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Excitotoxic neuronal injury in acute homocysteine neurotoxicity: Role of calcium and mitochondrial alterations

Elzbieta Zieminska, Ewa Matyja, Hanna Kozlowska, Aleksandra Stafiej, Jerzy W. Lazarewicz *

Medical Research Centre, Polish Academy of Sciences, 5 Pawinskiego Street, 02-106 Warsaw, Poland

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Abstract

In this study we tested if calcium imbalance and mitochondrial dysfunction, which have been implicated in the conventional mechanisms of excitotoxicity induced by glutamate (Glu), are also involved in homocysteine (Hcy) neurotoxicity. Primary cultures of rat cerebellar granule cells were incubated for 30 min in the presence of 25 mM D,L-Hcy or 1 mM Glu. At these concentrations both amino acids induced comparable neurodegeneration and chromatin condensation, evaluated after 24 h using the propidium iodide and Hoechst 33258 staining. These effects were partially prevented by cyclosporin A (CsA), but not FK506. Hcy-induced release of [^{3}H]inositol phosphates and increase in intracellular calcium level (evaluated with fluo-3 fluorescent probe) were weakly expressed. Hcy- and Glu-induced mitochondrial swelling was visualized under electron microscope, and the release of *Cytochrome c* was evaluated using immunocytochemical method and confocal microscopy. Comparing to Glu, the effects of Hcy were slightly less expressed and less sensitive to CsA, while FK506 did not modify mitochondrial alterations. These data indicate that mitochondrial alterations play a similar role in acute Hcy and Glu neurotoxicity, although the mechanisms triggering Glu- and Hcy-evoked mitochondrial dysfunction seem to differ, Hcy toxicity being less dependent on calcium.
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Keywords: Calcium; Cerebellar granule cells; *Cytochrome c*; Homocysteine; Mitochondria

1. Introduction

Increased concentration of homocysteine (Hcy) in body fluids was shown to be a risk factor in Alzheimer's disease (Ravaglia et al., 2005). Hcy is directly neurotoxic (for reviews see Molloy and Weir, 2001; Mattson and Shea, 2003). One of the suggested mechanisms of these effects is NMDA receptor-mediated neurotoxicity (Kim and Pae, 1996; Lipton et al., 1997). Previously, we demonstrated that in the rabbit hippocampus in vivo Hcy induces intracellular calcium release mediated by group I metabotropic glutamate receptor (mGluRs), and that both NMDA receptors and group I mGluRs participate in Hcy-evoked toxicity in primary cultures of rat cerebellar granule cells (CGC) (Lazarewicz et al., 2003; Zieminska et al., 2003). We have shown also that Hcy weakly stimulates calcium influx to CGC via NMDA receptors (Zieminska et al., 2003).

According to the hypothesis of excitotoxicity, glutamate (Glu) causes an excessive activation of the excitatory amino acid receptors, and a massive influx of Ca^{2+} into the neurons (Choi, 1985), the most important for neuronal damage being its entrance via NMDA receptors (Dubinsky and Rothman, 1991). More recently it has been shown that mitochondrial calcium overload inducing permeability transition (MPT) and the release from mitochondria of the apoptosis-inducing proteins, including *Cytochrome c*, are involved in Glu-evoked excitotoxicity (Ankarcrona et al., 1995; Kristal and Dubinsky, 1997; Nicholls et al., 1999). It is not clear if acute Hcy excitotoxicity, which requires simultaneous activation of the NMDA and mGluR GI receptors, but seems to be independent on the influx of extracellular calcium (Zieminska et al., 2003), follows such a pattern of pathogenic mechanisms described for Glu-evoked neuronal damage. Consequently, the aim of the present study was to verify if the intracellular calcium transients and mitochondrial dysfunction are involved in acute Hcy-mediated excitotoxicity in primary cultures of rat cerebellar granule cells.

* Corresponding author. Tel.: +48 22 6086528; fax: +48 22 6685423.
E-mail address: jerzyl@cmdik.pan.pl (J.W. Lazarewicz).

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2. Experimental procedures

2.1. Materials

L-Glutamate (Glu), *N*-methyl-D-aspartate (NMDA), D,L-homocysteine (Hcy), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), goat serum, Hoechst 33258 and propidium iodide dyes as well as the materials for cell culturing were purchased from Sigma Chemical Company (St. Louis, MO, USA). (S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) and *trans*-azetidine-2,4-dicarboxylic acid (*t*ADA) were obtained from Tocris Neuramin Ltd. (Bristol, Essex, UK). Immunosupresant cyclosporin A (CsA) was purchased from Novartis (Basel, Switzerland) and FK 506 from Calbiochem (Darmstadt, Germany). Fluo-3/AM was a product of Molecular Probes Inc. (Eugene, OR, USA). The anti-*Cytochrome c* mouse monoclonal antibody was purchased from PharMingen (Mississauga, Ont., Canada), while anti-mouse IgG1 antibody conjugated with fluorescein was a product of Southern Biotechnology (Birmingham, Ala., USA). ECL-kit was from Pierce Biotechnology Inc. (Rockford, IL, USA). All other chemicals were of analytical grade. Myo-[^{3}H]inositol was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA), and ^{45}Ca from the Radioisotope Research Development Centre (Swierk, Poland).

2.2. Cell cultures, evaluation of neurotoxicity

Primary cultures of cerebellar granule cells (CGC) were prepared from cerebella of 7-day-old rats and cultured exactly as was previously described (Zieminska et al., 2003). The cells at 7-day in vitro (DIV) were transferred to Locke 25 buffer and incubated for 30 min with 1 mM Glu or 25 mM D,L-Hcy alone or together with 0.5 μM CsA or 0.5 μM FK 506. Thereafter, Locke medium was replaced with the original growth medium and the cells were maintained for the next 24 h under standard conditions. For Hoechst 33258 staining, the neurons were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT), washed with PBS and stained with the fluorescent dye Hoechst 33258 (0.1 μg/ml in PBS) for 5 min at RT (Chan and Lin-Shiau, 2001). Changes in chromatine condensation were examined using fluorescent microscope Zeiss Axiovert 25 and analyzed by computer program KS 300. Neurons were additionally stained with propidium iodide (5 μg/ml). The evaluation of neurotoxicity using propidium iodide staining was performed as described previously (Zieminska et al., 2003).

2.3. Measurement of phoshoinositide hydrolysis

For measurements of the release of [^{3}H]inositol phosphates ([^{3}H]InsPs), CGC (4 million/wall) were incubated in 6-well plates under standard conditions in the medium containing 25 mM KCl, or in some experiments the standard growth medium was replaced for the last 2 days with the medium containing 5 mM KCl. Before the experiments, CGC were incubated for 24 h with 3 μCi/ml of myo-[^{3}H]inositol (specific activity 80–100 Ci/mmol), then the cells were washed, incubated with agonists alone or with group I mGluR antagonists for 30 min at 37 °C, extracted and analyzed for [^{3}H]InsPs as described by Gorman and Griffiths (1994).

2.4. Fluo-3/AM loading and fluorescence measurements

The CGC were incubated with 16 μM fluo-3/AM for 30 min in a buffer containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 5.6 mM glucose and 8.6 mM Hepes, pH 7.4, then the neurons were washed three times with the same medium and left in this buffer for further investigations (Berman and Murray, 2000). The basal fluo-3 fluorescence reflecting the steady-state level of intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored for 90 s and recorded at each 30 s, using the confocal microscope Zeiss LSM 510 with filter pairs of excitation and emission wavelength of 485 and 538 nm, respectively. Then 1 mM Glu or 25 mM D,L-Hcy were added and the fluorescence was recorded for 90 s using LSM 510 computer program (version 3.2).

2.5. Electron microscopy

The CGC cultured in 6-well plates with cover slips, after incubation for 30 min in the Locke 25 medium with 1 mM Glu or 25 mM D,L-Hcy alone or together with 0.5 μM FK 506 or 0.5 μM CsA, were fixed in 0.8% formaldehyde and 2.5% glutaraldehyde solution in phosphate buffer, pH 7.2, followed by 1% OsO_4 for 1 h in 4 °C. After dehydration in graded ethanol and embedding in Epon 812, ultra-thin sections were prepared and examined in JEM XB 1500 electron microscope.

2.6. Visualization of Cytochrome c in cytoplasm

The CGC cultured on cover slips (5 milllion cells/wall) were treated for 30 min with Glu or Hcy, then the neurons were fixed for 15 min at RT with 4% paraformaldehyde. The plasma membranes were permeabilized with 1% Triton X-100 in PBS. The non-specific binding was blocked by 1 h incubation at RT with 10% goat serum in PBS and 0.1% Triton X-100. Next, the cells were incubated for 24 h in the dark at 4 °C with anti-*Cytochrome c* mouse monoclonal antibody, diluted 1:200. The following day, after washing with PBS, the cells were incubated for 1 h with diluted 1:300 anti-mouse IgG1 antibody conjugated with fluorescein. Then the samples were washed in PBS and examined under Zeiss LSM 510 confocal microscope.

2.7. Data analysis

The results are presented as mean ± S.D. ($n = 6$). One-way ANOVA followed by Dunnett's test was used for comparisons between groups. Significance was taken at $P < 0.05$.

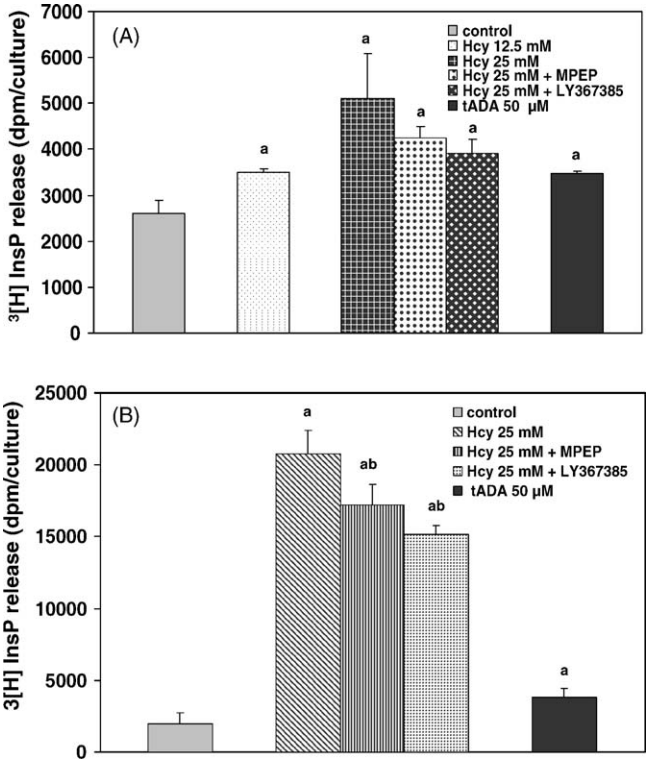

Fig. 1. Homocysteine (Hcy)- or *t*ADA-induced release of labeled inositol phosphates in cultured cerebellar granule cells, growing in standard 25 mM KCl containing medium (A) or for 2 days in low potassium medium (B). The cultures were pre-labeled overnight with [^{3}H]inositol, then incubated for 30 min with D,L-homocysteine (Hcy) or *t*ADA alone, or in the presence of 25 μM MPEP or LY367385. Data represent the release of [^{3}H]inositol phosphates in dpm/culture. The results are means ± S.D. ($n = 6$): a, means significantly different from control; b, means significantly different from effects of agonists alone ($P < 0.05$).

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