Nitric oxide synthase activity and endogenous inhibitors in rats recovered from allergic encephalomyelitis

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We have previously reported that in comparison with normal rats, the presence of experimental allergic encephalomyelitis (EAE) leads to decreased endogenous inhibitory activity (EIA) of Ca^{2+}-dependent nitric oxide synthase (NOS) in both brain and serum, and increased expression of protein 3-nitrotyrosine (NT) in brain. In this work we show that animals recovered from the clinical signs of EAE are not different from controls in terms of either brain NOS activity, EIA of NOS, or NT expression. These results suggest that parallel to the reversal of the disease symptoms, a normalization of the production of nitric oxide and related species occurs.

Key words: nitric oxide synthase - experimental allergic encephalomyelitis - nitrotyrosine

Experimental allergic (or auto-immune) encephalomyelitis (EAE) is an animal model for the study of human multiple sclerosis which can be induced in several suceptible animal species through the immunization with neuronal proteins.

The increased production of nitric oxide (NO) during the course of EAE is well documented; however, its role in the disease is controversial, considering that some studies show that NO has a toxic role in the formation of demyelinizing lesions (Merrill et al. 1993, Xiao et al. 1996), while others demonstrated that the NO produced by phagocytes can exert an immuno-supressor effect through the inhibition of T cells proliferation and leukocyte infiltration (Kubes et al. 1991, Okuda et al. 1997).

Regarding the presence of endogenous NOS inhibitors in EAE, a limited number of reports are available. Early in 1971, Baldwin and Carnegie described the existence of an enzyme responsible for the specific methylation of L-arginine. It was just more than 20 years later that it was shown that the renal excretion of dimethyl arginine (both symmetrical and assymmetrical, SDMA and ADMA, respectively) was 20% smaller in patients with MS in comparison with healthy subjects (Rawal et al. 1995).

In a previous work, we showed that a heat-sensitive endogenous inhibitory activity (EIA) of CA^{2+}-dependent nitric oxide synthase (cNOS) present in brain homogenates and sera from Lewis rats with EAE (stage III) was significantly lower than that observed in the control group, with no differences for the EIA of Ca^{2+}-independent NOS (iNOS; Teixeira et al. 2002). In this work, we investigated the endogenous inhibitory activity of NOS present in brain and serum samples from Lewis rats recovered from the clinical signs of EAE.

MATERIALS AND METHODS

Male Lewis rats (200-250 g) were obtained from the Cemib (Unicamp, SP). The animals received food and tap water ad libitum and were submitted to EAE induction as previously described (Teixeira et al. 2002). The experimental protocol was approved by the local Ethics Committee (CEEA, ICB/USP). The animals were daily weighed and examined for the presence of neurological signs, and 28 days after the immunization, those showing complete reversal of the clinical signs (EAE-r) were anesthetized for blood sample collection and removal of whole encephalic masses (WEMs) for further analysis.

NOS activity, nitrotyrosine expression and the presence of inhibitory activity present in the sera and WEM homogenates were assayed as previously described (Teixeira et al. 2002).

The expression of protein inhibitor of nNOS (PIN) in WEMs was analyzed by Western blot employing a primary mouse monoclonal antibody (250 ng/ml; Transduction Lab., US) after fixing the transferred protein onto the nitrocellulose membrane with 0.1% glutaraldehyde.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for NOS isoforms was performed according to the previously described protocol (Teixeira et. al. 2002). For RT-PCR analysis of PIN, the nucleotide sequence of the employed primers for PIN were selected according to Jaffrey and Snyder (1996).

Data are expressed as mean ± SEM (n = 4-6 samples per group) and comparisons among the experimental groups were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons.

RESULTS

As shown in the Table, no statistically significant differences were found between EAE-r and control animals in terms of either WEM NOS activity or NOS EIA (both thermostable and thermolabile) present in WEM and serum for the different NOS isoforms. Control and EAE animals also showed similar WEM PIN expression (either protein or mRNA; Fig. 1). No differences between EAE-r and control rat WEMS were found regarding the expres-

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TABLE

Values for whole encephalic mass (WEM) NOS activity and endogenous inhibitory activity (EIA) of NOS present in WEMs and sera obtained from control and EAE-r rats. No significant differences were observed between control and EAE-r groups for any of the measured variables

	Control	EAEr
WEM Ca^{2+}-dependent NOS activity (pmol/min/mg protein)	12.6 ± 3.7	12.3 ± 0.4
WEM Ca^{2+}-independent NOS activity (pmol/min/mg protein)	0.5 ± 0.2	0.4 ± 0.1
WEM thermolabile EIA of Ca^{2+}-dependent NOS (%)	30.4 ± 1.3	30.5 ± 1.5
WEM thermostable EIA of Ca^{2+}-dependent NOS (%)	21.9 ± 2.4	20.2 ± 1.5
Serum thermolabile EIA of Ca^{2+}-dependent NOS (%)	5.4 ± 0.9	8.8 ± 3.0
Serum thermostable EIA of Ca^{2+}-dependent NOS (%)	31.5 ± 2.6	35.8 ± 2.2
WEM thermolabile EIA of Ca^{2+}-independent NOS (%)	21.9 ± 2.6	25.0 ± 2.9
WEM thermostable EIA of Ca^{2+}-independent NOS (%)	20.1 ± 1.9	24.4 ± 2.5
Serum thermolabile EIA of Ca^{2+}-independent NOS (%)	12.8 ± 3.0	10.6 ± 2.6
Serum thermostable EIA of Ca^{2+}-independent NOS (%)	8.3 ± 2.3	10.4 ± 2.4

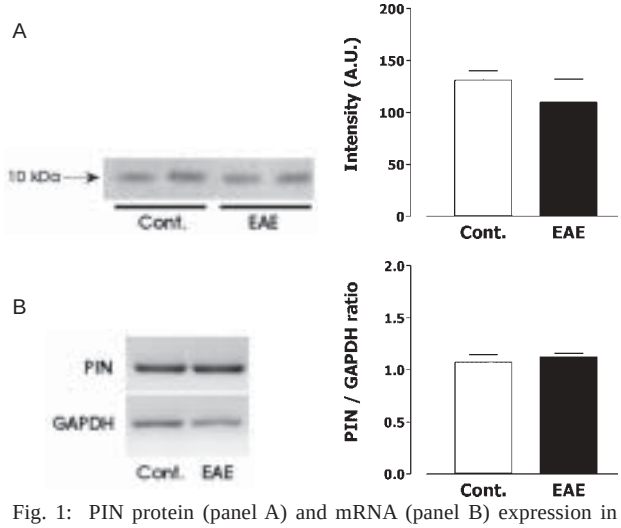

Fig. 1: PIN protein (panel A) and mRNA (panel B) expression in WEMs obtained from control and EAE rats. No significant differences were observed between the groups.

sion of 3-nitrotyrosine-containing proteins (molecular weights 28 and 53 kDA; Fig. 2, panel A). RT-PCR analysis revealed that WEMS from EAE-r and control rats are not different in terms of mRNA contents for either nNOS or iNOS; however, the EAE-r animals exhibited higher expression of mRNA for the endothelial NOS isoform (Fig. 2, panel B).

DISCUSSION

In a previous work, we showed that the heat-sensitive EIA of Ca^{2+}-dependent NOS present in brain homogenates and sera from Lewis rats with EAE (stage III) was significantly lower than that observed in the control group, with no differences for the EIA of Ca^{2+}-independent NOS, in addition to decreased Ca^{2+}-dependent NOS activity, increased iNOS mRNA and protein NT expression in brains from EAE rats (Teixeira et al. 2002). The results here presented show that parallel to the reversal of the EAE clinical signs, the normalization of the above mentioned variables occurs.

The lack of differences between EAE and control animals in terms of WEM PIN expression (either protein or mRNA) indicate that this protein is not related to the lower heat-sensitive EIA of Ca^{2+}-dependent NOS observed in EAE rats, and that other thermolabile factor (to our knowledge, not yet described) should account for this difference. However, the actual relevance of PIN as an endogenous nNOS inhibitor, still remains to be elucidated.

It is interesting to mention that except for eNOS, the expression of mRNA for both neuronal and inducible NOS isoforms were similar between the EAE-r and control groups. These observations are in partial contrast to those reported by Kim et al. (2000), who observed that the protein expression of all three NOS isoforms remain elevated in spinal cord obtained form animals recovered from the clinical signs of EAE. However, special care must be taken at the time of comparing these results, considering the differences in the studied tissues (WEM vs spinal cord), the time at which the samples were collected (28 vs 21 days after immunization) and the employed analytical techniques (Western blotting vs semiquantitative RT-PCR). Protein tyrosine nitration is usually related to the occurrence of peroxynitrite anion secundary to the production of high quantities of NO (mainly due to the iNOS isoform). On this point, consistency of our results can be verified, considering that NT proteins (molecular weights 28 and 53 kDA) and iNOS expression were increased during EAE (Teixeira et al. 2002) and normalized to control rat levels in the animals recovered from EAE symptoms.

Taken as a whole, the results herein presented suggest that except for eNOS mRNA expression, a normalization of the NO production pathways occur parallel to the reversal of the clinical signs of EAE in rats. However, the exact relationship between these two phenomena still deserve further investigation.

REFERENCES

Baldwin GS, Carnegie PR 1971. Specific enzymic methylation of na arginine in the experimental allergic encephalomyelitis protein from human myelin. *Science 171*: 579-581.

Jaffrey SR, Snyder SH 1996. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science 274*: 774-777.

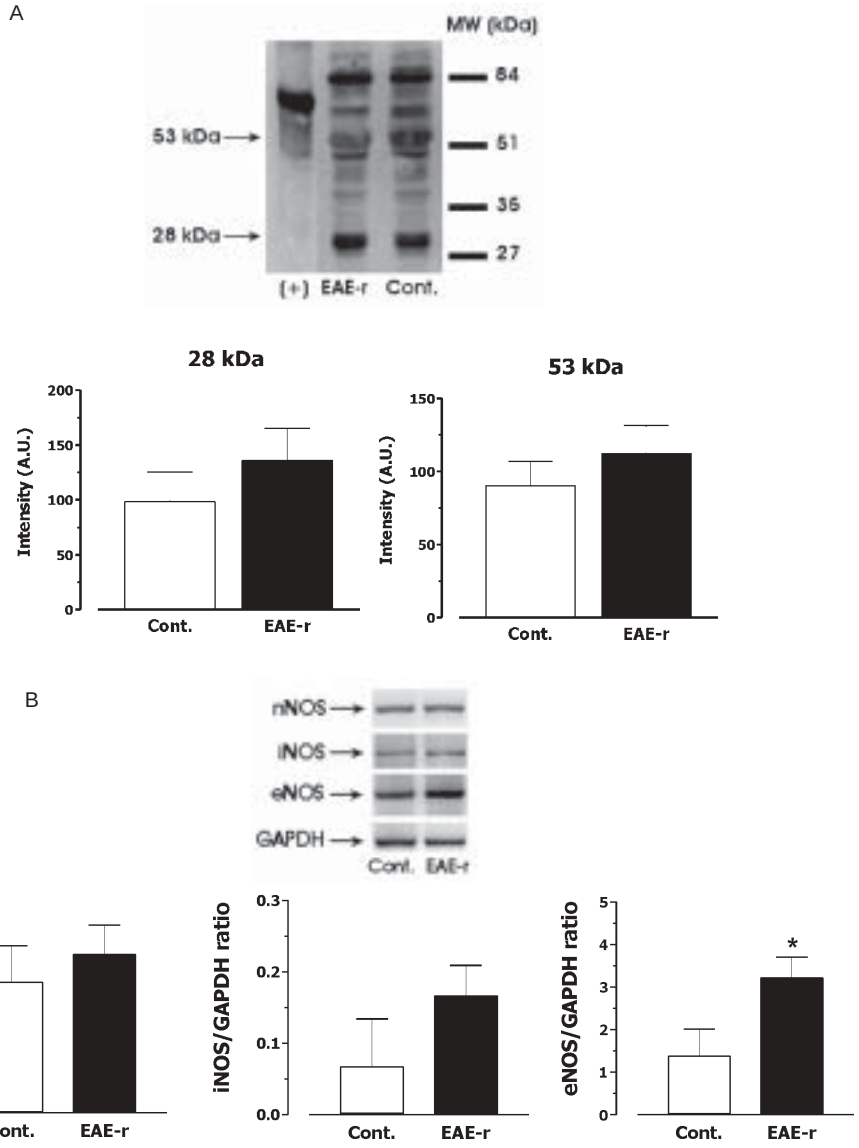

Fig. 2A: representative Western blot showing the expression of nitrotyrosine-containing proteins in WEMs obtained from control and EAE-r rats (panel A), and densitometric analysis of the bands at 28 and 53 kDa; B: RT-PCR analysis of mRNA for nNOS, iNOS and eNOS present in WEM samples obtained from control and EAE-r rats (GAPDH: internal control). *: $p < 0.05$ vs control group.

Kim S, Moon C, Wie MB, Kim H, Tanuma N, Matsumoto Y, Shin T 2000. Enhanced expression of constitutive and inducible forms of nitric oxide synthase in autoimmune encephalomyelitis. *J Vet Sci 1*: 11-17.

Kubes P, Suzuki M, Granger DN 1991. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA 88*: 4651-4655.

Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE 1993. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol 151*: 2132-2141.

Okuda Y, Sakoda S, Fujimura H, Yanagihara T 1997. Nitric oxide via na inducible isoform of nitric oxide synthase is a possible factor to eliminate inflammatory cells from the central nervous system of mice with experimental allergic encephalomyelitis. *J Neuroimmunol 73*: 107-116.

Rawal N, Lee YJ, Whitaker JN, Park JO, Paik WK, Kim S 1995. Urinary excretion of NG-dimethylarginines in multiple sclerosis patients: preliminary observations. *J Neurol Sci 129*: 186-191.

Teixeira SA, Castro GM, Papes F, Martins ML, Rogério F, Langone F, Santos LM, Arruda P, de Nucci G, Muscará MN 2002. Expression and activity of nitric oxide synthase isoforms in rat brain during the development of experimental allergic encephalomyelitis. *Brain Res Mol Brain Res 99*: 17-25.

Xiao BG, Zhang GX, Ma CG, Link H 1996. The cerebrospinal fluid from patients with multiple sclerosis promotes neuronal and oligodendrocyte damage by delayed production of nitric oxide in vitro. *J Neurol Sci 142*: 114-120.