The concentration of cGMP in serum and clinical activity of multiple sclerosis

Natalia Lubina-Dąbrowska¹*, Anna Magnuszewska², Adam Stępień¹ and Małgorzata Chalimoniuk²,³

¹Department of Neurology, Military Institute of Medicine, Warsaw, Poland.
²Cardinal Stefan Wyszyński University, Warsaw, Poland.
³Mossakowski Medical Research Centre Polish Academy of Sciences

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Multiple sclerosis (MS) is a demyelinating disease of the central nervous system of unknown etiology. The processes of demyelination accompanied by a local inflammatory process, which is the result of an autoimmune response to myelin antigens. The aim of this study was to analyze the levels of nitric oxide and cGMP in the serum of patients with multiple sclerosis (MS) during the relapse and remission. The study involved 27 persons, including 11 patients (K7/M4) during the MS relapse and 11 (K7/M4) patients with MS in remission, and 7 normal subjects (5K/2M). The concentration of cGMP and immunochemical method (ELISA) and the concentration of nitrite by Griess. The results showed higher levels of cGMP and nitrite in the serum of MS patients at relapse when compared to a control group and a group of MS patients in remission. Significant deterioration of neurological condition is a plan view of the disease evaluated by using the EDSS in patients with MS. The higher concentration of NO and cGMP levels in plan correlated with worse neurological status of patients with MS. The results suggest that nitric oxide and cGMP mediated MS causing neurologic deterioration of MS patients but not the main factors responsible for the changes taking place in the brain of MS.

Keywords: multiple sclerosis, cGMP, nitrogen oxide, EDSS

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that is characterized by the loss of myelin, mainly in the central nervous system. Demyelination is accompanied by a chronic inflammatory process, which is based on the infiltration of T lymphocytes, blood macrophage recruitment, and local microglia activation (Ranshoff et al., 1991). As a consequence, an impairment of axonal conduction occurs, resulting in neurological deficits and progression of disability (Bjartmar et al., 2003; Fassas et al., 2004). The disease most commonly occurs in people aged 20-40 and has a relapsing-remitting form. The severity of demyelination and inflammation depends, inter alia, on the balance between Th1 and Th2 cells, which produce pro-inflammatory and anti-inflammatory cytokines (Conti-Fine et al. 2006; Ekholm et al. 1997; Skapenko et al., 2005; Weiner et al., 2009) and antioxidative system disorders (Ljubisavljevic et al., 2012). Activation of microglia and astrocytes then occurs, leading to an increased concentration of pro-inflammatory cytokines in the brain, which has been observed in a rat MS model with autoimmune...
encephalomyelitis (EAE) (Sulkowski et al., 2013). An increase of pro-inflammatory cytokines causes nitric oxide synthase (iNOS) induction and increases the levels of nitric oxide (NO) in macrophages, microglia cells, and astrocytes (Giulivi et al., 2003). Studies carried out by Shen, et al. have shown that the INF-γ, IL-1β, and TNF-α cause induction of iNOS expression in astrocytes (Shen et al. 2005). Excess NO may react with the superoxide radical (O$_2^-$) to form the peroxynitrite anion (ONOO$^-$), which quickly disintegrates into a noxious, highly reactive hydroxyl radical (Beckman et al., 1994). Oxygen radicals and NO can have a strong cytotoxic effect on nerve cells through the inhibition of mitochondrial respiration (Kozubowski et al., 2004). It seems that NO may play a role in the pathogenesis of MS through its impact on demyelination and the formation of glial scars (Prinz et al., 2012). The direct impact of NO on increased permeability of the blood-brain barrier and the excitation and control of the inflammatory process (Giulivi et al., 2003) has been shown.

NO may participate in neurodegeneration of oligodendrocytes as well as causing functional and structural damage to the axons (Tiberio et al., 2005; Acar et al., 2003). However, Bo, et al. (1994) have shown that cGMP, which is synthesized by guanylyl cyclase (CG) activated by NO, accelerates the remyelination process (Bo et al., 1994). The administration of sildenafil, a phosphodiesterase type 5 inhibitor (PDE5 hydrolyzing cGMP), sildenafil, to rats improved their neurological condition and accelerated the process of remyelination (Pifarre et al. 2011, Polman et al. 2011). It was found that cGMP, through the activation of kinase cGMP-dependent (PKG), may participate in the rebuilding process of the myelin, but the mechanism is still unclear. Unfortunately, the literature shows controversies relating both to the role of NO and cGMP in the process of demyelination – remyelination (Bo et al., 1994; Tran et al., 1997; Nazliel et al., 2002, Pifarre et al. 2011). As has been previously demonstrated, NO and cGMP may show opposite effects (protective or neurotoxic) depending on their concentration. Maintaining physiological concentrations of cGMP is very important, since cGMP regulates many biochemical processes directly or indirectly through cGMP-dependent kinase. The aim of this study was to investigate the concentration of nitric oxide and cGMP in the serum of patients with multiple sclerosis (MS) at the time of relapse and remission.

**MATERIALS AND METHODS**

**Patients with MS and controls**

This study was conducted with a group of 29 people, including 22 MS patients and 7 healthy individuals. In the MS group, there were 14 women and 8 men. The diagnosis was established on the basis of the McDonald criteria of 2011 (Polman et al., 2011) and the exclusion criteria. Eleven patients were in disease relapse and 11 were in remission. This study did not include persons who were, in addition to the suspected MS, affected by other chronic diseases such as depression, liver, kidney, thyroid diseases, and abnormalities in blood morphology and biochemical tests (exclusion criteria). The group was homogeneous in terms of age, duration of disease, incidence of relapse, and remission and disability as assessed on the EDSS scale (Kurtzke’s Expanded Disabling Status Scale, EDSS). Those affected by the disease and the control group was of similar age (table 1). The age of patients during the disease remission phase averaged 31 ± 3.8, and those in the phase of relapse averaged 32 ± 4.6. In the control group, there were 7 healthy individuals (5 women and 2 men) with an average age of 23 ± 1.9. The relapsed patients had suffered from multiple sclerosis for an average of 3.8 years ± 1.8, and those in the remission phase, for 3.5 years ± 1.7 years (tab. 1). All patients with MS had undergone immunomodulation therapy with beta interferon. Patients with MS were administered, subcutaneously, every second day, IFN-â1b (Betaferon®, Bayer Schering Pharma, Berlin, Germany) at a dose of 250 µg. The relapsed patients came to follow-up appointments in order to be administered Solu-Medrol and blood collection. All relapses were treated intravenously with Solu-Medrol at a dose of 1 g/day for 3-5 days (Solu-Medrol, Pfizer, Switzerland). The participants of the study expressed their written consent to participate in the project. The study protocol was approved by the Bioethical Commission of the Military Medical Institute (Approval No. 34/WIM/2009).

The results are presented as mean values ± SEM, n = 11 for the MS relapse group, n = 11 for the MS remission group, n = 7 for the control group. Results are presented as mean ± SEM of n = 11 for group MS relapse, n = 11 for MS remission group, n = 7 for the control group.

**Table 1. Characteristics of patients with MS and the control group**

<table>
<thead>
<tr>
<th></th>
<th>Age [years]</th>
<th>Years of illness</th>
<th>Number of persons (sex m/f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23 ± 1.9</td>
<td>-</td>
<td>7 (2/5)</td>
</tr>
<tr>
<td>MS relapse</td>
<td>32 ± 4.6</td>
<td>3.8 ± 1.8</td>
<td>11 (4/7)</td>
</tr>
<tr>
<td>MS remission</td>
<td>31 ± 3.8</td>
<td>3.5 ± 1.7</td>
<td>11 (4/7)</td>
</tr>
</tbody>
</table>

**Serum collection**

Blood for testing was collected while fasting between 8:00 and 10:00 in the morning. Patients with MS took the last dose of medication at 20:00 on the previous day. Serum was obtained from the blood. After clotting at room temperature (30 minutes), the blood samples were
vortexed at 1500g for 15 min. The serum was stored at -70°C until the measurement of cGMP and nitrate concentration could be performed.

**Determination of cGMP concentration**

The concentration of cGMP was determined using an immunochemical method (ELISA) based on the competition between unmarked cGMP and cGMP marked with acetylcholinesterase to a certain number of specific spaces in the antibody against cGMP. To determine the concentration of cGMP, a commercial ELISA kit by Cayman Chemical Company was used (cat. no. 581 021, Ann Arbor, MI, USA) according to manufacturer’s instructions.

**Determination of nitrate concentration**

The nitrate concentration (nitrite, NO\(_2^-\)), measured as the exponent of the concentration of serum nitric oxide (NO), was marked using the Griess spectrophotometric method (Li et al., 1999).

**Statistical analysis**

The results were presented as an average value ± standard error of the mean (SEM) calculated from individual measurements assumed as the final result. The statistical analysis of the results was performed in Statistica 7.0. To determine the differences in values of clinical data: EDSS scale, duration of disease, age, concentration of cGMP, and nitrate in serum, the Kruskal-Wallis non-parametric test was used. Dependencies between the groups were tested using the U Mann-Whitney test. The results were considered statistically significant at the significance level p<0.05. The correlation of phenomena was developed by calculating the \(r\) Person's correlation coefficients, which are an expression of the strength of linearity in the correlations between the two variables. To be considered statistically significant, the values at p<0.05 were assumed.

**RESULTS**

The concentration of cGMP was determined at the time of relapse and remission in patients with MS and healthy individuals (control group; figure 1). The concentration of cGMP in the serum was 39.1 ± 6.6 pm/ml during relapse, 3.8 ± 1.0 pmol/ml during remission, and 14 ± 2.4 pmol/ml in healthy individuals (figure 1). Statistical analysis showed that the concentration of cGMP levels was significantly higher in patients during relapse compared to either those in the control group or patients with MS in remission (MS group - remission) (p<0.001). In addition, the concentration of cGMP in the serum of patients during remission was significantly lower compared to the control group (p<0.01; figure 1).

The level of cGMP were determined by ELISA. Results are presented as mean ± SEM of n = 11 for group SM a, n = 11 for MS remission group, n = 7 for the control group independent measurements. **\(p<0.01\), *** \(p<0.001\) compared to control, ### \(p<0.001\) compared to group the SM-remission.

The value of NO\(_2^-\) concentration in the serum was 106.3 ± 19.4 nmol/ml during relapse, 38.8 ± 4.4 nmol/ml during remission, and 44.8 ± 2.8 nmol/ml in the control group,(figure 2). Statistical analysis showed that the serum concentration of NO\(_2^-\) was higher in patients with MS during relapse compared to the control and also to the MS group in remission (p<0.001, figure 2).
The results of the conducted studies indicated the presence of higher concentrations of cGMP in the serum of MS patients during the relapse phase compared to healthy individuals and patients during remission. The changes in cGMP concentration were accompanied by an increase in the concentration of nitrites (the exponent of the NO concentration) in the serum of relapsed patients. Higher concentrations of nitrite and cGMP in the serum during the relapse correlated with aggravated disability as evaluated by the EDSS scale.

The demyelination processes in MS are accompanied by inflammation, resulting from the activation of CNS cells (astrocytes and microglia) as well as macrophages, monocytes, and lymphocytes. At the time of activation, the cells release various pro-inflammatory factors such as cytokines, and free radicals, such as ROS, both in the brain, blood, and cerebrospinal fluid (Brosnan et al. 1996; Golab et al., 2012). The released pro-inflammatory factors may cause the activation of pro-inflammatory enzymes such as induced nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), and thereby activate the demyelination process (Raine et al., 1997). This leads to a deceleration in neural conductivity and a degradation of the myelin sheaths ([Polman et al., 2011]. Numerous studies suggest that NO plays an important role in MS (Nazlief el et al., 2002; Miljkovic et al., 2002). Maciejek, et al. (1985) demonstrated that, in the initial stage of the disease, cGMP concentrations increased compared to the control group. However, in the later course of the disease, during the development of the various clinical forms of MS, cGMP concentrations returned to the control values (Maciejek et al., 1985). One of the main receptors of NO is soluble guanylyl cyclase (sGC), and its activation causes an increase in cGMP in neurons.

Cyclic GMP can be synthesized by the endothelial cells of blood vessels, blood cells, and other peripheral tissues, or it can be removed from the CNS completely (Sager et al., 2004). Throughout these processes, cGMP is released into the blood. It is known that an increase in the concentration of cGMP in the cortex of the brain occurs during the relapse of EAE. An increase of cGMP in the cortex was caused by both the activation of the soluble and mostly membrane CG. Studies have shown that iNOS expression occurs in the cerebral cortex of rats with EAE (Sulkowski et al., 2013). Previous studies have demonstrated an increased reactivity of iNOS protein and the level of mRNA in the areas of demyelination (deposits) in the cerebral tissue in patients (Bagasra et al., 1995). The expression of iNOS in the areas of demyelinating lesions in the brain in patients with MS may be caused by an increase in the release of pro-inflammatory cytokines: TNF-α, IFN-γ, and IL-1β in astrocytes and microglia (Golab et al., 2002). In vitro studies have indicated that the joint effect of pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ induces
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