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Hyperhomocysteinemia selectively alters expression and stoichiometry of intermediate filament and induces glutamate- and calcium-mediated mechanisms in rat brain during development

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ABSTRACT

The aim of the present work was to investigate the actions of a chemically induced chronic hyperhomocysteinemia model on intermediate filaments (IFs) of cortical and hippocampal neural cells and explore signaling mechanisms underlying such effects. Results showed that in hyperhomocysteinemic rats the expression of neural IF subunits was affected. In cerebral cortex, glial fibrillary acidic protein (GFAP) expression was donwregulated while in hippocampus high and middle molecular weight neurofilament subunits (NF-H and NF-M, respectively) were up-regulated. Otherwise, the immunocontent of IF proteins was unaltered in cerebral cortex while in hippocampus the immunocontent of cytoskeletal-associated low molecular weight neurofilament (NF-L) and NF-H subunits suggested a stoichiometric ratio consistent with a decreased amount of core filaments enriched in lateral projections. These effects were not accompanied by an alteration in IF phosphorylation. *In vitro* results showed that 500 μM Hcy-induced protein phosphatases 1-, 2A- and 2B-mediated hypophosphorylation of NF subunits and GFAP in hippocampal slices of 17-day-old rats without affecting the cerebral cortex, showing a window of vulnerability of cytoskeleton in developing hippocampus. Ionotropic and metabotropic glutamate receptors were involved in this action, as well as Ca^{2+} release from intracellular stores through ryanodine receptors. We propose that the mechanisms observed in the hippocampus of 17-day-old rats could support the neural damage observed in these animals.

1. Introduction

Tissue accumulation of homocysteine (Hcy) is the biochemical hallmark of homocystinuria, an inherited metabolic disorder caused by severe deficiency of cystathionine β-synthase (CBS, E.C.4.2.1.22) activity. Affected patients present alterations in various organs and systems, including central nervous and vascular systems (Kraus, 1998; Mudd et al., 2001). A variable degree of mental retardation and convulsions, whose pathophysiology is poorly understood, are also found in these patients (Malinow, 1990). There is a considerable body of evidence showing that an increase of Hcy levels in plasma is a strong and independent risk factor for the development of neurological and vascular diseases (Kuhn et al., 1998; Leblhuber et al., 2000; Mudd et al., 2001; Seshadri et al., 2002). In recent years, the mechanisms of Hcy-induced neural damage have been explored. It has been shown that Hcy inhibits Na^+K^+-ATPase activity (Matte et al., 2004; Streck et al., 2003b; Wyse et al., 2002), decreases energy metabolism (Streck et al., 2003a,c) and induces oxidative stress (Kruman et al., 2000). Moreover, Hcy activates an apoptotic cell death pathway in cultured rat hippocampal neurons (Kruman et al., 2000) and its metabolite, homocysteic acid, can induce cell death in the rat hippocampus *in vivo* (Langmeier et al., 2003). Despite the efforts to understand the molecular basis of the neurological dysfunction accompanying homocystinuria, the effects of high Hcy levels on the cytoskeleton of neural cells is poorly known.

Intermediate filaments (IFs) form extensive networks within the cytoplasm in most vertebrate cells. These networks extend

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radially in all directions coordinating cytoskeletal activities and relying information between the cell surface and the innermost compartments of the cell (Chang and Goldman, 2004). Neurofilaments (NFs) are highly conserved neuronal IF expressed in adult neurons, characterized on the basis of molecular weight—a low molecular weight 68-kDa isoform (NF-L), a middle molecular weight 160-kDa isoform (NF-M), and a highly phosphorylated high molecular weight 200-kDa isoform (NF-H). Lee et al. (1993) have demonstrated that despite the ability of NF-L alone to assemble into short filaments *in vitro*, NF-L cannot form filament arrays *in vivo*, requiring both NF-L and either NF-M or NF-H. While the exact etiology of neurofilament (NF) aggregate formation remains to be determined, alterations in the stoichiometry of NF in a variety of motor neuron degenerations are associated with NF aggregate formation (Ge et al., 2003; Lariviere and Julien, 2004). Despite NFs have been universally considered to be composed of three subunits, experimental evidence demonstrate that the composition of NF could include other proteins. In this context, α-internexin has been described to coassemble with all three NF proteins into a single network in optic axons of adult mice (Yuan et al., 2006). Glial fibrillary acidic protein (GFAP) is an IF protein that is known to be localized in astrocytes, although its precise contributions to astroglial physiology and function are not clear. Typically, the role of IF scaffolding is to impart resistance to incident mechanical stress, and loss of function or mutations of numerous IF genes have been linked to several disease states involving cellular fragility (Coulombe et al., 2001; Fuchs and Cleveland, 1998; Herrmann and Aebi, 2004; Omary et al., 2006). IF proteins are known to be phosphorylated on their head and tail domains and the dynamics of phosphorylation/dephosphorylation play a major role in regulating the structural organization and function of IFs in a cell- and tissue-specific manner (Grant and Pant, 2000; Helfand et al., 2004; Jones and Williams, 1982; Julien and Mushynski, 1982; Ksiezak-Reding and Yen, 1987; Nixon and Lewis, 1986; Nixon and Sihag, 1991; Omary et al., 2006). There are increasing evidence that site-specific phosphorylation of IF proteins can affect their assembly, and structural organization (Sihag et al., 2007).

We have shown that 100 μM Hcy, a plasma concentration related with mild hyperhomocysteinemia, induced IF hyperphosphorylation in hippocampal slices of 17-day-old rats and this effect involved the activation of NMDA receptors, voltage-dependent calcium channels, Gi-coupled receptors and the stimulation of $^{45}Ca^{2+}$ uptake (Loureiro et al., 2008). In agreement with this study, several mechanisms of toxicity have been reported including NMDA ionotropic receptor (iGluR) and group I metabotropic glutamate receptor (mGluR) mediated neurotoxicity (Ho et al., 2002; Zieminska et al., 2003). Animal models were used to better understand the pathophysiology of the diseases. In this context, Streck et al. (2002) have developed a chemical experimental rat model of hyperhomocysteinemia in which the plasma Hcy concentration is increased to levels that are similar to those found in human homocystinuria (Mudd et al., 2001). Thus, using this experimental model, we investigated the expression of NF and GFAP subunits as well as the immunoreactivity of these proteins in the high-salt Triton X-100 insoluble and soluble fractions, representing respectively the ratio of polymerized/aggregated and soluble IF subunits in cerebral cortex and hippocampus of rats chronically injected with Hcy. Taking into account that the structural organization of the IF network is spatially and temporally regulated by phosphorylation (Janosch et al., 2000), we also investigated the effect of *in vitro* exposure of tissue slices to 500 μM Hcy, a concentration described to induce neural injury (Ferlazzo et al., 2008) on the IF-associated phosphorylating system and some molecular mechanisms that could support the alterations observed in the chemically induced hyperhomocysteinemia.

2. Experimental procedures

2.1. Radiochemical and compounds

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil, D–L homocysteine, D-2-amino-5-phosphonopentanoic acid (D,L AP5), verapamil hydrochloride, dantrolene, calyculin A, okadaic acid, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, antibodies anti-NF-200 (clone N-52 and NE14), anti-NF-150 (clone NN-18), anti-NF-68 (clone NR-4), anti-GFAP (clone G-A-5) and peroxidase conjugated rabbit anti-mouse IgG were obtained from Sigma (St. Louis, MO, USA). The potent competitive non-NMDA iGluR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the non-selective group I/group II mGluR antagonist (R,S)-a-methyl-4-carboxyphenylglycine (MCPG) were purchased from Tocris Neuramin (Bristol, UK). FK506 was obtained from Calbiochem (La Jolla, CA, USA). TRIzol Reagent, SuperScript-II RT pre-amplication system and Platinum Taq DNA polymerase were from Invitrogen. SYBRgreen was from Molecular Probe. The chemiluminescence ECL kit was obtained from Amersham (Oakville, Ontario, Canada). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats (9, 12, 17, 21 and 29 days of age) from our breeding stock had free access to water and a 20% (w/w) protein commercial chow. They were maintained on a 12:12 h light/dark cycle and temperature of 22 ± 1 °C. The "Principles of Laboratory Animal Care" (NHI publication no. 85–23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre.

2.3. Experimental approaches

2.3.1. In vivo homocysteine treatment

For the *in vivo* study animals were chronically injected with Hcy from the 6th to the 28th day. Hcy was dissolved in saline buffer and the pH adjusted to 7.4 and was administered subcutaneously twice a day at 8 h intervals. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. Hcy doses were calculated from pharmacokinetic parameters previously determined (Streck et al., 2002). During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. One hour after the last injection, the rats were killed by decapitation without anaesthesia. Treated animals achieved maximal plasma Hcy concentrations (0.40–0.50 mmol/l) 15 min after subcutaneous injection of Hcy, according to Streck et al. (2002). Cerebral cortex and hippocampus were dissected onto Petri dishes, placed on ice and cut into 400 μm thick slices with a McIlwain chopper and phosphorylation assays were carried out as described below.

2.3.2. In vitro homocysteine incubation

For the *in vitro* studies, rats of 9, 12, 17, 21 and 29 days of age were killed by decapitation, the cerebral cortex and hippocampus were dissected onto Petri dishes, placed on ice and cut into 400 μm thick slices with a McIlwain chopper. Slices were initially preincubated as described below. After preincubation, 100, 200, 300, 400 or 500 μM Hcy was added to the incubation medium and phosphorylation assays were carried out as described below.

2.4. Preincubation of tissue slices

Tissue slices from the *in vivo* and *in vitro* treatments were initially preincubated at 30 °C for 20 min in a Krebs–HEPES medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na–HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin.

2.5. ^{32}P-orthophosphate incorporation

(a) *In vivo* model: after preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate.
(b) *In vitro* model: after preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate in the presence or absence of 100, 200, 300, 400 or 500 μM Hcy with or without addition of verapamil, dantrolene, DL-AP5, MCPG, CNQX, okadaic acid, calyculin A or FK506 when indicated.

The labelling reaction was normally allowed to proceed for 30 min at 30 °C and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, 50 mM Tris–HCl), pH 6.5, and the protease inhibitors described above. Slices were then washed twice with stop buffer to remove excess radioactivity.