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Insulin-dependent regulation of GLAST/EAAT1 in Bergmann glial cells

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ABSTRACT

Glutamate is the major excitatory neurotransmitter in the central nervous system. Ionotropic and metabotropic glutamate receptors are present in neurons and glial cells and are involved in gene expression regulation. A family of sodium-dependent glutamate transporters carries out the removal of the neurotransmitter from the synaptic cleft. In the cerebellum, the bulk of glutamate transport is mediated through the excitatory amino acids transporter 1 (EAAT1/GLAST) expressed in Bergmann glial cells. Proper transporter function is critical for glutamate cycling and glucose turnover, as well as prevention of excitotoxic insult to Purkinje cells. In order to gain insight into the regulatory signals that modify this uptake activity, we investigated the effects of insulin exposure. Using the well-defined chick cerebellar Bergmann glial cell culture model, we observed a time and dose-dependent decrease in [^{3}H]-D-aspartate uptake. As expected, this effect is mimicked by the tyrosine phosphatase inhibitor sodium orthovanadate, suggesting a receptor-mediated effect. Equilibrium [^{3}H]-D-aspartate binding experiments as well as a reverse transcriptase/polymerase chain reaction strategy demonstrated that the decrease in the uptake activity is related to reduced numbers of transporter molecules in the plasma membrane. Accordingly, the transcriptional activity of the chick *glast* promoter diminished upon insulin treatment. The present findings suggest the involvement of insulin in neuronal/glial coupling in the cerebellum.

L-Glutamate (Glu) is the main excitatory amino acid in the central nervous system (CNS) of vertebrates [34] and exerts its effects through specific membrane receptors expressed in neurons and glial cells. Glutamate receptors (GluRs) are classified as either ionotropic or metabotropic receptors. Ionotropic receptors are ligand-gated ion channels involved in fast synaptic transmission and include N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (KA) receptors. In contrast, metabotropic receptors are G-protein coupled receptors linked to phosphoinositide metabolism (group I) or inhibition of adenylate cyclase (groups II and III) [34].

Extracellular Glu concentrations are maintained below toxic levels by a family of Na^+-dependent excitatory amino acids transporters (EAATs) present in neurons and glial cells. Five different Glu transporters have been characterized: GLAST/EAAT1, GLT-1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5 [15,21]. Aside from preventing excitotoxic insult, Glu uptake stimulates glial lactate production [19], which is stoichiometrically coupled to the Glu/glutamine shuttle [28]. This observation has led to the proposal that upon local increases in glutamatergic activity, astrocytes feed neurons with lactate and provide them with glutamine, that is converted to Glu by glutaminase to replenish the synaptic vesicles [18]. The astrocyte/neuronal lactate shuttle implies that the release of lactate is via specific monocarboxylic transporters in glial cells and uptake occurs through similar transporters in the postsynaptic neuronal plasma membrane [1].

While GLT-1 is expressed throughout the CNS, GLAST is prominent in the cerebellum, and specifically in Bergmann glial cells (BGC) [5,27]. In fact, neither GLT-1 mRNA nor dihydrokainate-sensitive [^{3}H]-D-aspartate uptake activity is present in cultured chick BGC [22]. *In vivo*, BGC cells surround the glutamatergic synapses established between the terminals of the granule neurons (parallel fibers) and Purkinje cells in the molecular layer [11]. When Glu is released from the parallel fibers, BGC depolarizes and triggers a significant Ca^{2+} influx [16]. The biochemical transactions associated with this influx are suggested to be critical for neuronal/glial coupling [5,12,14].

Both insulin and functional insulin receptors are present in the CNS [24,25]. Insulin binds to specific and saturable receptors in glial cells [4]. Considering Glu uptake has a key role in brain energy

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metabolism and Glu cycling [18], and that insulin enhances glycogen synthesis in glial cells [10], we investigated the effects of insulin on Glu transport in the present study. Although similar studies have been undertaken [30], our use of cultured BGC allows interpretation of glial participation in glutamatergic neurotransmission [14].

Primary cerebellar BGC cultures were prepared from 14-day-old chick embryos as described previously [17]. Confluent monolayers were changed to a serum-free media 8 h before insulin or Glu treatment.

Uptake of a non-metabolizable analogue of Glu, [^{3}H]-D-aspartate (GE Healthcare, USA) was performed as detailed elsewhere [22]. Cells were solubilized with 0.1N NaOH and after determining protein amounts [3], radioactivity was measured by liquid scintillation counting.

Cell viability was assessed in the presence of the colorimetric substrate MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, USA). Non-stimulated or insulin-treated (Novo Nordisk A/S, Bagsvaerd, Denmark) BGC cultures were incubated with 40 μL of a 5 mg/mL MTT solution for 3 h at 37 °C. The purple insoluble precipitate was solubilized in isopropanol-HCl (4%) for measurement at 630 nm with a Dynatech MR400 spectrophotometer (Dynatech, USA).

For [^{3}H]-D-aspartate binding, untreated (control) or insulin-treated confluent BGC monolayers were incubated with [^{3}H]-D-aspartate (0.4 μCi/mL) for 1 h at 4 °C either in solution A (25 mM/L Hepes-Tris, 130 mM/L NaCl, 5.4 mM/L KCl, 1.8 mM/L $CaCl_2$, 0.8 mM/L $MgCl_2$, 33.3 mM glucose and 1 mM/L $NaHPO_4$, pH 7.4), or in solution A without Na^+ (NaCl was replaced with choline chloride), or in solution A with 100 μM D-aspartate. Monolayers were rapidly washed twice with solution A and solubilized with 0.1N NaOH. Non-specific binding was determined in the presence of 100 μM aspartic acid. [^{3}H]-D-aspartate binding to GLAST was defined as the difference between the specific binding in solution A containing normal Na^+ minus the specific binding in Na^+-free solution A.

Semi-quantitative RT-PCR was performed on total RNA isolated from treated cell monolayers using Trizol (GE Healthcare). First-strand cDNA was synthesized from 3 μg RNA using 200 U of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) and 40 pMol of oligo (dT) primer. Cycling conditions were: GLAST, 95 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min, for 25 cycles. The primers were sense: 5′ *GTCCTACCTCTGCTGTA* 3′; antisense: 5′ *CTCGGCATCCCTGTTCTT* 3′. The internal control S17 ribosomal protein mRNA [6] was co-amplified as previously described, and PCR products were resolved in 1.5% agarose gels stained with ethidium bromide.

Transient transfections were performed in 60% confluent BGC cultures using a calcium phosphate protocol [31] with 6 μg of p800GLASTCAT reporter plasmid (5′-non-coding region from the chick *glast* gene cloned into pCAT-BASIC vector; Promega, USA [12]). The transfection efficacy was close to 50% as determined by a transfection control (β-gal) in every cell batch. Insulin treatment (100 μM) was performed at 16 h post-transfection for 24 h.

For chloramphenicol-acetyl transferase assays, protein lysates were obtained by harvesting the cells in TEN buffer (40 mM/L Tris–HCl pH 8.0, 1 mM/L EDTA, 15 mM/L NaCl) followed by lysis from three freeze–thaw cycles in 0.25 mol/L Tris–HCl pH 8.0, and centrifugation at 12,000 × *g* for 3 min. Equal amounts of protein lysates (approximately 80 μg) were incubated with 0.25 μCi of [^{14}C]-chloramphenicol (50 mCi/mM, GE Healthcare) and 0.8 mM/L Acetyl-CoA (Sigma) at 37 °C. Acetylated protein forms were separated by thin-layer chromatography and quantified using a Typhoon PhosphoImager (GE Healthcare). Total CAT activity was calculated as the acetylated fraction corrected for activity in the pCAT-BASIC vector and expressed as relative activity to non-treated control cell lysates. All data are expressed as the mean ± S.E.M.

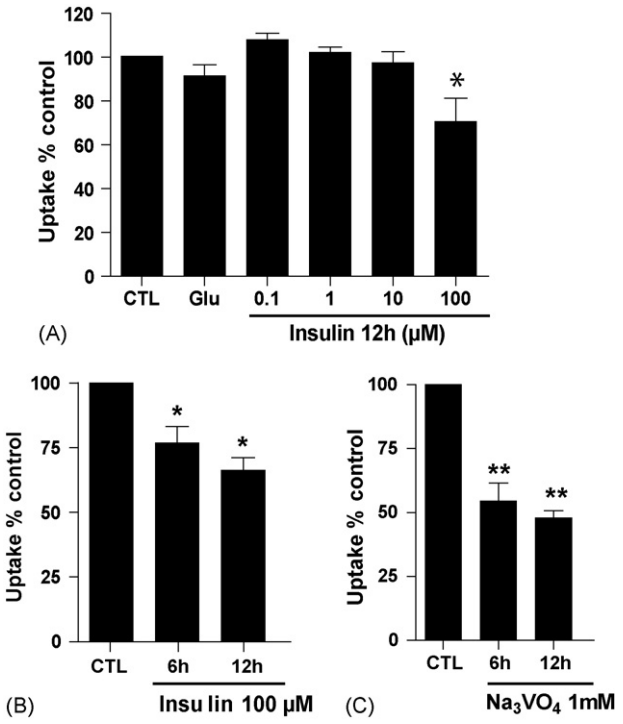

Fig. 1. Effect of insulin on Na^+-dependent aspartate uptake. (A) BGC cultured cells were incubated for 12 h at 37 °C in serum-free media with increasing concentrations of insulin (0.1–100 μM). Aspartate uptake activity was measured for 1 h in the presence of 0.4 μCi/mL [^{3}H]-D-aspartate. The time-dependent effect of insulin and sodium orthovanadate on [^{3}H]-D-aspartate uptake (performed as in A), was assessed in BGC monolayers incubated with 100 μM insulin (B) or with 1 mM sodium orthovanadate (C) for the indicated times in serum-free media. Mean values ±S.E.M. from three different experiments performed in quadruplicate. $^{**}P < 0.001$ *vs.* untreated, ANOVA and post-hoc Newman-Keuls tests (here and elsewhere ANOVA is stated).

For statistical analysis, one-way analysis of variance (ANOVA) was performed to determine the significant differences between conditions. Post-hoc Newman-Keuls multiple comparison tests were used to determine which conditions were significantly different from each other (Prism, GraphPad software, USA).

For electrophoretic mobility shift assays (EMSA), nuclear extracts were prepared as described [14]. All buffers contained a protease inhibitor cocktail to prevent nuclear factor proteolysis. Nuclear extracts (approximately 7.5 μg) from control or treated BGC were incubated on ice with 1 μg of poly [(dI-dC)] as non-specific competitor (GE Healthcare) and 1 ng of the following [^{32}P]-end labeled double stranded oligonucleotides:

RBPJk 5′ *CTAGGGTGTAAACACGCCGTGGGAAAAAATTTAT* 3′ [32]

YY1 5′ *CTAGAGGGTCTCCATTTTGAAGCGGG* 3′ [26]

Mixtures were incubated for 20 min on ice, then electrophoresed in 8% polyacrylamide gels with low ionic strength 0.5× TBE buffer. Dried gels were exposed to autoradiographic film.

Densitometry was performed by scanning bands with a Typhoon PhosphoImager and the images were quantified using ImageQuant software. Relative values denote data from treated cultures compared with data from untreated control cultures.

GLAST is the only Glu transporter expressed in cultured BGC [22]. Since Glu favors glucose uptake in these cells, we analyzed if insulin, another signal capable to induce glucose consumption, would modulate GLAST. To this end, we first assayed the insulin effect on [^{3}H]-D-aspartate uptake, a Glu non-metabolizable analogue. As shown in panel A of Fig. 1, a decrease in [^{3}H]-D-aspartate uptake was present after 12 h of insulin treatment. Short-term (minutes) insulin exposure did not modify the transport activity (not shown). We observed that treatment with 100 μM insulin