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Insulin-induced recurrent hypoglycemia exacerbates diabetic brain mitochondrial dysfunction and oxidative imbalance

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ABSTRACT

Intensive insulin therapy can prevent or slow the progression of long-term diabetes complications but, at the same time, it increases the risk for episodes of severe hypoglycemia. In our study, we used a protocol intended to mimic the levels of blood glucose that occur in type 1 diabetic patients under an intensive insulin therapy. Streptozotocin (STZ)-induced diabetic rats were treated subcutaneously with twice-daily insulin injections for 2 weeks to induce hypoglycemic episodes. Brain cortical and hippocampal mitochondria were isolated and mitochondrial bioenergetics (respiratory chain and phosphorylation system) and oxidative status parameters (malondialdehyde (MDA) levels, mitochondrial aconitase activity and enzymatic and non-enzymatic antioxidant defenses) were analyzed. The protein levels of synaptophysin, a marker of synaptic integrity, and caspase 9 activity were also evaluated in cortical and hippocampal homogenates. Brain cortical mitochondria isolated from hyper- and recurrent hypoglycemic animals presented higher levels of MDA and α-tocopherol together with an increased glutathione disulfide reductase activity, lower manganese superoxide dismutase (MnSOD) activity and glutathione-to-glutathione disulfide (GSH/GSSG) ratio. No significant alterations were found in cortical mitochondrial respiratory chain and oxidative phosphorylation system. Hippocampal mitochondria from both experimental groups presented an impaired oxidative phosphorylation system characterized by a decreased mitochondrial energization potential and ATP levels and higher repolarization lag phase. In addition, higher MDA levels and decreased GSH/GSSG, α-tocopherol levels, and aconitase, glutathione peroxidase and MnSOD activities were observed in both groups of animals. Hippocampal mitochondria from recurrent hypoglycemic animals also showed an impairment of the respiratory chain characterized by a lower state 3 of respiration, respiratory control ratio and ADP/O index, and a higher state 4 of respiration. Additionally, a non-statistically significant decrease in synaptophysin protein levels was observed in cortical homogenates from recurrent hypoglycemic rats as well as in hippocampal homogenates from hyperglycemic and recurrent hypoglycemic rats. An increase in caspase 9 activity was also observed in hippocampal homogenates from hyperglycemic and recurrent hypoglycemic animals. Our results show that mitochondrial dysfunction induced by long-term hyperglycemic effects is exacerbated by recurrent hypoglycemia, which may compromise the function and integrity of brain cells.

Introduction

Diabetes mellitus (DM) is a metabolic disorder of carbohydrate metabolism resulting from inadequate insulin release, which characterizes type 1 diabetes (T1DM), or insulin insensitivity that occurs in type 2 diabetes (T2DM), both of which result in hyperglycemia if not controlled. Although T1DM accounts for only 5–10% of all diabetes cases, it represents a significant public health concern. T1DM begins early in life and leads to long-term complications in several body systems including cardiovascular, renal, and nervous systems (Modi, 2007). Intensive insulin therapy, the standard treatment for individuals with T1DM, aims to provide a tight glycemic control. However, insulin treatment is unable to fully compensate for the tightly regulated insulin secretion of a normally functioning pancreas

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and has the common side-effect of producing periodic hypoglycemic episodes (Leese et al., 2003; McNay, 2005).

Although still controversial, growing evidence suggests an association between T1DM and cognitive performance impairments that is reflected in a mild to moderate slowing of mental speed and a diminished mental flexibility (Brands et al., 2005; Jacobson et al., 2011). By depriving the brain of glucose, severe hypoglycemia can cause brain damage leading to long-term impairments in learning and memory (Auer, 2004). And so, in diabetic patients, there have been concerns about the effects of recurrent hypoglycemia and chronic hyperglycemia on cognitive function (Jacobson et al., 2011).

Mitochondria are essential organelles for neuronal function because the limited glycolytic capacity of these cells makes them highly dependent on aerobic oxidative phosphorylation for their energetic needs (Moreira et al., 2010). However, it has been established that reactive oxygen species (ROS) production is also inherent to mitochondrial oxidative metabolism (Adam-Vizi and Starkov, 2010; Sullivan et al., 2004). It has been described that DM leads to an oversupply of electrons in the mitochondrial electron transport chain that results in membrane hyperpolarization and ROS formation, mitochondrial energy metabolism dysfunction and oxidative stress being recognized as the main players in diabetes-related complications (Maiese et al., 2007; Moreira et al., 2009a). A recent study demonstrated that recurrent hypoglycemia exacerbated cerebral ischemic damage in type 1 diabetic rats, the increased mitochondrial ROS production being suggested as the possible cause of the ischemic damage exacerbation (Dave et al., 2011). A previous study from our laboratory showed that an acute episode of hypoglycemia potentiated lipid peroxidation and the imbalance of the antioxidant defenses occurring in brain mitochondria isolated from streptozotocin (STZ)-induced diabetic rats (Cardoso et al., 2010). As far as we know, no studies exist regarding the effects of insulin-induced recurrent hypoglycemia in brain mitochondrial function and oxidative status. Therefore, this work aimed to evaluate the effects of long-term hyperglycemia and recurrent hypoglycemia induced by insulin in brain cortical and hippocampal mitochondrial bioenergetics and oxidative status. Several parameters, namely, respiratory chain parameters [states 3 and 4 of respiration, respiratory control ratio (RCR), and ADP/O index], phosphorylation system [transmembrane potential ($\Delta\Psi_m$), ADP-induced depolarization, repolarization lag phase, ATP levels], malondialdehyde (MDA) levels, mitochondrial aconitase activity, and non-enzymatic [glutathione-to-glutathione disulfide (GSH/GSSG) ratio and α-tocopherol levels] and enzymatic antioxidant [glutathione peroxidase (GPx), glutathione disulfide reductase (GR) and manganese superoxide dismutase (MnSOD)] defenses, were evaluated. The levels of synaptophysin, a marker of synaptic integrity, and caspase 9 activity were also analyzed in brain cortical and hippocampal homogenates.

Materials and methods

Chemicals

Streptozotocin was obtained from Sigma (St. Louis, MO, USA). Insulin (Humulin NPH) was obtained from Eli Lilly and Company (USA). Anti-α-tubulin antibody was obtained from Cell Signaling (Danvers, MA, USA). Ac-LEHD-pNA was obtained from Calbiochem, Merck KGaA (Darmstadt, Germany). All the other chemicals were of the highest grade of purity commercially available.

Animals treatment

Male Wistar rats (2-month-old) were housed in our animal colony (Laboratory Research Center, Faculty of Medicine, University of Coimbra) and maintained under controlled light (12 h day/night cycle) and humidity with free access to water and powdered rodent chow (except in the fasting period). Rats were deprived of food overnight and randomly divided into two groups. One group received an intraperitoneal (i.p.) injection of STZ (50 mg/kg body weight) freshly dissolved in 100 mM citrate, pH 4.5. The volume administered was always 0.5 ml/200 g body weight. The control group received an i.p. injection with an equal volume of citrate (vehicle). In the following 24 h, animals were orally fed with glycosylated serum in order to avoid hypoglycemia resulting from the massive destruction of β-cells and release of intracellular insulin associated with STZ treatment (Moreira et al., 2005). Three days after STZ administration, the tail vein blood glucose levels were measured in all animals and those presenting levels above 250 mg/dl were considered diabetic. After 3 months of the induction of diabetes, the STZ-induced diabetic rats were randomly divided into two groups and one group was subjected to recurrent hypoglycemia achieved by twice-daily subcutaneous injections of insulin (dose adjusted to blood glucose levels) for 2 weeks. Animal handling and sacrifice followed the procedures approved by the Federation of European Laboratory Animal Science Associations (FELASA).

Determination of blood glucose and glycated hemoglobin levels

Blood glucose concentration was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer, Portugal). Hemoglobin A1C (HbA1c) levels were determined using Systems SYNCHRON CX 4 (Beckman). This system utilizes two cartridges, Hb and A1c to determine A1c concentration as a percentage of the total Hb. The hemoglobin is measured by a colorimetric method and the A1c concentration by a turbidimetric immunoinhibition method.

Preparation of mitochondrial fraction

Brain cortical and hippocampal mitochondria were isolated from rats according to Moreira et al. (2001, 2002). In brief, the rat was decapitated, and the cortex and hippocampus were rapidly removed, washed, minced, and homogenized at 4 °C in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml BSA, pH 7.4) containing 5 mg of bacterial protease type VIII (Subtilisin). Single brain homogenates were brought to 20 ml and then centrifuged at 2500 rpm (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 5 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 10,000 rpm for 10 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended again in 10 ml of the medium and centrifuged at 10,000 rpm for 5 min. The pellet was resuspended in 10 ml of a washing medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4) and centrifuged at 10,000 rpm for 5 min. The final mitochondrial pellet was resuspended in the washing medium and mitochondrial protein was determined by the biuret method calibrated with BSA (Gornall et al., 1949).

Mitochondrial respiration measurements

Oxygen consumption of mitochondria was registered polarographically with a Clark oxygen electrode (Estabrook, 1967) connected to a suitable recorder in a thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of standard respiratory medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes and 10 μM EGTA; pH 7.4) with 0.5 mg of protein. State 3 of respiration (consumption of oxygen in the presence of substrate and ADP) was initiated with ADP (75 nmol/mg protein). States 3 and 4 (consumption of oxygen after ADP phosphorylation) of respiration, respiratory control ratio (RCR = state 3/state 4), and ADP/O index (a marker of the mitochondrial ability to couple oxygen consumption to ADP phosphorylation during state 3 of respiration) were determined according to Chance and Williams (1956).