Oxidative Status and Myelin Basic Protein Levels in Experimental Allergic Encephalomyelitis

DENEYSEL ALERJİK ENSEFALOMYELİT MODELİNDE OKSİDATİF DURUM VE MİYELİN BAZİK PROTEİN SEVİYELERİ

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Summary

Purpose: Central nervous system (CNS) tissue is particularly vulnerable to oxidative damage, suggesting that this could be an important factor in the pathogenesis of CNS inflammation in multiple sclerosis (MS) and its model experimental allergic encephalomyelitis (EAE). Free radical production is thought to be involved in inflammatory processes, both exacerbating inflammation and effecting tissue damage. Nitric oxide (NO) and thiobarbituric acid reactive substances mediated lipid peroxidation may be involved in myelin damage in EAE. We therefore examined indirect markers of oxidative stress in EAE. As an index of oxidative alteration, we measured malondialdehyde (MDA) levels in plasma as marker of lipid peroxidation and plasma levels of nitrite and nitrate, the end products of the L-arginine-NO pathway.

Materials and Methods: This study was undertaken at Dokuz Eylül University, Medical School Multidisciplinary Laboratories. Nitrite was measured by using the Griess reaction and nitrate levels were determined by the method of Bories. As a marker of myelin degradation which indicate escape of this myelin component out of the brain, blood myelin basic protein (MBP) levels were determined by RIA method. Blood MDA levels were measured according to the methods of Stocks and Dormandy.

Results: There was no statistically significant elevation in blood MDA or MBP in EAE. There were marked increases in nitrite and nitrate levels.

Conclusion: The reasons for having insignificant results for blood MBP concentrations in EAE could be explained as a consequence of rapid clearance by kidney. These findings may be significant since in comparison to MDA, the end products of the L-arginine-NO pathway, nitrite and nitrate, could be a more important effector molecule in the immunopathogenesis and sistemic activity of MS.

Key Words: Multiple sclerosis, Experimental allergic encephalomyelitis, Nitric oxide, Malondialdehyde, Myelin basic protein

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Özet

Amaç: Santral sinir sistemi (SSS) dokusunun özellikle oksidatif hasara karşı zedelenebilir oluşu nedeniyle, multipl skleroz ve onun modeli deneysel allerjik ensefalomyelit (EAE) patogenezinde önemli bir faktör olabilir. Serbest radikal oluşumunun, inflamatuar durumlara hem inflamasyonu alevlendirerek, hem de doku hasarını etkileyerek katıldığı düşünülmektedir. Nitrik oksit (NO) ve tiyobarbitürik asitle reaksiyona giren maddelerin aracılık ettiği lipid peroksidasyonu EAE'de myelin hasarına neden olabilir. Biz, bu yüzden EAE'de oksidatif stresin indirekt belirteçlerini araştırdık. Oksidatif değişikliğin göstergesi olarak, plazmada lipid peroksidasyon belirteci olan malondialdehit (MDA) ve L-arjinin-NO yolunun son ürünleri olan nitrit ve nitrat düzeylerini ölçtük.

Materyel ve Metod: Bu çalışma Dokuz Eylül Üniversitesi, Tıp Fakültesi Multidisiplin Laboratuarlarında yapılmıştır. Nitrit Griess reaksiyonu, nitrat düzeyleri ise Bories'in metoduna göre ölçüldü. Myelin yıkım ürünü olarak, beyinden myelin kaçışını gösteren kanda myelin bazik protein (MBP) düzeyleri RIA metoduyla ölçüldü. Kan MDA düzeyleri Stocks ve Dormandy'ye göre ölçüldü.

Bulgular: EAE grubunda, kan MDA ve MBP düzeylerinde anlamlı bir artış yoktu. Bununla birlikte, nitrit ve nitrat düzeylerinde anlamlı artışlar saptandı.

Sonuç: Kan MBP konsantrasyonlarının EAE grubunda anlamlı değişiklik göstermemesi, böbrekler tarafından hızlı temizlenmesi ile açıklanabilir. Bu bulgular MDA'ya oranla, L-arjinin-NO yolunun son ürünleri olan nitrit ve nitrat düzeyleri, MS'in sistemik aktivitesi ve immünpatogenezinde daha etkili bir molekül olabileceği için anlamlı olabilir.

Anahtar Kelimeler: Multipl skleroz, Deneysel allerjik ensefalomyelit, Nitrik oksit, Malondialdehit, Myelin bazik protein

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Experimental Allergic Encephalomyelitis (EAE) is an inflammatory and demyelinating autoimmune disease of the CNS which is commonly used as an experimental model to investigate disease mechanisms in MS (1). EAE can be induced by inoculation of animals with homogenized CNS tissue or highly purified myelin proteins such as MBP or proteolipid protein (PLP) (2).

Central nervous system (CNS) tissue is particularly vulnerable to oxidative damage, suggesting that this could be an important factor in the pathogenesis of CNS inflammation in multiple sclerosis (MS) (3). Nitric oxide (NO) has been demonstrated pivotal role in neurotransmission and regulation of neuroimmunologic system. Furthermore, NO released by microglial cells and infiltrating macrophages may participate in damage of oligodendrocytes and neurons (4).

NO and thiobarbituric acide reactive substances (TBARS) mediated lipid peroxidation may be involved in myelin damage in EAE (3,5). Myelin basic protein is exclusively located in the oligodendroglia-myelin complex and it is considered a possible marker in cerebrospinal fluid (CSF) for active demyelination (6). In addition to demyelination, blood-brain barrier (BBB) is broken in this disease. Consequently, MBP that is found in the structure of oligodendroglia-myelin complex penetrates the BBB (7).

The pathophysiologic mechanisms of human diseases suspected to be autoimmune in origin are generally poorly understood, and the diseases themselves are clinically unpredictable. The first aim of the study was to determine whether oxidative stress occurs or not in EAE. We therefore examined indirect markers of oxidative stress in EAE. As an index of oxidative alteration, we measured malondialdehyde (MDA) levels in plasma as marker of lipid peroxidation and we determined the plasma levels of nitrite and nitrate the end products of the L-arginine-NO pathway. In this study, our second aim was to determine, whether MBP crossed or not to blood through CSF. Another aspect of the second objective was also to use sera obtained in a less invasive way instead of CSF in our study.

Material and Methods

Animal

Ten- to 12 week-old, inbred, female Lewis rats, originally obtained from, Harlan, UK were purchased from the Experimental Medicine Research Center (DETAM) in Istanbul and housed in top filtered cages in a standart animal facility. The study consisted of 10 female Lewis rats with EAE and 5 female Lewis rats for control group.

During the experiment, two to three rats were housed in each cage. All animals were fed a regular diet and given water ad libitum without antibiotics.

Induction of EAE

Spinal cords from guinea pigs were obtained by insufflation. Guinea pig-spinal cord homogenate (GP-SCH) was prepared by homogenization in phosphate buffered solution (PBS) (1:1, V / V). The homogenates were lyophilized, reconstituted in PBS to a concentration of 300 mg/ml, and stored at -20°C until used. GP-SCH was diluted with PBS to a concentration of 100 mg/ml and emulsified 1:1(V / V) with Complete Freud Adjuvant (CFA) enriched with 10 mg/ml Mycobacterium hominis (H37Ra) (Difco Laboratories, Detroit, MI; USA). Rats were sensitized with 25 mg of GP-SCH emulsified with an equal volume of CFA containing 1 mg of H37Ra. The suspension, a total of 0.1 ml, was injected subcutaneously into the two footpads (1). On day 14 post-immunization (pi) 7 pairs of rats with identical clinical scores were identified. The other 5 rats received intraperitoneal placebo injections (0.89 % NaCl). Control group consisted of these Lewis rats which were treated with saline. At the time of death, anesthetized rats were sacrificed and the spinal cords were removed and the blood was also drawn by cardiac puncture. The experiments were approved by Animal Ethical Committee at the University of Dokuz Eylül.

Clinical Scoring of EAE

Animals were examined daily for signs of disease. The first clinical signs usually appeared on days 12 to 13 after immunization. The clinical severity of EAE was evaluated according to the following 3-point scale: 0-no abnormalitiy, 1-loss of tail tonicity, 2-tail paralysis and hind leg paresis, 3-quadriplegia or moribund state (1).

NO Determination

For deproteinisation of serum, 0.8 ml of distiled water and 0,6 ml of NaOH (0.3 M) were mixed into 0.2 ml of serum and then, 0.6 ml of $ZnSO_4$, 5% (w/v) was added and incubated at room temperature for 5 minutes. Afterthat, mixture was centrifuged at 2800 g for 20 minutes and supernatants were obtained for NO determination.

Nitric oxide was determined by measuring the formation of both the stable oxidation products of NO, namely nitrite (NO_2^-) and nitrate (NO_3^-). NO in oxygen-containing solutions was chemically unstable and underwent rapid oxidation to NO_2^-. The concentration of NO_2^- were determined by Griess reaction (8). In brief, an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphhalene diamine HCl, 2% H_3PO_4), was added to the 0.2 ml of tested supernatant. Following 10' of incubation at room temperature, the colour produced was measured at 550 nm with ELISA reader. To measure nitrate levels, nitrate content of serum was reduced to nitrite by incubation of the samples for 30' with nitrate reductase (NR) (0.1 U/ml, Boehringer Mannheim, Germany) by using the method of Bories (9). For reduction of nitrate to nitrite phosphate buffer (pH=7.5), FAD (0.2 mM) and NADPH were used and then, enzymatic reaction was started with NR (500 U/L). The amount of nitrate was obtained by subtracting the nitrite concentration from the total (nitrite+nitrate) concentration. The levels of NO were calculated from a standard curve of sodium nitrite concentrations of 2-100 μmol/L.

Measurement of malondialdehyde by thiobarbiturate (TBA) reaction

Plasma MDA levels were measured according to the methods of Stocks and Dormandy (10), and Jain et al (11) with slight modifications. Briefly: 0.2 ml of plasma was added to 0.8 ml of phosphate buffered saline pH:7.4. To this, 0.5 ml of 30% (W/V) TCA was added and tubes were vortexed and allowed to stand in ice 1h and then centrifuged at 1000 x g for 15'. One ml of the supernatant was transferred into another glass tube. To this was added 0.075 ml of EDTA (2 mM) and 0.25 ml of 2% (w/v) thiobarbiturate (TBA) in NaOH (0.05 nmol. L^{-1}). The mixture was vortexed and placed in a water bath at 95 °C for 15'. Absorbance was read against a blank at 532 nm after tubes were cooled to room temperature. The concentrations of MDA was calculated from a standard curve of 1,1,3,3,tetramethoxyproprane.

Determination of myelin basic protein (MBP) by radioimmunassay

MBP in serum were determined by using radioimmunassay kit (Diagnostic Systems Laboratories, Inc, DSL-1500, Texas, USA). Tests were made in double. Test follows the basic principle of RIA where there is competition between a radioactive and nonradioactive antigen for a fixed number of antibody binding sites. The amount of I^{125} - labelled MBP bound to the antibody is inversely proportional to the concentration of the MBP present. The approximate concentrations of MBP standart were between 0.5-32 ng/ml (0.03-1.73 nmol/L).

Statistics: The statistical evaluation was performed by computer with SPSS Software using One Way Anova and Student -Newman Kuels tests.

Results

As shown in Table 1, MDA concentrations in EAE (1.4±0.5 nmol/ml) were not significantly different than the control group (1.17± 0.59 nmol/ml) There were marked increases in nitrite (EAE: 17.4±7.8 nmol/ml; control: 8.2±4.8 nmol/ml; $p<0,05$) and nitrate (EAE: 58.1±28.6 nmol/ml; control: 17.2±9.7 nmol/ml; $p<0.01$) levels in EAE rats. Similarly, the levels of nitrite+nitrate in EAE (75.6±30.3 nmol/ml) were significantly different than control group (25.4±14.2 nmol/ml; $p<0.005$).

Table 1. Nitrite, nitrate, total nitrite+nitrate, MDA, MBP levels and clinical scores of EAE and control rats

	Control rats (n=6) mean±SD	EAE rats (n=10) mean±SD
Nitrite(nmol/ml)	8.2±4.8	17.4±7.8[a]
Nitrate(nmol/ml)	17.2±9.7	58.1±28.6[b]
Total nitrite+nitrate(nmol/ml)	25.4±14.2	75.6±30.3[c]
MDA(nmol/ml)	1.2±0.6	1.4±0.5
MBP(ng/ml)	5.2±1.7	9.3±8.8
Clinical score(points)	0	1.2±0.5[d]

[a]p<0.05, [b]p<0.01, [c]p<0.005, [d]p<0.000 significantly different from the control group.

Serum MBP concentrations were found increased in EAE group (9.3±8.9 ng/ml) compared to the control rats (5.2±1.7 ng/ml).

There were significant correlations between nitrate (r=0.532, p=0.011), total NO (r=0.664, p=0.001) and nitrite; and between nitrate and total NO (r=0.986, p=0.000); and also between nitrate (r=0.883, p=0.02), total NO (r=0.871, p=0.024) and EAE clinical score.

However, MDA and MBP concentrations did not show any correlation with neither NO parameters nor EAE clinical score.

Discussion

MBP in EAE

Antibodies to MBP are regularly found in CSF of patients with active MS as well as in CNS tissue of MS patients (12). Some cases of anti-MBP associated MS may have relatively less inflammation (13). It is important because it is likely to lead to specific therapies but its reason is not known.

Hohlfeld et al demonstrated that MBP -specific T cells produced neurotrophic factors and showed neuroprotective effects (14). Besides, Mancardi et al suggested that restricted immune responses lead to axonal damage and CNS demyelination (15).

A previous study has shown that MBP may penetrate into cerebrospinal fluid in patients with cerebrovascular disease (16). Cross et al showed in chronic relapsing EAE that the development of new lesions in previously uninvolved areas of the CNS involved the early entry of labeled MBP (+) cells which remained restricted to the perivascular space (17). It could be related with release of MBP from fresh lesions to the CSF and then to plasma. About 12 days after EAE induction, the BBB is permeable to macromolecules and protein growth factors may pass through disrupted BBB (18). In the study of Bashir and Whitaker, the metabolism of a peptid of human MBP in the rabbit pointed out how rapidly MBP is cleared from plasma and CSF by kidney (19). However, there is some clearance predominantly by kidney showing a half-life of 51.6±5.4 minutes that can be measured as urinary smaller peptid fragments to be a probable catabolic metabolism. Afterthat, Whitaker described urinary myelin basic protein-like material (MBPLM) which is cross reactive with human MBP (20). The levels of urinary MBPLM were found higher in secondary progressive disease-MS than relapsing remitting-MS. Thus, levels of urinary MBPLM may permit the examination of treatment tested to prevent relapsing remitting-MS from becoming progressive-MS (21). In addition to, Percy et al showed that urinary MBPLM could serve as a useful marker of myelination in the developing child and it exhibits a developmental profile that parallels the onset of normal myelination (22).

Under the light of literature, in this study, the reasons for having insignificant results for blood MBP concentrations in EAE group could be explained as a consequence of rapid clearance by kidney. We suggest that excess MBP concentrations may be an early event in the pathogenesis of demyelinating diseases of CNS and highlights the importance of animal models in therapies targeting repair.

NO and MDA in EAE

There is evidence that macrophages, T cells, and their secreted products such as tumour necrosis factors and leukotrienes play a central role in myelin damage and nerve disfunction which are involved in the pathogenesis of EAE (16,23,24).

However the mechanism by which the inflammatory cells induce neurologic dysfunction is not fully understood. Ding et al (25) revealed that an association between NO production and EAE severity. The free radical NO is considered a major mediator in immune and autoimmune function, since activated monocytes produce high levels of NO following induction of inducible nitric oxide synthase (iNOS) (26).

It is proposed that NO may mediate neuron and oligodendrocyte death and may participate in demyelination (28,29). In various virus encephalitis, NO induced by certain neurotrophic viruses may contribute to the pathogenesis of infection (29,30). Treatment of encephalitis virus- infected mice with aminoguanidine (an inhibitor of NOS) increased their survival (31). The free radical NO is considered to be a major mediator of immune function in host defense against tumors, bacteria, and parasites. There is evidence that the accumulation of NO me-

diates damage in inflamed tissue (26,29) and that the severity of the neurologic signs and the degree of inflamation in the brain of rats with Borna disease corralates with the induction of NOS (32). Spitsin et al revealed that peroxynitrite, the product of NO and superoxide elevated in EAE; and CNS inflammation in EAE were protected by natural peroxynitrite scavenger uric acid (33).

We found blood nitrate and nitrite levels elevated. Significant correlations between NO metabolites and total NO clinical score were found. There was no statistically significant increase in blood malondialdehyde (peroxidative damage indicator) or myelin basic protein (which indicates escape of this myelin component out of the brain).

In conclusion, slightly increased MDA concentrations may have facilitated NO production by sinergising with many cell adhesion molecules. In addition, the NO formation in circulation may arise from sistemic origin. The appropriate control may be a non-brain specific antigen such as peripheral prooxidant molecules such as methemoglobine or cyanohemoglobines. Besides, it is not certain whether excess NO is coming from microglial, astroglial or endothelial cells having nitric oxyde synthase.

These findings may be significant since in comparison to MDA, the end products of the L-arginine-NO pathway, nitrite and nitrate, could be a better marker molecule in the immunopathogenesis and sistemic activity of MS. One implication of this finding is that antioxidants may inhibit EAE development at several levels. Moreover, inhibitors of NOS can be used in the therapy of MS in humans.

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