ORIGINAL ARTICLE

Effect of vitamin D on the cerebral cortex proteomic profile in the mice with induced experimental allergic encephalomyelitis

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Abstract

BACKGROUND: Protective effect of vitamin D in the onset and progression of multiple sclerosis (MS) as well as its animal model- EAE (experimental allergic encephalomyelitis) has been documented in many studies.
AIM: Aim of our work was to analyze proteomic profile in the homogenate of cerebral cortex in mice with EAE treated with cholekalciferol (D3).
METHODS: In female C57/BL6 mice, EAE was induced by standard protocol using myeline oligodendrocyte glycoprotein (MOG 35-55), pertussis toxin and complet Freund adjuvans. Mice were divided into three groups – controls (4), EAE (4) and EAE+D3 (4). Vitamin D3 was daily taken as 100 ng of cholecalciferol within 4 weeks. Animals were daily inspected, with the determination of EAE scores and weight. Animal cerebral cortexes were taken after human decapitation and prepared as homogenates. Proteins of homogenates were separated by the 2D PAGE electrophoresis and 14 of 200 identified spots were analyzed by tandem mass spectrometry on MALDI-TOF/TOF by program PDQuest.
RESULTS: We have identified statistically significant changes in the level of three proteins, which might be linked to the effect of vitamin D . In mice with EAE in comparison to controls we detected 5.7 times higher level of calmodulin 1 (calm1, $p = 0{,}00013$), 14.8 times lower level of fructose -bisphosphate aldolase C (AldoC, $p = 0{,}003$), and 6.9 times lower level of aldehyddehydrogenase 1 (Aldh1a7, $p = 0{,}013$). In mice with induced EAE and treated with vitamin D3 we detected 1.5 times lower level of Calm1 ($p = 0{,}041$), 3.8 times increased level of AldoC ($p = 0{,}033$) and 4.3 times lower level of Aldh1and7 ($p = 0{,}034$) compared to the naive EAE animals.
CONCLUSIONS: Our results suggest that positive effect of vitamin D in the EAE etiopathogenesis includes at least partially an inhibition of calmodulin effect as well as the altera-

tions of glucose metabolism. Our results should be confirmed by the detailed additional studies.

Introduction

Multiple sclerosis is an autoimmune inflammatory-demyelinating disease of the central nervous system. Process of inflammation starts especially in the early stages of MS and later follows neurodegenerative processes. The disease is typical with many inflammatory infiltrations, especially in the white matter, where cells such as T-lymphocytes, macrophages, or B-lymphocytes progressivelly activate, proliferate into the final tissue to produce inflammatory lesions stimulated by pro-inflammatory cytokines. The result of the inflammation is damage of the myelin and oligodendrocytes in the lesion with the loss of the ability to spread electric impulses correctly. The activation of the microglia, subsequent oxidative stress, and excitotoxicity cause neurodegeneration with the loss of axons in white and gray matter leading to the cognitive deficit and disability (Compston & Coles 2008) .

Vitamin D is relevant factor with the important role in the etiology of SM (Kakalacheva & Lunemann 2011). After skin illumination by UV-B light, cholesterol is converted to the vitamin D precursor - vitamin D3 (cholecalciferol). This can be taken also in the diet perorally, it is a subject of hydroxylation in the liver to produce 25-hydroxycholecalciferol (calcidiol). Calcitriol (1,25-dihydroxycholecalciferol) is formed by calcidiol by hydroxylation in the carbon 1 in the immune cells, kidneys and other tissues (Pierrot-Deseilligny & Souberbielle 2010). Vitamin D participates in the regulation of diverse regulatory roles of immunocompetent cells, which triggers cascade of immunopathologic processes in the onset and progression of SM (Correale *et al.* 2009, Cantorna *et al.* 2015). The current studies examined vitamin D3 and the vitamin D3 hormone, calcitriol, as potential disease treatments in the MS as well as in the SM model disease, experimental autoimmune encephalomyelitis (EAE). The rationale for using vitamin D3 and calcitriol as potential pharmacological treatment derives from a large, diverse, and compelling body of evidence suggesting that low vitamin D levels and vice versa reduced calcitriol synthesis contribute causally to elevated MS risk, frequent MS relapses, and rapid MS disease progression (Mowry *et al.* 2012).

Protective effect of vitamin D is based on its immunomodulatory and neuroprotective influence documented in several studies (Munger *et al.* 2004, 2006; Compston & Coles 2008; Cadden *et al.* 2011; Mowry *et al.* 2012; Smolders *et al.* 2008; Runia *et al.* 2012; Ascherio *et al.* 2014). EAE as an animal model of SM is regularly induced by the animal immunization by the encephalolitogenic peptides (Devaux *et al.* 1997; Racke 2001; Tafreshi *et al.* 2005). Deficit of vitamin D causes an increased susceptibility of animals to the EAE development. Mice treated by calcitriol before immunization or even after the first EAE clinical signs, can eliminate development or suppress the scores of severity and clinical manifestation of EAE (Lemire *et al.* 1995; Cantorna *et al.* 1996). Vitamin D treatment to the mice with chronic EAE supresses proliferation of specific TH1 lymphocytes, inhibits production of IFN-γ dependent on the IL-12, limits disease relapses, decreases extent of perivascular inflammatory infiltration, supresses development of demyelinized focuses and axonal degeneration in the CNS (Mattner *et al.* 2000). Aim of our work was by the proteomic analysis of mice CNS treated with vitamin D, to explore protective molecular mechanisms of vitamin D in the animals with induced EAE and possible to translate our results into the clinical research of multiple sclerosis.

Materials and methods

Group of laboratory animals

The study includes 12 animals, female C57BL6 mice subdivided into control group (n = 4), group with induced EAE (n = 4), and group with induced EAE and treated with D_3 (n = 4).

Induction of EAE and vitamin D treatment

A standard protocol for induction of EAE has been used, which includes subcutaneous injection of 200 μg myelin oligodendrocyte proteinu (MOG 35-55) in solution with concentration of 1 mg/ml in the mixture of 200 μl complet Freund adjuvans (CFA) with the content of 10 mg/ml bacteria Mycobacterium tuberculosis (H37Ra, ATCC 25177). One hour and than 48 hours after MOG immunization was administered intraperitoneally *pertussis* toxin (400 ng in 200 μl of phosphate buffer in physiological solution (PBS, *phosphate-buffered saline*). Vitamin D was administered intraperitoneally in daily dosage 100 ng cholekalciferolu in duration of 28 days. Clinical status of animals was checked by experienced person and study was approved by the Etical commission of Comenius University and State veterinary commision No. Ro-3352/16-22.

Preparation of homogenate of brain tissue

After completion of in vivo experiment, animals was humanly decapitated and cerebral cortex was isolated and prepared as homogenate according to Dodd *et al.* (1981). Frozen tissue was homogenized in the Teflon-glass Potter-Elvehjem in homogenization buffer (5x volume of tissue) with 0.32 M sucrose, 5 mM HEPES (pH 7.0), 1 mM EDTA, 0.3 mM phenylmetylsulphonylfluorid. Samples were immediately frozen and stored at -70 °C. Protein concentration was detected by the Lowry method with the bovine serum albumin as standard (Lowry *et al.* 1951).

Tab. 1. Level of proteins in the group of animals with induced EAE and controls (CTL)

Protein	EAE (n = 4)		CTL (n = 4)		EAE vs. CTL		
	Average	SEM	Average	SEM	Increase of concentration	Decrease of concentration	T test p
Calm1	13.9	0.59	2.43	0.46	5.73 ×		0,0001
AldoC	0.15	0.05	2.23	0.45		14.83 ×	0,003
Aldh1and7	0.3	0.07	2.08	0.21		6.92 ×	0,0133

Tab. 2. Level of proteins in the group of animals with induced EAE and treated with vitamin D compared to the animals with EAE without vitamin D

Protein	EAE-D_3 (n = 4)		EAE (n = 4)		EAE-D_3 vs. EAE		
	Average	SEM	Average	SEM	Increase of concentration	Decrease of concentration	T test p
Calm 1	9.2	0.84	13.9	0.59		1.51 ×	0,04
AldoC	0.58	0.12	0.15	0.05	3.83 ×		0,03
Aldh1and7	1.28	0.14	0.3	0.07		4.25 ×	0,03

Treatment of homogenates and polyacrylamide electrophoresis

Proteins from homogenates were purified in the Eppendorf test tubes by using 200 μg of homogenate plus 4-times volume of acetone and stored for 2 hours at -20 °C. Samples were centrifugated at 13 000 rpm/RT/10 min, supernatant discarded and to the pellet was added 4 volumes of acetone, resuspended, incubated for additional hour at -20°C. After centrifugation at 13 000 rpm/10 min, supernatant discarded, remnant acetone was evaporated at room temperature for 10 minutes and pellet resuspended in 150 ul R/S of buffer. Prepared samples were loaded to the chambers of Sample/Re-hydratation tray, covered with 1 ml of mineral oil and soaked overnight at room temperature. Samples were analyzed by isoelectric focusing on strips with the length of 7 cm at pH 3-10 (Biorad) on 12 % gel with the 1 mm thickness, 15 mA/gel during 20 minutes. Electric current was than increased to 25 mA / gel during 1 h and 40 min. Later were samples fixated for 45 minutes by water solution of 40 % methanol and 10% acetic acid. Proteins were stained by Coomassie Blue Biosafe G-250 during 45 min and de-stained by distilled water over night.

Mass spectrometric analysis and statistical evaluation of results

De-stained 2D gels were scanned by the program Quantity One on Calibrated Densitometer G800. On the basis of programs PDQuest and Excel with T-test analysis were cut 14 spots, which have be treated to hydrolyze, and extracted peptides from the gel according to standard protocol, and analyzed by tandem mass spectrometry on MALDI-TOF/TOF with the loading of 0.75 μl of sample + 0.75 μl HCCA matrice and 0.5 μl of standard + 0.5 μl HCCA matrice.

Mass spectrometric analysis was done by the help of programes BioTools and Mascot search, quantitative analysis in the program PDQuest (T-Test: 95 % interval, multiple of concentration change) and for the prove was used T-Test in the MS Excel (T-Test: Two-Sample Assuming Equal).

Results

In our study we have analyzed levels of 14 proteins, which were chosen from 200 analyzed spots in the proteomic profile of cerebral cortex homogenate from control mice, mice with induced EAE and mice with induced EAE and treated with vitamin D. Statistically significant changes were detected in three proteins. These proteins were selected on the basis of comparison of changes in the tissue level between: i) controls ii) animals with induced EAE and iii) induced EAE with the vitamin D treatment and were identified by using of programes BioTools and Mascot search and by the quantitative analysis in the program PDQuest as follows: calmodulin 1 (Calm 1), fructose -bisphosphate aldolase C (AldoC) and cytosolic aldehyddehydrogenase 1 (Aldh1and7). In the group of animals with induced EAE in comparison to controls we detected 5.73 times higher concentration of calmodulin 1 (p = 0.0001), 14.83 times decrease in the level of fructose -bisphosphate aldolase C (p = 0.003) and 6.92 times lower concentration of cytosolic aldehyde dehydrogenase 1 (p = 0.0133) (Table 1). If compared tissue levels of above mentioned proteins in animals with induced EAE and treated with vitamin D and animals with EAE without vitamin D, we detected a 1.15 times lower level of calmodulin (p = 0.04). On the other side, tissue level of fructose -bisphosphate aldolase C (3.83-times, p = 0.03) was significatly higher and aldehyddehydroge-

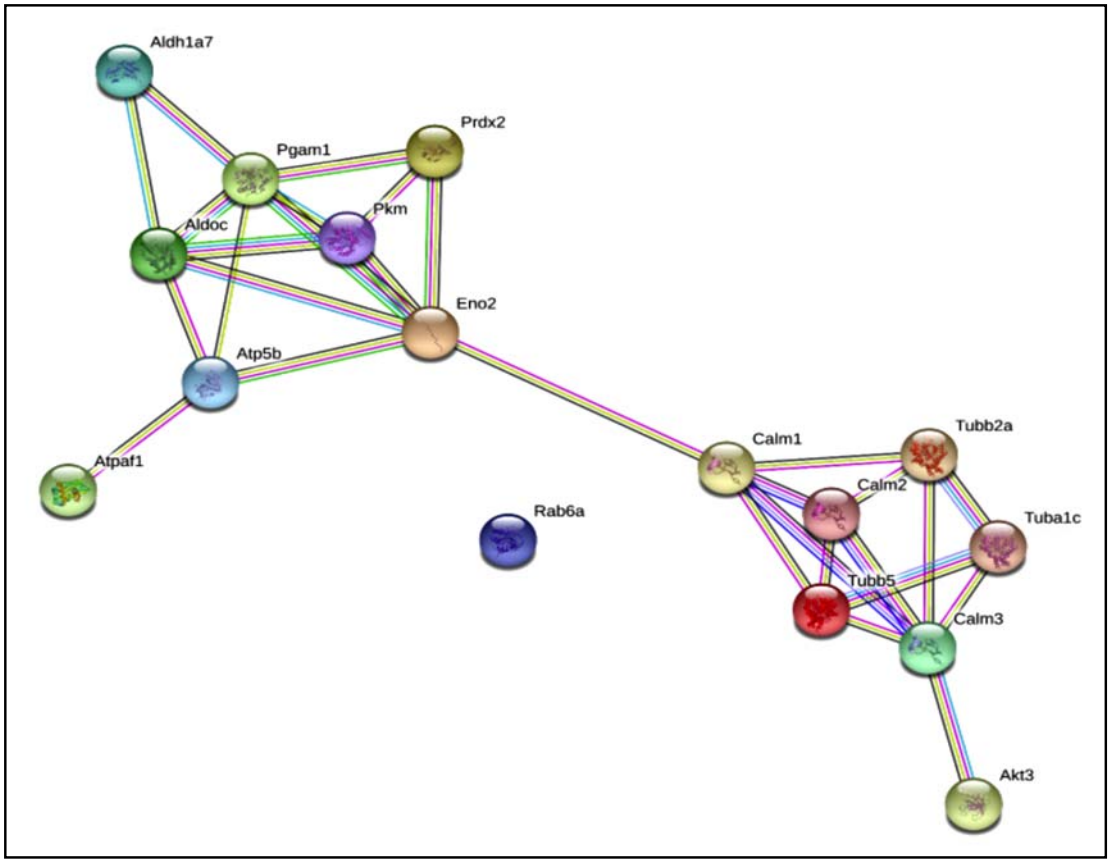

Fig. 1. Possible identified interactions between proteins.
Abbreviations: Calm 1, 2, 3 – calmodulin 1, 2, 3, AldoC - fructose-bisphosphate aldolase C, Aldh1a7 - cytosolic aldehyde dehydrogenase 1, Tubb2a – tubulin beta 2A, Prdx2 - peroxiredoxin 2, Eno2 – enolase 2, Atp5b - ATP synthase, Tuba1c – tubulin alfa 1C, Tubb5 - tubulin beta 5C, Akt3 – tymom viral proto-oncogen 3, Rab6a – member of RAS oncogenic family, Pgam1 – phosphoglycerate mutase 1, Atpaf1 – factor 1 of mitochondrial F1 complex of ATP synthase, Pkm – pyruvate kinase

nase 1 (4.25-times , $p = 0.03$) were significantly lower (Table 2).

Besides indicated changes in the protein concentration, we also try to identify possible mutual protein interactions, which may play a role in the mechanism of the effect of vitamin D in the animals with induced experimental allergic encephalomyelitis. Representative chart can be seen in the Fig 1.

Discussion and conclusions

This work was devoted to the proteomic analysis of particular proteins in the homogenates of cerebral cortex in mice to explore possible mechanisms of protective effect of vitamin D in the etiopathogenesis of experimental allergic encephalomyelitis and its clinical course. EAE is widely accepted animal model of human inflammatory and neurodegenerative disease- sclerosis multiplex (multiple sclerosis) (Racke 2001). Our results documented that at least three proteins were statistically changed in the cerebral cortex if we compared control animals with animals with induced EAE, and animals with EAE treated with vitamin D. Identified proteins, calmodulin 1, fructose-bisphosphate aldolase C and aldehyde dehydrogenase 1, might take part in the complex of signal pathways which are present in the immunomodulatory and neuroprotective effect of vitamin D (Cantorna *et al.* 2015). We propose that due to the changed level of calmodulin 1 in vitamin D treated animals, the cascades of its multiple biological effect seems partially affect the calcium dependent processes including immune responses. In fact, while MS is characterized by progressive demyelination, vitamin D is considered to have a role in the myelination and remyelination processes and displays many activities influencing brain function. Experimental studies showed that vitamin D could attenuate demyelination and induce remyelination through regulation of oligodendrocyte precursor cells (OPC) differentiation, stimulates microglia activation, promoting the clearance of myelin debris and favoring remyelination. Current studies explore the influence of vitamin D-related genes in MS susceptibility, reporting more than 12 vitamin D pathway genes. Among all, the gene codifying vitamin D receptor (VDR) is the most studied and others has been analyzed, including those with the proposed calcium/calmodulin susceptibility (Scazzone *et al.* 2021). On the other hand, identified decrease in

the level of fructose-bisphosphate aldolase C and aldehyddehydrogenase 1 can suggest dysregulations in the saccharidic and energetic metabolism in animals with induced EAE and treated with vitamin D, especially in the glycoprotein and myelin metabolism within the pathological processes (Cantorna *et al.* 2015). Paper also presents possible complex protein interactions between identified proteins and proteins which might play a role in the regulation of redox status (peroxiredoxin 2) in the affected brain region, its energetic homeostasis (ATP synthase, enolase 2, factor 1 of mitochondrial F1 complex of ATP synthase, phosphoglyceratemutase 1, pyruvate kinase), functions of microtubules (tubulin alpha 1C, tubulin beta 2A) and cellular tissue proliferation (tymom viral proto-oncogen 3, Rab6a – member of RAS oncogenic family). Likely all of these proteins might be included in the immune system dysregulation and consequtive neurodegeneration (Ascherio *et al.* 2014, Scazzone *et al.* 2021). In fact, the disease is typical with the inflammatory infiltrations, where plasma cells progressivelly activate to produce pro-inflammatory cytokines leading to the myelin damage. Activated microglia with the subsequent oxidative stress, and with the loss of axons in white and gray matter finalize to the cognitive deficit and disability and also signalling and metabolic dysregulation (Compston & Coles 2008) .

Conclusions

Our results suggest that positive effect of vitamin D in the EAE etiopathogenesis includes, at least, partially an inhibition of calmodulin effect as well as the alterations of glucose metabolism and provide a deeper molecular insights into the protective effects of vitamin D. However, our results should be confirmed by the detailed additional molecular and functional studies.

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