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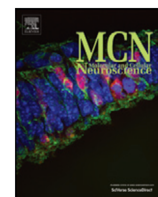

Excitotoxic potential of exogenous ferritin and apoferritin: Changes in ambient level of glutamate and synaptic vesicle acidification in brain nerve terminals

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ABSTRACT

Ferritin, an iron storage protein, is present in the serum and cerebrospinal fluid, has receptors on the cell surface, able to penetrate the brain–blood barrier, can be secreted from the cells, and leaks from destroyed cell in insult and brain trauma. The effect of exogenous ferritin on the key characteristic of glutamatergic neurotransmission was assessed in rat brain nerve terminals (synaptosomes). Exogenous ferritin (80 µg/ml, iron content 0.7%) significantly increased the ambient level of L-[^{14}C]glutamate (0.200 ± 0.015 versus 0.368 ± 0.016 nmol/mg of protein) and endogenous glutamate (fluorimetric glutamate dehydrogenase assay) in the nerve terminals. This increase was not a result of augmentation of tonic release because the velocity of tonic release of L-[^{14}C]glutamate was not changed significantly in ferritin-treated synaptosomes as compared to the control. Ferritin caused a decrease in synaptic vesicle acidification that was shown using fluorescent dye acridine orange. Iron-dependence of the effects of ferritin was analyzed with apoferritin (0.0025% residual iron). Apoferritin weakly affected the proton electrochemical gradient of synaptic vesicles but increased the ambient level and decreased the initial velocity of uptake of L-[^{14}C]glutamate by synaptosomes, nevertheless these effects were ~30% lesser than those caused by ferritin. Exogenous ferritin can provoke the development of excitotoxicity increasing the ambient level of glutamate and lowering synaptic vesicle acidification and glutamate uptake in the nerve terminals, however these effects are not completely iron-dependent. Thus, in the CNS exogenous ferritin can act as a modulator of glutamate homeostasis in iron-dependent and iron-independent manner.

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Introduction

The main function of an iron storage protein ferritin, which was found in many prokaryotic and eukaryotic organisms, is the sequestration of excess iron ions in an innocuous mineral form (Andrews et al., 1992; Kidane et al., 2006; Munro and Linder, 1978). Despite considerable differences in the amino acid sequences, the overall structure of ferritins is highly conserved (Chasteen and Harrison, 1999; D'Estaintot et al., 2004; Kidane et al., 2006). Ferritins are composed of 24 subunits, which form a spherical shell with a large cavity where up to 4500 three-valent iron ions can be deposited as compact mineral crystallites resembling ferrihydrite (Andrews et al., 1992; Chasteen and Harrison, 1999; D'Estaintot et al., 2004; Ford et al., 1984; Friedman et al., 2011; Kidane et al., 2006; Linder et al., 1989). Ferritin cores exhibit superparamagnetic properties, which are inherent to magnetic nanoparticles (Dubiel et al., 1999). Iron ions enter into the core of ferritin through hydrophilic intersubunit channels (Kidane et al., 2006), the average core diameter varies in different tissues from 3.5 nm to 7.5 nm (Dubiel et al., 1999; May et al., 2010). Ferritin stores cellular iron in a dynamic manner protecting the cell from potential iron-dependent radical damage and allowing the release of the metal according to demand (Friedman et al., 2011). Kidane et al. (2006) have shown that the release of iron from ferritin requires lysosomal activity, and when iron is needed, the metal is released from ferritin by lysosomal proteases.

Mammalian ferritins are found intracellularly in the cytosol, in the nucleus, the endo-lysosomal compartment and the mitochondria. Extracellular ferritins are detected in fluids such as serum, synovial and cerebrospinal fluid (CSF). Mouse serum ferritin is actively secreted by a non-classical pathway involving lysosomal processing (Meyron-Holtz et al., 2011). Experiments with intestinal Caco-2 cells indicate that enterocytes possess a ferritin receptor and absorb ferritin via a receptor-mediated pathway (Kalgaonkar and Lonnerdal, 2009). A ferritin receptor is also present on placental membranes (Liao et al., 2001). In insects and worms, ferritin belongs to classically secreted proteins that transport iron. Intracellular and extracellular ferritin may play a role in intra- and intercellular redistribution of iron (Meyron-Holtz et al., 2011).

Abbreviations: GDH, glutamate dehydrogenase; DL-TBOA, DL-threo-β-benzyloxyaspartate; NMDA, N-methyl-D-aspartate; AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; EAAT, excitatory amino-acid transporters.

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Nowadays, it is suggested that ferritin can be an important player in neurodegeneration (Friedman et al., 2011). Using a cell culture model of the blood–brain barrier, it was demonstrated that ferritin was transported across endothelial cells by transcytosis, and the mechanism of ferritin transportation was clathrin-dependent, similar to that previously identified for transferrin (Burdo et al., 2003; Fisher et al., 2007). Binding of exogenous ferritin to cell surface receptors has been implicated as an important iron delivery pathway in the brain. Receptor of H ferritin was identified on the cell surface of oligodendrocytes that could take up ferritin via receptor-mediated endocytosis (Fisher et al., 2007; Hulet et al., 2000; Wang et al., 2010). The authors suggest that iron delivered by ferritin is the major source of iron for oligodendrocytes (Wang et al., 2010). Disturbances in iron delivery to the brain and its regulation may cause abnormal iron distribution, and thus contribute to a variety of neurological disorders. Dávalos et al. (2000) showed that high plasma and CSF ferritin concentrations within the first 24 h from the onset of ischemic stroke were associated with early neurologic deterioration. In neuroblastoma, an increase in serum ferritin has been directly linked to the secretion of ferritin by the tumor. Human ferritins were detected in the sera of nude mice transplanted with human neuroblastoma (Hann et al., 1984; Wang et al., 2010).

Recently, Ruddell et al. (2009) proposed a new role for extracellular ferritin as a proinflammatory signaling molecule in hepatic stellate cells. Interestingly, this function is independent of the iron content of ferritin molecule suggesting that the role of exogenous ferritin may be entirely independent of its classical assignment as an iron storage protein (Wang et al., 2010). Also, in Alzheimer disease the three-fold increase of the concentration of ferritin is accompanied by a small enhancement in the total iron concentration. This finding may suggest that ferritin in Alzheimer disease has properties independent from its iron core (Friedman et al., 2011).

Taking into consideration the abovementioned facts, i.e. (i) presence of ferritin in the serum and CSF; (ii) ability of ferritin to penetrate the blood–brain barrier; (iii) existence of the receptors to ferritin on the cell surface; (iiii) secretion of ferritin from the cells, and also (iiiii) leakage of ferritin from destroyed cells during insult and brain trauma, it is clear that the effects of exogenous ferritin on the key characteristics of glutamatergic neurotransmission should be analyzed. Glutamate is not only a key excitatory neurotransmitter in the mammalian CNS, but also a potent neurotoxin. Ambient glutamate concentration is maintained at a low level between episodes of exocytotic release under normal physiological conditions, thereby preventing continual activation of glutamate receptors and protecting neurons from excitotoxic injury. In stroke, cerebral hypoxia/ischemia, hypoglycemia, traumatic brain injury, etc., the development of neurotoxicity is provoked by an increase in the concentration of ambient glutamate. Excessive extracellular glutamate overstimulates glutamate receptors initiating an excessive calcium entry through mainly N-methyl-D-aspartate ionotropic receptors, and causes excitotoxicity, neuronal injury and death. Ambient level of glutamate is set by a balance between the rate of tonic (unstimulated) release of glutamate and high-affinity Na^+-dependent glutamate uptake in neurons and glial cells. It is clear that an increase in ambient glutamate concentration has a potential for brain damage. The main questions we addressed were whether and how exogenous ferritin can provoke the development of pathogenic mechanisms underlying excitotoxicity? In rat brain nerve terminals, we assessed the effects of exogenous ferritin on: (i) the ambient level of glutamate; (ii) tonic release of glutamate; (iii) membrane potential; and (iiii) acidification of synaptic vesicles.

Alekseenko et al. (2008) showed that ferritin induced a decrease in high-affinity Na^+-dependent glutamate uptake by nerve terminals and considered ferritin as model protein shell-coated nanoparticles, which can serve as good tools to investigate possible toxic properties of synthetic metal nanoparticles coated by polymers. In this study, the effects of protein shell alone, i.e. apoferritin, on transporter-mediated glutamate uptake, the ambient level of glutamate and acidification of synaptic vesicles were assessed. Hereby, iron-dependence of ferritin-evoked effects was analyzed using iron-free apoferritin.

Results

The effect of ferritin on the ambient level of glutamate in the nerve terminals

In the experiments, we used isolated brain nerve terminals (synaptosomes), which retain all features of intact nerve terminals, e.g., ability to maintain the membrane potential, exocytotic release as well as accomplish uptake of the neurotransmitters. As stated in the Introduction section, the certain level of ambient glutamate is very important for proper synaptic transmission, whereas an increase in this level causes neurotoxicity. To determine the level of ambient glutamate in the nerve terminals, we used three methodological approaches, i.e. radiolabeled assay with L-[^{14}C]glutamate and D-[2,3-^{3}H]aspartate, a non-metabolized analog of L-glutamate, and fluorimetric assay of the analysis of endogenous glutamate concentration based on the assessment of glutamate dehydrogenase activity in the incubation medium.

Radiolabeled assay

The ambient level of L-[^{14}C]glutamate was measured after the addition of ferritin at a concentration of 80 μg/ml to the incubation medium of the synaptosomes (see Experimental methods section). It was significantly higher in the presence of ferritin as compared to the control and at 5 min time point consisted of 0.200 ± 0.015 nmol/mg of protein in the control and 0.368 ± 0.016 nmol/mg of protein in the presence of ferritin ($P \leq 0.05$, Student's *t*-test, $n = 5$) (Fig. 1, lines 1 & 3). Continuous monitoring showed that the ambient level of L-[^{14}C]glutamate remained enhanced in the presence of ferritin at least during 25 min time period. As Na^+/K^+ gradient is a driving force for glutamate uptake by transporters, a decrease in the extracellular [Na^+] is expected to inhibit transporter-mediated uptake of L-[^{14}C]glutamate and allow to measure the binding of L-[^{14}C]glutamate to the plasma membrane of synaptosomes. Standard Na^+-containing media is of 126 mM Na^+ (see Experimental methods section). Using monovalent organic cations N-methyl-D-glucamine for replacement of extracellular Na^+, we have shown that the binding of 10 μM L-[^{14}C]glutamate to synaptosomes in low - Na^+ medium was similar in the control and in the presence of ferritin (80 μg/ml). In additional control, we used albumin at the same concentration and did not reveal changes in the ambient level of

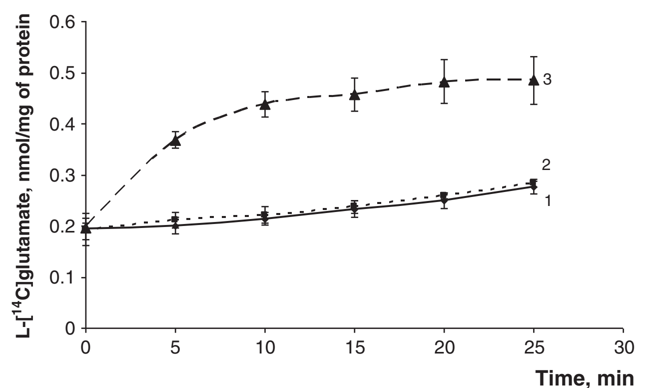

Fig. 1. The ambient level of L-[^{14}C]glutamate in the nerve terminals in the control (solid line 1), in the presence of albumin (80 μg/ml) (dotted line 2) and ferritin (80 μg/ml) (dashed line 3). The synaptosomes were loaded with L-[^{14}C]glutamate (1 nmol/mg of protein, 238 mCi/mmol) as described in the Experimental methods section. After loading, samples (0.5 mg of protein/ml) were preincubated at 37 °C for 8 min, and then after the addition of ferritin or albumin, the aliquots of the samples were collected at different time points, centrifuged, and L-[^{14}C]glutamate radioactivity was determined. Total synaptosomal L-[^{14}C]glutamate content was equal to 200,000 ± 15,000 cpm/mg protein. Data are means ± SEM of five independent experiments, each performed in triplicate.

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