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Administration of vitamin D_3 induces CNPase and myelin oligodendrocyte glycoprotein expression in the cerebral cortex of the murine model of cuprizone-induced demyelination

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Abstract

In the central nervous system (CNS) the main proteins of myelin are proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and CNPase. Myelin oligodendrocyte glycoprotein is a minor component of the myelin sheath, but is an important autoantigen linked to the pathogenesis of multiple sclerosis (MS). CNPase is expressed exclusively by oligodendrocytes in the CNS, and the appearance of CNPase seems to be one of the earliest events of oligodendrocyte differentiation and myelination. In this study the effects of vitamin D on total protein concentration, CNPase and MOG expression in the cerebral cortex of the murine model of cuprizone-induced demyelination was investigated. The mice were treated by cuprizone for five weeks in order to induce demyelination. The mice were then divided into 3 groups. The first group was injected intraperitoneally (IP) with vitamin D diluted in olive oil in the amount of 5 µg/kg/daily body weight. The second group (SHAM) was injected IP with olive oil and the third group was left without any injection as the control group (n = 11 for each group). After five weeks the mice were killed and the cerebral cortex was collected and the expression of CNPase and MOG was studied by Western blot. Total protein concentration in the vitamin D injected, SHAM and control groups were 0.918 ± 0.003, 0917 ± 0.004 and 0.916 ± 0.004 g/l, respectively (p > 0.05). However, a significant increase in the MOG and CNPase expression was seen in vitamin D injected group as compared to SHAM and control groups. It is concluded that vitamin D plays a role in the process of remyelination by increasing MOG and CNPase expression in the cortex.

Key words: *vitamin D, myelin, CNPase, MOG, cuprizone.*

Introduction

Multiple sclerosis (MS) is a neurodegenerative, inflammatory and demyelinating disease of the central nervous system (CNS) [11]. While the exact etiology of MS remains unknown, it is thought that many different genetic as well as environmental factors play a key role [18]. Hypovitaminosis D has long been considered as a risk factor for MS but there has recently been a sharp increase of interest in this factor [17,36]. The biologically active form of vitamin D is 1,25-dihydroxyvitamin D_3 that has a key role in the modulation of immune response [33]. This is suggested by the fact that many immune cells including

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macrophages, activated B and T cells and dendritic cells contain vitamin D receptor (VDR). Vitamin D_3 is a potent immune modulator that can even cure the animal model with MS [5]. It has been shown that vitamin D_3 acts on myelination via the activation of several myelin-associated genes [4]. It was demonstrated that vitamin D_3 could actually promote the repair process in the cuprizone model of mice [26]. Vitamin D which is a peripheral regulator of Ca^{2+} homeostasis, has numerous other physiological functions including protection against certain immune mediated disorders including MS [8]. It was shown that vitamin D_3 may be able to suppress the inflammatory ways that lead to the progression of MS [31]. Elevated levels of vitamin D have been shown to be associated with an improvement in experimental autoimmune encephalomyelitis (EAE) [32].

In myelin, a number of structural classes of proteins are present. These include proteolipid protein (PLP), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), peripheral myelin protein 2 (P2), myelin-associated glycoprotein (MAG), myelin-associated oligodendrocytic basic protein (MOBP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) [9]. Myelin oligodendrocyte glycoprotein is a transmembrane protein present in the CNS myelin and it is also one of the main autoantigens in MS [20]. Mutations and/or polymorphisms in the MOG gene may contribute to the development and progression of MS [15]. Myelin oligodendrocyte glycoprotein is a potent encephalophatogen that triggers strong T-cell and B-cell responses [14]. CNPase was shown to be implicated as an autoantigen in MS and is expressed in oligodendrocytes and considered a marker for myelin forming cells [25,27].

Myelin oligodendrocyte glycoprotein is a transmembrane protein expressed on the surface of oligodendrocyte cells and on the outermost surface of myelin sheaths and speculated to serve as a necessary adhesion molecule to provide structural integrity to the myelin sheath and is known to develop late on the oligodendrocyte [2]. Myelin oligodendrocyte glycoprotein is a target antigen that leads to autoimmune-mediated demyelination. Myelin oligodendrocyte glycoprotein has received much of its laboratory attention in studies dealing with MS. Several studies have shown a role of antibodies against MOG in the pathogenesis of MS [10]. The aim of this study was to examine the *in vivo* effects of vitamin D_3 on the CNPase and MOG expression in the cerebral cortex of the murine model of cuprizone-induced demyelination.

Material and methods

Animals

Balb/c mice were purchased from the Pasteur Institute, Tehran, Iran and maintained on the light-dark (12 : 12) cycle beginning at 8.00 am. They were kept at a constant temperature in mice boxes with unrestricted access to laboratory food and water. The colony was maintained through random pair mating. Cage maintenance was performed once a week and the animals were handled by the same individuals throughout the experimental period. Food and tap water was available *ad libitum* throughout the acclimatization and experimental period. The work was undertaken according to the provisions of the Declaration of Helsinki (as revised in Brazil 2013). All animal protocols used have been approved by the authors' institutional animal experimentation committee. 33 female Balb/c mice aged 6 to 8 weeks were included in this study (n = 11 for each group).

Injection of demyelination and treatment with vitamin D_3

Demyelination was induced by feeding 8-10-week-old mice a diet containing 0.2% cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich Inc.) mixed into ground standard rodent chow. The cuprizone diet was administered for 5 weeks for demyelination. The control group received breeder chow without cuprizone admixture. Animals were then put on standard rodent chow without cuprizone to induce remyelination. Cuprizone, a copper chelator, induces demyelination in the corpus callosum, hippocampus, and some other white matter regions of the rodent CNS. Its underlying mechanism of demyelination is not well understood, but cuprizone has been used to induce CNS demyelination for many decades. It has been noted that mouse strain, age, or gender impact the degree of demyelination [22]. The mice were then divided into three groups. The first group was injected intraperitoneally (IP) with vitamin D_3 for 6 weeks in the amount of 5 µg/kg body weight diluted in olive oil by gavage daily. The second group (SHAM) was treated with the equivalent olive oil and the third group was left without any injection as the control group. After four weeks the cerebral cortex

was harvested after euthanasia by an intraperitoneal injection of an overdose of anesthetic (sodium pentobarbitone) and the cerebral cortex were removed and processed as described. In total 33 animals were used in this study (n = 11 for each group).

Cell extract

Fresh tissue samples (10 mg each) were chopped into tiny pieces and suspended in 0.5 ml of protein lysis buffer [150 mM NaCl, 1.0% NP40, 20 mM Tris (pH 7.5), 5 mM EDTA, and Complete Mini protease inhibitor cocktail (Roche Diagnostics Ltd., West Sussex, UK)] and then mechanically homogenized by sonication. After centrifugation, the protein extracts were recovered and stored at –70°C until they were used.

Total protein concentration and Western blotting

The total protein concentration in the cerebral cortex extracts was determined by the Bio-Rad protein assay based on the Bradford dye procedure. For Western blot, protein extracts (50 µg/lane) were separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Ltd. Hertfordshire, UK). The membranes were blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 5% dry milk and probed either with polyclonal anti-CNPase antibody (Abcam plc, Cambridge, UK); Anti-CNPase antibody (ab27695) (1 : 1000 dilution), monoclonal anti-MOG antibody (Abcam plc, Cambridge, UK; ab109746) (1 : 1000 dilution) or a mouse monoclonal anti-β-tubulin antibody (as a loading control) (Abcam plc, Cambridge, UK) (1 : 10 000 dilution) and then treated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein was visualized using the Enhanced Chemiluminescence western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed by scanning immunoblots and quantitating protein bands using an image analyzer (Metaview Software, V4.6.8, Fryer Company Inc.).

Statistical analysis

In order to assess a possible distortion in allele frequencies between cases and controls, we performed a χ^2 test with one degree of freedom for both allelic and genotypic distributions between the groups of cases and controls. Significant association was defined by $p \leq 0.05$.

Results

Total protein concentration

The total protein concentration in the cerebral cortex extracts from vitamin D_3 injected, SHAM and control groups was determined by the Bio-Rad protein assay based on the Bradford dye mixture. The total protein contents of vitamin D_3 injected, SHAM and control were 0.91 ± 0.004, 0.91 ± 0.004 and 0.91 ± 0.003 (g/l), respectively. No significant increase in the total protein concentration was seen in the vitamin D_3-injected brain samples compared with those from the SHAM and control groups ($p > 0.05$).

Analysis of CNPase and MOG expression by Western blotting

Western blot analysis was performed to quantitatively evaluate CNPase and MOG expression in the cerebral cortical extracts. A western blot analysis using anti-CNPase and -MOG antibodies as a probe confirmed the presence of CNPase and MOG in all the extracts. An image analyzer was used to determine the intensities of the band in the respective lanes. Quantification of the western blot bands from repeated experiments (n = 11) showed that the amount of CNPase and MOG was significantly increased in the vitamin D_3-injected cerebral cortical extracts when compared with SHAM and control groups ($p < 0.0001$). In the vitamin D-injected group CNPase expression was increased approximately 1.70 and 1.71 times versus control and SHAM groups, respectively. Meanwhile, MOG expression was increased approximately 1.31 and 1.43 times in the vitamin D-injected extracts versus control and SHAM groups, respectively (Figs. 1 and 2).

Discussion

Multiple sclerosis is characterized by focal myelin damage, oligodendrocyte loss and infiltration of macrophages and T lymphocytes [1]. While the etiology of MS remains unknown, it is thought that many different genetic as well as environmental factors play a role [18]. It has been demonstrated that there is a significant association between latitude, deliberate sun exposure and vitamin D supplementation

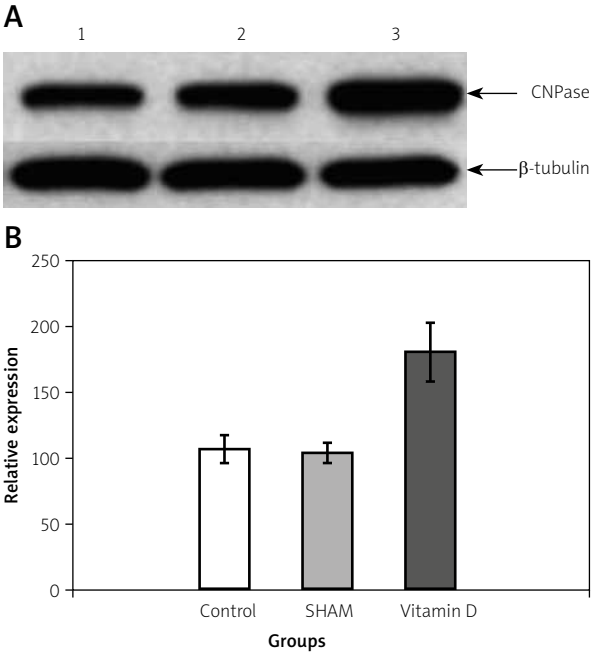

Fig. 1. A) CNPase expression in the cerebral cortex extracts from vitamin D_3 treated (Lane 3), SHAM (Lane 2) and control groups (Lane 1). β-tubulin (50-kDa) expression was determined as a protein loading control. **B**) Signal intensities from CNPase expression in the vitamin D_3 treated, SHAM and control cerebral cortex immunoblotting experiments were determined by densitometric analysis. In each of the experimental groups the number of animals investigated was n = 11. A significant increase in the CNPase expression was seen in vitamin D_3-injected group when compared with SHAM and control groups ($p < 0.0001$). No significant changes were seen between the SHAM and control group ($p = 0.91$).

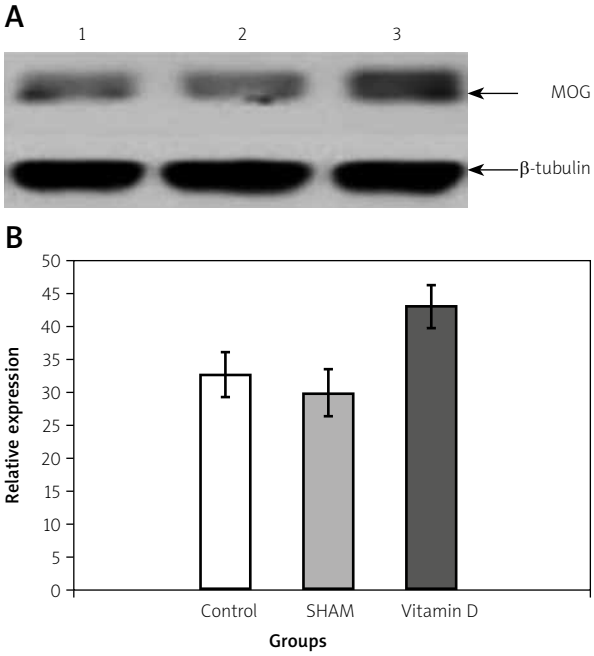

Fig. 2. A) MOG expression in the cerebral cortex extracts from vitamin D_3 treated (Lane 3), SHAM (Lane 2) and control groups (Lane 1). β-tubulin (50-kDa) expression was determined as a protein loading control. **B**) Signal intensities from MOG expression in the vitamin D_3 treated, SHAM and control cerebral cortex immunoblotting experiments were determined by densitometric analysis. In each of the experimental groups the number of animals investigated was n = 11. A significant increase in the MOG expression was seen in vitamin D_3-injected group when compared with SHAM and control groups ($p < 0.0001$). No significant changes were seen between the SHAM and control group ($p = 0.42$).

with MS [21]. In mice with EAE, only females could be treated with vitamin D_3 [32].

Nystad and colleagues showed that vitamin D could actually promote the repair process in a cuprizone-induced model of EAE mice, possibly by stimulating the effect on oligodendrocyte maturation and astrocyte activation [26]. It was also demonstrated that vitamin D_3 plays a positive effect on the remyelination process by endogenous progenitor cells and support its possible therapeutic effects in the context of demyelinating disease like MS [16]. Vitamin D has been shown to have a direct effect on neural stem cell proliferation, survival and neuron/oligodendrocyte differentiation, thus representing a novel mechanism underlying its remyelinating and neuroprotective effect in MS/EAE therapy [30]. A deficiency in vitamin D resulted in an increased susceptibility to EAE and vitamin D_3 or its analogs might potentially be important for treatment of MS [3]. It was demonstrated that vitamin D_3 acts on myelination by means of the activation of several myelin-associated genes [4]. The data reveal a role for vitamin D in the regenerative component of demyelinating disease and identify a new target for remyelination medicines [7].

Although many studies have demonstrated the positive role of vitamin D_3 in the remyelination process of the EAE mice model, but the mechanism underly-

ing its effect is not clearly understood. $1,25(OH)_2D_3$ has been shown to be potentially effective to block the development of autoimmune diseases [5].

It was suggested that CNPase may play an important role as a putative anti-inflammatory gene both in normal and injured brain and it might be a potential target self-antigen in MS [25,35]. It was suggested that the disturbances in CNPase activity may contribute, in some extent, to the changes in myelin morphology and CNPase may play a role in cellular processes requiring membrane structural reorganization [6,19].

Myelin oligodendrocyte glycoprotein is identified by monoclonal antibody 8-18C5. Myelin oligodendrocyte glycoprotein is localized on the surface of myelin and oligodendrocyte processes and its expression level may be modulated by the presence of compact myelin and/or MBP in the myelin sheath [24]. Several studies have shown a role of antibodies against MOG in the pathogenesis of MS [10]. Growth factors including insulin like growth factor were shown to be important in repair processes of demyelination [12]. Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) have been shown to have an important role in the process of remyelination by increasing Opalin (34 KDa) and MOG expression [23,29]. It is suggested that the activation of MOG transcription depends more on an intrinsic oligodendroglial maturation program of myelination. MOG mRNA expression is restricted to CNS tissue, and peak expression occurs during active myelination [13]. It was demonstrated that NKT cells are important mediators of $1,25D_3$-induced protection from EAE in mice and NKT cell-derived IL-4 may be an important factor in providing this protection [34]. It was shown that vitamin D receptor signaling regulates neuromuscular maintenance and enhances locomotive ability after physical exercise and Schwann cells and the neuromuscular junction are targets of vitamin D_3 signaling in locomotive ability [28].

The role of vitamin D_3 in the process of remyelination has been demonstrated [26]. In our knowledge this study is the first one to demonstrate the effect of vitamin D_3 on CNPase and MOG expression in the CNS of the cuprizone-induced mice model of MS. In this study we show that administration of vitamin D significantly increases CNPase and MOG expression in cuprizone induced mice cerebral cortex. As CNPase is a molecular marker for myelin forming cells, the increased CNPase expression in the cerebral cortex of a vitamin D-injected mouse may be due to increased differentiation of oligodendrocyte progenitor cells to mature oligodendrocyte. We have also shown that vitamin D increases MOG expression in the cerebral cortex which indicates the role of vitamin D in myelin formation.

The results of this study could have been anticipated with some certainty given the findings of other studies that have investigated the role of vitamin D in MS. It is also concluded that vitamin D_3 may have an important role in the process of remyelination by increasing CNPase and MOG expression.

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Disclosure

Authors report no conflict of interest.

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