Neuroscience 145 (2007) 873–886

SUSTAINED METABOLIC INHIBITION INDUCES AN INCREASE IN THE CONTENT AND PHOSPHORYLATION OF THE NR2B SUBUNIT OF *N*-METHYL-D-ASPARTATE RECEPTORS AND A DECREASE IN GLUTAMATE TRANSPORT IN THE RAT HIPPOCAMPUS *IN VIVO*

A. CAMACHO, T. MONTIEL AND L. MASSIEU*

Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, AP 70-253, México D.F. CP.04510, Mexico

Abstract—The concentration of glutamate is regulated to ensure neurotransmission with a high temporal and local resolution. It is removed from the extracellular medium by high-affinity transporters, dependent on the maintenance of the Na^+ gradient through the activity of Na^+,K^+-ATPases. Failure of glutamate clearance can lead to neuronal damage, named excitotoxic damage, due to the prolonged activation of glutamate receptors. Severe impairment of glycolytic metabolism during ischemia and hypoglycemia, leads to glutamate transport dysfunction inducing the elevation of extracellular glutamate and aspartate, and neuronal damage. Altered glucose metabolism has also been associated with some neurodegenerative diseases such as Alzheimer's and Huntington's, and a role of excitotoxicity in the neuropathology of these disorders has been raised. Alterations in glutamate transporters and *N*-methyl-D-aspartate (NMDA) receptors have been observed in these patients, suggesting altered glutamatergic neurotransmission. We hypothesize that inhibition of glucose metabolism might induce changes in glutamatergic neurotransmission rendering neurons more vulnerable to excitotoxicity. We have previously reported that sustained glycolysis impairment *in vivo* induced by inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), facilitates glutamate-mediated neuronal damage. We have now investigated whether this facilitating effect involves altered glutamate uptake, and/or NMDA receptors in the rat hippocampus *in vivo*. Results indicate that metabolic inhibition leads to the progressive elevation of extracellular glutamate and aspartate levels in the hippocampus, which correlates with decreased content of the GLT-1 glutamate transporter and diminished glutamate uptake. In addition, we observed increased Tyr^{1472} phosphorylation and protein content of the NR2B subunit of the NMDA receptor. Results suggest that moderate sustained glycolysis inhibition alters glutamatergic neurotransmission.

Key words: excitotoxicity, glutamate uptake, glutamate receptors, glycolytic metabolism, neurodegenerative diseases.

Glutamate uptake after its synaptic release stimulates glucose transport and its rate of oxidation in astrocytes (Hyder et al., 2006; Loaiza et al., 2003; Rothman et al., 2003; Swanson et al., 1994; Voutsinos-Porche et al., 2003). Administration of antisense oligonucleotides targeted to the glial glutamate transporter GLAST, or inhibition of glutamate transporters, prevents the stimulation of the glycolytic pathway mediated by excitatory amino acids (EAA) (Cholet et al., 2001; Debernardi et al., 1999). On the other hand, severe glycolysis inhibition impairs glutamate transport or even induces its reversed operation releasing glutamate to the extracellular space (Gemba et al., 1994; Ogata et al., 1995). These observations suggest that glycolytic metabolism is involved in the regulation of glutamate removal after its synaptic release, and as a consequence in the prevention of excitotoxicity.

We have shown previously that blockade of glutamate uptake *in vivo* does not induce neuronal death, despite increasing the extracellular levels of EAA (Massieu et al., 1995). However, when glycolytic metabolism is continuously inhibited by the systemic administration of iodoacetate (IOA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glutamate-mediated neuronal damage is enhanced (Massieu et al., 2000, 2003). In agreement, *in vitro* data show exacerbation of glutamate toxicity during glucose deprivation or glycolysis inhibition (Novelli et al., 1988; Zeevalk and Nicklas, 1992; Rego et al., 1999). However, the molecular mechanisms underlying this effect are not completely understood.

Decreased glucose metabolism is related to aging (Eberling et al., 1995; Moeller et al., 1996) and chronic neurodegenerative diseases (Mielke et al., 1998; Planel et al., 2004; Slosman et al., 2001; Stein et al., 1998). In addition, a moderate reduction in GAPDH activity is observed in brain and fibroblasts from Alzheimer's and Huntington's disease patients (Kish et al., 1998; Mazzola and Sirover, 2001). Moderate but progressive alterations in energy metabolism during the lifetime of patients might predispose neurons to excitotoxicity. In fact, an excitotoxic component has been suggested in the mechanism of neurodegeneration associated with these disorders (Schwarcz et al., 1983; Young et al., 1988; Csernansky et al., 1996). On the other hand, decreased glutamate uptake and expression levels of glutamate transporters have been observed in patients from Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Arzberger et al., 1997; Ferrarese et al., 2000; Heath and Shaw, 2002; Zoia

*Corresponding author. Tel: +52-55-5622-5761; fax: +52-55-5622-5607.
E-mail address: lmassieu@ifc.unam.mx (L. Massieu).

Abbreviations: DHK, dihydrokainic acid; EAA, excitatory amino acids; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IOA, iodoacetate; NMDA, *N*-methyl-D-aspartate; PBS-T, phosphate buffer 0.1 M, NaCl 0.9%, Tween 20 0.1%; RK, Ringer-Krebs; RKC, Ringer-Krebs containing choline chloride.

0306-4522/07$30.00+0.00 © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2006.12.069

et al., 2004). Similar results have been observed in transgenic mice of Huntington's disease (Behrens et al., 2002; Lievens et al., 2001; Shin et al., 2005). In addition to impaired glutamate removal, changes in the expression levels of NMDA receptor subunits might contribute to excitotoxicity. The presence of the NR2B subunit might be particularly relevant, because receptors containing this subunit show slower deactivation kinetics and high calcium permeability (Cull-Candy and Leszkiewicz, 2004; Monyer et al., 1994; Vicini et al., 1998). Altered expression of NMDA receptor subunits has been reported in postmortem tissue from Alzheimer's and Huntington's patients (Arning et al., 2005; Arzberger et al., 1997; Bi and Sze, 2002; Mishizen-Eberz et al., 2004). Furthermore, in a transgenic mouse model of Huntington's disease, increased vulnerability to excitotoxicity and enhanced amplitude of NMDA-mediated currents was recently reported (Li et al., 2003; Zeron et al., 2002). Moreover, cells expressing Huntingtin and the NR1/NR2B subunits show increased excitotoxic cell death (Zeron et al., 2001).

We have investigated whether enhancement of excitotoxic neuronal death during sustained glycolysis inhibition *in vivo*, involves changes in the extracellular levels of EAA, glutamate uptake and protein content of glutamate transporters and/or NMDA receptor subunits, in the rat hippocampus.

EXPERIMENTAL PROCEDURES

Microdialysis

Male Wistar rats (250–300 g) were used throughout the study. They were handled according to the National Institutes of Health Guide for Care and Use of Laboratory animals and the local Animal Care Committee approved all animal treatments. All efforts were made to minimize the number of animals used and their suffering. Animals were slightly anesthetized with ether before kill, and all microdialysis experiments were performed in anesthetized animals. Eight days before initiating the experiments rats were anesthetized with halothane (1.5%) and fixed on a stereotaxic frame for unilateral implantation of a guide cannula into the CA1 hippocampal region (coordinates: AP −3.6 from bregma, L +2.0 from midline, and V −2.0 from dura, according to Paxinos and Watson, 1986). The guide cannula was fixed to the skull with dental acrylic cement.

Seven days later, microdialysis experiments were initiated and carried out during the five following days. On the first day rats received an i.p. vehicle injection (phosphate buffer 10 mM), they were anesthetized and the microdialysis probe was inserted into the hippocampus in order to record extracellular amino acids basal values. During the three following days rats received an i.p. injection of the glycolysis inhibitor, IOA (15 mg/kg per day), and on the fifth day rats received again an i.p. vehicle injection. Microdialysis was performed daily 1 h after the corresponding i.p. injection. Probes were continuously perfused with a Ringer-Krebs medium (RK) containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, at a flux rate of 2 μl/min via a microinjection pump (BAS MD 1001, West Lafayette, IN, USA). After a 90-min equilibration period, three 25-μl fractions were collected and amino acids levels were determined as described below. At day 5, 24 hours after the end of the microdialysis experiment, animals were transcardially perfused for histological analysis.

In vitro amino acid membrane recovery was determined according to Massieu et al. (1995) and was calculated to be around 7–11%. Data represent values calculated from HPLC amino acid determination without correcting for membrane recovery since it was not determined *in vivo*.

Quantification of amino acid levels

Amino acid content in dialysates was determined by HPLC according to Antoine et al. (1999), with slight modifications as reported previously (Montiel et al., 2005). Briefly, the 25-μl collected fractions were derivatized with the same volume of *o*-ophthalaldehyde and 3 min later a 10-μl volume of this mixture was injected into an HPLC system (Waters 600, Milford, MA, USA) equipped with an ODS column (25 cm×4 mm internal diameter, Supelco, Inc., Bellefonte, PA, USA). The mobile phase consisted of 18% methanol: 22% acetonitrile: 14% isopropanol: 46% phosphate buffer (60 mM, pH=6.65)/phosphate buffer (60 mM pH=6.65) and a flux rate of 1 ml/min was used in a linear gradient of 33 min duration from 10 to 90% solvent mixture. Amino acid concentration was calculated by comparison with a standard mixture of amino acids equally processed. Values from the three fractions collected daily were pooled. Data in Table 1 and Fig. 2 represent means±S.E.M. of the number of animals indicated in figure legends.

Histological analysis

In order to verify the site of the probe insertion 24 hours after the end of the microdialysis experiment animals were transcardially perfused under deep anesthesia with 200 ml 0.9% saline followed by 200 ml 5% formaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were removed and left in 5% formaldehyde for additional 24 h. They were transferred to 10, 20 and 30% sucrose (24 h each) and 40-μm sections were obtained in a cryostat and stained with Cresyl Violet.

Determination of GAPDH activity

Rats were treated either with vehicle (phosphate buffer 10 mM) or IOA (15 mg/kg) during one, two or three consecutive days. Two hours after each injection and 24 h after the latest, the animals were ether-anesthetized, killed by decapitation and the hippocampus extracted. The left hippocampus was used for immunoblot analysis (see below) and the right hippocampus for the determination of GAPDH activity. This was determined as previously reported with minor modifications (Ikemoto et al., 2003; Montiel et al., 2006). Tissue was homogenized in 1:10 (wt/vol) Tris–HCl pH 8.5 and GAPDH activity was monitored in a reaction mixture (1 ml total volume) containing (in mM): 1.7 arsenic acid, 20 sodium

Table 1. Basal levels (μM) of glutamine, taurine, alanine and glycine in the hippocampus and their changes induced by IOA treatment

Amino acid	Basal level	Peak concentration during IOA treatment	Level 24 h after IOA treatment
Glutamine	58.55±4.32	64.14±7.14	61.98±6.69
Taurine	33.31±5.05	32.95±2.67	34.48±4.47
Alanine	18.41±1.93	24.16±3.39	18.56±1.88
Glycine	41.62±5.18	59.31±7.57	62.76±13.9
GABA	0.326±0.03	0.369±0.09	0.291±0.03

Amino acid levels were determined in collected microdialysates by HPLC as described in the experimental procedures. Levels at peak represent the maximal amino acid concentration observed during IOA treatment. Data are means±SEM from 5–7 animals.