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Effects of Hemin on Ca^{2}+ Permeability in Neurons of C57BL/6 Mouse Brain

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Abstract

Excitotoxicity results in a significant increase in Ca^{2}+ influx; essentially from open N-methyl-D-aspartate receptors (NMDARs) channels that cause a secondary rise in the intracellular Ca^{2}+ concentration. It is correlated with neuronal death induced by Ca^{2}+ overload. Dysfunction of NMDARs is associated with excitotoxic neuronal death in neurodegenerative disorders. In this study, the effects of hemin on Ca^{2}+ permeability in neurons of C57BL/6 mouse brain examined. Isolated from 1-dayold C57BL/6 mice, were cultured in serum-free media. Cells were maintained in growth medium at 37°C in 95% air/5% CO2 for 2 weeks in vitro before treatment. Primary neurons were cultured in serum-free media were treated with hemin (0, 12.5, 25, 50, 75,100 μM) for 18 (h). Intensity of calcium fluorescence was reduced in treated cultures with hemin (100, 86, 78.5, 60, 56, 46%, respective to the concentrations stated previously; $P<0.05$ for all). Hemin increased Ca^{2}+ influx in cultured neurons. NMDAR stimulation by hemin increased the activating of NMDARs and Ca^{2}+ influx in the cultured neurons. Therefore, hemin is cytotoxic due to increase of intracellular Ca^{2}+ influx.

Keywords: Excitotoxicity; Ca^{2}+ overload; NMDA receptor; Hemin; Mammalian cells; Neurodegenerative disorders; Excitotoxicity; Cytoplasmic calcium; Neuronal death; Mammals; Neurotransmission; Brain

Introduction

At rest, the cytoplasmic concentration ($[Ca^{2+}]i$) in mammalian cells is very low (50-100 nM) [1]. Excitotoxicity results in a significant increase in Ca^{2+} influx essentially from open N-methyl-D- aspartate receptors (NMDARs) channels that cause a secondary rise in the intracellular Ca^{2+} concentration [2], Glutamate is capable of to killing neurons with excitotoxicity [3]. This secondary overload is correlated with neuronal death induced by Ca^{2+} overload [4]. Dysfunction of NMDARs is associated with excitotoxic neuronal death in neurodegenerative disorders [5]. Glutamate excitotoxicity is a critical factor in brain damage leading to brain and neurodegenerative disorders, such as; Alzheimer's disease. The mechanism of excitotoxicity is causally linked to an intense elevation in cytoplasmic calcium concentration ($[Ca^{2+}]c$) that leads to cell injury and neuronal death [6-8]. Correspondingly, stabilizing low cytosolic Ca_{2+} by chelation with BAPTA [(1,2-bis(o-aminophenoxy) ethane-N, N, N, N-tetra acetic acid;)] increases the survival rate of neurons exposed to excitotoxic glutamate [9,10].

In the central nervous system of mammals, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPARs) and NMDARs primarily mediate fast excitatory neurotransmission. Ca_{2+} permeability; allows NMDARs to play a vital role in brain damage, neuropathology, and neuroplasticity [11]. The glutamate receptors of AMPA and NMDA are permeable to Ca_{2+} and are expressed in the brain regions responsible for cognitive functions, such as neocortex and hippocampus [12]. The receptors are heterothermies comprising a combination of GluN1, GluN2A-D and GluN3A-B subunits [13]. NMDARs receptors activation leads to opening of an ion channels that is permeable for cations, resulting in the influx of Na^{+} and Ca^{2+} ions and efflux of K+ ions [14]. During excitatory neurotransmission, presynaptic release of glutamate activates glutamate receptors in the postsynaptic membrane, resulting in the generation of an excitatory postsynaptic potential [15]. Phosphorylation of glutamate receptor regulates the Ca^{2+} channels and Ca^{2+} permeability [16].

NMDARs have a high affinity to stimulate L-glutamate [17]. L-glutamate is the strongest NMDA agonist (EC50 2.3 μM) [18]. Hemin is an iron–protoporphyrin molecule consisting of four pyrrole rings [19]. Hemin is cytotoxic due to its ability to contribute to the generation of reactive oxygen species [20]. As respects, hemin play an crucial role in correlated with neuronal death induced by Ca^{2+}, In this study, the effects of hemin on Ca^{2+} permeability in neurons of C57BL/6 mouse brain examined.

Methods

Cell culture

Twenty-eight 1day-old C57BL/6 male mice were prepared from Animal Center of School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. They were kept cages (4 mice per cage) at room temperature and provided with a balanced diet, Light cycle with 12h light, 12h dark. The mice randomly divided into 6 groups; and anesthetized with Ketamine (87 mg/kg) and Xylazine (13 mg/kg). All mice were killed, and their brain and neocortex were removed. Neurons isolated by neutrosphere method and were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific). Cells were maintained in growth medium at 37 °C in 95% air/5% CO_2 for 2 weeks in vitro before treatment. Primary postnatal neurons cultured in serum-free media were treated with hemin (Frontier Scientific; 0, 12.5, 25, 50, 75,100 μM) for 18 h [21].

Calcium imaging

The neurons were incubated in medium with (Fluo-8 AM) (ATT Bio quest, Inc) for 1 h at 37°C. The neurons were then washed twice with Hank's Balanced Salt Solution (Frontier Scientific), containing $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM). The intensity of Flu-8 AM fluorescence was evaluated after adding NMDA (100 μM) and Nifedepine (10 μM) to neurons, with hemin. The intensity of fluorescence was measured of 482 nm and 505-530 nm (emission reverse microscopy) (Leica HC PL FLUOTAR 20×/0.50 objective, Lambda DG-5 Plus) [22].

Statistical analysis

Statistical analysis was carried out using ANOVA; with SPSS 19. One-Way ANOVA was used to test for differences in mean values from multiple samples (calcium imaging data) with comparison tests. Statistical difference between two means was analyzed by two-tailed unpaired or paired Student's t-test. Differences were considered significant if $p<0.05$. All data are expressed as mean±SEM.

Results

Primary postnatal neurons cultured in serum-free media were treated with hemin (0, 12.5, 25, 50, 75,100 μM) for 18 h. fluorescence was reduced in treated cultures with hemin (100, 86, 78.5, 60, 56, 46%, respectively) (P < 0/05 for all) (Figure 1 & Table 1).

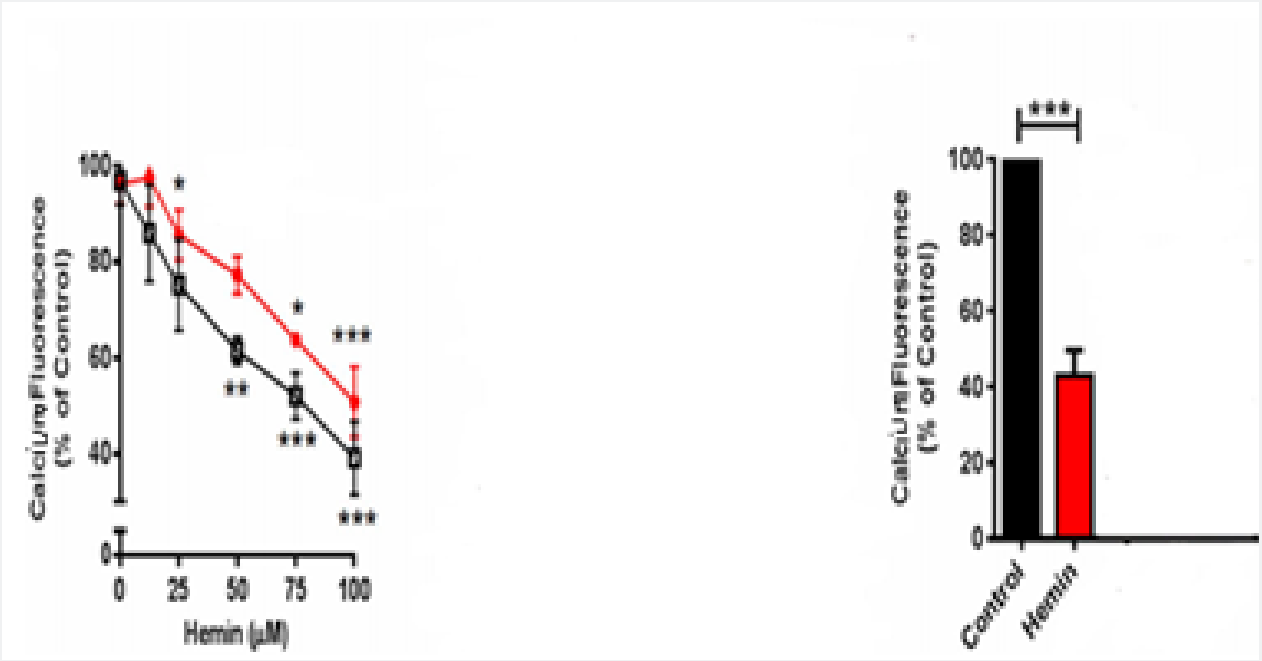

Figure 1: The Intensity of Calcium Fluorescence in Treated Cultures with Hemin. Primary Neurons Cultured in Serum-Free Media were Treated with Hemin (0, 12.5, 25, 50, 75,100 μM) for 18 h. Intensity of Calcium Fluorescence was Reduced in Treated Cultures with Hemin (100, 86.5, 78.5, 60, 56,46%). [*P < 0/05; **P < 0/01; ***P < 0/001].

Table 1: Reduction in Calcium Florescence in Postnatal Neurons Treated with Various Concentrations of Hemin.

Group	Hemin (μM)	Calcium Fluorescence (%)	P Value
N=1	0	100	0.043
N=2	12.5	86.5	0.038
N=3	25	78.5	0.024

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N=4	50	60	0.0034
N=5	75	56	0.00028
N=6	100	46	0.00012

Discussion

Ca^{2+} influx is regulateds by NMDARs subunits [23]. NMDAR stimulation increases Ca^{2+} influx [24]. Stimulation of NMDAR activates Ca^{2+} signaling pathway in neurons [25]. Conversely, inhibition of NMDARs inhibits it [26]. Hemin suppresses NMDAR activity [27], by regulating the GluN1, GluN2A and GluN2B subunits of NMDA [28]. NMDAR stimulation by hemin was increased activating of NMDARs and Ca^{2+} influx in the cultured neurons.

Data availability

All data relating to the present study are contained in Table.

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