Biochemical Pharmacology 92 (2014) 326–335

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Regulation of ceramide synthase 6 in a spontaneous experimental autoimmune encephalomyelitis model is sex dependent

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ARTICLE INFO

Article history:
Received 8 July 2014
Accepted 13 August 2014
Available online 27 August 2014

Keywords:
Ceramide synthase 6
Experimental autoimmune encephalomyelitis
Multiple sclerosis
Tumor necrosis factor alpha receptor 2
Interferon gamma
17ß-estradiol

ABSTRACT

Ceramides (Cer) are mediators of inflammatory processes. In a chronic experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS), we observed a significant elevation of C16-Cer and its synthesizing enzyme, ceramide synthase(CerS)6, in the lumbar spinal cord. In the present study, we have confirmed that C16-Cer and CerS6 are also upregulated in the lumbar spinal cord in a spontaneous relapse-remitting EAE model, using SJL mice overexpressing a transgenic T cell receptor (TCR1640). CerS6 was found to be expressed in macrophages, T cells and B cells in EAE lesions. In macrophages, we demonstrated that interferon gamma (IFN-γ)-induced CerS6 upregulation was amplified by 17ß-estradiol, an action that was further accompanied by increased upregulation of tumor necrosis factor alpha (TNF-α). Accordingly, CerS6 and TNF-α expression was upregulated predominantly in the spinal cord in female TCR1640 mice, which usually develop the relapse-remitting form of EAE, while male TCR1640 mice showed an attenuated regulation of CerS6 and TNF-α and exhibit mostly chronic disease progression. Furthermore, expression of TNFR2, one of two receptors of TNF-α, which is linked to neuroprotection and remyelination, was also upregulated to a greater extent during EAE in female TCR1640 mice in comparison to male TCR1640 mice. Taken together, our results confirm the upregulation of CerS6 and C16-Cer in an adjuvant-independent, physiological EAE model and further suggest an anti-inflammatory role of CerS6 in the regulation of the disease course in female TCR1640 mice via TNF-α/TNFR2.

1. Introduction

Multiple sclerosis (MS) and its corresponding animal model, experimental autoimmune encephalomyelitis (EAE), are induced by autoimmune responses against myelin components in the central nervous system (CNS). Activated autoreactive T-cells proliferate and secrete proinflammatory cytokines, which in turn stimulate microglia, macrophages and astrocytes, and recruit B cells, ultimately resulting in damage to myelin, the myelin forming oligodendrocytes and axons [1]. In the relapse-remitting form of MS, the demyelination is followed by a remyelination phase, leading to partial recovery from the symptoms. Important players in the development and resolution of inflammation are the cytokines interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), which act dichotomously in a pro- and

Abbreviations: Cer, ceramide; dhCer, dihydroceramide; CerS, (dihydro)ceramide synthase; EAE, experimental autoimmune encephalomyelitis; ES, 17β-estradiol; IFN-γ, interferon gamma; M-CSF, macrophage colony-stimulating factor; MOG, myelin oligodendrocyte protein; MRM, multiple reaction monitoring; MS, multiple sclerosis; PMA, phorbol myristate acetate; PTX, pertussis toxin; TNF-α, tumor necrosis factor alpha; TNFR1, TNF-α receptor 1; TNFR2, TNF-α receptor 2; WT, wild type.

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http://dx.doi.org/10.1016/j.bcp.2014.08.016
0006-2952/

anti-inflammatory manner [2,3]. IFN-γ secreted from activated Th1-cells activates macrophages and initiates the synthesis of tumor necrosis factor alpha (TNF-α). TNF-α can mediate reduction of autoreactive T cells and thereby suppresses the inflammatory process in the CNS [2]. These data indicate an important role of TNF-α in the induction of the recovery phase during the peak phase of EAE.

Sphingolipids are known mediators of apoptosis, proliferation, growth arrest and inflammation, depending upon cell- and receptor-types and downstream targets [4]. Sphingolipids also play a role in the disease process of MS [5]. Ceramides form the backbone to several complex sphingolipids, like sphingomyelins and glycosylceramides and can be generated by de-novo synthesis or by degradation of complex sphingolipids. A rate limiting step in the biosynthesis of ceramides is the attachment of various acyl-CoA side chains to a sphingoid base by the ceramide synthases (CerS). The ceramide synthases (CerS1-6) act in a chain length specific manner and introduce side chains to form C14–C30 ceramides. Thus, CerS1 synthesizes mainly C18-Cer, CerS4 synthesizes C18-/C20-Cer; CerS5 and CerS6 synthesize mostly C16-Cer, CerS2 synthesizes mainly C22/C24-Cer and CerS3 synthesizes very long chain ceramides [6]. In addition to de-novo synthesis, the salvage pathway also supplies ceramides, mainly via the activation of sphingomyelinases (SMase) [7].

Several EAE models exist which are distinguishable on the basis of their disease courses (chronic or relapse-remitting) or in the procedure for disease induction (active induction using various autoantigens or overexpression of T cell receptors directed against components of the myelin sheath). Recently, we investigated the role of CerS6/C16-ceramide in a chronic EAE model induced by the autoantigen myelin oligodendrocyte protein $(MOG)_{35-55}$ peptide [8]. In the present study, we used a spontaneous EAE model, which more closely mimics the disease in human multiple sclerosis and exhibits a more physiological pathogenesis when compared with actively induced models. The TCR1640 mice overexpress a transgenic T cell receptor, which is expressed by 99% of $CD4^+CD8^-$ thymocytes and is targeted against the MOG_{92-106} peptide, leading to the spontaneous development of EAE in adult mice. The female mice mainly develop the relapse-remitting form of EAE, while the male TCR1640 mice mainly develop the chronic form [9].

We demonstrated that C16-Cer and CerS6 are upregulated in macrophages and astroglia in the lumbar spinal cord of chronic EAE mice and that IFN-γ-mediated synthesis of TNF-α in macrophages is CerS6 dependent [8]. Here we investigated whether CerS6 and thereby C16-Cer are also important mediators in the TCR1640 mouse model and whether this regulation is sex-dependent (females have a twofold higher risk of developing MS). Therefore, we examined the C16-Cer/CerS6 expression profiles in male and female TCR1640 mice.

2. Materials and methods

2.1. Cells and reagents

THP-1 monocytes were purchased from Sigma–Aldrich (Schnelldorf, Germany) and were cultured and incubated in RPMI 1640 medium containing 10% FCS (charcoal stripped) and 1% penicillin/streptomycin. Primary monocytes were cultured in RPMI 1640 medium–GlutaMAX containing 10% FCS (charcoal) and 1% penicillin/streptomycin. Since normal FCS contains estrogen, FCS without estrogen (FCS charcoal stripped) was used for our experiments. Cells were cultured at 37 °C in an atmosphere containing 5% carbon dioxide. IFN-γ (human), IFN-γ (murine), macrophage colony-stimulating factor (M-CSF) and phorbol myristate acetate (PMA) were purchased from PeproTech (Hamburg, Germany). 17ß-estradiol was ordered from Sigma–Aldrich (Schnelldorf, Germany). Sphingolipids were purchased from Avanti Polar Lipids (Alabaster, USA).

2.2. T cell receptor (TCR) transgenic SJL/J mice

In all experiments, the ethical guidelines for investigations in conscious animals were followed and the experiments were approved by the local Ethics Committee for Animal Research.

The TCR1640 transgenic mice are bred on a SJL/J genetic background and carry a transgene encoding for Vα8.3 and Vß4 TCR chains under the control of the transgenic MHC class I H2-K^b promoter that is targeted against MOG_{92-106} in the context of I-A^s. 99% of $CD4^+CD8^-$ thymocytes express the transgenic TCR, as determined by flow cytometry [9]. Hemizygous expression of the TCR1640 is sufficient to induce spontaneous EAE development. The TCR transgenic mice were a kind gift from the group of H. Wekerle (Max-Planck-Institute of Neurobiology, Martinsried, Germany).

Mice described as "healthy" were age-matched, female $TCR1640^{-/-}$ control mice lacking an inherent ability to develop EAE. Clinical symptoms were classified by clinical scores as follows: (0) no signs, (0.5) tail hypotonia, (1) flaccid tail, (1.5) flaccid tail and weakness of one hind limb, (2) flaccid tail and weakness of both hind limbs, (2.5) flaccid tail and paralysis of one hind limb and weakness of one hind limb, (3) paralysis of both hind limbs.

2.3. Preparation of tissue for histology studies

Terminally anesthetized mice were perfused transcardially with phosphate buffered saline (PBS) (for mRNA, protein and sphingolipid analysis) followed by 4% paraformaldehyde (for immunohistochemistry). Spinal cord (lumbar segments) was extracted and stored at −80 °C for mRNA, protein and sphingolipid analysis. The tissue for immunohistochemistry (spinal cord) was kept in 4% paraformaldehyde for 1 h, placed overnight in 20% sucrose, imbedded in tissue freezing medium (Jung, Leica Microsystems GmbH, Nussloch, Germany), quickly frozen on dry ice and stored at −80 °C.

2.4. Immunohistochemistry

Twelve micrometer sections were permeabilized in PBS containing 0.1% Triton X-100 for 10 min and then blocked in PBS containing 3% bovine serum albumin and 0.1% Triton X-100 for 30 min at RT. The sections were incubated with the primary antibody at 4 °C overnight, followed by fluorescence labeled antibodies, diluted 1:800 for 2 h in PBS containing 0.1% Triton X-100. The following antibodies were used in the dilution indicated: anti-CerS6 (1:50), anti-CD4 (1:200), anti-CD20 (1:100) and anti-F4/80 (1:100). The CerS6 (goat polyclonal; sc-65127) antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), while anti-CD20 (rat polyclonal; 14-0201-82) was ordered from eBioscience (Frankfurt, Germany). The antibodies against F4/80 (rat polyclonal; MCA497RT) and CD4 (rat monoclonal; MCA1767) were from Serotec (Düsseldorf, Germany). The secondary antibodies AlexaFlour488 chicken-anti-goat IgG (H+L) (A21467) and AlexaFlour555 goat-anti-rat IgG (H+L) (A21434) were purchased from Life Technologies GmbH (Darmstadt, Germany).

2.5. Preparation of crude protein extracts

Tissue samples from spinal cord were homogenized in Tris–CHAPS–buffer (10 mM Tris–HCl/20 mM CHAPS, pH 7.4) supplemented with protease inhibitors. The homogenate was centrifuged at 4 °C and 20,000 × *g* for 30 min. Supernatants were collected and stored at −80 °C. Protein concentrations were assessed using the Bradford method.