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Homocysteine-induced acute excitotoxicity in cerebellar granule cells *in vitro* is accompanied by PP2A-mediated dephosphorylation of tau

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ABSTRACT

Our results demonstrate that acute exposure of primary rat cerebellar granule cell cultures to homocysteine at millimolar concentrations induces a glutamate receptor-mediated decrease in tau protein phosphorylation, which is accompanied by excitotoxic neuronal damage. This contrasts with the previously reported hyperphosphorylation of tau in homocysteine-treated neurons, and with the assumption that hyperhomocysteinemia is one of the risk factors in Alzheimer disease, in which abnormal hyperphosphorylation of tau protein leads to neurofibrillary degeneration. The mechanisms of homocysteine neurotoxicity have been explained mainly either by disturbances in methylation processes, that may also lead to the accumulation of phosphorylated tau due to reduced activity of tau-selective protein phosphatase 2A, or by excitotoxicity. Since the relationships between homocysteine excitotoxicity and tau phosphorylation are unclear, the aim of this study was to characterize these processes in neurons acutely treated with homocysteine at neurotoxic concentrations, and to link them to the activities of glutamate receptors and protein phosphatase 2A. Within 24 h following a 30 min exposure of neuronal cultures to 20 mM D,L-homocysteine, significant neurotoxicity was induced. This could be reduced by treatment with an uncompetitive NMDA receptor antagonist, MK-801 (0.5 μM), or by mGlu1 and mGlu5 receptor antagonists, LY367385 and MPEP, respectively (both at 25 μM). Western blot analysis showed a rapid decrease in immunostaining of phospho-tau, 2 h after incubation of cell cultures with 15 mM D,L-homocysteine, which persisted for 6 h after the insult. Application of MK-801, LY367385 or okadaic acid (100 nM), an inhibitor of protein phosphatases 1 and 2A, significantly prevented dephosphorylation of tau, implying a role for the activation of glutamate receptors and protein phosphatase 2A. The phosphorylation of tau may be increased or reduced by treatment with homocysteine, and the nature of the cellular response to this sulfur-containing amino acid depends on the neuronal phenotype.

1. Introduction

Alzheimer's disease (AD) is a late-onset progressive neurodegenerative disorder that has been linked to genetic as well as acquired factors (Reddy, 2006). Apart from deposits of β-amyloid proteins, one of the main features of AD is progressive, intraneuronal accumulation of fibers referred to as paired helical filaments (PHF), which is followed by the generation of neurofibrillary tangles (NFT). It is believed that PHF formation is a late event in AD progression and is related to extensive losses of neurons and synapses in selected areas of the brain (Alonso et al., 2001). The major component of PHF is abnormally hyperphosphorylated full-length tau. Tau is a member of the microtubule-associated phosphoprotein (MAP) family that plays an essential role in the regulation and stabilization of neuronal microtubule assembly in the central nervous system and is involved in axonal transport (Terry, 1998). The degree of tau phosphorylation modulates its affinity for microtubule binding sites and regulates the dynamics of microtubules. Hyperphosphorylated tau protein isolated from the brains of AD patients lacks the ability to promote microtubule assembly (Iqbal et al., 1986). Abnormal phosphorylation of tau protein, which leads to PHF and subsequently NFT formation observed in AD brains (Iqbal et al., 1994), might result from multiple metabolic abnormalities. The pivotal dysfunction appears to be an imbalance in kinase and phosphatase activities resulting from dysregulation of the phosphorylation/dephosphorylation system (Gong et al., 2006). Several studies have shown attenuated expression and/or activity of protein phosphatases, particularly protein phosphatase 2A (PP2A), in brain tissues from patients with AD, and the critical role of decreased PP2A activity in tau hyperphosphorylation has been demonstrated in model

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experiments (Gordon-Krajcer et al., 2000; Vogelsberg-Ragaglia et al., 2001; Sontag et al., 2004).

Homocysteine (Hcy) is a sulfur-containing amino acid, which is an endogenous product of methionine metabolism in the course of the transmethylation pathway. Methionine is metabolized to *S*-adenosylmethionine (SAM), which serves as the proximal methyl donor for most methylation reactions (Kruman et al., 2000; Shi et al., 2003). SAM-dependent methylation reactions result in the production of *S*-adenosylhomocysteine (SAH), which is a potent inhibitor of methyltransferase enzymes. In the presence of high concentrations of Hcy, SAH accumulates in cells, leading to global inhibition of methylation processes (Medina et al., 2001; Obeid and Herrmann, 2006). In adults, the normal range for total plasma Hcy is 5–15 μM (Kang et al., 1992). High methionine intake, Hcy metabolism abnormalities (Medina et al., 2001) and/or dietary folate and vitamin B deficiencies (Mattson and Shea, 2003) can lead to hyperhomocysteinemia, where total plasma levels can reach 50–200 μM. Epidemiological studies have shown that elevated level of Hcy in tissue fluid is associated with cardiovascular diseases (Biasioli and Schiavon, 2000; Kang et al., 1992). It is also a risk factor in AD (Leblhuber et al., 2000; Miller, 2000; Morris, 2003; Shea et al., 2002).

Data from recent studies link tau pathology with Hcy (Vafai and Stock, 2002; Morris, 2003; Sontag et al., 2007; Luo et al., 2007). Hcy-induced disturbances in methylation processes may lead to downregulation of PP2A, which in turn results in hyperphosphorylation of tau protein (Vafai and Stock, 2002; Obeid et al., 2007; Sontag et al., 2007). Alternatively, homocysteine-induced activation of excitatory amino acid receptors may influence the level of tau phosphorylation (Ho et al., 2002). The role of NMDA receptors and group I metabotropic glutamate receptors (mGluRs GI) in homocysteine-induced neurodegeneration has been repeatedly demonstrated (Lipton et al., 1997; Zieminska et al., 2003, 2006; Obeid and Herrmann, 2006; Zieminska and Lazarewicz, 2006). Excitotoxicity-evoked disturbances in calcium-mediated signaling, that may alter the equilibrium in protein phosphorylation and dephosphorylation systems are among the putative contributory factors involved in abnormal tau phosphorylation (Mattson, 2003). Thus, excitotoxicity triggered by Hcy might be among the mechanisms interfering with the phosphorylation of tau.

Hcy-induced tau phosphorylation has been demonstrated in the studies of Ho et al. (2002) and Chan et al. (2008), using primary cultures of neurons submitted to a prolonged treatment with Hcy or a folate-deprived neuroblastoma cell line, respectively. Both investigations demonstrated the accumulation of phosphorylated tau protein linked to Hcy-mediated activation of NMDA receptors and mitogen-activated protein kinase (MAPK). However, the increased tau phosphorylation evoked by prolonged exposure to Hcy may result not only from NMDA receptor- and calcium-mediated activation of kinases. Also SAM deficit and a reduction in phosphatase activity may be involved. Acute exposure of neurons to Hcy under conditions promoting excitotoxicity, but minimizing its interference with methylation processes, might distinctly affect phosphorylation of tau. The role of PP2A activity in Hcy-induced effects also remains unclear.

The aim of the present study was to characterize changes in tau phosphorylation in neurons acutely treated with Hcy at neurotoxic concentrations, and to relate these changes to NMDA receptor and mGluRs GI activation, and to the activity of PP2A. Primary cultures of rat cerebellar granule cells were acutely exposed to D,L-homocysteine in the absence or presence of NMDAR and mGluR antagonists, and these were examined for neuronal viability and changes in tau protein phosphorylation.

2. Experimental procedures

2.1. Materials

D,L-homocysteine (Hcy), *N*-methyl-D-aspartate (NMDA), okadaic acid (OA) and materials for cell culture were purchased from Sigma (St. Louis, MO, USA). (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) were obtained from Tocris Neuramin Ltd. (Bristol, UK). (+)-5-Methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) was obtained from RBA (Natic, MA, USA).

2.2. Cell cultures

Primary cultures of granule neurons were prepared from cerebella of 8-day-old Wistar rats as described by Schousboe et al. (1985). Use of the rat pups was in accordance with Polish and international regulations. The procedure was approved by the First Local Ethical Committee in Warsaw. All efforts were made to reduce the number of animals used and minimize their suffering. Briefly, the brains were removed from decapitated heads, cerebella were dissected and meninges with the vessels were stripped off. Then the cerebella were chopped into 400 μm cubes. The tissue was then incubated for 15 min at 37 °C in the medium containing 120 mM NaCl, 5 mM KCl, 25 mM HEPES (pH 7.4) and 9.1 mM glucose supplemented with 0.025% trypsin and 0.05% DNase I. The incubation was terminated by the addition of trypsin inhibitor (0.04%) and the mixture centrifuged for 1.5 min at 480 g and 4 °C. The pellet was triturated, and after further centrifugation the cells were suspended in basal Eagle's medium supplemented with heat-inactivated 10% fetal calf serum (Sigma), 25 mM KCl, 4 mM glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml). Granule neurons were seeded at a density of 2×10^6 cells per well in 12-well plates (NUNC) or at a density of 15×10^6 cells per poly-L-lysine-coated culture bottle. Cultures were supplemented with 7.5 μM cytosine arabinofuranoside 36 h after plating. The cells were used for experiments after 7 days in culture at 37 °C in a humidified atmosphere containing a 5% CO_2.

2.3. Experimental procedure and sample preparation

Acute Hcy-induced neurotoxic damage was evoked *in vitro* as described by Ankarcrona et al. (1995). The growth medium was replaced by Locke 25 incubation buffer (134 mM NaCl, 25 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, pH 7.4, 2.3 mM $CaCl_2$, 5 mM glucose). Glutamate receptor antagonists were added for 5 min before 30-min incubation with Hcy. The Locke medium was then removed and the cells returned to the original growth medium and cultured for a further 24 h under standard conditions. The viability of cultures was estimated morphologically using 5% propidium iodide staining and live/dead neurons were counted using a Zeiss Axiovert 25 fluorescence microscope. The proportion of live cells was expressed as a percentage of the total cell number.

To study effects of Hcy on tau phosphorylation, the cells were treated as described above, using Locke 25 incubation buffer. To examine the role of PP2A in alterations of the phosphorylation state of tau, neurons in Locke 25 buffer were pretreated with okadaic acid for 1 h before Hcy administration. Control cells were incubated in Locke 25 buffer without additions. Thereafter, the Locke 25 buffer was removed and the cells were cultured in the original growth medium. The cells were harvested just after incubation (0 h), and 2, 4 or 6 h after removing the pharmacological agents. According to the method of Valerio et al. (1995), the cells were collected in 300 μl of ice-cold lysis buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, plus 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Protease Inhibitor Cocktail, 0.5% nonidet-P40 and 0.5% Na-deoxycholate (all from Sigma). The suspension was homogenized and centrifuged at 10,000 × *g* for 20 min at 4 °C. The resulting supernatant was used for further analysis.

2.4. Immunochemical analysis of tau protein abundance and phosphorylation

For Western immunoblot analysis, monoclonal antibody AT-8, purchased from Innogenetics Laboratories (Ghent, Belgium, dilution 1:400) or monoclonal antibody Tau 46 obtained from Zymed Laboratories (CA, USA, dilution 1:500) were used. Secondary antibody (GAM) conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) was used at 1:1000 dilution. Electrophoresis of protein lysates (70 μg) was performed on 10% SDS-polyacrylamide gels (SDS-PAGE). The separated proteins were electrophoretically transferred onto nitrocellulose membranes and these were incubated with 5% non-fat dried milk in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl (TBS) for 1 h at room temperature to block non-specific binding sites. The blots were then incubated overnight with primary antibodies at 4 °C. After washing in TBS containing 0.05% Tween-20, the membranes were incubated with secondary antibody for 2 h at room temperature. Both the primary and secondary antibodies were diluted in 5% dried milk-TBS. Specific immunoreactivity was detected by enhanced chemiluminescence staining (ECL), placing the blots processed with Amersham ECL reagents against Amersham Hyperfilm ECL. The intensity of immunostained bands was quantified by densitometric scanning of the autoradiographs using an Image Scanner III with LabScan 6.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).