Neuroscience 165 (2010) 28–38

SELECTIVE VULNERABILITY OF BRAIN REGIONS TO OXIDATIVE STRESS IN A NON-COMA MODEL OF INSULIN-INDUCED HYPOGLYCEMIA

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Abstract—Insulin-induced hypoglycemia causes the death of neurons in particular brain regions including the cerebral cortex, the striatum and the hippocampus, while the cerebellum and the brain stem are more resistant. The mechanisms underlying this selective vulnerability to hypoglycemic damage are unknown. In the present study we have analyzed the presence of lipoperoxidation products and nitrosilated protein residues in different rat brain regions during and after the induction of hypoglycemia. Insulin-injected hypoglycemic rats were sacrificed before the onset of the isoelectric period or infused with glucose to end hypoglycemia, and then sacrificed at different times. Increased lipoperoxidation levels were observed before the onset of the isoelectric period, while 3-nitrotyrosine (NT) residues in proteins and NT-positive cells were only observed after glucose reperfusion. These changes were found only in vulnerable brain regions, while none of them was evident in the cerebellum, suggesting a correlation between oxidative damage and vulnerability to hypoglycemic neuronal death in selective brain regions. Results suggest that a pro-oxidant state is promoted in certain brain regions during hypoglycemia and after the glucose reperfusion phase, which might result from the activation of several oxidative stress pathways and may be related to subsequent cell death.

Key words: cerebral cortex, lipoperoxidation, nitrotyrosines, oxidative damage.

Severe hypoglycemia is a serious complication of insulin therapy in diabetic patients exceeding insulin administration, and hypoglycemic episodes are frequent in many people with type 1 diabetes mellitus and advanced type 2 diabetes mellitus (Cryer, 2004). Furthermore, the ability to sense a reduction in blood glucose levels and the counterregulatory mechanisms responsible of its correction are impaired in patients with type 1 diabetes, which make them susceptible of suffering from hypoglycemia (Becker and Ryan, 2000; Jones and Davis, 2003; Cryer, 2006). Hypoglycemic episodes in diabetic patients might induce cognitive impairment in children (Ryan et al., 1985; Rovet et al., 1987; Jones and Davis, 2003; Naguib et al., 2009) and adults (Akyol et al., 2003; Carroll et al., 2003; Roberts et al., 2008), and in rodent models of hypoglycemia and type 1 diabetes hippocampal damage has been associated with impairment in learning and memory tests (Suh et al., 2003; Alvarez et al., 2009). Brain ischemia is another pathological condition associated with severe impairment of glucose brain supply due to the disruption of cerebral blood flow, and hypoglycemia has been suggested to be an important component of the ischemic neuropathology (Suh et al., 2008a).

Insulin administration in rodents is the most common experimental model of hypoglycemia (Auer et al., 1984a). In these animals, when blood glucose concentration is reduced to 20 mg/dl or less, brain activity ceases and the hypoglycemic coma (also known as isoelectric period) takes place. This period is terminated by the i.v. infusion of glucose. After 30–60 min of isoelectricity, neuronal damage develops in selective brain areas such as the cortex, the hippocampus and the caudo-putamen, while other areas like the cerebellum and the brain stem are more resistant (Auer et al., 1984b; Auer, 1986). However, the mechanisms underlying the differential brain vulnerability to hypoglycemic damage have not been elucidated. Pioneer studies associated hypoglycemic neuronal death with the massive release of the excitatory amino acids, aspartate and glutamate, accompanied by energy failure and ionic imbalance, early after the onset of the isoelectric period (Wieloch, 1984; Harris et al., 1984; Sandberg et al., 1986). However, the biochemical events preceding isoelectricity, which might contribute to the later development of selective brain damage, have not been completely elucidated. Data obtained from animals sacrificed before the onset of coma, reported no changes in brain ATP levels (Gorell et al., 1976), while increased aspartate levels in hippocampal and striatal nerve endings were found (Gundersen et al., 2001). On the other hand, Patočková et al. (2003) showed the presence of lipoperoxidation in mouse brain homogenates before the initiation of coma. However, whether these changes correlate with the regional brain vulnerability to hypoglycemic damage still remains unknown.

Oxidative stress is known to be present in different pathological conditions in the CNS such as ischemia and various neurodegenerative diseases (for review see: Margaill et al., 2005; Halliwell, 2006). The presence of oxidative stress during hypoglycemia has been recently sug-

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Abbreviations: EDTA, ethylenediaminetetracetic acid; EEG, electroencephalogram; FITC, fluorescein isothiocyanate; FJB, fluorojade B; GFAP, glial fibrillary acidic protein; NADPH, nicotinamide adenine dinucleotide phosphate; NT, 3-nitrotyrosine; PBS, phosphate buffer saline; ROS, reactive oxygen species; RR, righting reflex; SEM, standard error of the mean; TBARS, thiobarbituric acid-reactive substances.

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2009.10.003

gested (Patočková et al., 2003; Singh et al., 2004; Suh et al., 2007), although its temporality and regional distribution in brain have not been explored in detail. Recent studies show that oxidative stress develops mainly after the isoelectric period during the glucose perfusion phase, and that its presence is related to the subsequent death of neurons (Suh et al., 2007). However, it is not known whether oxidative stress can be triggered by severe hypoglycemia in the absence of isoelectricity, and if it is related to the vulnerability of certain brain regions to hypoglycemic damage. Thus, in the present study we have evaluated the presence of two oxidative stress markers, lipoperoxidation and 3-nitrotyrosine (NT) modified protein residues in a model of insulin-induced hypoglycemia without isoelectricity. The presence of these markers was evaluated at different times before the onset of the isoelectric period and after glucose reperfusion, in the vulnerable regions, hippocampus, cortex and striatum as well as in the more resistant region, the cerebellum. Results suggest a correlation between oxidative stress and selective brain vulnerability to hypoglycemic damage.

EXPERIMENTAL PROCEDURES

Male Wistar rats (320–380 g) obtained from the animal house at the Instituto de Fisiología Celular (Universidad Nacional Autónoma de México) were used throughout the study. They were handled according with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and with the local Animal Care Committee approval. All efforts were made to minimize the number of animals used and their suffering. Rats were housed under controlled conditions of temperature and light (12 h cycle) with *ad libitum* access to food and water, unless otherwise stated. To induce hypoglycemia rats were fasted overnight and received an i.p. injection of 30 U bovine insulin (Sigma, St. Louis, MO, USA) as described (Haces et al., 2008). Blood obtained from the tip of the tail was used to monitor blood glucose concentration with a glucometer (Abbott Laboratories, Bedford, MA, USA) at different intervals after insulin administration and before glucose infusion: 15 min, 1 h and 2.0–3.0 h (Fig. 2); every hour during the 3 h glucose infusion period (not shown in Fig. 2); and 0, 9, 21 and 45 h after the end of glucose infusion (corresponding to 6, 12, 24 and 48 h post-insulin in Fig. 2).

Interhemispheric electroencephalogram (EEG) recordings were obtained from rats (n=6) implanted with epidural electrodes

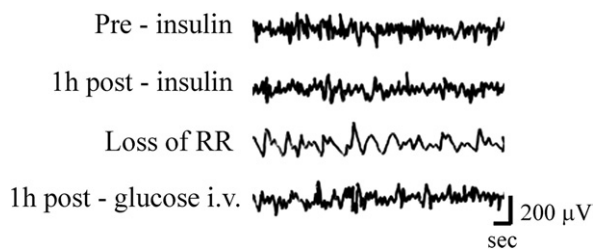

Fig. 1. Representative EEG recording showing the changes in brain electrical activity during hypoglycemia. Rats were implanted with epidural electrodes 1 week before insulin treatment. The day of the experiment a control EEG was obtained before insulin administration and electrical activity was followed until glucose infusion was concluded. At the time of the loss of the righting reflex (RR) high amplitude and low frequency waves were present. After glucose infusion the normal electrical activity is recovered.

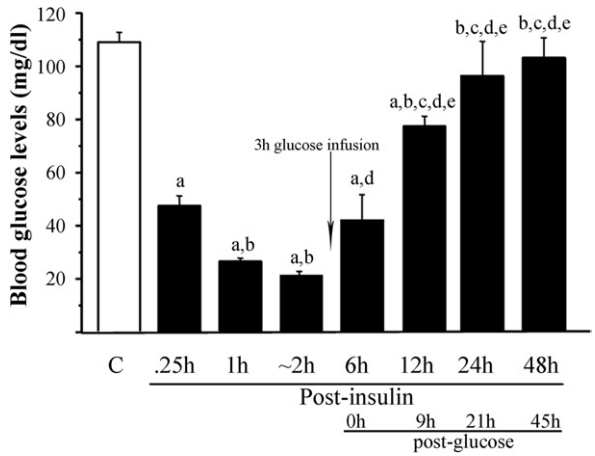

Fig. 2. Blood glucose levels in control and insulin-treated animals before and after glucose infusion. Black bars represent groups of animals that received only insulin or insulin+glucose infusion to prevent the isoelectric period. The white bar represents control animals. Samples were obtained at the indicated times (n=5–9 animals per group). [a] $P<0.05$ relative to control animals, [b] $P<0.05$ relative to 15 min after insulin administration, [c] $P<0.05$ relative to 1 h after insulin injection, [d] $P<0.05$ relative to the time of the loss of the RR (~2 h), [e] $P<0.05$ relative to insulin-treated animals analyzed 6 h after insulin administration.

1 week before insulin injection. The EEG was recorded before and after insulin injection and was followed until the end of glucose infusion. The EEG of the hypoglycemic animals exhibited all the progressive stages characteristic of hypoglycemia, as described by others (Auer et al., 1984a; Suh et al., 2003). Between 2 and 3 h after insulin injection, electrical brain activity was impaired, revealing an increase in the amplitude and a slowing of the theta (4–8 Hz) and delta (1–4 Hz) waves (compare lines 1 and 2 with line 3 in Fig. 1). At this stage animals are very drowsy and loose the righting reflex (RR), as previously reported (Gundersen et al., 2001). At this moment the rat hind limb is firmly hold, a polyethylene catheter is rapidly inserted through the femoral vein and fixed with adhesive tape for glucose infusion. This surgery takes about 2–5 min and glucose is infused through a perfusion pump (Harvard Apparatus 22, South Natik, MA, USA) (0.2 ml of 50% glucose followed by continuous infusion of 25% glucose, 1.5 ml/h for 3 h, as reported by Suh et al. (2004)). Animals were ambulatory about 20 min after the initiation of glucose administration, and recovery of normal brain electrical activity was observed 1 h after the loss of the RR (Fig. 1). When glucose infusion is finished and animals are completely recovered, the catheter is removed and the animals are left in separate cages. A 4.5±0.3 min (n=6) period elapsed between the loss of the RR and the i.v. glucose infusion. Only one out of six animals receiving glucose infusion showed an isoelectric period of 5.25 min characterized by the complete absence of electrical brain activity (flat or isoelectric EEG), while the rest of the animals never showed a flat EEG. EEG recordings were taken again 24 h later in order to verify the normal pattern of the EEG at this time (not shown). Afterwards, brains were extracted and sections obtained for Fluoro-Jade B (FJB) and Hoechst staining, and for glial fibrillary acidic protein (GFAP), NT and NeuN immunocitochemistry (see below).

Lipoperoxidation

In a separate group of animals we assessed the production of thiobarbituric acid-reactive substances (TBARS), which are products of lipid peroxidation, in tissue homogenates according to Gluck et al. (2000) as described in Haces et al. (2008). Animals were sacrificed at different times after insulin injection (0.25 and