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Enhanced expression of three forms of nitric oxide synthase in autoimmune central nervous system disease

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Abstract

To elucidate the role of nitric oxide synthase (NOS) in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), we analyzed the expression of constitutive neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS), an enzyme of NO production, 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 NO production in the brain.

Western blot analysis shows that three isoforms of NOS significantly increased in the spinal cords of rats at the peak stage of EAE, while minimal amounts of these same enzymes are identified in normal rat spinal cords. Immunohistochemical study showed that brain cells including neurons and astrocytes revealed an increased

expression of either nNOS and/or eNOS during the peak and recovery stages of EAE, while 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 data suggests that both constitutive nNOS and eNOS as well as iNOS, possibly producing NO, plays an important role in the recovery of EAE.

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

Introduction

Nitric oxide (NO) is a readily diffusible apolar gas synthesized from L-arginine via nitric oxide synthase (NOS) 1. The enzyme responsible for NO formation exists in two forms: (1) a constitutive, Ca2+-dependent form, which is rapidly activated by agonists that elevate intracellular free Ca2+ 2 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 2, 3. In the central nervous system (CNS) tissues, constitutive NOS is thought to synthesize NO, which is important for intracellular signaling, neurotransmission, and vasoregulation 4, 5. Unless activated, iNOS is not expressed in brain cells 5-7. In the CNS, the local generation of toxic concentrations of NO via nNOS and iNOS has been implicated in mediating excitotoxic neuronal injury (nNOS) 8, 9, hypoxic-ischemic brain damage (nNOS, iNOS) 10-14, traumatic brain injury (nNOS) 15, and autoimmune disorders (iNOS) 16-20.

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS used to study human demyelinating diseases such as multiple sclerosis 21. 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 22, 23 and apoptotic elimination of inflammatory cells leading to recovery 24, 25.

Several studies have shown that iNOS is an important mediator of CNS inflammation through the generation of NO in the course of EAE 16, 26-30 as well as in human multiple sclerosis

lesions 31. Contrary to the 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 aggravates EAE progression depending on the stage of inflammation 32-35 and because EAE is exacerbated in mice lacking the NOS2 gene 36. Furthermore, even in the increase of nitric oxide and iNOS in EAE, animals with EAE recover from paralysis 28, suggesting that iNOS may have a capacity to prevent immunologically privileged CNS from the invading inflammatory cells in EAE. More recently, Gonzalez-Hernandez and Rustioni 37 reported that three isoforms of NOS including nNOS, eNOS and iNOS, generating NO, exert a beneficial effect on peripheral nerve regeneration.

The aim of this study was to examine the quantitative changes of three isoforms of NOS (nNOS, eNOS and iNOS) in the course of hyeracute EAE by Western blot analysis, and to examine the structural interaction between apoptotic cells and brain cells which constitutively express either nNOS or eNOS in EAE lesions by immunohistochemistry.

Materials and Methods

Animals

Lewis rats were obtained from the Korea Research Institute of Bioscience and Biotechnology, KIST (Taejeon, Korea) and bred in our animal facility. Male rats weighting 160-200 g/ea, aged 7-12 weeks, 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 Freunds adjuvant(CFA; *Mycobacterium tuberculosis* H37Ra, 5 mg/ml; Difco). Immunized rats were further given *Bordetella pertussis* toxin (2 g/ea) (Sigma Chemical Co., St. Louis, MO) intraperitoneally, and 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) 22. Control rats were immunized with CFA only. Five rats were killed under ether anesthesia at various stages of the EAE.

Tissue sampling

In this study, tissue sampling was performed on days 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 (-70C) 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 (5 sec at RT) and centrifuged at 12,000g for 10 minutes (min). The supernatant was diluted with electrophoretic sample buffer to obtain a protein concentration of 3 g/l, and heated at 100C for 5 min. Samples were electrophoresed under denaturing conditions in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using a discontinuous procedure 38. 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 g protein per well. The protein concentration was estimated using the method of Bradford 39. Samples containing standard markers of nNOS (155kDa), eNOS (140 kDa) and iNOS (130 kDa) (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 (25 mM TRIS, 192 mM 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 4C and 30 Volts. The transferred proteins were visualized by staining the membrane for 10 min with Brilliant Blue R-250 (Sigma, St. Louis, MO), and incubating the nitrocellulose membrane in TBS-5% BSA (50 mM TRIS/HCl, 20 nM NaCl, pH 7.4 containing 5% bovine serum albumin) for 2 hours (hrs) at RT blocked non-specific sites. The blot was then rinsed with TBS-T (TBS with 0.1% Triton X-100). The iNOS, nNOS and eNOS binding was detected by incubating the membrane in a moist chamber overnight at 4C, 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, Upstat

Immunohistochemistry

Five-micron sections of 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 1 hr 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'-diaminbenzidine 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 fragmentation was detected by *in situ* nick end-labeling as described in the manufacturers instructions (Oncor, London, UK) 41. In brief, the paraffin sections were deparaffinized, rehydrated, and washed in PBS. The sections were treated with 0.005% pronase (Dako, Denmark) for 20 min at 37C and subsequently incubated with TdT buffer solution (140 mM sodium cacodylate, 1 mM cobalt chloride, 30 mM Tris-HCl, pH 7.2) containing 0.15 U/l TdT and 0.004 nmol/l digoxigenine-dUTP for 60 min at 37C, and then in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min. Then, they 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 labelling of TUNEL+ cells and either astrocytes and 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 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). Histological examination showed that a large number of inflammatory cells infiltrated the perivascular lesions and parenchyma of the spinal cord of rats with EAE at the peak stage. In the normal and CFA-immunized control spinal cords, no infiltrating cells were detected in the spinal cord parenchyma (data not shown).

Western blot analysis of three isoforms of NOS in EAE

The expression of both nNOS (Fig. 2, A), eNOS (Fig. 2, B) and iNOS (Fig. 3) was assessed semiquantitatively using densitometry.

Intense immunoreactivity of both nNOS and eNOS was identified at the peak stage (day 13 PI, G3) of EAE (Fig. 2), and remained until the recovery stage of EAE (day 21 PI, R0) (Fig. 2). Although little nNOS and eNOS was identified in normal spinal cords, its expression increased in 5CFA treated rat spinal cord (day 13 PI), as compared with normal control (Fig. 2). This increased expression of nNOS and eNOS compared with normal rat spinal cords was evident by densitometric semiquantitative analysis (Fig. 2, graphs).

Unlike the expression of both nNOS and eNOS in the spinal cords with rat EAE, a few iNOS was identified in normal spinal cords but its expression slightly increased in 5CFA treated group, as compared with normal control (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 EAE-affected rats increased significantly compared with that in normal rat spinal cords. Increased expression of iNOS persisted through the EAE recovery stage (day 21 PI, R0). These data indicate that the induction of EAE upregulates both constitutive nNOS and eNOS in addition to iNOS. In addition, NT immunoreactivity was recognized during the peak and recovery stages of EAE, but not in normal or 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.

Immunohistochemical localization of nNOS, 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 expressed in some small neurons and a granular pattern in the spinal cord parenchyma which were presumed as neuropils. In addition to the constitutive expression of nNOS in neuronal tissues, it was expressed in some inflammatory cells in the EAE lesions of the spinal cord (Fig. 4D). In the same lesions, eNOS immunoreactivity was detected in the endothelial cells of blood vessels and some astrocytes (Fig. 4E). iNOS immunoreactivity (Fig. 4F) was found predominantly in infiltrating cells stained with ED1 and some astrocytes in the EAE lesions.

In the control group, nNOS (Fig. 4A), eNOS (Fig. 4B), and iNOS (Fig. 4C) were rarely identified in the parenchyma of spinal cords of normal and adjuvant-immunized rats.

Structural interaction between apoptotic cells and brain cells.

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

Discussion

This study reports that both constitutive nNOS and eNOS is significantly increased in the hyperacute autoimmune CNS inflammation, suggesting that constitutive NOS is stimulated by the inflammatory cell in the pathogenesis of EAE, as does the inducible isoform of NOS in EAE 29. However, this study does not support the finding of EAE in iNOS knockout mice in which both nNOS and eNOS were not increased 42.

As far as nNOS is concerned in EAE, this study is a first quantitative

identification of nNOS increase in the spinal cords of rat EAE. Brain cells including neurons and some astrocytes exhibited increased expression of nNOS in the course of EAE. Subsequently, we identified the intimate structural interaction between apoptotic cells and either neurons or astrocytes which were potent cell types to express nNOS and eNOS respectively. Although the functional role of both nNOS and eNOS in either neurons and/or astrocytes in CNS diseases has not been fully understood, nNOS has involved in either the tissue destruction in traumatic brain injury 15, or in the neuronal cell survival in vesicular stomatitis virus infections 43.

Taken these dual effects of nNOS in brain injury, we prefer to compromise that both nNOS and eNOS, producing NO, might mediate either stasis of T cell proliferation in the spinal cord parenchyma out of neurons 22, 44 and/or survival of neuronal cells in EAE. Our findings are further supported by the observation that brain cells, such as oligodendroglia, do not undergo apoptosis in the murine EAE model, while homing inflammatory cells are selectively vulnerable to apoptotic process 45.

A question remains to be explained in EAE, why few apoptotic figures are found in brain cells which are potent cell types of NOS expression. In a recent study using a murine EAE model, brain cells including oligodendroglia and astrocytes were proven to escape from apoptosis 46, 47. We suppose that additional activation of caspase family 45 and/or Fas-Fas ligand interaction 48, 49 would be necessary to induce apoptosis of T cells in EAE, although endogenously generated NO via either eNOS or iNOS may be involved in the process of apoptosis 50.

Combined, our study shows that 3 isoforms of NOS including nNOS, eNOS and iNOS are increased in the initiation of EAE, and suggests that 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 brain cells including neurons and astrocytes, has a beneficial role by removing inflammatory cells through the stasis of T cell proliferation and eventually apoptosis of inflammatory cells in EAE.

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Legends for figures

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

Figure. 2. Western blot analysis of constitutive neuronal NOS (nNOS) (A) and endothelial NOS (eNOS) (B) in the spinal cords of rats with normal, complete Freunds adjuvant (supplemented with *Mycobacterium tuberculosis* H37Ra, 5 mg/ml) treated group (5CFA, day 13 PI), peak (G.3) and recovery stage (R.0) of EAE. The molecular mass of nNOS (155 kDa) and eNOS (140 kDa) was indicated respectively. *Graphs*, A semiquantitative analysis of nNOS and eNOS at different clinical stages was made using optical density (OD) measurements on Western blot signals. Representative data of 3 separate experiments.

Figure 3. Western blot analysis of iNOS in the spinal cords of rats with EAE. The molecular mass of inducible NOS (130 kDa) was indicated. *Graph*, 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.

Figure 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 normal 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 some inflammatory cells in the parenchyma of 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 spinal cord (G3, days 13 PI). Original magnification: x200. 4A and 4D, rabbit anti-nNOS; 4B and 4E, rabbit anti-eNOS; 4C and 4F, rabbit anti-iNOS antisera.

Figure 5. Double labeling of TUNEL method and either astrocytes or macrophages in EAE lesions. Apoptotic cells (brown) were commonly detected around neurons (5A) and some GFAP (+) processes (blue) identical to astrocytes (5B). Some apoptotic cells (brown) were colocalized with ED1 (+) cells (5C, blue). TUNEL and ABC-alkaline phosphatase reaction. A-C: EAE G.3, days 13 PI, original magnification 132. A: TUNEL and hematoxylin; B and C: TUNEL and either rabbit anti-GFAP (B) or ED1 (C).