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Expression Profiles of Metallothionein I/II and Megalin in Cuprizone Model of De- and Remyelination

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Abstract—Copper chelator cuprizone (CPZ) is neurotoxicant, which selectively disrupts oligodendroglial respiratory chain, leading to oxidative stress and subsequent apoptosis. Demyelination is, however, followed by spontaneous remyelination owing to the activation of intrinsic CNS repair mechanisms. To explore the participation of metallothioneins (MTs) in these processes, in this study we analyzed the expression profiles of MT-I/II and their receptor megalin (low-density lipoprotein receptor related protein-2) in the brain of mice subjected to different protocols of CPZ feeding. Experiments were performed in female C57BL/6 mice fed with 0.25% CPZ during 1, 3 and 5 weeks. They were sacrificed immediately after feeding with CPZ or 2 weeks after the withdrawal of CPZ. The data showed that CPZ-induced demyelination was followed by high astrogliosis and enhanced expression of MTs and megalin in white (corpus callosum and internal capsule) and gray matter of the brain (cortex, hippocampus, and cerebellum). Moreover, in numerous cortical neurons and progenitor cells the signs of MT/megalin interactions and Akt1 phosphorylation was found supporting the hypothesis that MTs secreted from the astrocytes might directly affect the neuronal differentiation and survival. Furthermore, in mice treated with CPZ for 5 weeks the prominent MTs and megalin immunoreactivities were found on several neural stem cells and oligodendrocyte progenitors in subgranular zone of dentate gyrus and subventricular zone of lateral ventricles pointing to high modulatory effect of MTs on adult neuro- and oligodendrogenesis. The data show that MT I/II perform important cytoprotective and growth-regulating functions in remyelinating processes activated after toxic demyelinating insults.

Key words: adult neurogenesis, Akt1 phosphorylation, cuprizone, endocytosis, low-density lipoprotein receptor-related protein-2, metallothioneins I/II.

INTRODUCTION

The complex molecular mechanisms contributing to the de- and remyelination in multiple sclerosis (MS) and other inflammatory and neurodegenerative diseases are often investigated in the cuprizone (bis-cyclohexanone-oxalyldihydrazone, CPZ) model of intoxication, since it leads to reproducible induction of acute or chronic demyelinating lesions followed by spontaneous remyelination after withdrawal of cuprizone feeding (Kipp et al., 2009; Bénardais et al., 2013; Gudi et al., 2014; Praet et al., 2014). It is assumed that induced pathology and the selective vulnerability of mature oligodendrocytes (OLGs) to apoptosis originate from copper deficiency due to Cu chelation or from Cu entrapment within the cell (Messori et al., 2007), since this leads to detrimental effects on mitochondrial function and induce the disturbance of energy metabolism in oligodendroglia that require vast amounts of energy for myelin synthesis (reviewed by (Torkildsen et al., 2008; Kipp et al., 2009, 2012; Skripuletz et al., 2011; Zendedel et al., 2013; Praet et al., 2014). Subsequently, demyelinated axons became more vulnerable to attacks by brain intrinsic and extrinsic immune cells and inflammatory mediators owing to the lack of trophic support by myelin sheaths and higher energy demand for the conduction of action potentials in the presence of higher $Na+$ channel density (Smith and Lassmann, 2002; Craner et al., 2004; Zendedel et al., 2013). Demyelination is, however, transitory and followed by spontaneous remyelination which depends on numerous factors that protect injured cells and/or stimulate the proliferation of remaining oligodendrocyte progenitor cells (OPCs) and their differentiation into mature OLGs that interact with the denuded axons (Franklin and French-Constant, 2017). In this context it was also emphasized that the remyelination might be diminished not only due to precursor cell depletion, but also owing to the presence of a nonpermissive local

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Abbreviations: CPZ, copper chelator cuprizone; DG, dentate gyrus; GCL, granular cell layer; LDL-R, low-density lipoprotein receptor; MTs, metallothioneins; OLGs, oligodendrocytes; OPCs, oligodendrocyte progenitor cells; PBS, phosphate-buffered saline; PLA, performed proximity ligation assay; ROIs, regions of interest; SVZ, subventricular zone.

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environment and defective activation of signaling pathways that provide protective effects following CPZ intoxication (Kipp et al., 2009; Praet et al., 2014).

A large body of evidence implies that to the later events also contribute cysteine-rich metallothioneins (MTs), which during CNS injury show both neuroprotective and neuroregenerative properties (Hidalgo et al., 2001; Aschner and West, 2005; Penkowa, 2006; Fitzgerald et al., 2007; West et al., 2008; Pedersen et al., 2009). Their function has been attributed not only to intracellular free radical scavenging and to zinc and copper regulation in injured cells (Coyle et al., 2002; Maret, 2011) but also to the ability of secreted MT to bind on surface receptors belonging to the family of low-density lipoprotein receptor (LDL-R)-related proteins (LRP), such as LRP-2/megalin and LRP-1, which in turn activate the signal transduction pathways that support neurite outgrowth and survival (Ambjørn et al., 2008; Chung et al., 2008b; Asmussen et al., 2009; West et al., 2011; Auderset et al., 2016). In this regard it was shown that MT and its synthetic analog EmtinB might directly stimulate neurite outgrowth and promote survival of cerebellar granule neurons *in vitro* (Ambjørn et al., 2008), as well as that native MT-2A might block copper-mediated deposition of insoluble extracellular β-amyloid (Aβ) plaques and toxicity in rat cortical neurons (Chung et al., 2010). Accordingly, it was suggested that MTs might have a significant therapeutic potential in megalin-mediated mechanisms, which are involved in the pathogenesis of Alzheimer's (AD) and other neuropathological diseases (Ambjørn et al., 2008; Chung et al., 2008b; Asmussen et al., 2009; West et al., 2011; Auderset et al., 2016). Moreover, implying that similar anti-oxidant and anti-inflammatory mechanisms might be activated also in CPZ model of demyelination, in glial cells and in OLGs and OPCs were found an overexpression of MT-I/II mRNA and proteins, elevated levels of TGF-β and IL-10 and increased activities of NAD-linked cytoplasmic oxidoreductase and glycerolphosphate-3 dehydrogenase (Biancotti et al., 2008).

In an attempt to provide further insights into the potential neuroprotective function of MTs, in the present study we made an immunohistochemical spatio-temporal profiling of MT-I/II expression and distribution throughout CNS tissue during acute and chronic CPZ demyelination, induced by different CPZ-feeding protocol, with a focus on possible interaction of MT-I/II with megalin/ LRP-2 and their involvement in processes of de- and remyelination.

EXPERIMENTAL PROCEDURES

Experimental animals

Experiments were performed on female C57BL/6 mice (9–10 weeks of age). They were housed 4–5 per cage under standard conditions of light, temperature and humidity with unlimited access to food and water. Experimental procedures involving animals complied with Croatian laws and rules (NN 135/06; NN 37/13; NN 125/13; NN 055/2013) and with the guidelines set by European Community Council Directive (86/609/EEC). Experimental protocol was approved by the Ethics Committee of the University of Rijeka.

Cuprizone administration

The cuprizone model was performed according to the previously described protocols (Torkildsen et al., 2008; Kipp et al., 2009; Zendedel et al., 2013). To induce demyelination mice were fed with a 0.25% (w/w) cuprizone/CPZ) [finely powdered oxalic bis (cyclohexylidene-hydrazide); Sigma–Aldrich C9012-25G; Germany] homogeneously blended in the standard food for laboratory animals. Feeding lasted 1, 3 or 5 weeks and mice were sacrificed next day after the expiration of the dietary intoxication protocol or 2 weeks after the withdrawal of CPZ given for 5 weeks (group 5 + 2 weeks). Control mice received the same chow without cuprizone. At the end of the experiment mice were anesthetized by combination of Ketamine (80 mg/kg) and Xylasine (5 mg/kg), given by intraperitoneal (i.p.) injection, transcardially perfused with cold phosphate-buffered saline (PBS, 10 mM, pH 7.4) and 4% paraformaldehyde (PFA, SIGMA, Germany) and subsequently sacrificed by exsanguinations in deep anesthesia, according to the guidance of European Community Council Directive (86/609/EEC) and recommendation of National Centre for the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk).

Tissue preparation for paraffin slices

Tissue samples of the brain were rapidly removed from six animals/group, coronally or sagittally dissected after perfusion and fixed in 4% paraformaldehyde solution (Sigma, Germany) during 24 h. Tissue was embedded in paraffin wax and cut using HM 340E microtome (Microtom, Germany). The tissue sections (4 μm) were deparaffinized, rehydrated and subjected to heat-induced antigen retrieval (0.01 M Sodium Citrate pH 6.0).

Immunohistochemistry

Immunohistochemical labeling of MT I + II and megalin proteins was performed on paraffin embedded tissues using DAKO EnVision + System, Peroxidase (DAB) kit according to the manufacturer's instructions (DAKO Cytomation, USA), as previously described (Jakovac et al., 2011). Briefly, slices were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, mouse monoclonal anti-MT I + II IgG1 (clone E9; Dako Cytomation, USA; diluted 1:50 with 1% BSA in PBS), rabbit polyclonal anti-megalin IgG (H-245, Santa Cruz Biotechnology, USA; diluted 1:200 with 1% BSA in PBS), rabbit polyclonal anti-GFAP IgG (Abcam, UK; diluted 1:5000 with 1% BSA in PBS) or rabbit polyclonal anti-myelin PLP IgG antibodies (Abcam, UK, diluted 1:1000 with 1% BSA in PBS) were added to tissue samples and incubated overnight at 4 °C in a humid environment, followed by a 45-min incubation with peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulins containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreactions' product was visualized by adding

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