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Research report

Expression and activity of nitric oxide synthase isoforms in rat brain during the development of experimental allergic encephalomyelitis

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Abstract

The activity and expression of nitric oxide synthase (NOS) isoforms and protein nitrotyrosine (NT) residues were investigated in whole encephalic mass (WEM) homogenates during the development of experimental allergic encephalomyelitis (EAE) in Lewis rats. EAE stages (0–III) were daily defined by clinical evaluation, and in the end of each stage, WEMs were removed for analysis of NOS activity, protein NT residues and mRNA for the different NOS isoforms. In the presence of NADPH, WEMs from EAE-III rats showed lower Ca^{2+}-dependent NOS activity than those from control group. These differences disappeared in the presence of exogenous calmodulin, flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and NADPH. Of all the cofactors, just the omission of FAD caused comparable decrease of Ca^{2+}-dependent NOS activity from both groups. Ca^{2+}-independent NOS activity from EAE-III animals was insensitive to the omission of any of the cofactors, while in control animals this activity was significantly inhibited by the omission of either FAD or BH_4. Increased levels of both iNOS mRNA and protein NT expression were observed in animals with EAE, which also showed lower levels of a thermolabile NOS inhibitor in WEM homogenates and sera than controls. In conclusion, during late EAE stages, constitutive Ca^{2+}-dependent NOS activity decreases concomitantly with iNOS upregulation, which could be responsible for the high protein NT levels. The differential dependence of iNOS activity on cofactors and the absence of an endogenous thermolabile NOS inhibitor in animals with EAE could reflect additional control mechanisms of NOS activity in this model of multiple sclerosis.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Other neurotransmitters

Keywords: Nitric oxide; Experimental allergic encephalomyelitis; Multiple sclerosis; Nitrotyrosine; Lewis rat

1. Introduction

Experimental allergic encephalomyelitis (EAE) is an autoimmune demyelinating disease that has been widely characterized and proposed as a valid animal model for the study of human multiple sclerosis [35,36].

The pathological mechanisms involved in the etiology of these disorders are multifactorial and only partially understood or defined. T cells and macrophages constitute the majority of inflammatory cells infiltrating the central nervous system (CNS) in animals with EAE. Upon stimulation, macrophages can release several molecules that act as pro-inflammatory mediators (such as the cytokines IFNγ, TNFα, TNFβ and others) or tissue-damaging agents (such oxygen and nitrogen-derived free radicals). The first

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demonstration of the participation of the free radical nitric oxide (NO) in EAE was reported for MacMicking et al. [26], who showed increased production of reactive nitrogen intermediates as well as reactive oxygen intermediates by both systemic and CNS-derived peripheral blood mononuclear cells and polymorphonuclear leukocytes from rats with EAE. By means of reverse transcriptase polymerase chain reaction (RT–PCR), the presence of mRNA for iNOS (the inducible Ca^{2+}-independent nitric oxide synthase isoform) was later demonstrated in brains from animals with EAE [22]. NO was subsequently localized to the spinal cord of mice with EAE using electron paramagnetic resonance spectroscopy [24], leading to the conclusion that NO must play a role in the disease process.

The involvement of NO overproduction in the pathogenesis of EAE has been investigated and a variety of damaging effects has been proposed. NO can react with superoxide anion ($O_2^{\cdot}$) to form peroxynitrite ($ONOO^-$), a potent oxidant which can cause tissue damage through lipid peroxidation and nitration of proteins containing tyrosine residues (3-nitrotyrosine-modified proteins [2]). On this point, several studies demonstrated the presence of nitrotyrosine (NT)-modified proteins as a biochemical marker of peroxynitrite-induced damage in EAE [9,33,45].

In this work we studied the expression and activity of the NOS isoforms (Ca^{2+}-dependent endothelial [eNOS] and neuronal [nNOS], and Ca^{2+}-independent inducible [iNOS]) and NT-containing protein expression in whole encephalic masses of rats along the the different stages of EAE development.

2. Materials and methods

2.1. Experimental allergic encephalomyelitis (EAE) induction in rats

Lewis rats (both sexes, weighing 200–250 g) were obtained from the UNICAMP Animal Care facilities. The animals received food and tap water ad libitum and were submitted to EAE induction by a single s.c. injection of 100 μl of an emulsion containing guinea-pig myelin basic protein (GP-MBP, 250 μg/ml; Sigma Chemical Co., USA) and *Mycobacterium tuberculosis* (1.5 mg/ml; Difco, USA) emulsified with complete Freunds complete adjuvant into each hind foot pad under ether anesthesia. Rats were weighed and examined daily for the presence of neurological signs. Symptoms of EAE were evaluated at different stages represented by 0 (no clinical signs), I (flaccid tail), II (partial hind limb paralysis) and III (complete hind limb paralysis).

In the end of the different stages the animals were anesthetized, sacrificed and whole encephalic masses (WEM) were removed and immediately frozen in liquid nitrogen for further analysis (detailed below).

2.2. Ex vivo NOS activity quantification

NOS activity in WEM homogenates was estimated by the rate of conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in the presence of NADPH, as previously described [11]. Pharmacological controls of the enzyme activity were performed in parallel and consisted in either the omission of $CaCl_2$ and addition of 1 mM EGTA or the addition of 1 mM L-NAME to the incubation medium.

To characterize the dependence of NOS isoforms from either control or EAE rats on cofactors, Ca^{2+}-dependent and independent NOS activities were measured in the presence of exogenously added 10 μM flavin adenine dinucleotide (FAD), 10 μg/ml calmodulin (CaM) and 100 μM tetrahydrobiopterin (BH_4) according to Hiki et al. [17].

To study the presence of endogenous NOS inhibitors, we added native and denatured WEM homogenates or sera obtained from either control or EAE-III animals to a NOS preparation obtained from normal rat WEM. The thermal stability of the collected samples was studied by heating the homogenates and sera at 100 °C during 10 min and 2 min, respectively.

2.3. Western blot analysis for nitrotyrosine (NT)-containing proteins

WEM homogenate proteins (50 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 10% polyacrylamide) according to Laemmli [23] and electrophoretically transferred to a nitrocellulose membrane. After blocking nonspecific sites with 0.2% casein, the membranes were incubated overnight at 4 °C with the primary mouse monoclonal antibodies raised against NT-modified KLH (500 ng/ml; Upstate, USA). Membranes were washed with Tris-buffered saline containing 0.2% Tween 20 and incubated with alkaline phosphatase-conjugated rabbit anti-mouse antibody. A chemiluminescent assay (Immun-Star; Bio-Rad, USA) was used to detect immunoreactive NT-containing proteins. Intensities of the bands were estimated by densitometric analysis.

2.4. Determination of serum nitrite/nitrate concentrations

Blood samples from either control or EAE (stage III) rats were collected for the determination of serum nitrite (NO_2^-) and nitrate (NO_3^-) concentrations by high-performance liquid chromatography (HPLC) as previously described [30].

2.5. RT–PCR for NOS isoforms

Total RNA from WEMs was extracted by the TRIZOL

reagent method, according to the manufacturer protocol (Life Technologies, GIBCO-BRL, USA). cDNA was synthesized from 15 μg of total RNA using Superscript II (Life Technologies) according to the manufacturer's protocol. cDNA samples were stored at −20 °C until use.

Nucleotide sequence of the employed primers have previously been reported by Ferraz et al. for eNOS and iNOS [12] and Swain et al. for nNOS [42]. PCR reactions were performed in a final volume of 50 μl, containing 5 μl of cDNA solution, 5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 μM of each oligonucleotide pair (for specific amplification of either nNOS, iNOS or eNOS), 3 μM of the internal control primer pair (glyceraldehyde-3-phosphate dehydrogenase; GAPDH), and 2 units of *Taq* DNA polymerase (Life Technologies). Amplification cycle was done with denaturation for 5 min at 94 °C followed by 33 cycles of amplification consisting of a denaturation step at 94 °C for 1 min, a primer-annealing step at 65 °C for 45 s, and an extension step at 72 °C for 1.5 min. After the last amplification cycle, samples were incubated at 72 °C for 7 min for extension. Aliquots of PCR reaction products (approximately 20 μl), previously normalized to give equivalent amounts of the GAPDH control product in all samples, were electrophoresed on 1.5% agarose gels. Gels were visualized under UV light and images were captured using the EagleEye apparatus (Amersham, USA). Bands for nNOS, iNOS and eNOS sized 560 bp, 651 bp and 224 bp, respectively. Intensities of the ethidium bromide-stained bands were determined using the FLA3000 Fluorescent Analyzer (Fujifilm) to determine the relative expression level for each NOS isoform.

2.6. Statistical analysis

Data are expressed as mean±S.E.M. and comparisons among the experimental groups were analyzed by one-way ANOVA followed by the Student's Newman–Keul's test for multiple comparisons. Statistical significance is considered for *P*-values less than 0.05.

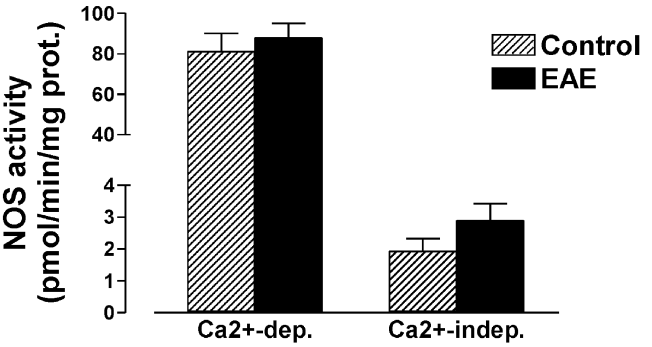

Fig. 2. NOS activity content measured in WEM homogenates obtained from rats with EAE at stage III in the presence of the cofactors NADPH, FAD, CaM and BH_4. No significant differences were observed between the groups for either Ca^{2+}-dependent or Ca^{2+}-independent NOS activity.

3. Results

Fig. 1 shows that in the absence of any exogenously added cofactor (with the exception made for NADPH), Ca^{2+}-dependent NOS activity was significantly decreased in rats with EAE at stage III (5.9±0.3) when compared with either control (7.8±0.7), EAE-0 (7.8±0.3) or EAE-I groups (7.8±0.4 pmol L-cit/min/mg protein). However, and as shown in Fig. 2, no significant differences in Ca^{2+}-dependent NOS activities were observed between control and EAE-III rats when assayed in the presence of all the NOS cofactors tested: NADPH, FAD, CaM and BH_4 (81.0±9.1 and 87.9±7.2 pmol L-cit/min/mg protein, respectively). Under these conditions, and as shown by Fig. 2, Ca^{2+}-independent NOS activity in WEM homogenates from EAE-III rats was not significantly different than that measured in control animals (2.9±0.5 and 1.9±0.4 pmol L-cit/min/mg protein, respectively).

Fig. 3 shows that Ca^{2+}-dependent NOS activities measured in WEM homogenates obtained from either control or EAE-III rats were similarly inhibited (~30%) by the absence of FAD in the incubation media. In contrast, Ca^{2+}-independent NOS activity in WEM homogenates

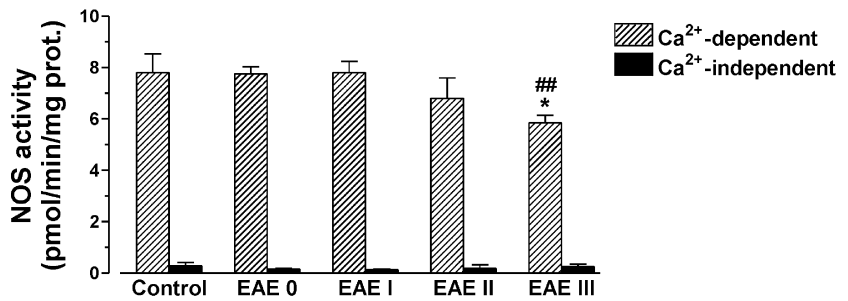

Fig. 1. NOS activity content measured in total brain homogenates obtained from rats with EAE at different stages. No significant differences were observed for Ca^{2+}-independent NOS activity among the groups. *$P<0.05$ vs. Control and EAE-I, and $^{\#\#}P<0.01$ vs. EAE-0.

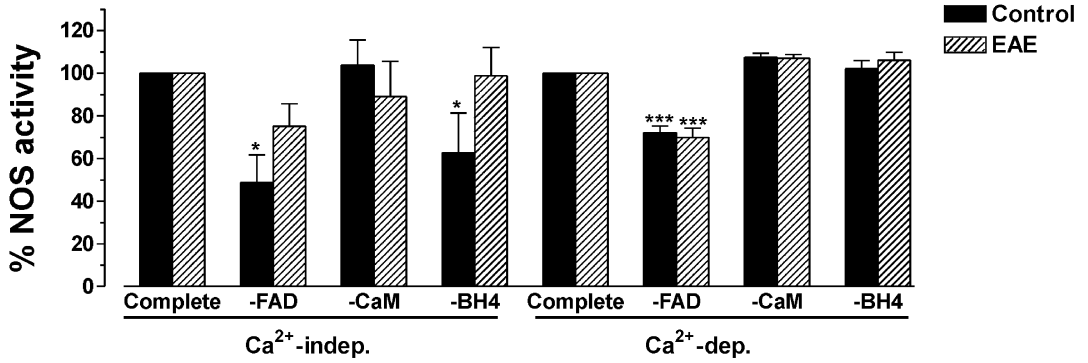

Fig. 3. Effect of the omission of each of the cofactors FAD, CaM and BH_4 in the incubation media on Ca^{2+}-dependent and Ca^{2+}-independent NOS activity in homogenates of WEM obtained from control rats and EAE (stage III). *$P<0.05$ and ***$P<0.001$ vs. the respective Complete group.

from EAE-III rats was not affected by the absence of any of the exogenously added cofactors, while the omission of either FAD or BH_4 from the incubation media significantly inhibited Ca^{2+}-independent NOS activity from control animals (48.7±13.0% and 62.7±18.6%, respectively).

Fig. 4 shows that both WEM homogenates and sera from control animals inhibited NOS (from normal rat WEM homogenate) to a higher degree than that observed with WEM homogenates and sera from EAE-III rats (WEM, control: 20.11±3.23; EAE-III: 12.13±2.07%, $P<0.05$; serum, control: 53.10±1.84%; EAE-III: 38.66±1.93%, $P<0.001$). These differences in NOS inhibiting activities between the experimental groups disappeared when both the WEM homogenates or sera were heat-denatured (Fig. 4).

Western blot analysis of NT-containing proteins in WEM homogenates showed two main bands (approximate mol. wts of 53,000 and 28,000 ; Fig. 5A), and their expressions were significantly increased in rats with EAE during the stages II and III compared with control group (Figs. 5B, C).

Serum NO_3^- levels were significantly higher in rats with EAE at stage III than in control animals (104.6±9.8 and 33.2±6.75 μM, respectively; $P<0.01$), however no significant differences between the groups were observed for serum NO_2^- (Fig. 6).

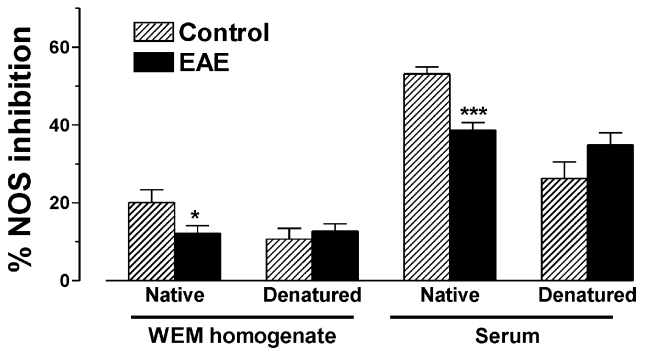

Fig. 4. Effect of the presence of whole encephalic mass (WEM) homogenates and sera obtained from control and EAE rats at stage III on a NOS preparation obtained from normal rats. *$P<0.05$ and ***$P<0.001$ vs. the respective Control.

RT–PCR analysis revealed increased levels of iNOS mRNA in WEM homogenates obtained from EAE-III rats in comparison with control animals (Fig. 7). On the other hand, no significant changes in either nNOS or eNOS mRNA were observed.

4. Discussion

Our results show that Ca^{2+}-dependent NOS activity present in WEM homogenates obtained from rats with EAE gradually lowers with the severity of the disease when compared with the activity measured in control animals in the absence of any exogenously added NOS cofactor (with the exception made for NADPH). Under these conditions, no significant differences were observed for Ca^{2+}-independent activity among the groups. The lack of differences observed between the groups in terms of either eNOS or nNOS protein expression when analyzed by Western blot (data not shown), led us to investigate about either the presence of inhibiting factor(s) that could affect Ca^{2+}-dependent NOS activity, or the deficiency of any of the NOS cofactor(s) in WEMs from EAE rats.

In the presence of all of the essential cofactors for NOS (NADPH, FAD, CaM and BH_4) we observed that Ca^{2+}-dependent NOS activity present in WEMs from EAE rats was restored to levels present in controls, and that Ca^{2+}-independent NOS activity was still similar between the groups, despite the tendency to be higher in the EAE group and the significant upregulation of mRNA for this isoform (Fig. 7). The omission of FAD from the incubation medium led to similar decreases of Ca^{2+}-dependent NOS activity in WEMs from either EAE or control animals.

Significant decreases of Ca^{2+}-independent NOS activity were observed when either FAD or BH_4 were omitted from the incubation media of control rat WEMs; on the contrary, no significant changes in the activity of this isoform present in WEMs from EAE animals were de-

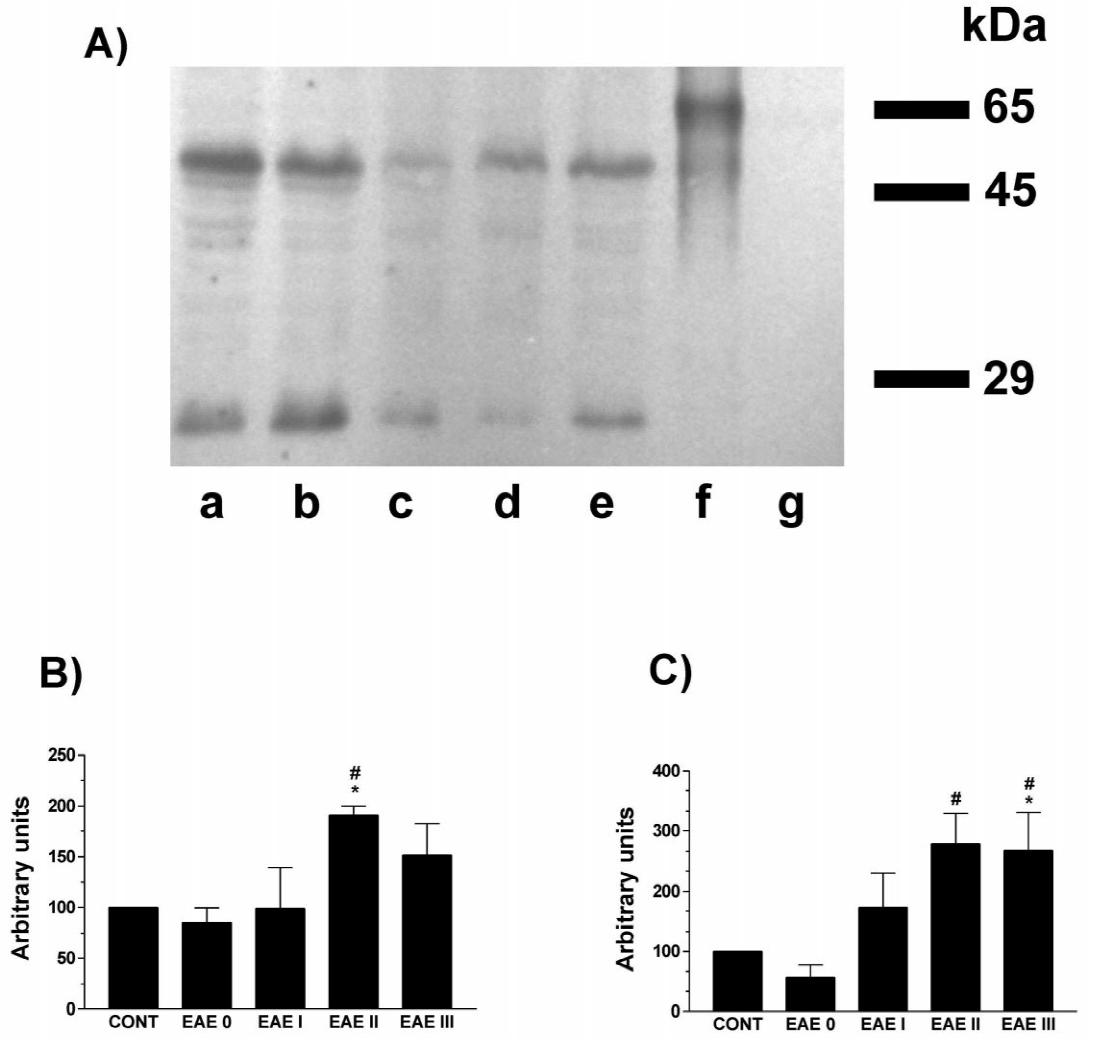

Fig. 5. (A) Representative Western blot ($n=4$) for nitrotyrosine-containing proteins (NT) in WEM homogenates obtained from rats with EAE at different stages (50 μg/lane) using monoclonal anti-nitrotyrosine antibody (500 ng/ml). Lane a: stage III; b: stage II; c: stage I; d: stage 0; e: control; f: nitro-albumin (2.5 μg); g: native albumin. (B,C) Densitometric analysis of the bands shown in panel A correspond to proteins with mol. wt=28,000 and 51,000, respectively. *$P<0.05$ vs. control group; #$P<0.05$ vs. stage 0.

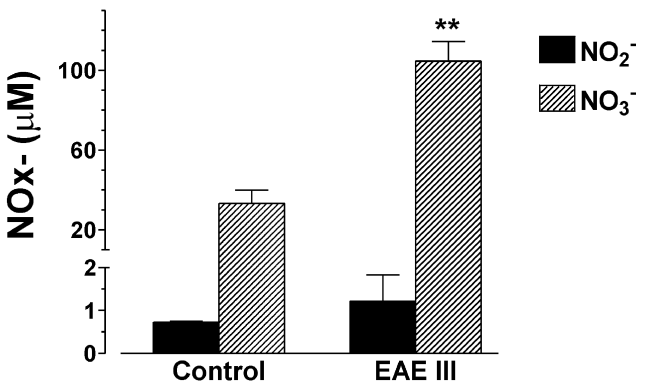

Fig. 6. Serum nitrite (NO_2^-) and nitrate (NO_3^-) concentrations in control ($n=3$) and EAE rats at stage III ($n=6$). No significant differences were observed for NO_2^- between the groups. **$P<0.01$ vs. Control group.

tected in the absence of any of the added cofactors (with the exception made for NADPH).

Both FAD and BH_4 are essential cofactors for the functional activity of NOS, given their participation in the electron transfer processes that take place during the two steps involved in NO biosynthesis [21,27–29,41].

The occurrence of iNOS in the EAE model in rats was initially reported by Koprowski et al. [22]. Moreover, Okuda et al. [32] showed that the levels of either mRNA or protein expression for both iNOS and some pro-inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-6, IFN-γ, TNF-α and TNF-β) measured in the spinal cord of rats were positively correlated with the degree of severity of the illness, apart from the fact of the lack of alteration of the immunoregulatory cytokines IL-4, IL-10 or TGF-β.

At this point, it is worth mentioning that previous results show that pro-inflammatory cytokines stimulate BH_4 production in both endothelial cells [19] and vascular smooth

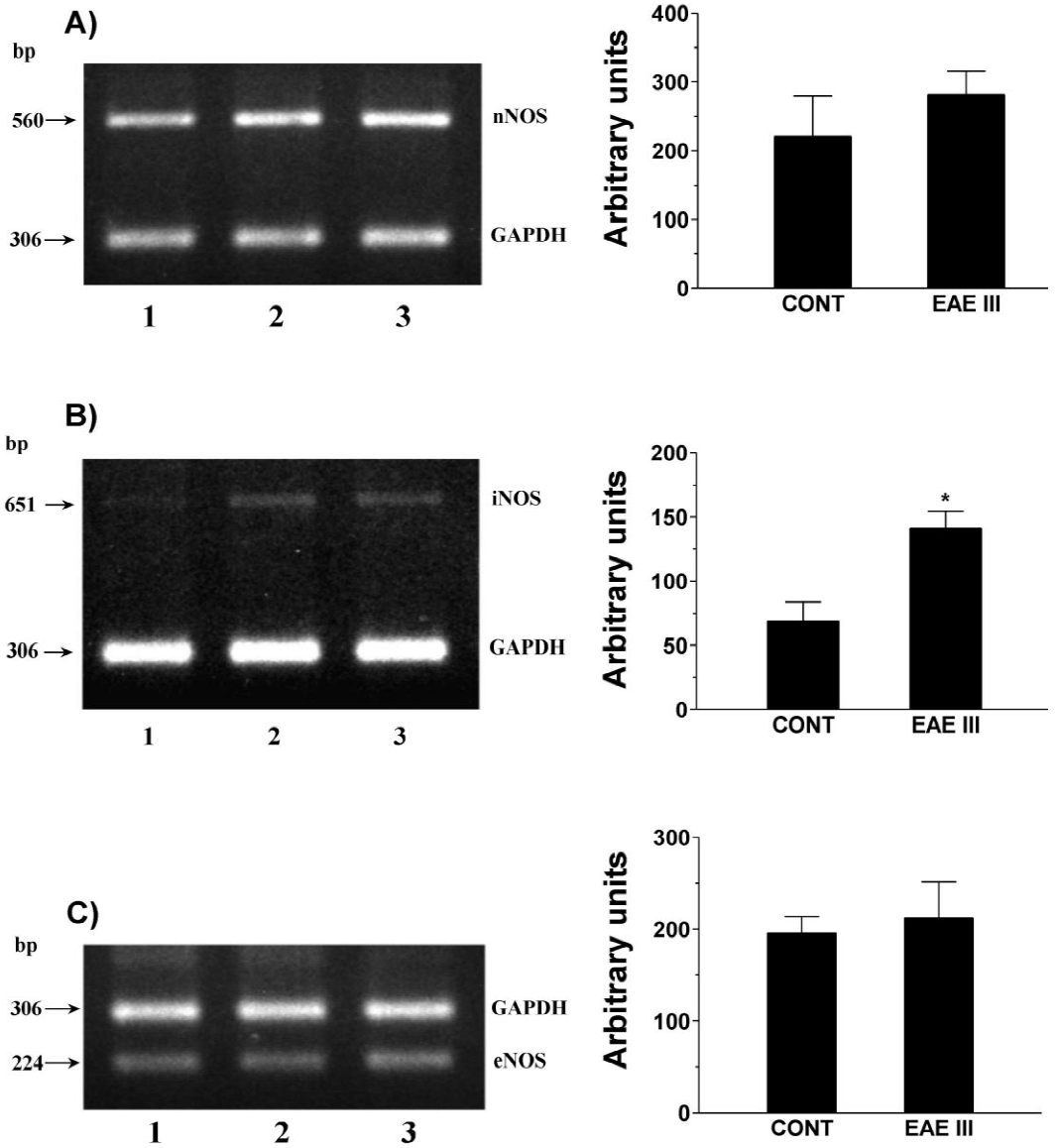

Fig. 7. Representative RT–PCR amplification of mRNA for (a) nNOS, (b) iNOS and (c) eNOS obtained from WEM homogenates from control animals and rats with EAE at stage III. GAPDH was used as the internal standard. Lane 1: Control, Lanes 2 and 3: EAE at stage III. On the right, the respective mRNA levels estimated by densitometry of the bands. *$P<0.001$ vs. control group.

muscle [16]. Walter et al. [46] showed that TNF-α is the most potent stimulus for BH_4de novo synthesis. Considering this relationship and that pro-inflammatory cytokine production is upregulated in animals with EAE at the peak of severity of the disease, we can hypothesize that high endogenous BH_4 levels in rats with EAE are responsible for the lack of significant effects observed on iNOS activity when exogenous BH_4 was not present in the incubation media (Fig. 3).

On the other hand, elevated glutathione reductase activity (an index of riboflavin status) has been reported in cerebrospinal fluid from patients with multiple sclerosis [5]. Similarly to the observations above with regard to BH_4, these clinical findings could explain why the omission of exogenous FAD from the incubation media was devoid of significant effects on the activity of Ca^{2+}-independent NOS present in WEMs from rats with EAE, but inhibited by ~50% the enzyme activity present in control rat WEMs (Fig. 3). However, we do not have a satisfactory explanation for the effects of omission of this cofactor on Ca^{2+}-dependent NOS activity, where similar decreases were observed in WEMs obtained from both groups of animals.

Despite the inability to detect higher Ca^{2+}-independent NOS activity in WEM homogenates from rats with EAE (stage III), higher levels of plasma nitrate, WEM iNOS mRNA and nitrotyrosine-modified proteins were found in this group of animals, thus concluding on the exacerbated

NO production in comparison with the control group. This conclusion is strengthen by previous reports showing high levels of NO end-products in both serum samples obtained from EAE rats [7,9,31] and cerebrospinal fluid samples collected from patients with multiple sclerosis [13,48].

As shown in Fig. 4, both WEM homogenates and sera from animals with EAE inhibited a WEM source of Ca^{2+}-dependent NOS to a lower degree than that observed with samples obtained from control animals. However, these differences between the groups disappeared when either the WEM homogenates or the serum samples were heat-denatured. Interestingly, this denaturing process was effective in lowering just the inhibitory activity present in samples from control animals, thus suggesting that endogenous levels of heat-sensitive NOS inhibitors present in control rats are, at least, decreased in rats with EAE. These results are in principle contradictory considering the significant decrease in Ca^{2+}-dependent NOS activity observed in rats with EAE which cannot be explained in terms of enzyme cofactor deficiency. Several works report that protein arginine residues can be methylated by an enzyme family known as *N*-methyltransferases [6] which catalyze the transference of a methyl group to one of the arginine guanidinium nitrogens resulting in N^G-monomethyl-L-arginine (L-NMMA), N^G,N^G-dimethyl-L-arginine (asymmetric dimethyl-L-arginine; ADMA) and N^G,N^G-dimethyl-L-arginine (symmetric dimethyl-L-arginine; SDMA). After proteolysis, these modified L-arginine residues are released [18] and have been effectively detected in cells of the immune system [20], neurons [43] and human plasma [44]. L-NMMA and ADMA (but not SDMA), are nonspecific NOS inhibitors [34], and high ADMA concentrations have been reported to occur in plasma samples obtained from either patients or experimental animals with hyperlipidemia [49], renal failure [44] and arteriosclerosis [25], as well as in schizophrenic patients [10].

Rawal and coworkers [37] showed that the renal excretion of ADMA and SDMA in patients with multiple sclerosis is approximately 20% lower than that found in control subjects. Despite the fact that the latter observation could, at least in part, justify our findings, a valid physiological interpretation remains to be established.

NO can react with superoxide anion to form peroxynitrite, a very strong oxidant and nitrating agent that can either promote lipid peroxidation, protein nitration in tyrosine residues or even be cleaved to render the highly toxic hydroxyl radical [2]. The role of NO at the CNS level has been studied in this animal model of multiple sclerosis, however the results are not conclusive. NO can cause damage in specific regions in a dose- and time-dependent manner, but it can also contribute to significantly attenuate the disease state (for revision see [47]). Pasquet and co-workers [34] showed that peroxynitrite can exert an efficient feedback control of NOS activity by preferentially promoting oxidation of reduced thiol groups over the nitration of tyrosine residues of the enzyme. In the other hand, several works have previously shown that NO can inhibit NOS activity by itself [1,4,15,38,39].

Pharmacological approaches employing NOS inhibitors also lead to contradictory conclusions on the role of NO on the clinical status of animals with EAE. The first studies suggesting a role for NO in EAE were performed by Cross and coworkers [8], which were later confirmed by Brenner et al. [3]. These authors observed that the administration of aminoguanidine (a preferentially iNOS inhibitor) was able to avoid the clinical symptoms of the disease in SJL mice. Similar results were obtained from rats with EAE [50], thus concluding on the pathogenicity of high iNOS-derived NO levels during the disease. On the other hand, Zielasek et al. [51] showed that the administration of different NOS inhibitors had no significant therapeutic effects on EAE in rats. However, these authors describe that aminoguanidine slightly worsened the clinical status of the animals, an observation which was later confirmed by Cowden et al. [7], employing both aminoguanidine and L-NMMA. Strengthening these conclusions, other authors also showed that the administration of either non-selective NOS inhibitors, such as L-NAME and L-NMMA [40], or highly selective iNOS inhibitors like L-NIL [14], had negative clinical effects, suggesting that the beneficial effects of NO could be explained by the basis of its immunosuppressing activity.

Taken the above considerations, and based on the results shown herein, we can hypothesize that upregulation of iNOS occurs during the late stages of EAE, which can, in turn, be responsible for the increased production of NO and the appearance of nitrotyrosine-containing proteins at the CNS level. Parallel to these events, a decrease in constitutive Ca^{2+}-dependent NOS activity occurs, probably due to inhibition by the excessive amounts of NO/peroxynitrite formed, but not related to the presence of other endogenous inhibitor(s) or to any cofactor deficiency. On the other hand, the lack of effect of the omission of either FAD or BH_4 on the ex vivo Ca^{2+}-independent NOS activity present in WEMs from rats with EAE, as well as the absence of a circulating thermolabile NOS inhibitor in these animals, could account for a physiological mechanism tending to potentiate the immunosuppressor activity of iNOS-derived NO. However, additional studies are required in order to confirm these hypotheses.

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