CFA; *Mycobacterium tuberculosis* H37Ra, 5 mg/ml; Difco). Immunized rats were further given *Bordetella pertussis* toxin (2 µg/ea) (Sigma Chemical Co., St. Louis, MO) intraperitoneally and 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) [34]. Control rats were immunized with CFA only. Five rats were killed under ether anesthesia at the various stages of the EAE.

Tissue sampling

In this study, tissue sampling was performed on day 13 and 21 post-immunization (PI) during the peak and recovery stages of EAE, respectively. Five rats in each group were killed under ether anesthesia. The spinal cords of rats 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).

Western blot analysis

Frozen spinal cords with EAE were thawed at room temperature (RT), minced, weighed, placed in PBS (1 : 4 w/v), and homogenized with a Tissue-Tearor (Biospec, USA). The homogenate was sonicated three times for 5 sec at RT and centrifuged at 12,000 × g for 10 min. The supernatant was diluted with electrophoretic sample buffer to obtain a protein concentration of 3 µg/µl, and then

heated at 100°C for 5 min. The heated samples were electrophoresed under denaturing conditions in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using a discontinuous procedure [25]. 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 30 µg protein per well. The protein concentration was estimated using the method of Bradford [5]. Samples containing standard markers of nNOS (155 kDa), eNOS (140 kDa), and iNOS (130 kDa) (Transduction Laboratories, Lexington, KY) were run at 100 Volts/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 (25 mM Tris, 192 mM glycine, 20% methanol (v/v), 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°C and 30 Volts. To visualize the transferred proteins, the nitrocellulose membrane was stained with Brilliant Blue R-250 (Sigma, St. Louis, MO) for 10 min and subsequently incubated in TBS (50 mM Tris/HCl, 20 mM NaCl, pH 7.4) containing 5% bovine serum albumin for 2 hrs at RT to block non-specific sites. The blot was then rinsed with TBS-T (TBS with 0.1% Triton X-100). The iNOS, nNOS and eNOS bindings were 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 (1 : 100 in dilution, Upstate Biotechnology Inc., NY). The finding of nitrotyrosine (NT) indicates the generation of peroxynitrite and the potential damage of proteins by nitration [2]. 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) for 3 hrs at RT. Visualization was achieved using 1% 3,3'-diaminobenzidine-HCl in 0.1% TBS. Immunoblot signals were quantified with a densitometer (M GS-700 imaging Densitometer, Bio-Rad, U.K.).

Immunohistochemistry

Five-micron sections of the paraffin-embedded spinal cords were deparaffinized and treated with 0.3% hydrogen peroxide in methyl alcohol for 30 min to block endogenous peroxidase. After three washes with PBS, the sections were exposed to 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) (Transduction Laboratories, Lexington, KY) for 60 min at RT. For the identification of astrocytes and macrophages, rabbit anti-glial fibrillary acidic protein (GFAP) (Sigma Chemical Co., St. Louis, MO) and ED1 (Serotec, London, U.K.) were applied. 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'-diaminobenzidine and 0.01% hydrogen peroxidase in 0.05 M Tris buffer). Before being mounted, the sections were counterstained with hematoxylin.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL)

DNA fragments were detected by *in situ* nick end-labeling as described in the manufacturers instructions (Oncor, London, UK) [16]. In brief, the paraffin sections were deparaffinized, rehydrated, and washed with PBS. The sections were treated with 0.005% pronase (Dako, Denmark) for 20 min at 37°C and incubated in a TdT buffer solution (140 mM sodium cacodylate, 1 mM cobalt chloride, 30 mM Tris-HCl, pH 7.2, 0.004 nmol/μl digoxigenine-dUTP) containing 0.15 U/μl TdT for 60 min at 37°C. After another incubation in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at 37°C, the sections were reacted with peroxidase-labeled anti-digoxigenine antibody for 60 min. Positive cells were visualized using a diaminobenzidine substrate kit (Vector) and counterstained with hematoxylin.

Double labeling of TUNEL and either astrocytes or macrophages

In the first step, apoptotic cells were detected by the TUNEL method when DAB developed a brown color. After thorough washing, the slides were stained for microglia or astrocytes using an avidin-biotin alkaline phosphatase kit (Vector). Alkaline phosphatase was developed in blue using BCIP/NBT (Sigma). The antisera used for double labelling were rabbit anti-GFAP for astrocytes and ED1 for macrophages/activated microglia.

Results

Clinical observation of EAE

The clinical course of EAE is shown in Fig. 1. EAE rats immunized with the spinal cord homogenates showed floppy tail (G1) on days 9-10 PI and severe paresis (G3) on days 11-15 PI. All the rats were recovered after day 17 PI (Fig. 1). Histological examination showed that a large number of inflammatory cells were infiltrated into the perivascular lesions and the parenchyma of the spinal cord of rats with EAE at the peak stages. In normal rats and CFA-immunized control rats, the infiltration of inflammatory cells was not found in the spinal cord parenchyma (data not shown).

Western blot analysis of three isoforms of NOS in EAE

The expression of nNOS (Fig. 2A), eNOS (Fig. 2B) and

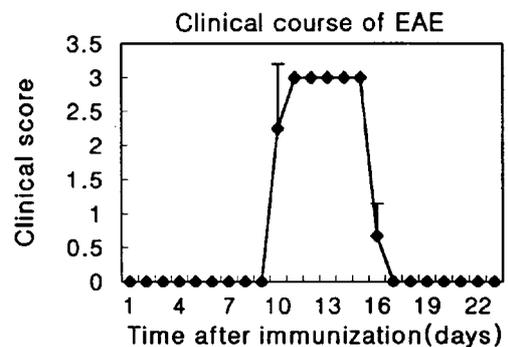


Fig. 1. The clinical course of rat spinal cord homogenate-induced experimental autoimmune encephalomyelitis (EAE) in Lewis male rats.

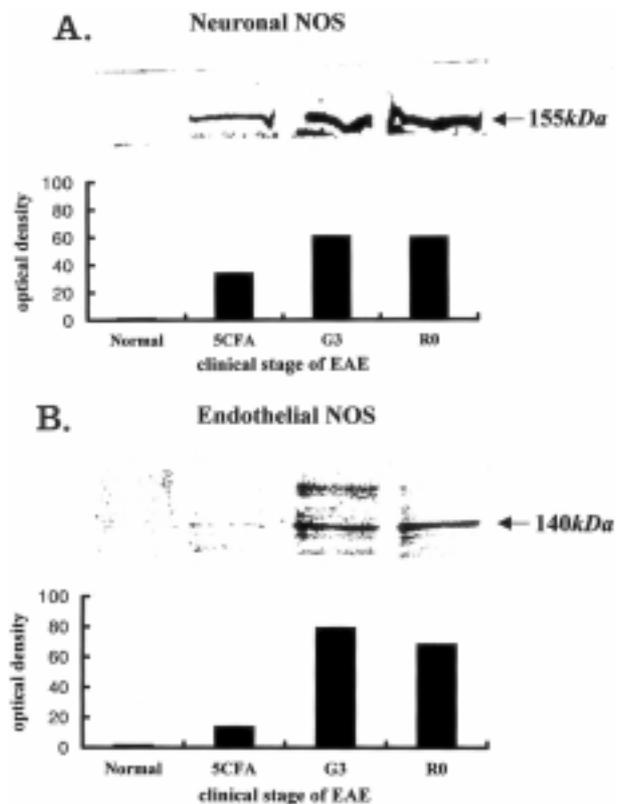


Fig. 2. Western blot analysis of constitutive neuronal NOS (nNOS) (A) and endothelial NOS (eNOS) (B) in the spinal cords of rats. Normal: control rats, 5CFA: complete Freund's adjuvant (supplemented with *Mycobacterium tuberculosis* H37Ra, 5 mg/ml) treated rats (day 13 PI), G3: peak stage of EAE, and R0: recovery stage of EAE. The molecular mass of nNOS (155 kDa) and eNOS (140 kDa) was indicated respectively. A semiquantitative analysis of nNOS and eNOS at different clinical states was performed by optical density (OD) measurement on Western blot signals. A representative data of 3 separate experiments.

iNOS (Fig. 3) was assessed semiquantitatively by densitometry. The intense immunoreactivity of both nNOS and eNOS was identified at the peak stage (day 13 PI, G3) of

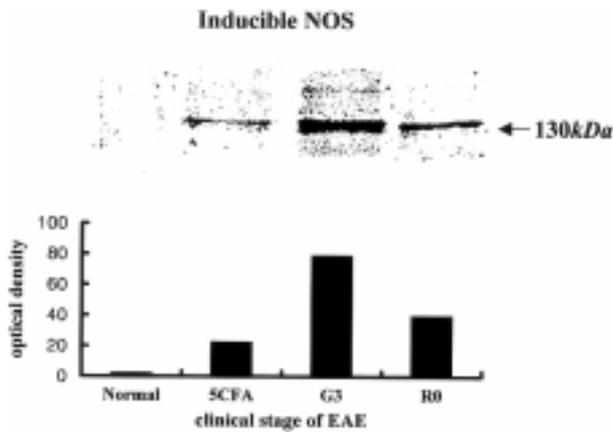


Fig. 3. Western blot analysis of iNOS in the spinal cords of rats with EAE. Normal: control rats, 5CFA: complete Freund's adjuvant (supplemented with *Mycobacterium tuberculosis* H37Ra, 5 mg/ml) treated rats (day 13 PI), G3; peak stage of EAE, and R0: recovery stage of EAE. The molecular mass of inducible NOS (130 kDa) was indicated. A semiquantitative analysis of iNOS at different clinical states (normal, 5CFA, peak stage, recovery stage) represents significant changes in the EAE-induced spinal cord versus the normal spinal cord. A representative data of 3 separate experiments.

EAE (Fig. 2), and remained until the recovery stage of EAE (day 21 PI, R0) (Fig. 2). Although little nNOS and eNOS were identified in the normal spinal cords, their

expression was increased in the spinal cord of 5CFA-treated rats (day 13 PI), as compared with normal control rats (Fig. 2). The increase in the expression of nNOS and eNOS was evident by the densitometric semiquantitative analysis (Fig. 2, graphs).

Unlike the expression of both nNOS and eNOS in the spinal cords of rats with EAE, small amounts of iNOS were identified in the normal spinal cords but its expression slightly increased in the spinal cord of 5CFA-treated rats, as compared with normal control rats (Fig. 3). Increased iNOS immunoreactivity was evident during the peak (G3) and recovery stages (R0) of EAE (Fig. 3). Using densitometric semiquantitative analysis (Fig. 3, graph), iNOS immunoreactivity in the spinal cord of rats with EAE significantly increased compared with that in the spinal cord of normal rats. The increased expression of iNOS persisted through the EAE recovery stage (day 21 PI, R0). These data indicate that the induction of EAE upregulates three isoforms of NOS. In addition, NT immunoreactivity was recognized during the peak and recovery stages of EAE, but not in the normal or the CFA-immunized spinal cords (data not shown). The increased expression of NT during the peak stage of EAE suggests that peroxynitrite or NO is generated in the autoimmune spinal cord lesions.

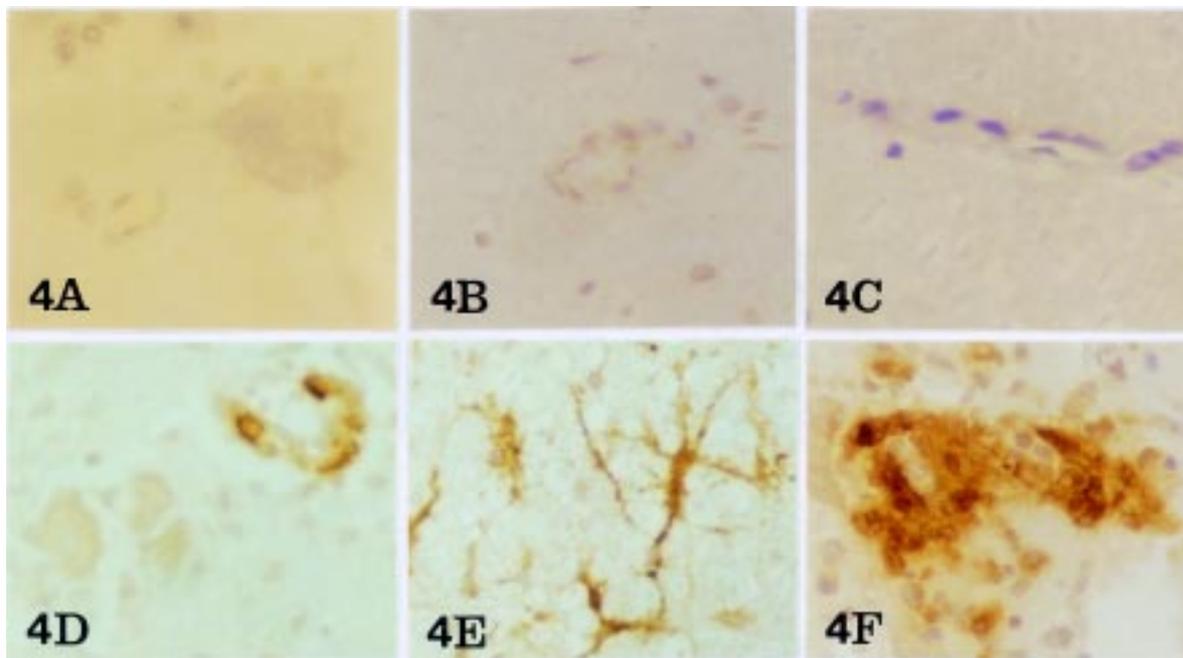


Fig. 4. Immunostaining of three isoforms of NOS in the spinal cords of normal (4A-4C) and EAE-affected rats (4D-4F). The immunoreactivity of nNOS (4A), eNOS (4B), and iNOS (4C) was scarcely identified in the spinal cords of control rats. At the peak stage of EAE, nNOS-positive cells were seen in neuronal cell bodies in the gray matter and in some inflammatory cells in the parenchyma of the spinal cord (4D). The eNOS-positive cells were found in vessels and some astrocytes (4E). Oval type iNOS-positive cells were found mainly in perivascular lesions (4F). Counterstained with hematoxylin. 4A, 4B, and 4C: normal rat spinal cords. 4D, 4E and 4F: EAE-affected rat spinal cord (G3, day 13 PI). Original magnification: x200. 4A and 4D: rabbit anti-nNOS, 4B and 4E: rabbit anti-eNOS, 4C and 4F: rabbit anti-iNOS antisera.

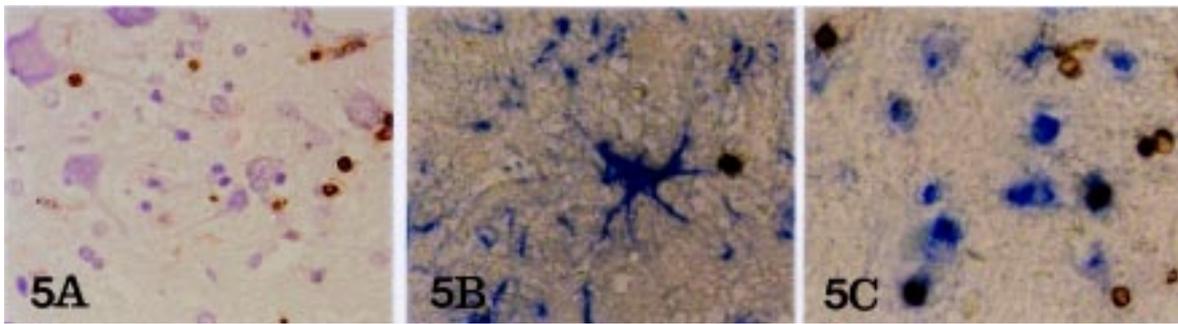


Fig. 5. Double labeling of TUNEL method on either astrocytes or macrophages in EAE lesions on days 13 PI. Apoptotic cells (brown) were commonly detected around neurons (5A) and some GFAP (+) processes (blue) identical to astrocytes (5B). Some apoptotic cells were co-localized with ED1 (+) cells (5C, blue). TUNEL and ABC-alkaline phosphatase reaction. Original magnification: A, $\times 33$; B and C, $\times 132$. A: TUNEL and hematoxylin, B: TUNEL and either rabbit anti-GFAP, C: TUNEL and rabbit anti-ED1.

Immunohistochemical localization of nNOS, eNOS, and iNOS in EAE

In the spinal cords of rats with EAE, the expression of nNOS was found in some small neurons and in the spinal cord parenchyma with a granular pattern. In addition, the expression of nNOS was also found in some inflammatory cells in the EAE lesions of the spinal cord (Fig. 4D). The expression of eNOS was observed in the endothelial cells of blood vessels and some astrocytes (Fig. 4E). The expression of iNOS (Fig. 4F) was found predominantly in infiltrating cells stained with ED1 and some astrocytes in the EAE lesions. Meanwhile, the expression of nNOS (Fig. 4A), eNOS (Fig. 4B), and iNOS (Fig. 4C) were rarely identified in the parenchyma of spinal cords of normal or adjuvant-immunized rats.

Structural interaction between apoptotic cells and brain cells.

In rats with EAE, the majority of apoptotic cells were distributed in the parenchyma, but scarcely found in the perivascular cuffs of the spinal cords. Double labeling showed that the apoptotic cells were commonly found in the area adjacent to neurons (Fig. 5A) and some GFAP-positive processes were identical to astrocytes (Fig. 5B). In some cases, the apoptotic cells were co-localized with ED1 (+) cells, suggesting that macrophages undergo apoptosis (Fig. 5C). The apoptotic cells were barely seen in the neurons and glial cells in the spinal cords of rat with EAE.

Discussion

In this study, the expression of both nNOS and eNOS was significantly increased in the hyperacute autoimmune CNS inflammation, suggesting that the constitutive NOS is stimulated by the inflammatory cells in the pathogenesis of EAE, as does iNOS in EAE [45]. However, our study did not support the finding of EAE in iNOS knockout mice in which both nNOS and eNOS were not increased³⁹.

The brain cells including neurons and some astrocytes

exhibited an increased expression of nNOS in the course of EAE. There was an intimate structural interaction between apoptotic cells and either neurons or astrocytes, which are potent cell types to express nNOS and eNOS, respectively. Although the functional role of both nNOS and eNOS in neurons and/or astrocytes in CNS diseases has not been fully understood, nNOS may be involved in either the tissue destruction in traumatic brain injury [41] or in the survival of neuronal cells in vesicular stomatitis virus infections [24].

Taken these dual effects of nNOS in the brain injury, we prefer to compromise that both nNOS and eNOS might mediate either stasis of T cell proliferation in the spinal cord parenchyma out of neurons [34, 46] or survival of neuronal cells in EAE. Our findings are further supported by the observation that the brain cells such as oligodendroglia do not undergo apoptosis in the murine EAE model, while homing inflammatory cells are selectively vulnerable to the apoptotic process [4].

A question remains to be explained in EAE. Why are few apoptotic figures found in brain cells that are potent cell types of NOS expression? In a recent study using a murine EAE model, the brain cells including oligodendroglia and astrocytes were proven to escape from the apoptosis [3, 30]. We suppose that additional activation of caspase family [4] and/or Fas-Fas ligand interaction [9, 47] would be necessary to induce the apoptosis of T cells in EAE, although endogenously generated NO via either eNOS or iNOS may be involved in the process of apoptosis [40].

In conclusion, our results showed that the three isoforms of NOS including nNOS, eNOS, and iNOS were increased in the initiation of EAE and suggested that the brain cells including neurons and astrocytes are possible sources for either nNOS or eNOS in the course of EAE. We postulate that NO, produced via both constitutive nNOS and eNOS from the brain cells, has a beneficial role by removing inflammatory cells through the stasis of T cell proliferation and eventually by the apoptosis of inflammatory cells in

EAE.

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