



Differential Expression of Klotho in the Brain and Spinal Cord is Associated with Total Antioxidant Capacity in Mice with Experimental Autoimmune Encephalomyelitis

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Abstract

Recently, we reported a positive correlation between Klotho, as an anti-aging protein, and the total antioxidant capacity (TAC) in cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients. However, there is no information about the Klotho and TAC changes within the central nervous system (CNS). Thus, the current study aimed to employ an experimental autoimmune encephalomyelitis (EAE) model in C57BL/6 mice using MOG_{35–55} peptide to examine the relationship between Klotho and TAC within the CNS. To this end, the brain and spinal cord were obtained at the onset and peak stages of EAE as well as non-EAE mice (sham/control groups). The Klotho expression was assessed in the brain and spinal cord of different experimental groups at mRNA (qPCR) and protein (ELISA) levels. Also, TAC level was determined in the tissues of different experimental groups. The results showed that Klotho expression in the brain at the onset and peak stages of EAE were significantly lower than that in non-EAE mice. Conversely, Klotho expression in the spinal cord at the onset of EAE was significantly higher than that of non-EAE mice, while Klotho was comparable at the peak stage of EAE and non-EAE mice. The pattern of TAC alteration in the brain and spinal cord of EAE mice was similar to that of Klotho expression. In conclusion, for the first time, this study demonstrated a significant positive correlation between Klotho and TAC changes during the pathogenesis of EAE. It is suggested that Klotho may have neuroprotective activity through the regulation of redox system.

Keywords Klotho · Experimental Autoimmune Encephalomyelitis (EAE) · Total Antioxidant Capacity (TAC) · Brain · Spinal cord

Introduction

Multiple sclerosis (MS) is a debilitating chronic inflammatory disease in which the myelin sheath of the central nervous system (CNS) is destroyed and demyelinated by auto-reactive immune cells (Steinman 1996).

Inflammatory pathways in MS patients and relevant animal models lead to oxidative injury of CNS and imbalance in redox system, which are considered as important pathological hallmarks of MS (Grigoriadis and Pesch 2015; Zargari et al. 2007). Free radical production and oxidative damage in MS disease could be attributed to the activation of the CNS macrophages (microglial cells) and also the excessive production of glutamate (Gilgun-Sherki et al. 2004). In addition, it has been shown that oligodendrocytes, which contribute to the production of myelin sheath, are highly susceptible to oxidative damage (Gilgun-Sherki et al. 2004). Hence, identification of substances with antioxidant ability against MS-induced oxidative injury is a noteworthy area of research. Klotho, as an anti-aging protein is a good case in point, because of its anti-oxidative (Kuro-o 2008), anti-inflammatory (Maekawa et al. 2009), and neuroprotective (Zeldich et al. 2015) characteristics. Klotho was accidentally discovered in an attempt to make transgenic mice. It was subsequently concluded that mice lacking Klotho gene (Klotho^{-/-} mice) show phenotypes

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resembling human aging (Kuro-o et al. 1997). This protein is highly expressed in choroid plexus of the brain, parathyroid gland, and kidneys (Kuro-o et al. 1997; Li et al. 2004). Klotho is a type 1 transmembrane protein which is shed and circulates in blood, cerebrospinal fluid (CSF), and urine of human and mouse (Ahmadi et al. 2016; Aleagha et al. 2015; Imura et al. 2004).

Research evidence indicates the involvement of Klotho in regulation of oxidative stress in the CNS (Kuro-o 2008; Nagai et al. 2003). Treatment of Klotho knockout mice with antioxidant factors such as vitamin E could reduce cognitive problems, suggesting that Klotho may act as a neuroprotective agent whose loss is associated with oxidative stress and cell death of neurons (Nagai et al. 2003). Furthermore, Klotho exerts its neuroprotective effects on hippocampal neurons through the regulation of redox system (Zeldich et al. 2014). Recently, we demonstrated that a decrease in the circulating form of Klotho in CSF samples of patients with relapsing-remitting MS (RRMS) is associated with the total antioxidant capacity (TAC) in human CSF samples (Aleagha et al. 2015). However, there is no information about the Klotho and TAC changes within the central nervous system (CNS). Thus, the aim of the present study was to examine the possible association between Klotho expression and antioxidant system within the CNS during experimental autoimmune encephalomyelitis (EAE) induction in mice. To this end, the Klotho expression at mRNA (qPCR) and protein (ELISA) levels along with TAC were assessed in the brain and spinal cord of different experimental groups.

Materials and Methods

Animals

Female C57BL/6 mice (~7 weeks old) weighing 23 ± 2 g were obtained from Pasteur Institute, Tehran, Iran. The mice were maintained under standard condition (a quiet environment, without excessive noise or vibration, 12 h light:12 h dark cycle, standard humidity and temperature) in the animal house of Tarbiat Modares University. The animals were fed with standard pellet food and water ad libitum. For acclimatization to new condition, the animals were kept for about 5 weeks in the animal house prior to treatments. This animal study was approved by the Medical Ethics Committee of Tarbiat Modares University.

EAE Induction and Experimental Groups

Forty mice were divided randomly into three groups as follows:

Group 1, EAE-induced ($n = 20$):

Hooke Kit™ (Hooke laboratories, Cat No. EK-2110, USA) was used to induce EAE, following the instruction given by the company. In brief, animals were initially anesthetized by isoflurane inhalation. They were then immunized with myelin oligodendrocyte glycoprotein (MOG_{35–55}) antigen in an emulsion with complete Freund's adjuvant (CFA). The MOG_{35–55}/CFA emulsion was administered subcutaneously (s.c.) into the flanks of each mouse (0.1 ml/flank or 0.2 ml/mouse). Subsequently, each mouse was injected intraperitoneally (i.p.) with two equal doses of pertussis toxin (PTX; 400 ng PTX in 0.1 ml of sterile phosphate-buffered saline (PBS)). The first injection was given 2 h after immunization followed by the second injection 24 h later.

Group 2, sham-treated ($n = 10$):

The mice in this group were treated similar to what was described for group 1 except that sterile PBS alone was injected instead of MOG_{35–55}/CFA emulsion and PTX.

Group 3, controls ($n = 10$):

The control group mice were treated with CFA and PTX as described for group 1, except that they were administered CFA emulsion alone (without MOG_{35–55}).

Clinical Scoring of EAE

Scoring was performed daily following EAE induction to monitor any sign of disability. A 6-point scoring system was used according to the procedure described by Hooke™ laboratories: score 0—no obvious changes in motor function compared to non-immunized mice; 1—limp tail; 2—limp tail and weakness of hind legs or mouse appears to be at score 0, but there are obvious signs of head tilting when the walk is observed; 3—limp tail and complete paralysis of hind legs or limp tail with paralysis of one front and one hind leg; 4—limp tail, complete hind leg and partial front leg paralysis; 5—severe paralysis or dead due to paralysis.

Tissue Preparation and Processing

Mice assigned to onset stage were sacrificed on days 9 and 10, while mice at peak stage together with animals in sham and control groups were sacrificed 15 days after immunization. Intracardial perfusion with PBS (0.1 M, pH 7.4) was performed for each mouse under anesthesia with xylazine and ketamine. Then brain (cerebrum) and spinal cord tissues were harvested, rinsed with PBS, and crushed on ice into tiny portions. Each sample was divided into two parts: one portion was used for RNA extraction and the other one was utilized for preparing homogenate (10% w/v) for assays.

Estimation of TAC in the Brain and Spinal Cord

TAC was estimated by FRAP assay based on ferric reducing antioxidant power (FRAP) of the sample. The 10% homogenates (*w/v*) of the brain and spinal cord in PBS were used to estimate TAC level. Tissue homogenates were centrifuged for 15 min at 1500*g* at 4 °C (Eppendorf centrifuge, 5810R, Germany) and then the supernatants were immediately used for FRAP assay. The assay was performed according to the procedure described by Benzie and Strain (Benzie and Strain 1996) using 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ; Merck, Germany) reagent.

RNA Extraction and qPCR

Total RNA was extracted from brain and spinal cord using TRIZOL™ reagent (Invitrogen, USA). The cDNA biosynthesis was performed using HyperScript™ First Strand Synthesis kit (GeneAll Biotech, Korea).

The genes were amplified using specific primers for Klotho (target gene) and hypoxanthine guanine phosphoribosyl transferase (HPRT; endogenous gene). The sequence of primers for the genes is as follows: Klotho, forward: 5'-AATGGCTGGTTTGTCTCGG-3' and reverse: 5'-CCCATCCAGTCTGATTGCTT-3'; HPRT, forward: 5'-ATTATGCCGAGGATTTGGA-3' and reverse: 5'-ACTTATAGCCCCCCTTGA-3'. The qPCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, USA). PCR for Klotho and HPRT genes was performed under the following condition: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR reactions were performed in duplicate and relative quantification was obtained using $2^{-\Delta\Delta C_t}$ method.

Estimation of Tissue Klotho Protein

Klotho was estimated in the brain and spinal cord homogenate using commercial ELISA kit (MyBiosource, USA; code no.

MBS015591). The sensitivity of the kit was 0.1 pg/ml with a detection range of 1.56 to 50 pg/ml. Tissues were homogenized (10% *w/v*) in sterile PBS containing 1 mM EDTA, 1 mM PMSF, and 0.125% Triton™ X-100 (Sigma-Aldrich Co., USA). The ELISA procedure was performed according to package insert of the commercial kit. The calculated concentration of Klotho (pg/ml) in each tissue homogenate was divided by the total protein concentration (mg/ml) of the corresponding homogenate. The calculated data were presented as picograms per milligram protein. The total protein concentration was measured using Bradford method (Bradford 1976).

Statistical Analysis

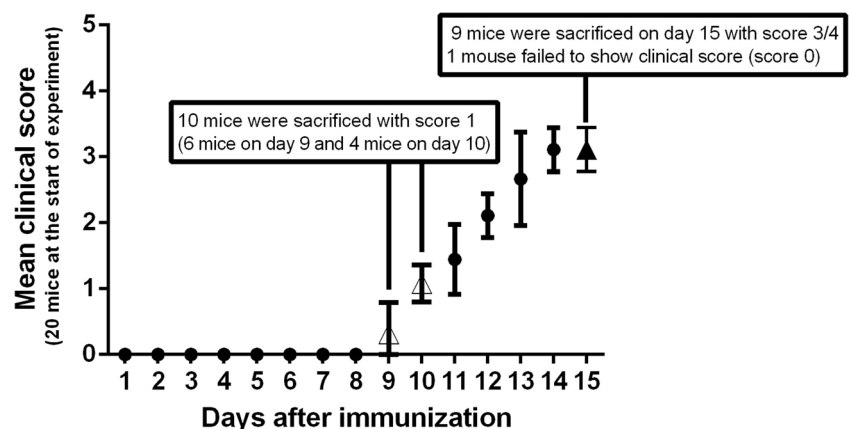
Data were analyzed using GraphPad Prism (version 6.01) program. Paired samples *t* test was applied for comparison of means between the brain and spinal cord of different experimental groups. One-way ANOVA was further applied to compare the mean values of different assays. Pairwise group comparisons were carried out using Tukey's test (post hoc analysis). Pearson product moment correlation was also conducted to gauge the association between different variables. The data were presented as mean \pm SD, and statistical significance was considered less than 0.05 ($P < 0.05$).

Results

The Rate of EAE Incidence

In this study, 95% of the mice (19/20) were affected by immunization as judged by distinct signs and clinical scores (Fig. 1). EAE onset was noticed on days 9–10 and was associated with a gradual increase in clinical scores until day 14. The mice were stable on day 15 (the clinical score of mice did not change on day 15), with the obtained values being considered as the highest clinical scores (peak). Finally, 10 mice were assigned to “onset” stage (Score 1) and 9 were assigned

Fig. 1 EAE incidence in immunized mice and the relative clinical scores



to “Peak” stage (Score 3/4). Sham and control groups without any sign of EAE were considered score zero (0).

TAC Levels in the Brain and Spinal Cord

As indicated in Fig. 2a, TAC (FRAP assay) was approximately 4.5 times greater in the brain of normal (non-EAE; sham and control groups) mice as compared to that measured in the spinal cord tissue ($P < 0.0001$). In addition, TAC was nearly 1.5-fold greater in the brain of EAE mice (onset and peak groups) than that of the spinal cord of these mice (Fig. 2a; $P < 0.05$).

The results showed that FRAP levels in the brain of sham (7.78 $\mu\text{mol FeSO}_4/\text{g wet tissue} \pm 0.62$) and control (7.98 $\mu\text{mol FeSO}_4/\text{g wet tissue} \pm 0.57$) groups were in the same range (Fig. 2b; $P = 0.920$). Moreover, we found that FRAP levels significantly declined in the brain of mice at the onset (4.10 $\mu\text{mol FeSO}_4/\text{g wet tissue} \pm 0.48$) and at the peak (2.71 $\mu\text{mol FeSO}_4/\text{g wet tissue} \pm 0.49$) of EAE when compared to the sham and control groups ($P < 0.003$; Fig. 2b).

In case of spinal cord samples, there was no significant difference in FRAP between sham and control groups ($P = 0.92$; Fig. 2c). FRAP levels remained within the normal range in mice at the peak stage, whereas there was a twofold increase in FRAP levels in the spinal cord of the onset group (Fig. 2c; $P < 0.001$).

Klotho Gene Expression in the Brain and Spinal Cord

Klotho gene expression (qPCR) in the brain of sham/control mice was ~ 140 times higher than that measured in the spinal cord (Fig. 3a; $P < 0.0001$). The expression of Klotho in the brain of EAE mice at the onset stage was two times and at peak stage was four times higher than that in the corresponding spinal cords (Fig. 3a, $P < 0.01$).

Klotho gene expression in the brain samples obtained from sham/control groups was in the same range (Fig. 3b). The expression of Klotho markedly decreased (~ 12 -fold depletion; $P < 0.0001$) in the brain at the onset stage when compared to the sham/control groups (Fig. 3b), and it was further depleted (~ 25 -folds) in mice at peak stage compared to sham/control groups (Fig. 3b; $P < 0.0001$).

There was a surge in Klotho expression (~ 3.5 -folds) in the spinal cord of mice at the onset of EAE when compared to sham/control groups (Fig. 3c; $P < 0.0001$). Klotho expression remained unchanged in the spinal cord of mice at the peak stage (Fig. 3c).

Klotho Protein Concentration in the Brain and Spinal Cord

As shown in Fig. 4a, the protein concentration of brain Klotho in EAE mice at the onset stage was six times greater than that in the spinal cord. Figure 4b indicates that the means of Klotho protein concentration in the brain tissue of sham-treated group was 2.36 pg/mg protein ± 0.23 , while it was 2.30 pg/mg protein ± 0.31 ($P = 0.951$) in controls. The brain Klotho concentrations at protein level at the onset and peak stages declined to about 2.5- and 7.5-folds, respectively, compared to samples obtained from control/sham groups (0.97 pg/mg protein ± 0.08 at the onset vs. 0.30 pg/mg protein ± 0.05 at the peak of EAE) (Fig. 4b; $P < 0.0001$).

Except for mice at the onset stage of EAE (0.17 pg/mg protein ± 0.04), the Klotho protein concentrations in the spinal cord of other experimental groups were very low and below the detection range of the ELISA kit (Fig. 4a, c).

Correlation Studies

There was a significant correlation between Klotho expression and FRAP level in the brain of EAE mice (Table 1). Likewise,

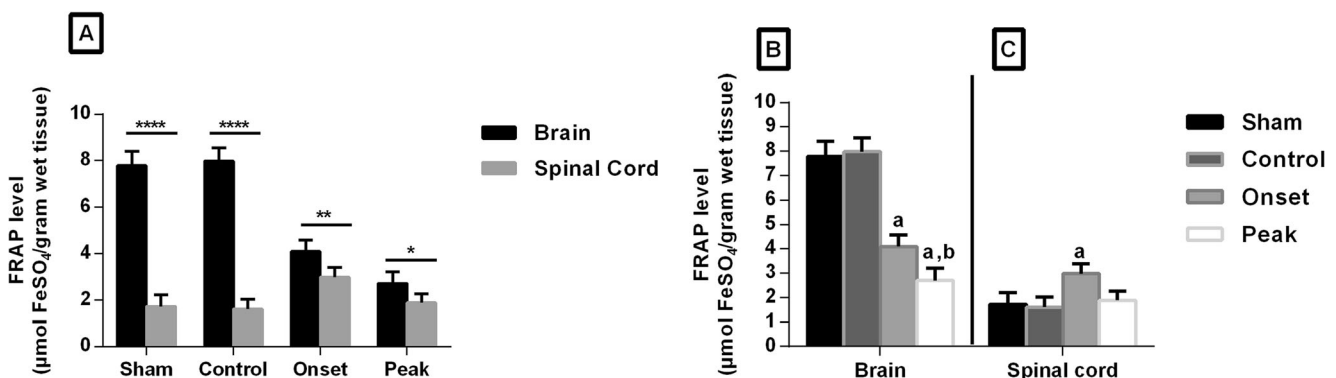


Fig. 2 Total antioxidant capacity (TAC) in the brain and spinal cord. Comparison of TAC levels (FRAP assay) between the brain and spinal cord of different experimental groups (a). TAC was measured in the brain (b) and spinal cord (c) of mice using FRAP assay. *means $P < 0.05$,

means $P < 0.01$ and **means $P < 0.0001$. The “a” sign means significantly different ($P < 0.05$) compared to sham and control groups. The “b” sign shows significantly different ($P < 0.05$) compared to mice at the onset of EAE

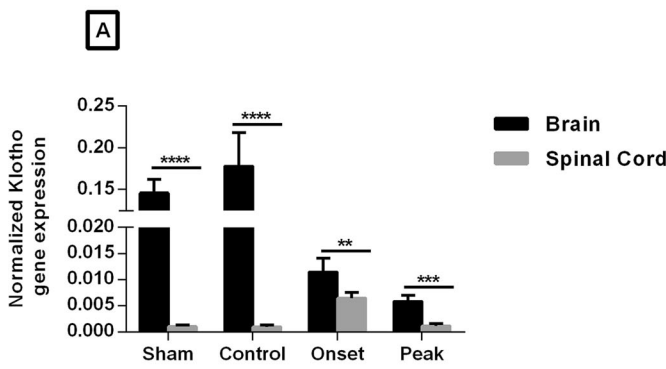
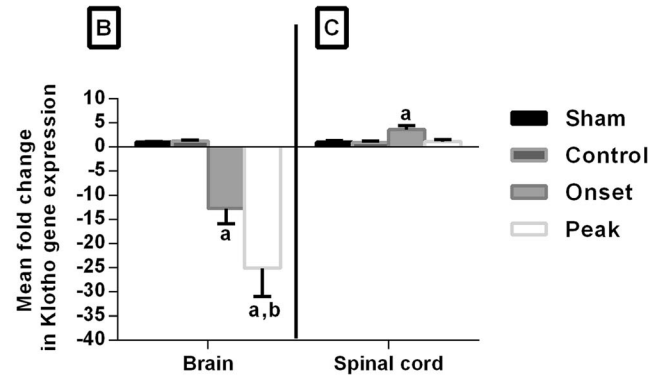


Fig. 3 Klotho gene expression in the brain and spinal cord. Comparison of normalized Klotho gene expression (data are shown as $2^{-\Delta Ct}$) between the brain and spinal cord of different experimental groups (a). Changes in Klotho expression at mRNA levels are shown as the mean fold in the brain (b) and spinal cord (c) determined by qPCR. The fold change of the



Klotho gene was calculated using $2^{-\Delta\Delta Ct}$ method. **means $P < 0.01$, ***means $P < 0.001$ and ****means $P < 0.0001$. The “a” sign means significantly different ($P < 0.05$) compared to sham and control groups. The “b” sign shows significantly different ($P < 0.05$) compared to mice at the onset of EAE

these parameters were positively correlated in the spinal cord as well as the brain tissues (Table 1). The correlation between Klotho protein concentration and FRAP level in the spinal cord was also positive, though statistically insignificant (Table 1).

Discussion

Klotho is a newly discovered multi-functional protein, the function of which is not well understood particularly in response to disease condition. In the present study, using the EAE model, we could verify the possible interplay of brain and spinal cord in terms of Klotho expression and antioxidant factors. Data presented in this paper showed that upon induction of EAE in mice, the pattern of Klotho expression at mRNA and protein levels is substantially altered in the brain and spinal cord. This finding, together with changes in antioxidant activity of CNS of mice during EAE progression, may

suggest a protective role for Klotho in this chronic inflammatory disease.

In the present study, using qPCR and ELISA techniques, we found a very high expression of Klotho in the brain of non-EAE (sham/control groups) mice compared to that measured in the spinal cord (Figs. 3 and 4). Similar results were obtained regarding Klotho protein determination in the spinal cord of non-EAE mice using immunohistochemistry method (data not shown). This finding was in agreement with the Chen’s report (Chen et al. 2013) showing a higher expression of Klotho in the mice brain compared to that detected in the spinal cord. The differential expression of Klotho was further confirmed by Chen et al. (2013), who showed that Klotho knockout mice exhibited impaired myelination in corpus callosum and optic nerve, while the spinal cord remained unaffected. The considerable different pattern of Klotho expression in the brain and spinal cord of normal (non-EAE) mice may reflect the region-specific function of this protein within the CNS.

Regarding the Klotho expression and TAC level, another interesting result of this study was that the brain and spinal

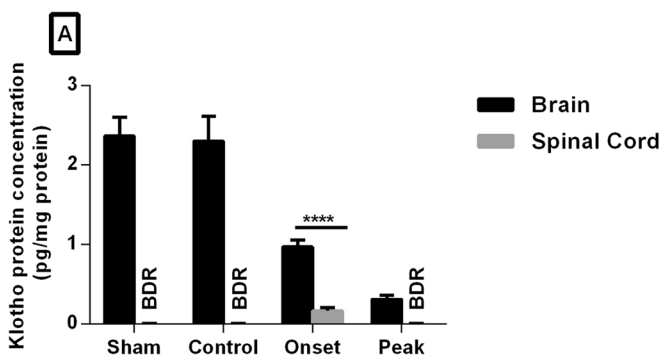
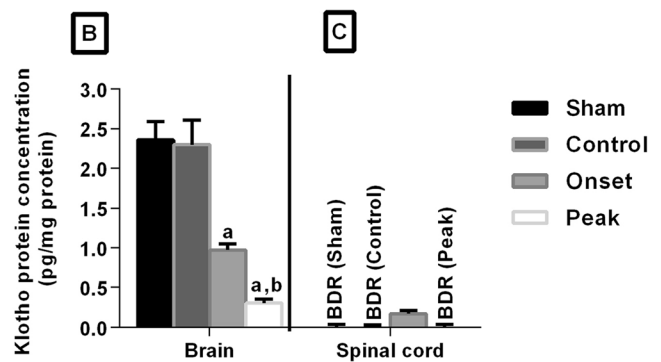


Fig. 4 Klotho protein concentration in the brain and spinal cord. Klotho was estimated at protein level using sandwich ELISA kit. The Klotho concentration was compared between brain and spinal cord of different experimental groups (a). Changes in Klotho protein concentration are shown in the brain (b) and spinal cord (c) of mice. ****means $P <$



0.0001. The “a” sign means significantly different ($P < 0.05$) compared to sham and control groups. The “b” sign shows significantly different ($P < 0.05$) compared to mice at the onset of EAE. “BDR” stands for below the detection range of the commercial ELISA kit

Table 1 Correlation between total antioxidant capacity (TAC) and Klotho expression in the brain and spinal cord of EAE mice

Correlations	Brain	Spinal cord
Klotho gene expression and TAC	$r^a = 0.760$ $P^b = 0.004$	$r = 0.774$ $P = 0.003$
Klotho protein concentration and TAC	$r = 0.828$ $P = 0.0009$	$r = 0.464$ $P = 0.353$

^a Means “correlation coefficient”

^b Means “*P* value.” The significance was considered less than 0.05 (*P* < 0.05)

cord tissues responded differently to the EAE induction. In this line, previous experiments revealed that EAE development is associated with a considerable inflammation in the brain, suggesting that, in addition to the spinal cord, the brain tissue is affected during EAE development (Bernardes et al. 2013; Kuersten et al. 2007; Marques et al. 2012; R  ther et al. 2017; Yao et al. 2012). In the brain, the clinical scores of EAE mice are associated with infiltration of CD4⁺ T cells, whereas in the spinal cord, the granulocyte infiltration is correlated with clinical scores (Kuersten et al. 2008).

Interestingly, we revealed that Klotho exhibited different patterns of gene expression and protein concentration in the brain and spinal cord of EAE mice. In this connection, a substantial decrease in Klotho expression in the brain tissue of EAE animals during the onset and peak stages may be considered as the consequence of inflammatory reactions in the CNS. Of note, there are several studies indicating that Klotho expression is influenced by inflammatory reactions (Karami et al. 2017; Moreno et al. 2011; Teocchi et al. 2013; Thurston et al. 2010; Witkowski et al. 2007). For example, an inverse relationship has been displayed between Klotho expression and proinflammatory cytokines, e.g., tumor necrosis factor- α (TNF- α) (Moreno et al. 2011). Likewise, previous research has indicated the downregulation of Klotho expression by TNF- α in patients with temporal lobe epilepsy (Teocchi et al. 2013). On the other hand, accumulation of a relatively high concentration of Klotho in choroid plexus region of the brain implies that Klotho may be involved in maintaining the CSF-blood barrier (CBB) and CSF production (Li et al. 2004). Accordingly, as CBB is disrupted during the course of EAE (Christy et al. 2013), it can be assumed that along with escalation of EAE-induced damage to CBB, there is a suppression in the brain Klotho expression.

Surprisingly, we found that Klotho expression in the spinal cord of mice at the onset of EAE was significantly higher than that of normal mice and also mice at the peak stage of EAE (Figs. 3 and 4). This result may support the idea that, in EAE mice, inflammatory pathways are different in the spinal cord compared to the brain. Simmons et al. revealed that the brain and spinal cord of EAE mice exhibited distinct sensitivities to cellular mediators of tissue damage such as IFN- γ and IL-17

(Simmons et al. 2014). Moreover, as Klotho is highly expressed in choroid plexus of the brain ventricles (Li et al. 2004), it is plausible to assume that Klotho in the spinal cord at onset stage is elevated in compensation of the shortage of the Klotho, which is normally supplied by the choroid plexus region (Cararo-Lopes et al. 2017). Nevertheless, it appears that the overexpression of Klotho in the spinal cord at the onset stage does not majorly prevent or delay the progression stage of the disease. The region-specific function of Klotho was also demonstrated by showing that overexpression of Klotho in cuprizone-induced demyelination in a mice model could enhance re-myelination of the brain tissue, with no alleviation in EAE-related symptoms (Zeldich et al. 2015).

In addition, similar changes in Klotho expression were observed in relation to TAC (FRAP level) in the brain of normal and EAE mice (Fig. 2). In this line, previous studies showed that Klotho protects hippocampal neurons from oxidative damage in cell culture medium through the induction of the thioredoxin/peroxiredoxin system (Zeldich et al. 2014). Moreover, Klotho overexpression increases the activation of Nrf2 transcription factor, which is able to induce the expression of antioxidant response elements (AREs) (Balasubramanian and Longo 2010; Maltese et al. 2017). Therefore, it is reasonable to expect that Klotho changes may lead to alteration of the TAC.

The significant positive correlation detected between the antioxidant defense of the brain and spinal cord with the expression of Klotho in response to EAE induction in mice further attests to this finding (Table 1). Perhaps, the results obtained in EAE mouse model are consistent with our recent observation in MS patients showing a positive and significant relationship between Klotho changes and TAC in the CSF samples (Aleagha et al. 2015). The mode of action of Klotho in EAE-induced oxidative damage is not fully understood because, during the MS/EAE progression, different enzymatic and non-enzymatic antioxidant factors may contribute to the TAC to protect CNS against the oxidative stress factors (Zargari et al. 2007). Regardless of the role of individual antioxidant factors, the association between Klotho and TAC may be a positive sign of neuroprotective activity of the Klotho in the CNS in response to inflammatory reactions. In addition, appearance of Klotho expression in the spinal cord tissues obtained from the low score (onset) group together with elevation in the TAC may suggest that this protein is induced as an early marker of the disease which is probably involved in balancing the oxidative stress factors.

Conclusion

The correlation between Klotho expression in the brain and spinal cord and TAC during the course of EAE induction revealed that this anti-aging factor may play a role in

antioxidant activities of the CNS. Despite the correlation between TAC and Klotho levels, claiming a causal relationship between these biochemical changes is premature and needs further verification. Although the exact mechanism(s) of action of Klotho on MS/EAE are not yet fully understood, its neuroprotective activity through the regulation of redox system can be suggested.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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