INCREASED GLUTAMATE RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF INSULIN INDUCED HYPOGLYCEMIC AND STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract—Hypoglycemia causes brain fuel deprivation, resulting in functional brain failure and brain death. It is a serious complication of insulin therapy in diabetic patients. A single intrafemoral dose of streptozotocin was administered to induce diabetes. Hypoglycemia was induced by appropriate doses of insulin s.c. in control and diabetic rats. Glutamate content and glutamate receptor kinetics were studied using [^{3}H]glutamate. [^{3}H]MK 801 was used to study the NMDA receptor kinetics. NMDA2B and metabotropic glutamate receptor (mGluR) 5 subunits receptor gene expressions were done using real time PCR. There was a significant ($P<0.001$) increase in the glutamate content in the cerebral cortex of hypoglycemic and diabetic rats when compared with control with more glutamate content in the hypoglycemic group. Scatchard analysis using [^{3}H]glutamate and [^{3}H]MK 801 in the cerebral cortex showed a significant ($P<0.001$) increase in the maximal binding (B_{max}) in both hypoglycemic and diabetic rats when compared with control with no significant change in equilibrium dissociation constant. The glutamate and NMDA receptor binding parameters were significantly ($P<0.001$) enhanced in the hypoglycemic rats compared with hyperglycemic rats. Real time PCR analysis also showed a significant increase ($P<0.001$) in the gene expression of NMDA2B and mGluR5 subunits of glutamate receptor. This increased gene expression of NMDA2B and mGluR5 glutamate receptor subunits confirmed the enhanced mRNA of receptor subunits and subsequently at the protein level from the receptor kinetic studies. The enhanced glutamate receptors were more prominent in hypoglycemic group which is of significance in this study. Up-regulation of glutamate leads to Ca^{2+} overload in cells, potentially leading to cell damage and death. This functional damage during hypoglycemia is suggested to contribute to cognitive and memory deficits which has immense clinical relevance in the therapeutic management of diabetes. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hypoglycemia, glutamate excitotoxicity, cerebral cortex, NMDA2B, mGluR5.

Hypoglycemia is a relatively common episode primarily affecting diabetic patients receiving treatment with insulin or other hypoglycemic drugs and patients suffering from insulinoma. Hypoglycemia impose alterations upon both the central (CNS) and peripheral (PNS) nervous systems. Hypoglycemia can lead to brain damage and long-term cognitive impairment (Wieloch, 1985; Gazit et al., 2003). The brain and other tissues require glucose in order to function properly. Glutamate is essential for synaptic communication in the CNS, but inadequate regulation of extracellular glutamate and glutamate receptor agonists can cause toxicity in the nervous system (Olney, 1989; Choi, 1992; Coyle and Puttfarcken, 1993; Greene and Greenamyre, 1996; Doble, 1999) leading to neurodegenerative disorders. Acute or chronic hypoglycemia leads to neurological dysfunction and injury. Prolonged insulin-induced hypoglycemia (IIH) causes widespread loss of neurons and permanent brain damage with irreversible coma (Kleihues et al., 1986). It impairs simple brain functions associated with the ability to meet memory and cognitive challenges. Children and adults exposed to hypoglycemia can develop long-term impairment of cognitive function (Blattner, 1968; Karp, 1989; Ryan et al., 1985; Hawdon, 1999; Vannucci and Vannucci, 2001) and are at risk of epilepsy (Kaufman, 1998). Severe hypoglycemia triggers a cascade of events in vulnerable neurons that may culminate in neuronal death even after glucose normalization. Glutamate receptor activation and excitotoxicity has long been recognized as an upstream event in this cascade (Wieloch, 1985). In brain, glutamate accumulation is reported to cause neuronal degeneration (Berman and Murray, 1996; Budd and Nichols, 1996; Atlante et al., 1997). The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA kainate (Choi, 1988). Thus hypoglycemia has become the limiting factor in achieving good glycemic control in patients with diabetes. Recent studies from our laboratory described the regulatory role of the neurotransmitters and their receptor subtypes in diabetes and pancreatic regeneration (Asha and Paulose, 1999; Jackson and Paulose, 2000; Renuka et al., 2004, 2005; Mohanan et al., 2005a,b, 2006; Ani et al., 2006; Eswar et al., 2006, 2007).

The cerebral cortex is the seat of our highest forms of intelligence. It plays a central role in many complex brain functions including memory, attention, perceptual awareness, thought, language, and consciousness. Glutamate triggers neuronal death when released in excessive concentrations by overexcitation of its receptors (Vizi, 2000). Glutamate receptor subtypes are critical in gating the plasticity and memory formation. NR2B is expressed selec-

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tively in the forebrain, with high levels in the cerebral cortex, hippocampal formation, septum, caudate-putamen, olfactory bulbs, and thalamus. An increased NMDA2B mRNA level was found in the postmortem brain of Huntington's disease patients showing neuronal degeneration due to glutamate excitotoxicity (Arzberger et al., 1997). Metabotropic glutamate receptors (mGluRs) have various functions on neuronal excitability in the CNS (Pin and Duvoisin, 1995). Group I mGluRs are positively coupled to phosphoinositide hydrolysis and the mobilization of intracellular Ca^{2+} leading to excitotoxic cell death. Metabotropic glutamate (mGlu) regulates synaptic glutamate release both *in vitro* (Herrero et al., 1994) rat brain slices (Croucher et al., 1997; Thomas et al., 2000) and *in vivo* (Patel and Croucher, 1997). The role of NMDA2B and mGluR5 receptors in hypoglycemic brain damage is not reported before. Our present studies on NMDA2B and mGluR5 receptor gene expressions will definitely enlighten the involvement of glutamate in diabetes and hypoglycemia. The present work was carried out to study the changes in the glutamate content, glutamate and NMDA receptor kinetics. Also, gene expression of glutamate receptor subunits NMDA2B and mGluR5 were carried out in the cerebral cortex of control, insulin-induced hypoglycemic and streptozotocin-induced diabetic rats.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats of 200–250 g body weight were purchased from Kerala Agriculture University, Thrissur, Kerala, India and Amrita Institute of Medical Sciences, Kochi, Kerala, India and used for all experiments, Kochi and used for all experiments. Animals were divided into the following groups as (i) control [C], (ii) diabetic [D], (iii) IIH in diabetic rats [diabetic+IIH] and (iv) IIH in control rats [control+IIH]. Each group consisted of six to eight rats. They were housed in separate cages under 12-h light/dark periods and were maintained on standard food pellets and water *ad libitum*. All animal care and procedures were in accordance with Institutional and National Institutes of Health guidelines. All efforts were made to minimize the number of animals used and their suffering.

Induction of diabetes and hypoglycemia

Diabetes was induced by a single intrafemoral dose (55 mg/kg body weight) of streptozotocin prepared in citrate buffer, pH 4.5 (Arison et al., 1967; Hohenegger and Rudas, 1971). The diabetic+IIH group received daily two doses (10 unit/kg body weight) and control+IIH group received daily two doses (1.5 unit/kg body weight) of regular human insulin (Actrapid) (Flanagan et al., 2003). Diabetic+IIH and control+IIH group had daily two episodes of IIH for 10 days. Control rats were injected with citrate buffer. Glucose was measured by GOD-POD glucose estimation kit (Biolab Diagnostics Pvt. Ltd.). The glucose was estimated at 0, 30, 60, 120, 180, 240, 300 min after the insulin administration.

Tissue preparation

The rats were maintained in hypoglycemic condition for 10 days and were killed on the 11th day by decapitation. The cerebral cortex was dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966). The tissues were stored at −70 °C until assay.

Quantification of glutamate

Glutamate contents in the cerebral cortex of experimental groups were quantified by displacement method using modified procedure of Enna and Snyder (1976). Tissue was homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris–HCl and 1 mM $MgCl_2$ buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 27,000×g for 15 min. The supernatant were pooled and used for the assay. The incubation mixture contained 1 nM [³H] glutamate (Amersham Biosciences, Little Chalfont, UK) with and without glutamate at a concentration range of 10^{-9} M to 10^{-4} M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for nmol/g wt. of the tissue.

Glutamate receptor binding studies using [³H] radioligand

Membranes were prepared according to the modified method of Timothy et al. (1984). Tissue was homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris–HCl and 1 mM $MgCl_2$ buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 1000×g for 15 min at 4 °C and the pellets were discarded. The supernatants were pooled and centrifuged at 27,000×g for 15 min. The resulting pellet was lysed in a 10 mM Tris–HCl buffer, pH 7.4, for 30 min and centrifuged at 27,000×g for 15 min. The resultant pellet was washed three times in 10 mM Tris–HCl buffer, pH 7.4, and centrifuged at 27,000×g for 15 min. All steps were carried out at 4 °C.

Membranes were incubated in 0.25 ml reaction mixture containing 25 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$ and 20 nM to 350 nM of [³H]glutamate containing 0.2 mg to 0.3 mg protein concentrations. Incubation was carried out at 30 °C for 15 min and the reaction was stopped by centrifugation at 27,000×g for 15 min. The pellet and the wall of the tube were quickly and carefully washed with ice-cold distilled water. SDS (0.1%) and scintillation liquid were added to the dry pellet and radioactivity incorporated was determined with a Wallac scintillation counter. All the assays were carried out in triplicate. Nonspecific binding was determined by adding 350 μM nonradioactive glutamate to the reaction mixture in a parallel assay. Specific binding was considered to be the difference between total and nonspecific binding.

NMDA receptor binding studies using [³H] MK 801

The membrane fractions were prepared by a modification of the method described by Hoffman et al. (1996). Brain cerebral cortex was homogenized in a 0.32 M sucrose buffer solution containing 10 mM Hepes/1 mM EDTA buffer (pH 7.0). The homogenate was centrifuged at 1000×g for 10 min, and the supernatant was centrifuged at 40,000×g for 1 h. The pellet was resuspended and homogenized in 10 mM Hepes buffer containing 1.0 mM EDTA (pH 7.0) and centrifuged at 40,000×g for 1 h. The final pellet was suspended in Hepes/EDTA buffer and stored at −80 °C until binding assays were performed. The [³H]MK-801 (Perkin Elmer Inc., Boston, MA, USA) binding saturation assay was performed in a concentration range of 0.5–50 nM at 23 °C in an assay medium containing 10 mM Hepes (pH 7.0), 200–250 μg of protein, glycine (100 μM), and glutamate (100 μM). Specific [³H]MK-801 binding was obtained by subtracting nonspecific binding in the presence of 100 μM unlabeled MK-801 from the total binding. After 1 h of incubation, the reaction was stopped by filtration through GF/B filters and washed with additional buffer. Bound radioactivity was counted with cocktail-T in a liquid scintillation counter (Wallac 1409). The nonspecific binding determined showed 20–30% in all our experiments.

The maximal binding (B_{max}) and equilibrium dissociation constant (K_d) were derived by linear regression analysis according to Scatchard (1949) by plotting the specific binding of the radioligand