UCP2 and ANT differently modulate proton-leak in brain mitochondria of long-term hyperglycemic and recurrent hypoglycemic rats

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Abstract A growing body of evidence suggests that mitochondrial proton-leak functions as a regulator of reactive oxygen species production and its modulation may limit oxidative injury to tissues. The main purpose of this work was to characterize the proton-leak of brain cortical mitochondria from long-term hyperglycemic and insulin-induced recurrent hypoglycemic rats through the modulation of the uncoupling protein 2 (UCP2) and adenine nucleotide translocator (ANT). Streptozotocin-induced diabetic rats were treated subcutaneously with twice-daily insulin injections during 2 weeks to induce the hypoglycemic episodes. No differences in the basal proton-leak, UCP2 and ANT protein levels were observed between the experimental groups. Mitochondria from recurrent hypoglycemic rats presented a decrease in proton-leak in the presence of GDP, as specific UCP2 inhibitor, while an increase in proton-leak was observed in the presence of linoleic acid, a proton-leak activator, this effect being reverted by the simultaneous addition of GDP. Mitochondria from long-term hyperglycemic rats showed an enhanced susceptibility to ANT modulation as demonstrated by the complete inhibition of basal and linoleic acid-induced proton-leak caused by the ANT specific inhibitor carboxyatractyloside. Our results show that recurrent-hypoglycemia renders mitochondria more susceptible to UCPs modulation while the proton-leak of long-term hyperglycemic rats is mainly modulated by ANT, which suggest that brain cortical mitochondria have distinct adaptation mechanisms in face of different metabolic insults.

Keywords ANT · Cortical brain mitochondria · Long-term hyperglycemia · Proton-leak · Recurrent hypoglycemia · UCP2

Introduction

Coincident with the use of oxygen for aerobic respiration is the generation of reactive oxygen species (ROS), whose levels are controlled by endogenous cellular antioxidants to keep oxidative homeostasis. This is especially relevant at the mitochondrial level since the respiratory chain is the major site for ROS production (Naudi et al. 2012). Besides the antioxidant defense system, the respiratory chain itself can modulate mitochondrial ROS production through specific proteins (Leloup et al. 2011). Mitochondrial proton-leak is a basal or induced permeability of the mitochondrial inner membrane, resulting in partial dissipation of the transmembrane electrochemical gradient ($\Delta$Ψ$_m$) and uncoupling of substrate oxidation from ATP synthesis (Brookes 2005). Since mitochondrial-derived ATP is essential for cellular functions such uncoupling is often regarded as being detrimental. However, a growing body of evidence suggests that
a low level of proton-leak may have a cytoprotective role (Mattiaison et al. 2003; Teshima et al. 2003; Hoeter et al. 2004), possibly because mild $\Delta \Psi_m$ dissipation diminished ROS generation, and prevented mitochondrial Ca$^{2+}$ overload (Korshunov et al. 1997; Votyakova and Reynolds 2001; Miwa and Brand 2003). The initial connection between UCPs and ROS came from a study made by Nègre-Salvayre et al. (Negre-Salvayre et al. 1997) revealing that the inhibition of UCP1 activated the formation of ROS in brown fat mitochondria. Subsequent data suggested that UCP activity may lead to an increase in proton conductance through the interaction with superoxide anion ($O_2^•−$) (Echtay et al. 2002) or ROS products (Echtay et al. 2003). The suggestion that an increased ROS production would lead to mild-uncoupling that, in turn, would decrease ROS formation, placed neuronal UCPs as possible important brain damage modifiers in protecting against oxidative stress (Cardoso et al. 2011). Likewise, mitochondrial proton-leak mediated by ANT has been reported to occur in the presence of lipid peroxidation products leading to a feedback mechanism to protect against ROS damage (Echtay et al. 2003).

Chronic hyperglycemia is the hallmark of diabetes and associated complications. High glucose-induced excessive ROS production has been considered to play an important role in the onset and progression of diabetes mellitus (DM) (Rains and Jain 2011). DM is a complex metabolic disorder characterized by defects in the body’s ability to control glucose and insulin homeostasis (Rains and Jain 2011). Accumulating evidence shows that DM is often associated with complications in the central nervous system which can lead to cognitive deficits and dementia (Gispen and Biessels 2000). The increased risk for dementia in diabetic patients may result from direct effects of hyperglycemia, hypoglycemia and disrupted insulin signaling in the brain or indirect ischemic effects of diabetes-promoted cerebrovascular disease (Craft 2009; Wrighten et al. 2009). Despite the presence of three UCPs isoforms in the brain, UCP2 has been the major focus of studies concerning brain damage and neurodegeneration. It was previously demonstrated that an increase in brain UCP2 expression was correlated with the survival of cortical neurons in conditions of oxygen and glucose deprivation, a situation that was associated with a decrease in mitochondrial ROS production (Mattiaison et al. 2003). Despite this evidence, the role of UCP2 in diabetes-induced brain damage remains elusive.

In the present study, our main goal was to characterize the proton-leak of brain cortical mitochondria, through UCP2 and ANT modulation, exposed to insulin-induced recurrent hypoglycemia and/or long-term hyperglycemia, conditions that occur in type 1 diabetic patients under insulin therapy.

### Materials and methods

#### Chemicals

Streptozotocin was obtained from Sigma (Portugal). Insulin (Humulin NPH) was obtained from Eli Lilly and Company (USA). Anti-UCP2 antibody was obtained from Calbiochem-EMD Chemicals (Gibbstown, USA). 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 were 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 were randomly divided in two groups. One group received an i.p. injection of STZ (50 mg/kg body weight) freshly dissolved in citrate 100 mM, pH4.5. The volume administered was always 0.5 ml/200 g animal 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 $\beta$-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-diabetic rats were randomly divided in two groups and one group was subjected to recurrent hypoglycemia achieved by twice-daily injections of insulin [s.c., dose adjusted to blood glucose levels] during 2 weeks. Animals 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 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 mitochondria were isolated from rats according to (Moreira et al. 2001, 2002). In brief, the rat was decapitated, and the cortex was 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, pH7.4) containing 5 mg of bacterial protease type II.