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# Insulin therapy modulates mitochondrial dynamics and biogenesis, autophagy and tau protein phosphorylation in the brain of type 1 diabetic rats

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ABSTRACT

The main purpose of this study was to examine whether streptozotocin (STZ)-induced type 1 diabetes (T1D) and insulin (INS) treatment affect mitochondrial function, fission/fusion and biogenesis, autophagy and tau protein phosphorylation in cerebral cortex from diabetic rats treated or not with INS. No significant alterations were observed in mitochondrial function as well as pyruvate levels, despite the significant increase in glucose levels observed in INS-treated diabetic rats. A significant increase in DRP1 protein phosphorylated at Ser616 residue was observed in the brain cortex of STZ rats. Also an increase in NRF2 protein levels and in the number of copies of mtDNA were observed in STZ diabetic rats, these alterations being normalized by INS. A slight decrease in LC3-II levels was observed in INS-treated rats when compared to STZ diabetic animals. An increase in tau protein phosphorylation at Ser396 residue was observed in STZ diabetic rats while INS treatment partially reversed that effect. Accordingly, a modest reduction in the activation of GSK3β and a significant increase in the activity of phosphatase 2A were found in INS-treated rats when compared to STZ diabetic animals. No significant alterations were observed in caspases 9 and 3 activity and synaptophysin and PSD95 levels. Altogether our results show that mitochondrial alterations induced by T1D seem to involve compensation mechanisms since no significant changes in mitochondrial function and synaptic integrity were observed in diabetic animals. In addition, INS treatment is able to normalize the alterations induced by T1D supporting the importance of INS signaling in the brain.



## 1. Introduction

Type 1 diabetes (T1D) is a metabolic disease that originates from the autoimmune destruction of β-cells due to lymphocytic infiltration of pancreatic islets, resulting in the permanent dependency of patients on exogenous insulin (INS) to survive [1].

Cognitive deficits, such as impaired learning, memory, problem solving, and mental flexibility have been recognized as being more common in T1D subjects than in the general population [2,3]. It has also been demonstrated that T1D exacerbates tau protein hyperphosphorylation and amyloid beta (Aβ) formation contributing to the deposition of neurofibrillary tangles and Aβ plaques, the two major pathological hallmarks of Alzheimer's disease (AD), in the amyloid precursor protein (APP) transgenic mice [4].

INS has been proven to exert a role in synaptic plasticity and memory consolidation through the modulation of the activity of excitatory and inhibitory receptors such as those for glutamate and GABA, and by triggering signal transduction cascades leading to the alteration of gene expression [5,6]. Furthermore, INS and insulin-like growth factors (IGFs) have been shown to protect neurons against Aβ toxicity [7,8]. Likewise, insulin reduced tau protein phosphorylation and promoted its binding to microtubules, the effects of INS being mediated through the inhibition of glycogen synthase kinase-3β (GSK-3β) via the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway [9].

Mitochondria account for more than 90% of the cellular energy production [10]. This bioenergetic production assumes its maximum importance in the brain since neurons have a high energy demand and a limited glycolytic capacity, making them highly dependent on aerobic oxidative phosphorylation [11].

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Mitochondrial network is maintained through the fine balance between mitochondrial fission and fusion. Mitochondrial fission is governed by dynamin-like protein 1 (DRP1), a large cytosolic GTPase that is recruited to the mitochondrial membrane upon a fission-like stimuli, and by Fis1, a small mitochondrial molecule located in the outer membrane [12]. In turn, mitochondrial fusion is directed by three large GTPases, Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), both located in the mitochondrial outer membrane, and optic atrophy 1 (OPA1) protein, located in the inner mitochondrial membrane [12].

Mitochondrial biogenesis results from an intricate crosstalk between both nuclear and mitochondrial genomes. The molecular machinery underlying mitochondrial biogenesis is constituted by the nuclear respiratory factor 1 (NRF 1) and nuclear respiratory factor 2 (NRF 2), which control the nuclear genes that encode mitochondrial proteins, and mitochondrial transcription factor A (TFAM) that drives transcription and replication of mitochondrial (mt) DNA [13,14]. The expression of NRF1, NRF2, and TFAM is regulated by the peroxisome proliferator activator receptor gamma-coactivator 1α (PGC-1α) [15].

Autophagy is an evolutionarily conserved housekeeping process that enables cells to get nutrients through the digestion of their own components and, at the same time, degrades misfolded proteins and aggregates, damaged organelles and invading microorganisms [16]. Autophagy is a tightly regulated process in a multistep manner. To the level of vesicle nucleation/initiation two main proteins are involved: the mammalian target of rapamycin (mTOR), which is an autophagic repressor; and Beclin 1, which is an autophagic inducer [17]. Regarding membrane elongation, cytosolic LC3-I is transformed to a membrane-bound form, LC3-II [17]. In the autophagic degradation of ubiquitinated protein aggregates in mammalian cells, LC3 interacts with p62, which is a ubiquitin-binding protein therefore being considered an autophagic substrate [17].

The main aim of this study was the evaluation of the effects of streptozotocin (STZ)-induced T1D and INS treatment on brain cortical mitochondria, autophagy and tau protein phosphorylation. We evaluated several mitochondrial parameters: respiration [respiratory control ratio (RCR), and ADP/O index], phosphorylation system [transmembrane potential (ΔΨm), ADP-induced depolarization, repolarization lag phase], fission/fusion protein levels (DRP1, Fis 1 and OPA1, MFN1, MFN2, respectively), and biogenesis (NRF1, NRF2, TFAM and the number of copies of mtDNA). Autophagy (mTOR, Beclin1, LC3 and p62 protein levels), the activity of several kinases and phosphatase 2A that modulate tau protein phosphorylation, activity of caspases 3 and 9 and protein levels of synaptophysin and PSD95 were also evaluated.

## 2. Material and methods

### 2.1. Reagents

STZ was obtained from Sigma Aldrich (St. Louis, MO, USA). INS (Humulin NPH) was obtained from Eli Lilly and Company (USA). All the chemicals used were of the highest grade of purity commercially available.

### 2.2. Animal housing and treatment

Seventeen male Wistar rats (2-month-old) purchased from Charles River were housed in our Animal Facility (Laboratory Research Center, Faculty of Medicine, University of Coimbra) and maintained under controlled light (12 h day/night cycle) and humidity with ad libitum 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 of eleven animals 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 (six animals) received an i.p. injection with an equal volume of citrate (vehicle solution). In the following 24 h, animals were provided with free access to glycosylated serum in order to avoid hypoglycemia resulting from the massive destruction of β-cells and consequent release of intracellular insulin associated with STZ treatment [18]. 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. Two months after the induction of diabetes with STZ, diabetic rats were randomly divided into two groups and one group of six animals was subjected to daily subcutaneous (s.c.) injections of INS, in order to lower the systemic levels of glucose (dose adjusted to blood glucose levels as follows: if blood glucose levels were ≤200 mg/dL, 2 U INS were administered to rats; if blood glucose levels were >200 mg/dL an extra 2 U INS per each 100 mg/dL blood glucose were given to rats), during one month. Three months after the induction of diabetes, the rats were sacrificed by cervical displacement and decapitation. Animal handling and sacrifice followed the procedures approved by the Federation of European Laboratory Animal Science Associations (FELASA).

### 2.3. Measurement of blood glucose and hemoglobin A1C levels

Blood glucose was determined immediately after sacrifice by a glucose oxidase reaction, using a glucometer (Glucometer-Elite, Bayer). 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.

### 2.4. Measurement of brain INS, glucose and pyruvate levels

Brain cortical tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing 0.1 M phenylmethylsulfonyl fluoride (PMSF), 0.2 M dithiothreitol (DTT), and protease and phosphatase inhibitors (commercial protease and phosphatase inhibitor cocktails from Roche Applied Science). The crude homogenate was incubated on ice for 15 min, frozen and defrozen 3 times to favor disruption, and centrifuged at 14000 rpm (Eppendorf centrifuge 5415C) for 10 min, at 4 °C, and the resulting supernatant was collected and stored at −80 °C.

INS quantification was performed using an ELISA kit (BertinPharma, France) according to the manufacturers' instructions. The principle of the kit is based on the competition between unlabeled rat insulin and acetylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific Guinea-Pig anti-rat insulin antiserum sites.

Glucose quantification was performed using a PicoProbe™ glucose fluorometric assay kit (BioVision, USA) according to manufacturers' instructions. The principle of the kit is based on the enzymatic oxidation of D-glucose to form a product which reacts with a colorless probe to generate fluorescence. The fluorescence generated is directly proportional to the amount of glucose present in the sample.

Pyruvate quantification was performed using a pyruvate colorimetric assay kit (BioVision, USA) according to the manufacturers' instructions. The principle of the kit is based on the enzymatic oxidation of pyruvate by pyruvate oxidase to generate color upon reaction with a pyruvate probe. The color intensity is proportional to pyruvate content; therefore the pyruvate concentration can be accurately measured.

### 2.5. Preparation of mitochondrial fractions

Brain cortical mitochondria were isolated from rats by the method of Moreira et al. [19], using 0.02% digitonin to allow the release of mitochondria from the synaptosomal fraction. Briefly, after animal decapitation, the cortex was immediately separated 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 the bacterial protease (Sigma). Single brain homogenates were brought to 30 ml and then centrifuged at 2500 rpm (Sorvall Evolution RC Superspeed