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Correlation of nitric oxide levels in the cerebellum and spinal cord of experimental autoimmune encephalomyelitis rats with clinical symptoms

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Experimental autoimmune encephalomyelitis (EAE) is a well-established cell-mediated autoimmune inflammatory disease of the CNS, which has been used as a model of the human demyelinating disease. EAE is characterized by infiltration of the CNS by lymphocytes and mononuclear cells, microglial and astrocytic hypertrophy, and demyelination which cumulatively contribute to clinical expression of the disease. EAE was induced in female Sprague-Dawley rats, 3 months old (300 g ± 20 g), by immunization with myelin basic protein (MBP) in combination with Complete Freund's adjuvant (CFA). The animals were divided into 7 groups: control, EAE, CFA, EAE + aminoguanidine (AG), AG, EAE + N-acetyl-L-cysteine (NAC) and NAC. The animals were sacrificed 15 days after EAE induction, and the level of nitric oxide ($NO^{\cdot}$) production was determined by measuring nitrite and nitrate concentrations in 10% homogenate of cerebellum and spinal cord. Obtained results showed that the level of $NO^{\cdot}$ was significantly increased in all examined tissues of the EAE rats compared to the control and CFA groups. Also, AG and NAC treatment decreased the level of $NO^{\cdot}$ in all tissues compared to the EAE group. The level of $NO^{\cdot}$ is increased significantly in the spinal cord compared to the cerebellum. The clinical course of the EAE was significantly decreased in the EAE groups treated with AG and NAC during the development of the disease compared to EAE group and its correlates with the $NO^{\cdot}$ level in cerebellum and spinal cord. The findings of our work suggest that $NO^{\cdot}$ and its derivatives play an important role in multiple sclerosis (MS). It may be the best target for new therapies in human demyelinating disease and recommend the new therapeutic approaches based on a decreased level of $NO^{\cdot}$ during the course of MS.

Key words: nitric oxide, experimental autoimmune encephalomyelitis

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a well-established cell-mediated autoimmune inflammatory disease of the CNS. Pathologically, EAE is characterized by infiltration of the CNS by lymphocytes and mononuclear cells as a consequence of breakdown in blood brain barrier (BBB) permeability, microglial and astrocytic hypertrophy, and demyelination, which cumulatively contribute to clinical expression of disease (Raine et al. 1980). Because the clinical and pathological aspects of this disease have significant similarities to the human demyelinating disease multiple sclerosis (MS) it has been used as a model of that disease (Raine et al. 1980, Willenborg and Staykova 1998, Jack 2005).

In physiological conditions, nitric oxide ($NO^{\cdot}$) is produced from the oxidation of the terminal guanidine nitrogen of arginine, by a NADPH-dependent enzyme, $NO^{\cdot}$ synthase (NOS). There are three NOS isoforms – neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Moncada et al. 1991, Knowles and Moncada 1994, Buchwalow 2001). Nitric oxide mediates many biological functions, including regulation of vascular tone, platelet activation, and acting as a neurotransmitter of nonadrenergic, noncholinergic innervations. $NO^{\cdot}$ is tumoricidal and microbicidal, and plays a

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role of transsynaptic retrograde messenger in the brain, thus participating in synaptic plasticity (Nathan and Xie 1994, Willenborg and Staykova 1998, Jack et al. 2005). It has also been shown to be a part of a number of immunopathologies including EAE and MS (Nathan and Xie 1994). The role of $NO^{\cdot}$ in the immune system comprises both regulatory and effector functions. The regulatory functions include immunosuppressive effects (inhibition of lymphocyte proliferation), while effector functions include immunopathologic effects (tissue destruction) and immunoprotective activities (apoptosis of autoreactive T cells) (Nathan and Xie 1994, Xu et al. 2001).

In pathological conditions, when $NO^{\cdot}$ is produced in high levels, it leads to a rapid reaction with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), or with other biomolecules (proteins, DNA, lipids) (Beckman et al. 1994) that play an important role in neuronal tissue damage, inducing mitochondrial dysfunction, lipid peroxidation, protein nitration, ion channel disability and electrolyte imbalance (Beckman and Koppenol 1996, Marques et al. 2008, Pautz et al. 2010).

In the recent studies that evaluate the role of $NO^{\cdot}$ in development of EAE, several authors use iNOS inhibitors or an oxidant-scavenger to treat EAE. Aminoguanidine (AG) is equipotent to N^G-monomethyl-L-arginine (L-NMA), an inhibitor inducible isoform of $NO^{\cdot}$ synthase. In our previous work we have reported that AG inhibits the nitrosative stress in whole encephalitic mass (WEM) and clinical signs of EAE (Ljubisavljevic et al. 2011), but others have found that there can be aggravation and prolongation of the disease upon AG treatment (Ruuls et al. 1996, Brenner et al. 1997). Also, it has been shown in our previous work that N-acetyl-L-cysteine (NAC), an oxidative scavenger, is beneficial against reactive nitrogen species (RNS) and reactive oxygen species (ROS) generation in WEM, decreasing the level of nitrosative and oxidative damage and clinical course of EAE (Ljubisavljevic et al. 2011).

Unfortunately, current efforts fail to clearly define the $NO^{\cdot}$ role underlying EAE and MS pathology and its association with neurological dysfunction. Because of confusing results of some studies using NOS inhibitors and/or oxidative scavengers to treat EAE, which have reported $NO^{\cdot}$ proinflammatory (promoting cytotoxicity), and/or antiinflammatory roles (suppressing the immune response) (Mitrovic et al. 1994, Farias et al. 2007) we have evaluated the level of $NO^{\cdot}$ in different regions of CNS (cerebellum, spinal cord) of EAE rats and its correlation with clinical expression, using AG and NAC as a potential nitrosative stress modulators.

METHODS

Animals

Female Sprague Dawley rats, 3 months old, weighing 300 ± 20 g, were housed in the Biomedical Research Centre animal care facility of the Medical Faculty of Nis throughout the experiment under a 12:12 h light-dark cycle. The rats were kept in plastic cages and fed on a standard diet and water ad libitum. All animals received human care in strict accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH publication 80-23, revised 1985). The experimental protocols were reviewed and approved by the Faculty Ethical Committee.

Induction of EAE

Experimental autoimmune encephalomyelitis was induced by the subcutaneous injection of myelin basic protein, bovine type (50 μg), dissolved in phosphate buffered saline (PBS) emulsified in the volume equal to the complete Freund's adjuvant (CFA), on days 0 and 7 in the hind foot pad of the animals under anesthesia. Two intraperitoneal injections of 200 ng Pertussis toxin were given on days 0 and 1. Each of 49 animals was randomly assigned to seven groups: control (PBS 0.3 ml/i.p/daily), EAE (PBS 0.3 ml/i.p/daily after EAE induction), CFA (PBS 0.3 ml/i.p/daily), EAE and AG (AG 100 mg/kg body weight/daily after EAE induction), AG (100 mg/kg body weight/daily), EAE and N-acetyl-L-cysteine (150 mg/kg body weight/daily after EAE induction) and NAC (100 mg/kg body weight/daily).

All animals were tested daily for clinical signs of EAE (healthy = 1; loss of tail tone = 2; hindlimb weakness = 3; hindlimb paralysis = 4; hindlimb paralysis plus forelimb weakness = 5; moribund or dead = 6; Sajad et al. 2009). The animals were sacrificed 15 days after EAE induction and the cerebellums and spinal cords were dissected, washed in PBS, placed on ice and 10% homogenates of all the tissue were stored at −20°C for later biochemical analysis. Nitrite and nitrate concentration as a measure of nitric oxide production was determined as follows.

Determination of nitrate and nitrate concentration

After deproteinization, the production of NO· was evaluated by measuring nitrite and nitrate concentrations. Nitrites were assayed directly spectrophotometrically at 543 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl)ethylendiamine dihydrochloride in distilled water). However, nitrates were previously transformed into nitrites by cadmium reduction (Navaro-Gonzalvez et al. 1998).

Protein content

Protein content was measured according to the Lowry procedure using bovine serum albumin as standard (Lowry et al. 1951).

Chemicals

Chemicals were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade. All drug solutions were prepared on the day of the experiment.

Statistical analysis

All the data presented were mean ± SD. Normal distribution was verified using Kolmogorov-Smirnov test. The significance of the difference between experimental and control groups was analyzed using analysis of variance (ANOVA) followed by paired samples *t* test, Bonferroni test and Chi Square test using the statistical program SPSS version 13. An α-level of 0.05 was used for statistical significance.

RESULTS

As we have reported in our previous paper, AG and NAC treatment of EAE rats during the development of the disease significantly decreased the clinical score of EAE compared to EAE group (Ljubisavljevic et al. 2011). That clinical score significantly correlated with NO· levels in examined tissues – cerebellum and spinal cord (c=0.71; c=0.72, respectively; $P<0.01$).

The obtained results showed that the nitrate and nitrite level, as a measure of NO· production, was significantly increased in all examined tissues (cerebellum, spinal cord) of EAE rats compared to the control

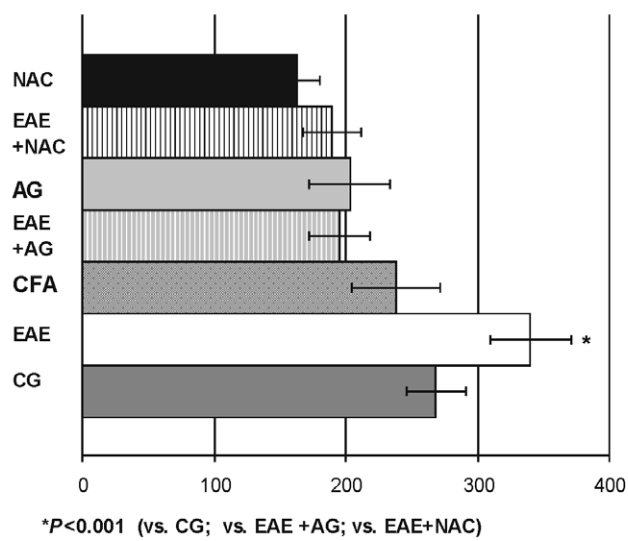

Fig. 1. NO_2 and NO_3 concentration (nmol/mg prot.) in rat cerebellum. (CFA) Complete Freund's adjuvant; (AG) aminoguanidine; (NAC) N-acetyl-L-cysteine; (CG) control group; (EAE) rats with experimental autoimmune encephalomyelitis; (CFA) rats treated with CFA; (EAE + AG) EAE rats treated with AG; (AG) rats treated with AG; (EAE + NAC) EAE rats treated with NAC; (NAC) rats treated with NAC.

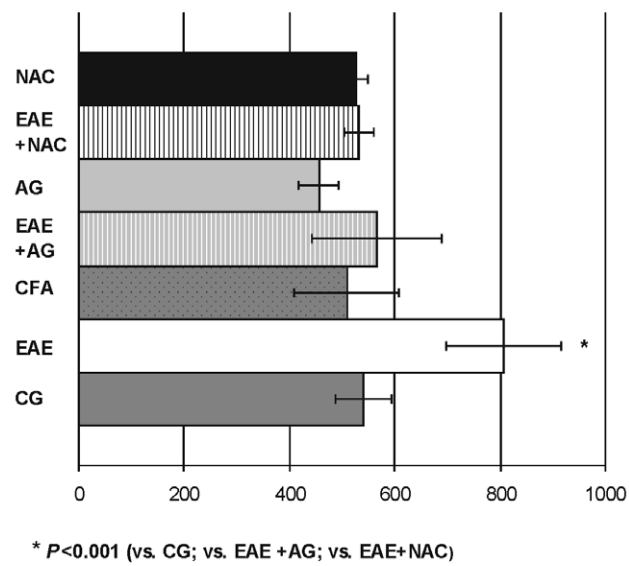

Fig. 2. NO_2 and NO_3 concentration (nmol/mg prot.) in rat spinal cord. (CFA) Complete Freund's adjuvant; (AG) aminoguanidine; (NAC) N-acetyl-L-cysteine; (CG) control group; (EAE) rats with experimental autoimmune encephalomyelitis; (CFA) rats treated with CFA; (EAE + AG) EAE rats treated with AG; (AG) rats treated with AG; (EAE + NAC) EAE rats treated with NAC; (NAC) rats treated with NAC.

and CFA groups (t_5=7.73; t_5=7.61, respectively; P<0.001– Fig. 1, Fig. 2, respectively).

AG treatment decreased the level of NO· products in all tissues (cerebellum, spinal cord) compared to the EAE group (t_6=12.328; t_6=8.468, respectively; P<0.001 – Fig. 1, Fig. 2, respectively).

Also, NAC treatment decreased the level of NO· products in all tissues (cerebellum, spinal cord) compared to the EAE group (t_5=13.85; t_5=10.19, respectively; P<0.001 – Fig. 1, Fig. 2, respectively).

NO· increase is the most pronounced in the spinal cord compared to the whole encephalitic mass (Ljubisavljevic et al. 2011) and cerebellum (t_7=15.98; t_8=18.8, respectively; P<0.001 – Fig. 3).

DISCUSSION

Our results show increased NO· production in all estimated CNS regions. As has been previously proposed, that may be the consequence of activated microglia, astrocytes, macrophages, and other immune cell types, which infiltrate CNS tissue due to NO· mediated vasodilatation and increased BBB permeability (Smith et al. 1999, Thiel and Audus 2001). In EAE, NO· can have proinflammatory, cytotoxic, and/or anti-inflammatory roles, suppressing the immune response (Mitrovic et al. 1994, Farias et al. 2007).

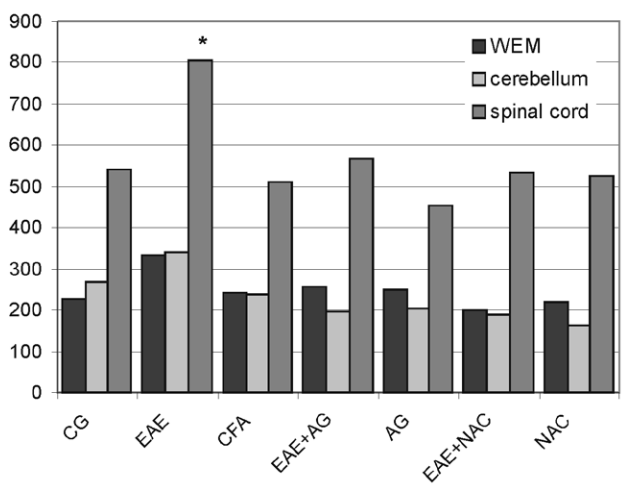

Fig. 3. NO_2 and NO_3 concentration (nmol/mg prot.) in rat CNS different regions. (CFA) Complete Freund's adjuvant; (AG) aminoguanidine; (NAC) N-acetyl-L-cysteine; (CG) control group; (EAE) rats with experimental autoimmune encephalomyelitis; (CFA) rats treated with CFA; (EAE + AG) EAE rats treated with AG; (AG) rats treated with AG; (EAE + NAC) EAE rats treated with NAC; (NAC) rats treated with NAC.

The major Th1 cytokines, such as interferon-γ (IFN-γ), as well as tumor necrosis factor-α (TNF-α), and interleukin-1-b (IL-1b), induce immune cell iNOS *in vitro*, apparently by transcriptional modulation (Napoli and Neumann 2010). On the other hand, these proinflammatory cytokines activate endothelia and modulate the BBB, inducing the expression of endothelial cell adhesion molecules (Losy et al. 1999) and in this way promote immune cell infiltration of nerve tissue. The recent reports indicate that NO· also induces the production of TNFα (Bishop et al. 2009, Henderson et al. 2009).

The increased secretion of reactive nitrogen intermediates (RNI) by inflammatory leukocytes and high levels of NO· and iNOS mRNA, documented in CSF of MS patients (Cross et al. 1998) and in the peripheral blood of rats with hyperacute EAE, correlates directly with disease severity as we have shown in our work (De Groot et al. 1997, Yamashita et al. 1997, Ljubisavljevic et al. 2011).

In the context of EAE clinical expression, NO· secreted by iNOS plays a dual role. On one side, it is thought that NO· mediates protection, because EAE symptoms are exacerbated in iNOS-/- mice (Fenyk-Melody et al. 1998). It may function to increase T helper 1 response (Kahl et al. 2003) or to eliminate inflammatory cells from the CNS by promoting apoptosis or downregulation of adhesion molecules (Okuda et al. 1998). On the other hand, our data are consistent with the hypothesis that NO· has cytotoxic effects that include oligodendroglia disruption and impairment of the ability of myelin supporting cells to maintain and produce myelin during early EAE phase. Calcium-dependent NOS (eNOS and nNOS) activity in the spinal cord was reported to remain unchanged or decrease during EAE concomitantly with iNOS upregulation (Calabrese et al. 2001, Kahl et al. 2003). Following these, Okuda and coauthors (1998) have used AG, a selective iNOS inhibitor, in mice with actively induced EAE. Administration of AG during the early development of EAE, as we have proposed in our work (Ljubisavljevic et al. 2011), produced a significant delay in EAE onset, but AG administration after the onset of clinical EAE enhanced the clinical severity and mortality rate and provoked the onset of relapse. These data suggested that NO· plays different roles during the induction and progression phases of EAE. However, the role of NO· in EAE is not the same in different phases

of the disease, changing according to the immunological status (Xu et al. 2001). Since NOS activity represents the combined activity of all three NOS isoforms, it is evident that each isoform could play a different role in EAE. It has been found in some studies that NO· levels in the spinal cord are about 20% lower in eNOS-/- mice than in control groups, decreasing in parallel with both strains during EAE progression (Lin et al. 1993, Muzhou and Tsirka 2009). We have demonstrated a higher spinal cord NO· level compared to other examined CNS regions (Fig. 3), which directly correlates with clinical expression of EAE (hindlimb paralysis with/without forelimb weakness) in EAE animals. The neurological expression is the consequence of nitrosative stress in the spinal cord, more pronounced than in the cerebellum and brain. It may be the result of more lesions per the spinal cord volume unit (Saito et al. 1994 , Kim et al. 2006) compared to brain and cerebellum. However, Blanco and colleagues (2010) suggest that there are changes affecting the cerebellar NO/NOS system during this disorder, as a result of the changes in iNOS cellular distribution, but not its expression.

Although demyelination has been defined as the cause of neurological dysfunction in MS, the recent work has suggested that neuronal (axonal) degeneration is responsible for irreversible neurological disability. Inflammation and demyelination, potentially reversible pathologies, have been attributed to the relapsing remitting course of MS (RR-MS), while irreversibile neuronal damage has been shown in primary progressive and secondary progressive course of MS (PP, SP MS), as a consequence of oxidative and nitrosative stress (Kornek et al. 2000, Muzhou and Tsirka 2009).

Even if the inhibition of NO· production can be shown to prevent demyelination, NO· may not directly damage myelin or myelinating cells, but rather act as the means of induction or enhancement of other factors (Smith et al. 1999). It is possible that the negative outcomes triggered by NO· production could ensue from its conversion to the toxic metabolite peroxynitrite ($ONOO^-$) through the reaction with O_2^-. Peroxinitrite is formed very early in EAE, exerting a wide variety of effects on cellular systems by modifying protein structure, and thereby function, through the formation of nitrotyrosine adducts and, when present at sufficiently high levels, it induces excitotoxicity, DNA damage, and apoptosis (Brown and Bal-Price 2003). This increased nitrotyrosine reactivity is present in MS brains, particularly in areas of demyelination and inflammation (Bo et al. 1994, Kahl et al. 2003). NO· can also damage DNA directly by deamination, and inhibit the repair activity of the enzyme DNA ligase, leading to cell death (Bo et al. 1994).

NO· has been shown to inhibit several enzymes, including protein kinase C and enzymes involved in mitochondrial respiration, including aconitase, NADH-ubiquinone oxidoreductase and succinateubiquinone oxidoreductase (Boullerne et al. 1995). The effects on the mitochondrial respiratory chain may be expected to cause deficits in cellular energy supplies. ATP content is reduced in neurons exposed to NO· and ONOO-, which results in neuronal and axonal damage. This is supported by our results, as the animals with decreased NO· levels had lower EAE clinical intensity (Ljubisavljevic et al. 2011). Also, NO·/ONOO- may have adverse consequences in the production of neoepitopes which may provoke an immune reaction. There is evidence to suggest that this phenomenon can explain the EAE clinical course amelioration (Boullerne et al. 1995, Okuda et al. 1997).

Aminoguanidine (AG), an iNOS inhibitor, has been shown to delay the disease onset and decrease EAE severity (Brenner et al. 1997, Ljubisavljevic et al. 2011). Our recent results show that the maximum clinical severity of EAE and the duration of illness were significantly reduced by the application of AG (Ljubisavljevic et al. 2011), due to an inhibition of iNOS. NAC treatment of EAE rats also reduced the severity of EAE clinical symptoms which could be explained by the suppression of mononuclear cell infiltration into CNS and the decrease of proinflammatory Th1 cytokine response (IFN-γ) (Pahan et al. 1998, Malabendu and Kalipada 2005, Ljubisavljevic et al. 2011).

CONCLUSION

The findings of our work suggest that NO· and its derivatives may play an important role in MS. It may be the best target for new therapies in human demyelinating disease and may suggest new therapeutic drugs based on decreased levels of NO· following MS clinical expression.

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