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Hepcidin and metallothioneins as molecular base for sex-dependent differences in clinical course of experimental autoimmune encephalomyelitis in chronic iron overload ☆

Božena Ćurko-Cofek *, Tanja Grubić Kezele, Vesna Barac-Latas

Department of Physiology and Immunology, Medical Faculty, University of Rijeka, B. Branchetta 20, 51 000 Rijeka, Croatia

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ABSTRACT

Multiple sclerosis is a chronic demyelinating disease of the central nervous system characterised by inflammatory and degenerative changes. It is considered that disease arises from the influence of environmental factors on genetically susceptible individuals. Recent researches, using magnetic resonance imaging, connected iron deposits in different brain regions with demyelinating process in multiple sclerosis patients. Although iron is an essential trace element important for many biological functions it could be harmful because iron excess can induce the production of reactive oxygen species, development of oxidative stress and lipid peroxidation which leads to demyelination. In experimental autoimmune encephalomyelitis model, the most common experimental animal model for multiple sclerosis, we recently found that chronic iron overload influences the clinical course of disease in Dark Agouti rats. In female rats iron overload accelerated the onset of disease, while in male rats it accelerated the progression of disease and increased mortality rate. We hypothesize that those differences arise on molecular level in different expression of stress response proteins hepcidin and metallothioneins in male and female iron overloaded rats. They are both upregulated by metal ions in both sexes. Hepcidin is additionally upregulated by estrogen in female rats and therefore causes higher degradation of iron exporter ferroportin and sequestration of iron in the cells, lowering the possibility for the development of oxidative stress. Antioxidative effect of metallothioneins could be increased in female rats because of their ability to reversibly exchange metal ions with the estrogen receptor. In case of iron excess metallothioneins release zinc, which is normally bound to them. Zinc binds to estrogen receptor and leaves metallothioneins binding domains free for iron, causing at least provisional cytoprotective effect. To test this hypothesis, we propose to determine and compare serum levels of hepcidin and estrogen using ELISA essay as well as expression and distribution of acute stress response proteins hepcidin and metallothioneins, iron and estrogen receptor in the brain and spinal cord tissue using immunohistochemistry in control and chronic iron overloaded male and female rats in experimental autoimmune encephalomyelitis model. It would be also possible to perform the same immunohistochemistry in the brain tissue of multiple sclerosis patients *post mortem*. The results of experiments could contribute to better understanding of cytoprotective mechanisms in chronic iron overload that could have possible therapeutic applications in iron disturbances. In order to elucidate whether common measure of systemic iron status, like ferritin, haemoglobin concentration and transferrin saturation levels, may be used to distinguish physiologic from potentially harmful iron levels in local disease, for example multiple sclerosis and Still's disease, well-designed clinical trials would be of great interest.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of central nervous system (CNS) characterised by inflammatory and degenerative changes in the brain and spinal cord [1]. The estimated number of people with MS in the world is about 2,5 million [2]. Prevalence of MS varies considerably within regions; the highest is in North America and Europe and it is rare in Asia and in trop-

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* Corresponding author.
E-mail address: bozena.curko.cofek@medri.uniri.hr (B. Ćurko-Cofek).

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ics and subtropics [3]. It is the most common disease of CNS to cause permanent disability in young adults [4] and therefore not only health but also socioeconomic problem.

Despite years of research etiopathogenesis of MS is not completely understood. Many studies point to a dual influence of genetics and environmental elements [5]. Genetic susceptibility is connected to several haplotypes, especially with HLA-DRB1*15 haplotype [6]. Among the environmental risk factors with the strongest evidence for involvement in MS are: Epstein-Barr virus [7], vitamin D connected with latitude [8] and smoking [9]. It is currently considered that disease arises from the influence of the environmental factors on the genetically susceptible individuals [10].

Recent researches aided with magnetic resonance imaging point to iron as one of the factors that influence the development of MS and connect iron deposits in different brain regions with the demyelinating process in MS [11–13].

Iron is an essential trace element important for many biological functions such as oxygen transport, DNA synthesis, and mitochondrial oxidation [14]. Its ability to change from ferrous (Fe^{2+}) to ferric (Fe^{3+}) state support the redox potential critical for oxygen and energy metabolism [15]. The largest iron pool is in the heme and it is required for oxygen transport in hemoglobin, oxygen storage in myoglobin, and electron transport for cytochrome function in aerobic respiration [16]. The second largest iron pool is in the non-heme form stored in ferritin. When needed, iron can be released from ferritin but at the same time this mechanism limits excess of free iron able to generate reactive oxygen species (ROS) [17].

The body has no effective means of excreting iron [18], therefore it is very important to maintain iron homeostasis to fulfil needs for iron and avoid the toxicity caused by excess of ROS [19]. The production of ROS is catalysed by ferrous iron with the decomposition of H_2O_2 and formation of extremely toxic hydroxyl radical (OH^*) [20]. ROS can produce membrane damage, changes of protein structure and function, lipid peroxidation and structural damage to DNA [21].

The brain is especially susceptible to the effects of ROS because it has large oxygen metabolism, relatively feeble protective antioxidant mechanisms and high iron levels [22]. The highest iron levels in CNS are in myelin and oligodendrocytes [23]. In these cells, iron is needed for the enzymes involved in lipid and cholesterol synthesis, as well as for the enzymes required for the high metabolic demands of myelination [24]. Brain has a large amount of polyunsaturated fatty acids which can undergo the lipid peroxidation process caused by ROS and oxidative stress and produce toxic compounds such as aldehydes (e.g. malondyaldehide) and dienals (e.g. 4-hydroxinonenal – 4-HNE) which may cause demyelination and neuronal apoptosis [25].

In our recently published article we have shown that chronic iron overload has influence on clinical course of experimental autoimmune encephalomyelitis (EAE) [26], the most commonly used experimental model for MS [27] because of their immune, histopathological, genetic and clinical similarities [28]. In this experiment, we used Dark Agouti (DA) rat strain because it develops relapsing-remitting course of disease, characterised by acute relapses separated with periods of clinical remission [29], which can be found in about 80% MS patient [30]. The animals were subjected to chronic iron overload for two consecutive weeks before the induction of disease with encephalitogen (brain bovine homogenate in complete Freund's adjuvant). Our results showed sexual dimorphism with milder effects on female rats. In female rats iron overload accelerated the onset of disease and the first peak of clinical symptoms. In male rats iron overload accelerated the second peak of the disease and significantly increased mortality rate (85% male, 25% female rats). The differences in the clinical course were accompanied with higher expression of lipid peroxidation marker (4-HNE) in the CNS tissue and more pronounced demyelinating changes in male rats, especially in spinal cord [26].

Hypothesis

We hypothesize that sex-dependent differences in the clinical course of EAE in iron overloaded rats are caused on molecular level by upregulation of acute stress response proteins hepcidin and metallothioneins (MTs) in female rats. Both proteins have the ability to lower the concentration of non-transferrin bound iron (NTBI) and consequently the production of ROS. This results in a more expressed cytoprotective effect and consequently different clinical course and lower mortality rate in female rats.

Our hypothesis is supported by the following findings: (I) upregulation of hepcidin causes the sequestration of iron and decrease NTBI, a possible source of oxidative stress [31]; (II) estrogen induces production of hepcidin in hepatocytes through the estrogen response element (ERE) in the promoter region of hepcidin gene [32]; (III) MTs play a protective role against oxidative stress by scavenging free radicals [33]; (IV) reversible metal exchange occurs between MTs and estrogen receptor (ER) [34].

Discussion and evaluation of hypothesis

During our previous research of chronic iron overload in rat EAE model, we found that iron overloaded female DA rats sequester more iron in the form of ferritin than iron overloaded male DA rats [26]. This acts as a cytoprotective effect because ferritin reduces the concentration of free (NTBI) iron and could be mediated by increased expression of hepcidin in female rats.

Hepcidin is an acute-phase peptide [35] synthesised in liver [36], which functions as the main regulator of iron homeostasis on systemic level [35]. In case of hepcidin excess, like in chronic inflammation, iron absorption is decreased and the lack of available iron creates unfavourable conditions for development of infection, giving hepcidin antimicrobial properties [36]. However, the lack of available iron also causes anaemia of inflammatory disease [19]. In contrast, low hepcidin expression may lead to iron overload that is harmful to many organs [15]. For example, iron storage within cardiomyocytes in patients with untreated hereditary hemochromatosis or transfusion-associated hemosiderosis leads to cardiac failure which is a leading cause of death among these patients [37].

Hepcidin acts by reducing the release of iron from the cells in the extracellular space by down-regulation of iron exporter ferroportin [38]. It binds to ferroportin on the plasma membrane of enterocytes, macrophages, hepatocytes and other cells, promoting its JAK-dependent phosphorylation, internalization and lysosomal degradation [39]. Ferroportin is important as the only exporter of inorganic iron in mammalian cells [40]. When hepcidin is upregulated and causes internalization of ferroportin, more iron is sequestering in the cells and deposit as ferritin [41]. Iron sequestered in ferritin can't participate in redox reactions [42]. Consequently, less iron is available as non-transferrin bound iron (NTBI), a possible source of oxidative stress [31].

Hepcidin synthesis is mainly induced by iron loading and inflammation and it is suppressed by erythropoietic activity [43]. Regulation of hepcidin synthesis by iron involves bone morphogenic protein (BMP) [44], haemojuvelin (coreceptor for BMP) and transferrin receptor [45]. Production of BMP6 isoform is increased when iron concentration in liver is high, therefore BMP6 may be the signal that reflects the iron stores [46].

Hepcidin-inducing steroids don't activate known pathways, including BMP, but act through progesterone receptor membrane component-1 using an evolutionarily conserved mechanism [47].