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**Cortical and hippocampal mitochondria bioenergetics and oxidative status during
hyperglycemia and/or insulin-induced hypoglycemia**

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Abstract

This study was undertaken to evaluate the effects of streptozotocin (STZ)-induced hyperglycemia and insulin-induced hypoglycemia in cortical and hippocampal mitochondria bioenergetics and oxidative status. For that purpose we used, citrate (vehicle)-treated Wistar rats, STZ-treated rats [i.p., 50mg/kg body weight] and STZ-treated rats injected with insulin [s.c., dose adjusted to blood glucose levels] 1h prior to sacrifice to induce an acute episode of hypoglycemia. Several parameters were analyzed: respiratory chain, phosphorylation system, thiobarbituric acid reactive substances (TBARS) levels, hydrogen peroxide (H₂O₂) production rate, and non-enzymatic and enzymatic antioxidant defenses. Cortical mitochondria from insulin-induced hypoglycemic rats present a significant decrease in the ADP/O index, a significant increase in the repolarization lag phase and a decrease in GSH/GSSG ratio when compared with STZ and control mitochondria. Both STZ-induced diabetes and insulin-induced hypoglycemia promote a significant increase in TBARS levels and a decrease in glutathione disulfide reductase activity. Diabetic cortical mitochondria present a significant decrease in glutathione peroxidase (GPx) activity compared to control mitochondria. In turn, insulin-induced hypoglycemia induced a significant increase in GPx and manganese superoxide dismutase (MnSOD) activities. In hippocampal mitochondria, insulin-induced hypoglycemia increases the respiratory control ratio whereas both situations, hyper- and hypoglycemia, potentiate H₂O₂ production and decrease the activity of MnSOD. These results suggest that the poor glycemic control that occurs in type 1 diabetic patients undergoing insulin therapy may have detrimental effects in brain areas involved in learning and memory.

Keywords: Cortex; Hippocampus; Hyperglycemia; Insulin-induced hypoglycemia; Mitochondria; Oxidative stress

1. Introduction

Diabetes mellitus is an endocrine disorder of carbohydrate metabolism resulting from inadequate insulin release (type 1 diabetes) or insulin insensitivity (type 2 diabetes) [1]. Type 1 diabetes represents a significant health concern, given that it begins early in life and leads to long-term complications including cardiovascular, renal and central nervous system (CNS) complications [2]. Management of type 1 diabetes requires special and continued insulin administration, blood glucose monitoring and meal planning [2]. Nevertheless, patients with type 1 diabetes experience daily fluctuations in blood glucose levels, ranging from low (hypoglycemia) to high (hyperglycemia) blood glucose levels [3].

In normal situations, glucose is the brain's main fuel and therefore because the brain is dependent on a continuous delivery of glucose, it cannot survive more than a few minutes of glucose deprivation [3]. Insulin therapy brings the risk of hypoglycemic brain injury that is the main obstacle occurring in insulin-dependent diabetic patients. Hypoglycemia, depending on its severity, can cause irritability, impaired concentration, neurological deficits, seizures, coma and, ultimately, neuronal death [4]. Hypoglycemic neuronal death is most prominent in neurons from specific brain regions such as in the hippocampus and superficial layers of the cortex and striatum [5-7], which may lead to deficits in memory and learning [8]. A previous epidemiological study reported that cognitive function in type 1 diabetic patients is generally well-maintained even at elevated blood glucose levels but during hypoglycemia significant decrements in several areas of cognition occur, namely those involved in associative learning skills [9]. Indeed, type 1 diabetic patients present impairments in memory, learning and cognition [10]. Moreover, data show that memory and learning processes are already affected after 20 days of diabetes [11] and that after 10 weeks of STZ-induced diabetes,

diabetic rats present an impaired performance in the Morris water maze test, which evaluate hippocampal-dependent learning [12].

Mitochondrial oxidative phosphorylation (OXPHOS) generates the energy necessary to fuel cellular function whereas the dysfunction of mitochondria leads to reduced ATP production, impaired calcium buffering and the formation of reactive oxygen species (ROS) [13,14]. Mitochondria are both sources and targets of ROS actions. Situations in which occurs a free radical overload will result in the damage of proteins, DNA and membrane phospholipids. The impairment of mitochondrial function together with increased oxidative stress has been implicated in the pathogenesis of a wide range of diseases including diabetes. Growing evidence show increased levels of lipid peroxidation and a decrease in antioxidant defenses in brain mitochondria from STZ-induced diabetic rats [15-17]. It was also shown that mitochondrial permeability transition pore opening and increased capacity to generate ROS are involved in the hypoglycemic-induced cell death [18,19,20].

In light of those findings, and due to the scarcity of information concerning mitochondrial bioenergetics and oxidative status in hyper- and/or insulin-induced hypoglycemic conditions, this study was undertaken to investigate how cortical and hippocampal mitochondria are affected by STZ-induced diabetes, a model of type 1 diabetes, and insulin-induced acute hypoglycemia. We evaluated respiratory parameters [states 3 and 4 of respiration, respiratory control ratio (RCR), and ADP/O index], phosphorylation system [transmembrane potential ($\Delta\Psi_m$), repolarization level, repolarization lag phase], thiobarbituric acid reactive substance (TBARS) levels, H_2O_2 production rate, non-enzymatic (GSH/GSSG ratio) and enzymatic antioxidant [glutathione peroxidase (GPx), glutathione disulfide reductase (GR) and manganese superoxide dismutase (MnSOD)] defenses.

2. Materials and Methods

Chemicals - STZ was obtained from Sigma (Portugal). Insulin (Actrapid Penfill) was obtained from Novo Nordisk A/S (Denmark). All the other chemicals were of the highest grade of purity commercially available.

Animals treatment - Male Wistar rats (3-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, pH 4.5. The volume administered was always 0.5ml/200g animal body weight. The control group received an i.p. injection with an equal volume of citrate (vehicle). In the following 24 hours, 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 [15]. 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 one month of the induction of diabetes, the STZ-diabetic rats were randomly divided in two groups and one group received a s.c. injection of insulin (adjusted to blood glucose levels; ≈ 30 U / >300 mg/dl) 1h prior to sacrifice to induce an acute episode of hypoglycemia. 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 CX4 (Beckman). This 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 - Briefly, after animal decapitation, hippocampus and cortex were rapidly separated and homogenized in 10 ml of homogenization medium (0.32 M sucrose, 10 mM HEPES, and 0.5 mM EGTA-K⁺, pH 7.4). The homogenate was centrifuged at 747 g (Sorvall Evolution RC Ultra Centrifuge) for 10 min and the supernatant was again centrifuged at 11952 g for 10 min. The pellet was resuspended in 10 ml of washing medium (0.32 M sucrose, 10 mM HEPES, pH 7.4) and centrifuged at 11952 g for 10 min. The final pellet was resuspended in 200 μ l of washing medium and mitochondrial protein was determined by the biuret method calibrated with BSA [21].

Mitochondrial respiration measurements - Oxygen consumption of the brain mitochondria was registered polarographically with a Clark oxygen electrode [22] 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 KH₂PO₄, 5 mM Hepes and 10 μ M EGTA; pH 7.4) with 0.8 mg of protein. Cortical and hippocampal mitochondria (0.8 mg/ml) were energized with 5 mM succinate (substrate of complex II) in the presence of

2 μM rotenone. State 3 of respiration were initiated with ADP (155 nmol/mg protein). Respiratory control ratios (RCR = state 3/state 4), respiratory states, and ADP/O ratios were determined according to Chance and Williams [23].

Mitochondrial membrane potential measurements - The transmembrane potential ($\Delta\Psi_m$) was monitored by evaluating transmembrane distribution of the lipophilic cation TPP^+ (tetraphenylphosphonium) with a TPP^+ -selective electrode prepared according to Kamo *et al.* [24] using an Ag/AgCl-saturated electrode (Tacussel, model MI 402) as reference. TPP^+ uptake has been measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Linear 1200 recorder. The voltage response of the TPP^+ electrode to $\log[\text{TPP}^+]$ was linear with a slope of 59 ± 1 , in a good agreement with the Nernst equation. Reactions were carried out in a chamber with magnetic stirring in 1ml of the standard medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4 , 5 mM Hepes and 10 μM EGTA; pH 7.4) containing 3 μM TPP^+ . This TPP^+ concentration was chosen in order to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria [25]. The $\Delta\Psi_m$ was estimated by the equation: $\Delta\Psi_m$ (mV) = $59 \log(v/V) - 59 \log(10^{\Delta E/59} - 1)$ as indicated by Kamo *et al.* [24] and Muratsugu *et al.* [26]. v , V , and ΔE stand for mitochondrial volume, volume of the incubation medium and deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation, and that the law of mass conservation is applicable. A matrix volume of 1.1 $\mu\text{l/mg}$ protein was assumed. No correction was made for the “passive” binding contribution of TPP^+ to the mitochondrial membranes, because the

purpose of the experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate a slight overestimation on $\Delta\Psi_m$ values. However, the overestimation is only significant at $\Delta\Psi_m$ values below 90 mV, therefore, far from our measurements. Cortical and hippocampal mitochondria (0.8 mg/ml) were energized with 5 mM succinate in the presence of 2 μ M rotenone in order to activate the mitochondrial electron transport chain. After a steady-state distribution of TPP⁺ had been reached (ca. 1 min of recording), $\Delta\Psi_m$ fluctuations were recorded.

Measurement of thiobarbituric acid reactive substances (TBARS) levels -

TBARS levels were determined by using the thiobarbituric acid assay (TBA), according to a modified procedure described by Ernster and Nordenbrand [27]. The amount of TBARS formed was calculated using a molar coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol TBARS/mg protein.

Measurement of hydrogen peroxide (H₂O₂) production rate -

H₂O₂ levels were measured fluorimetrically using a modification of the method described by Barja [28]. Briefly, cortical and hippocampal mitochondria (0.2 mg) were incubated at 37°C with 10 mM succinate in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH₂PO₄, 3 mM MgCl₂, 145 mM KCl, 30 mM Hepes, 0.1 mM homovalinic acid and 6 U/ml horseradish peroxidase. After 15 min, the reaction was stopped with 0.5 ml cold stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12). Fluorescence was determined at 312 nm excitation and 420 nm emission wavelengths. The H₂O₂ levels were calculated using a standard curve of H₂O₂ and expressed as pmol/mg protein/15 min.

Measurement of glutathione (GSH) and glutathione disulfide (GSSG) levels

- GSH and GSSG levels were determined with fluorescence detection after reaction of the supernatant containing $\text{H}_3\text{PO}_4/\text{NaH}_2\text{PO}_4$ -EDTA or $\text{H}_3\text{PO}_4/\text{NaOH}$, respectively, of the deproteinized homogenates solution with ophthalaldehyde (OPT), pH 8.0, according to Hissin and Hilf [29]. In brief, freshly isolated cortical and hippocampal mitochondria (1 mg) resuspended in 1.5 ml phosphate buffer (100 mM NaH_2PO_4 , 5 mM EDTA, pH 8.0) and 500 μl H_3PO_4 4.5% were rapidly centrifuged at 50 000 rpm (Beckman, TL-100 Ultracentrifuge) for 30 min. For GSH determination, 100 μl of supernatant was added to 1.8 ml phosphate buffer and 100 μl OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 nm and 350 nm emission and excitation wavelength, respectively. For GSSG determination, 250 μl of the supernatant were added to 100 μl of N-ethylmaleimide and incubated at room temperature for 30 min. After the incubation 140 μl of the mixture were added to 1.76 ml NaOH (100 mM) buffer and 100 μl OPT. After mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 nm and 350 nm emission and excitation wavelength, respectively. The GSH and GSSG levels were determined from comparisons with a linear GSH or GSSG standard curve, respectively.

Measurement of glutathione peroxidase (GPx) activity - GPx activity was determined spectrophotometrically at 340 nm by following the method of Flohé and Gunzler [30]. Briefly, the activity of GPx was measured upon a 5 min incubation, in the dark, of 0.1 mg of each sample with 0.5 mM phosphate buffer (0.25 M KH_2PO_4 , 0.25 M K_2HPO_4 and 0.5 mM EDTA, pH 7.0), 0.5 mM EDTA, 1 mM GSH and 2.4 U/ml

glutathione reductase. The quantification occurred after the addition of 0.2 mM NADPH and 1.2 mM tert-butyl hydroperoxide, at 30°C with continuous magnetic stirring, for 5 min, in a Jasco V560 UV/VIS Spectrophotometer. The measurements were made against blanks prepared in the absence of NADPH. GPx activity was determined using the molar extinction coefficient $6220 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol/min/mg protein.

Measurement of glutathione disulfide reductase (GR) activity - For the activity of GR, 0.1 mg of each sample were incubated for 1 min with 0.2 mM phosphate buffer (containing 0.2 M K_2HPO_4 and 2 mM EDTA, pH 7.0) and 2 mM NADPH. The measurements were made at 340 nm and initiated with the addition of 20 mM GSSG, at 30°C, with continuous magnetic stirring, for 4 min, against blanks prepared in the absence of GSSG, using a Jasco V560 UV/VIS Spectrophotometer [31]. GR activity was determined using the molar extinction coefficient $6220 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol/min/mg protein.

Measurement of manganese superoxide dismutase (MnSOD) activity - MnSOD activity was determined spectrophotometrically, at 550 nm [32]. After the incubation of 0.1 mg of protein with 0.07 mM of phosphate buffer (50 mM K_2HPO_4 and 100 μM EDTA, pH 7.8), 0.025 mM hypoxanthine, 0.025 Triton X-100, 0.1 mM nitrobluetetrazolium (NBT) and 1.33 mM KCN, the reaction was started with the addition of 0.025 U/ml xanthine oxidase, and the reaction was allowed to continue for 3 minutes at 25°C, with continuous magnetic stirring. The measurements were performed in a Jasco V560 UV/VIS Spectrophotometer, against a blank, prepared in the absence of hypoxanthine. The activity of MnSOD was calculated using a standard curve, prepared with different concentrations of superoxide dismutase.

Statistical analysis - Data were analyzed using SPSS 16.0 for Windows. Results are presented as mean \pm SEM of the indicated number of experiments. Statistical significance between groups was defined using the Mann-Whitney U-test. A p-value < 0.05 was considered significant.

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3. Results

Characterization of the experimental animals

STZ rats present significantly higher values of glycemia and glycosylated hemoglobin (HbA_{1C}) and a significant decrease in body weight when compared with control rats (Table 1), confirming the diabetic state of STZ animals. As expected, insulin-induced hypoglycemia decreased significantly blood glucose levels when compared with STZ and control rats and the glycosylated hemoglobin values are similar to those of STZ rats (Table 1).

Effects of diabetes and insulin-induced hypoglycemia in the respiratory chain and phosphorylation system of cortical and hippocampal mitochondria

$\Delta\Psi_m$ is fundamental for the phenomenon of OXPHOS, which results in the conversion of ADP to ATP via ATP synthase. Mitochondrial respiratory chain pumps H⁺ out of the mitochondrial matrix across the inner mitochondrial membrane. The H⁺ gradient originates an electrochemical potential (Δp) resulting in a pH (ΔpH) and a voltage gradient ($\Delta\Psi_m$) across the inner membrane. Insulin-induced hypoglycemia promoted a significant decrease in ADP/O index (a marker of the mitochondrial ability to couple oxygen consumption to ADP phosphorylation during state 3 of respiration) (Fig.1) and an increase in repolarization lag phase (time necessary for ADP phosphorylation) (Table 2) in cortical mitochondria when compared with STZ-induced diabetic and control rats. No statistical differences were observed in the other parameters evaluated in cortical mitochondria (Figs. 1 and 2 and Table 2). In hippocampal mitochondria insulin-induced hypoglycemia significantly increased RCR (ratio between states 3 and 4 of respiration) when compared with control and STZ rats (Fig.3). However, no statistical differences were found in the phosphorylation system parameters: $\Delta\Psi_m$, repolarization level

(capacity of the mitochondria to recover $\Delta\Psi_m$ after ADP phosphorylation) and repolarization lag phase (Table 3).

Effects of diabetes and insulin-induced hypoglycemia in the susceptibility of cortical and hippocampal mitochondria to oxidative stress and damage

Lipid peroxidation is a marker of oxidative damage. Figs.4A and 4B show that cortical and hippocampal mitochondria from the three groups of rats do not have significant alterations in TBARS levels in the absence of the pro-oxidant pair ADP/Fe²⁺ (basal conditions). In the presence of ADP/Fe²⁺ (Px), cortical mitochondria of STZ and insulin-induced hypoglycemic rats present a significant increase in TBARS levels when compared with control cortical mitochondria (Fig.4A). However, no significant alterations were observed in hippocampal mitochondria in the presence of ADP/Fe²⁺ (Px) (Fig.4B).

The production of H₂O₂ by mitochondria gives an indication of the pro-oxidant state of mitochondria. Fig.5B shows that hippocampal mitochondria from STZ and insulin-induced hypoglycemic rats present a significant increase in H₂O₂ production when compared with control mitochondria. Additionally, hippocampal mitochondria from insulin-induced hypoglycemic animals produce significantly higher levels of H₂O₂ when compared with STZ hippocampal mitochondria (Fig.5B). However, H₂O₂ production remained statistical unchanged in cortical mitochondria isolated from the three groups of experimental animals (Fig.5A).

Effects of diabetes and insulin-induced hypoglycemia in the antioxidant defenses of cortical and hippocampal mitochondria

Mitochondria possess a multi-leveled ROS defense network that includes non-enzymatic antioxidants like GSH and antioxidant enzymes such as GPx, GR and MnSOD. Fig.6A shows that insulin-induced hypoglycemic cortical mitochondria present a significant decrease in GSH/GSSG ratio when compared to diabetic and control situations. In hippocampal mitochondria no statistical changes in the GSH/GSSG ratio were detected (Fig.6B).

GPx activity is significantly decreased in cortical mitochondria isolated from STZ diabetic rats when compared with control mitochondria (Fig.7A). However, insulin-induced hypoglycemia increased the activity of this enzyme when compared with diabetic mitochondria (Fig.7A). No differences were observed in hippocampal mitochondria from all experimental groups, although it is visible a slight decrease in the activity of this enzyme in diabetic and insulin-induced hypoglycemic situations (Fig.7B). STZ-induced diabetes and insulin-induced hypoglycemia significantly decreased GR activity in cortical mitochondria (Fig.8A) but had no significant effects in hippocampal mitochondria (Fig.8B). MnSOD activity increased significantly in insulin-induced hypoglycemic cortical mitochondria (Fig.9A) but decreased significantly in hippocampal mitochondria from STZ and insulin-induced hypoglycemic rats when compared with control animals (Fig.9B). No significant alterations were observed in the citrate synthase activity of mitochondria isolated from the different groups of experimental animals (data not shown).

4. Discussion

This study shows that STZ-induced diabetes and/or insulin-induced hypoglycemia affects differently hippocampal and cortical mitochondria bioenergetics and oxidative status suggesting that these brain areas have specific differences in mitochondrial function and defense mechanisms. These differences may underlay the different susceptibilities of distinct brain areas to metabolic insults.

Characterization of STZ animals was made by measuring blood glucose and HbA_{1c} levels and body weight. Diabetic rats presented a significant increase in glucose and HbA_{1c} levels and a significant decrease in body weight when compared with control rats (Table 1). This is in agreement with biochemical characteristics of type 1 diabetic patients [33]. Insulin therapy despite being used to ameliorate diabetes-induced complications is also associated with hypoglycemic episodes [34]. In *in vivo* studies intended to evaluate hypoglycemia effects, it is necessary to adequate the insulin dosage to the purpose of the study. When the goal of the work is to evaluate an acute episode of hypoglycemia, insulin dosage used often leads to blood glucose levels of about 30-50 mg/dl [35,36]. Accordingly, we adjusted the levels of insulin administered to diabetic animals in order to achieve blood glucose levels around 33 mg/dl (Table 1).

Brain requires a continuous glucose delivery [37] and, consequently, episodes of hypoglycemia, chronically elevated or fluctuating glucose levels, may compromise brain structure or the proper functioning of specific pathways or regions in the brain leading to cognitive decline [3]. Neuronal processes are critically dependent on mitochondrial function. This is reflected by the high oxygen demand of CNS that consumes 20% of all inspired oxygen although it accounts only for 2% of total body weight [38]. In addition, OXPHOS provides 90% of the ATP required for the normal functioning of neurons [14]. Previous data from our laboratory show that 4 weeks of

STZ-induced diabetes do not affect the OXPHOS capacity neither the respiratory chain of total brain mitochondria [39]. Accordingly, the present study shows that cortical mitochondria isolated from STZ diabetic rats do not show differences in the OXPHOS (Table 2) or in the respiratory chain parameters (Figs.1 and 2).

Insulin is an important neuromodulator, being involved in several neurobiological processes such as energy homeostasis and cognition [40]. It has been previously shown that insulin exerts neuroprotection against oxidative stress and modulates synaptosomal γ -aminobutyric acid and glutamate transport under oxidizing and/or diabetic conditions [41,42]. A previous study from our laboratory showed that total brain mitochondria from STZ-diabetic rats treated with insulin are more resistant to the deleterious effects promoted by the amyloidogenic peptide A β 1-40 [15]. Although insulin does not affect basal mitochondria function, it prevents the decline in OXPHOS efficiency and avoids the increase in oxidative stress promoted by A β 1-40 [15]. In the present study we used insulin to induce an acute episode of hypoglycemia. Under those conditions a significant decrease in ADP/O index (Figs.1 and 2) and an increase in lag phase (Table 2) were observed indicating that insulin-induced hypoglycemic cortical mitochondria consume more oxygen and spend more time to phosphorylate the amount of ADP present in the medium. It has been previously demonstrated that when hypoglycemia is prolonged from 30 to 60 min, the number of irreversible damaged cells increase substantially [43]. Furthermore, a significant decrease in ADP/O index was observed in brain mitochondria exposed to 60 min of severe hypoglycemia [44].

The OXPHOS system is a major source of free radicals including H₂O₂, hydroxyl (HO[•]), and superoxide anion (O₂^{•-}). ROS, besides being important signaling molecules involved in synaptic transmission and acting as second messengers in several

signaling pathways, are key players in neuronal death. Mitochondrial ROS production has been suggested to be involved in the aging process and in the pathogenesis of neurodegenerative disorders and diabetes [13,45]. Excessive ROS production, which could be caused by hyperglycemia [46] and/or hypoglycemia [47], is associated with decreased activity of antioxidant systems [48]. The antioxidant defense system is comprised by antioxidant enzymes that catalyses the cellular detoxification of reactive species. MnSOD is an intracellular enzyme that dismutates $O_2^{\bullet-}$ into H_2O_2 , which can be converted to H_2O through the action of catalase or GPx in the presence of GSH. GR ensures the replenishment of GSH, one of the most important cellular antioxidant. Indeed, data show that hypoglycemia and diabetes lead to a decrease in glutathione content and MnSOD and catalase activities and to an increase in lipid peroxidation in several brain areas [49]. Accordingly, we observed that cortical mitochondria isolated from insulin-induced hypoglycemic and STZ-induced diabetic rats present a significant increase in TBARS levels in the presence of the pro-oxidant pair ADP/ Fe^{2+} (Fig.4A). This increase in lipid peroxidation could be an indicator of the degree of oxidative stress during insulin-induced hypoglycemia and/or STZ-induced diabetes [49]. In STZ-induced diabetes we found a significant decrease in GPx (Fig.7A) and GR (Fig.8A) activities when compared with control mitochondria. These decreases correlate with the GSH/GSSG ratio, which remained statistically unchanged (Fig.6A). Also, Baydas and colleagues [50] reported an increase in lipid peroxidation levels in hippocampus, cortex and cerebellum of STZ-induced diabetic rats while the GSH levels suffer no significant alterations. Previous data from our laboratory show that the maintenance of GSH content in total brain mitochondria isolated from STZ rats could be correlated with the preservation of $\Delta\Psi_m$ [15], since $\Delta\Psi_m$ contributes for keeping matrix NADPH/ $NADP^+$ pool in the reduced form, which in turn helps to maintain the matrix glutathione in the

reduced state [14]. Similarly, we found no differences in the OXPHOS of cortical mitochondria from STZ diabetic rats (Table 2). Moreover, although cortical mitochondria from STZ diabetic rats are more prone to lipid peroxidation, the high levels of GSH help to maintain H_2O_2 production levels unchanged (Fig.5A) maybe in an attempt to keep normal mitochondrial function.

GSH/GSSG ratio decreased significantly in the insulin-induced hypoglycemic cortical mitochondria compared with STZ and control mitochondria (Fig.6A). GSH is an antioxidant that protects mitochondria from lipid peroxidation and when depleted causes brain mitochondria enlargement and degeneration [51]. In agreement, we also found a significant decrease in GR activity compared with control rats (Fig.8A) and an increase in GPx activity compared with STZ-induced diabetic animals (Fig.7A). The increase in GPx activity (Fig.7A) may indicate an adaptive mechanism developed during the insulin-induced hypoglycemic insult to counteract the overproduction of H_2O_2 promoted by the increased activity of MnSOD (Fig.9A). Accordingly, H_2O_2 production levels remained statistically unchanged (Fig.5A). Therefore, during the insulin-induced hypoglycemic insult, due to the attempt to counterbalance the increased production of H_2O_2 , the GSH pool was depleted and, since the cells are unable to replace the GSH pool they become more vulnerable to the metabolic insult.

In hippocampal mitochondria we observed a significant decrease in MnSOD activity (Fig.9B) under hyperglycemic and insulin-induced hypoglycemic situations. This may cause an accumulation of $O_2^{\bullet-}$ potentiating the formation of other radicals disturbing the cellular redox status. The role of MnSOD is critical in protecting neurons from free-radical damage. In the matrix, MnSOD activity should be in balance with the activity of H_2O_2 detoxifying enzymes. Matrix GSH redox cycle in coordination with MnSOD mediated scavenging of $O_2^{\bullet-}$ is crucial for preventing excessive H_2O_2

accumulation [52]. However, the hyper- and insulin-induced hypoglycemic conditions also lead to a significant increase in H₂O₂ production levels (Fig.5B) and a non-statistically significant decrease in GPx activity (Fig.7B). Similar results were obtained by Aragno and co-workers [53] that reported an increase in H₂O₂ levels and a decrease in GPx and catalase activities in the hippocampus of STZ-diabetic rats. H₂O₂ in the presence of reduced transition metals, such as Fe²⁺, can be converted to toxic HO[•] through Fenton and/or Haber Weiss reactions [14]. The lack of correlation between MnSOD (Fig.9B), GPx (Fig.7B) and H₂O₂ production levels (Fig.5B) may suggest that H₂O₂ production in hippocampus does not result (only) from the dismutation of mitochondrial O₂^{•-}. Since our preparation consisted in a mitochondrial crude preparation the H₂O₂ can also be originated from an extra-mitochondrial source [54]. Hino and colleagues [55] evaluated the effects of insulin-induced hypoglycemia in hippocampal cells and glucose-deprived medium in cultured neurons and they found a significant increase in GSSG levels in both experimental situations. However, we found no differences in the GSH/GSSG ratio in hippocampal mitochondria (Fig.6B) that could be correlated with TBARS levels (Fig.4A). We should take into account our experiments were performed in mitochondrial crude preparations while the above-mentioned studies were performed in cells. Also, although not statistically significant, we observed a slight increase of GR activity (Fig.8B). Therefore, and compared to cortical mitochondria, both hyperglycemia and insulin-induced hypoglycemia promote distinct effects in the oxidative status of hippocampal mitochondria: a decrease in MnSOD activity and an increase in H₂O₂ levels associated with the maintenance of the GSH/GSSG ratio and GPx and GR activities.

The literature shows that glucose deprivation in hippocampal neurons and cultured cerebellar granule neurons exacerbates the production of ROS, which is

accompanied by an increase in Ca^{2+} concentration and decrease in $\Delta\Psi_m$ [56,57]. In this study we found no differences in the OXPHOS (Table 3) but, interestingly, we found a significant increase in RCR in insulin-induced hypoglycemic hippocampal mitochondria (Fig.3). The rate of H_2O_2 production usually depends on the $\Delta\Psi_m$ that, in turn, depends on the coupling and metabolic state of mitochondria [58]. Furthermore, evidence show that the maximum rate of H_2O_2 production in heart mitochondria requires a high $\Delta\Psi_m$ and respiring, coupled mitochondria [59]. Moreover, Starkov and co-workers [60] proposed that the rates of H_2O_2 production in the rat brain mitochondria could not be directly related to relative rates of states 3 or 4 of respiration.

It has been described that different brain regions have distinct vulnerabilities to oxidative stress and that the cortex possess lower basal level of antioxidant enzymes and therefore, is more prone to oxidative damage [61]. A previous study showed that cortical areas and hippocampus of 20 weeks old Wistar rats possess different levels of cooper (Cu), iron (Fe) and zinc (Zn), trace elements known to be involved in neurological degenerative disorders, which are higher in cortex compared to the hippocampus [62]. In fact, studies show that hyperglycemia differently affects cortical and hippocampal morphology and biochemistry [63]. Additionally, Xu and colleagues [64] demonstrated that cortical neurons and astrocytes are more vulnerable to oxygen-glucose deprivation than cells from striatum and hippocampus. Bree and co-workers [65] demonstrated that diabetes uniquely increases the cortex susceptibility to hypoglycemic damage. The differences in cortical and hippocampal mitochondrial bioenergetics and antioxidant defenses observed in the present study may explain the different susceptibilities found in different brain areas.

5. Conclusions

The cerebral cortex and the hippocampus are the seat of our highest forms of intelligence. Although both situations of hyperglycemia and insulin-induced hypoglycemia lead to an increase in ROS levels associated with a decrease in antioxidant defenses in cortical and hippocampal mitochondria, we observed that both brain areas behave differently in response to a metabolic insult. Furthermore, we show that an acute episode of hypoglycemia induced by insulin potentiates the effects of STZ-induced diabetes having detrimental effects in cortical mitochondria bioenergetics and making the antioxidant defenses unable of overcome the increased oxidative stress. Therefore, the poor glycemic control that occurs in diabetic patients undergoing insulin therapy may have a negative impact in brain mitochondria, namely those of the cortex, predisposing the brain to degenerative events and cognitive impairment.

6. Acknowledgements

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Legends

Figure 1: Effect of STZ-induced diabetes and insulin-induced hypoglycemia in the cortical mitochondria respiratory parameters: states 3 (A) and 4 (B) of respiration, RCR (C) and ADP/O index (D). Data are the mean \pm SEM of 8-10 animals from each condition studied. Statistical significance: ** $p < 0.01$ when compared with control cortical mitochondria; ⁺ $p < 0.05$ when compared with STZ cortical mitochondria.

Figure 2: Representative traces of oxygen uptake in cortical mitochondria from each condition studied. Freshly isolated cortical brain mitochondria (0.8 mg) in 1 ml of the reaction medium were energized with 5 mM succinate in the presence of 2 μ M rotenone. State 3 of respiration were initiated with ADP (155 nmol/mg protein).

Figure 3: Effect of STZ-induced diabetes and insulin-induced hypoglycemia in the hippocampal mitochondria respiratory parameters: states 3 (A) and 4 (B) of respiration, RCR (C) and ADP/O index (D). Data are the mean \pm SEM of 6-10 animals from each condition studied. Statistical significance: * $p < 0.05$ when compared with control hippocampal mitochondria; ⁺ $p < 0.05$ when compared with STZ hippocampal mitochondria.

Figure 4: Effect of STZ-induced diabetes and insulin-induced hypoglycemia on thiobarbituric acid reactive substances (TBARS) formation induced by the pro-oxidant pair ADP/Fe²⁺ (Px) in cortical (A) and hippocampal (B) mitochondria. Freshly isolated mitochondria were incubated at 1 mg/ml under standard conditions as described in the Material and methods section. Data shown represent mean \pm SEM from six to eight independent experiments. Statistical significance: *** $p < 0.001$; * $p < 0.05$ when compared with control cortical mitochondria in the presence of ADP/Fe²⁺ (Px).

Figure 5: Effect of STZ-induced diabetes and insulin-induced hypoglycemia on hydrogen peroxide (H₂O₂) production. Cortical (A) and hippocampal (B) mitochondria were incubated under the conditions described in the Material and methods section. Data are the mean \pm SEM from six to ten independent experiments. Statistical significance: * $p < 0.05$ when compared with control hippocampal mitochondria; ⁺ $p < 0.05$ when compared with STZ hippocampal mitochondria.

Figure 6: Effect of STZ-induced diabetes and insulin-induced hypoglycemia on glutathione content in cortical (A) and hippocampal (B) mitochondria. Data shown represent mean \pm SEM from six to eight independent experiments. Statistical significance: ** $p < 0.01$ when compared with control brain mitochondria. ⁺ $p < 0.05$ when compared with STZ cortical mitochondria.

Figure 7: Effect of STZ-induced diabetes and insulin-induced hypoglycemia on glutathione peroxidase (GPx) activity in cortical (A) and hippocampal (B) mitochondria. Data are the mean \pm SEM of 6-8 animals from each condition studied. Statistical significance: ** $p < 0.01$ when compared with control cortical mitochondria; ⁺ $p < 0.05$ when compared with STZ cortical mitochondria.

Figure 8: Effect of STZ-induced diabetes and insulin-induced hypoglycemia on glutathione reductase (GR) activity in cortical (A) and hippocampal (B) mitochondria. Data are the mean \pm SEM of 6-8 animals from each condition studied. Statistical significance: ** $p < 0.01$ when compared with control cortical mitochondria.

Figure 9: Effect of STZ-induced diabetes and insulin-induced hypoglycemia on superoxide dismutase (MnSOD) activity in cortical (A) and hippocampal mitochondria (B). Data are the mean \pm SEM of 6-8 animals from each condition studied. Statistical

significance: * $p < 0.05$ when compared with control; + $p < 0.05$ when compared with STZ hippocampal mitochondria.

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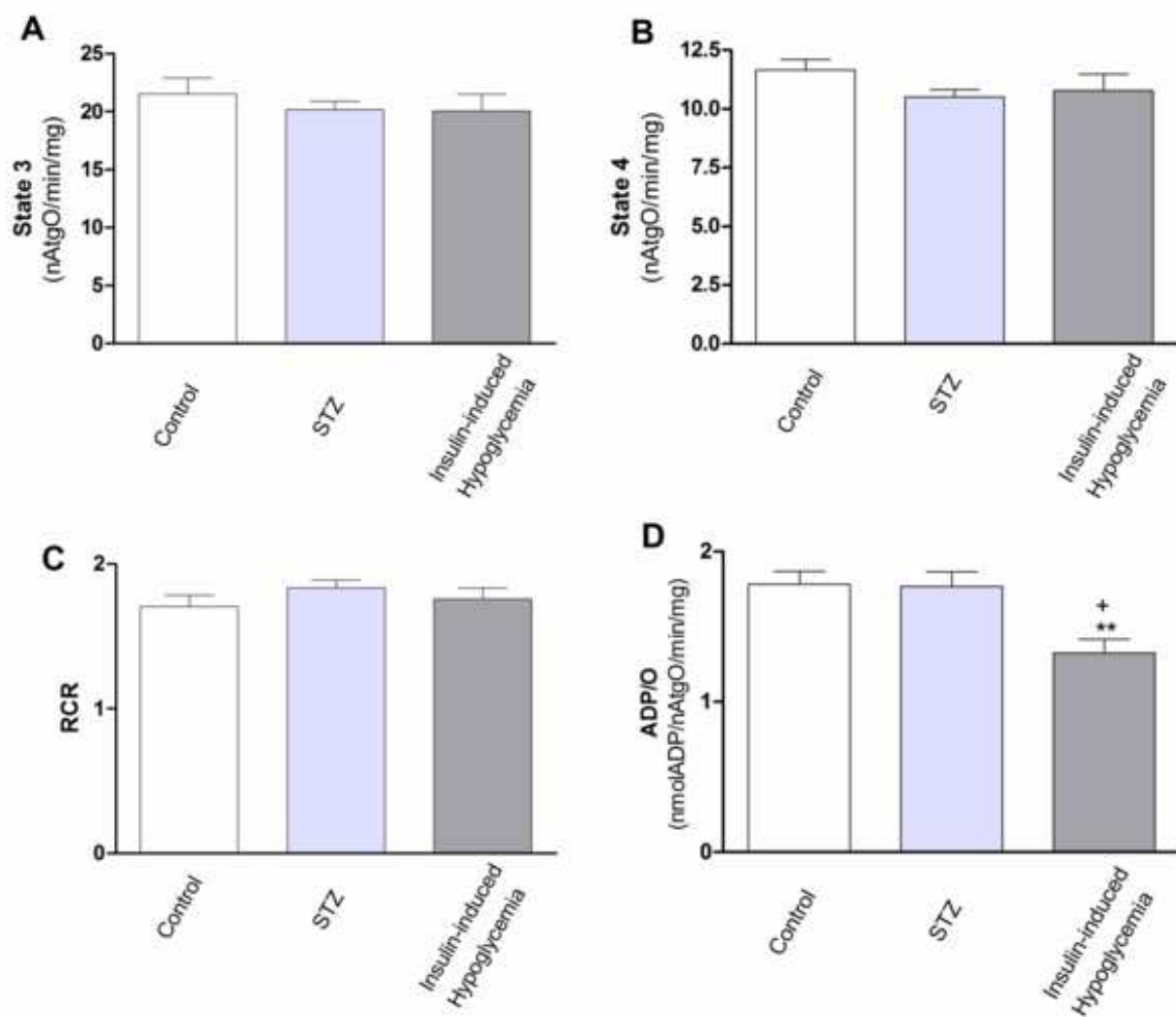


Figure 1

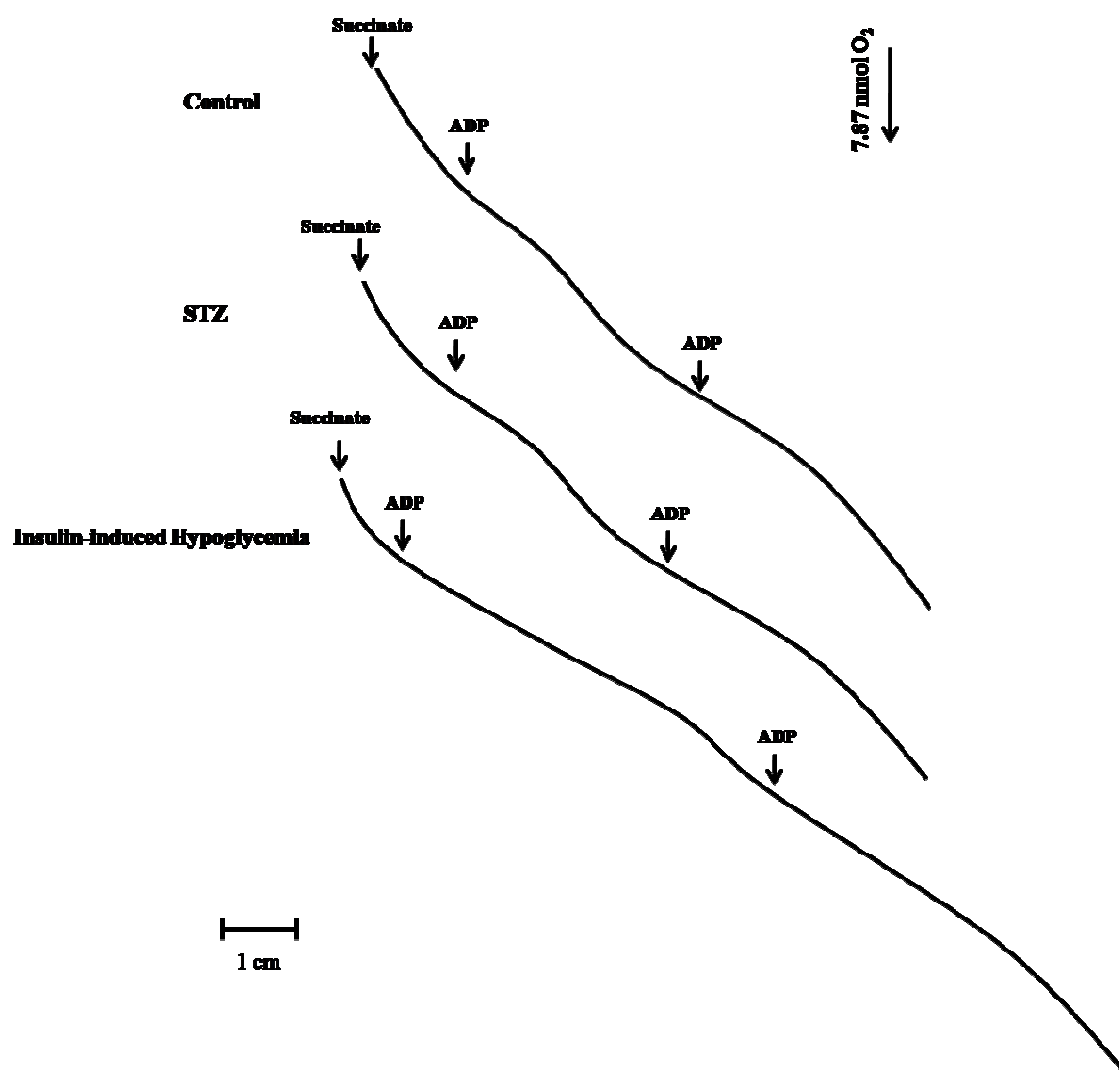


Figure 2

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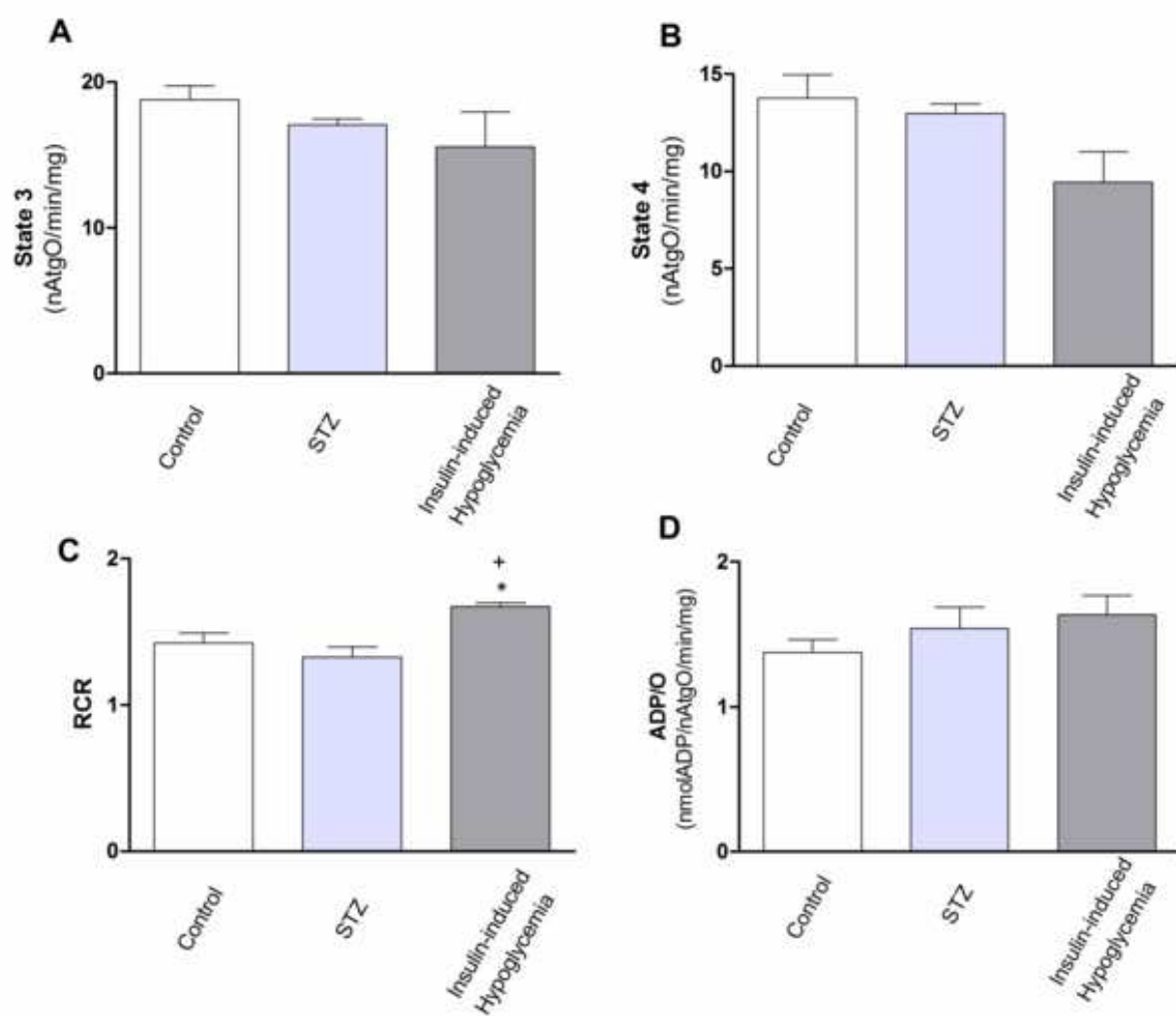


Figure 3

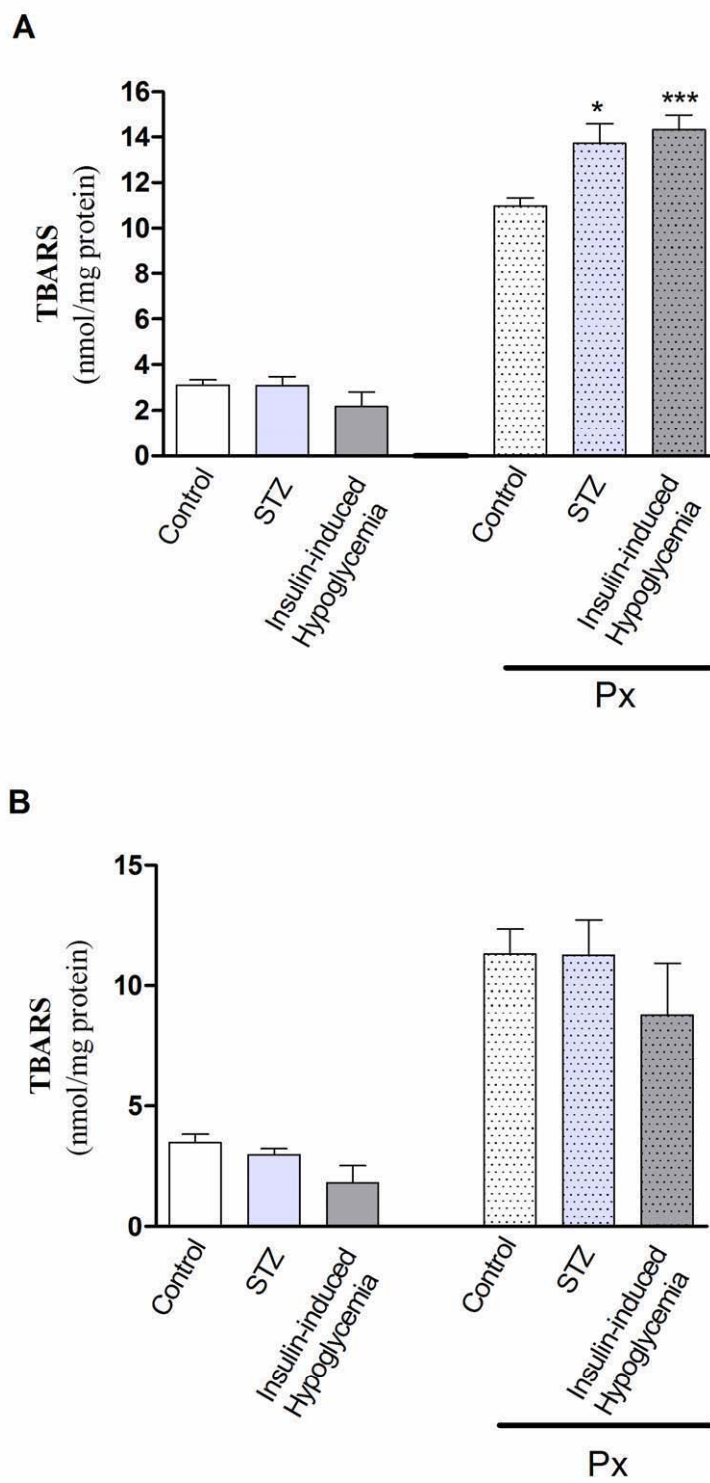


Figure 4

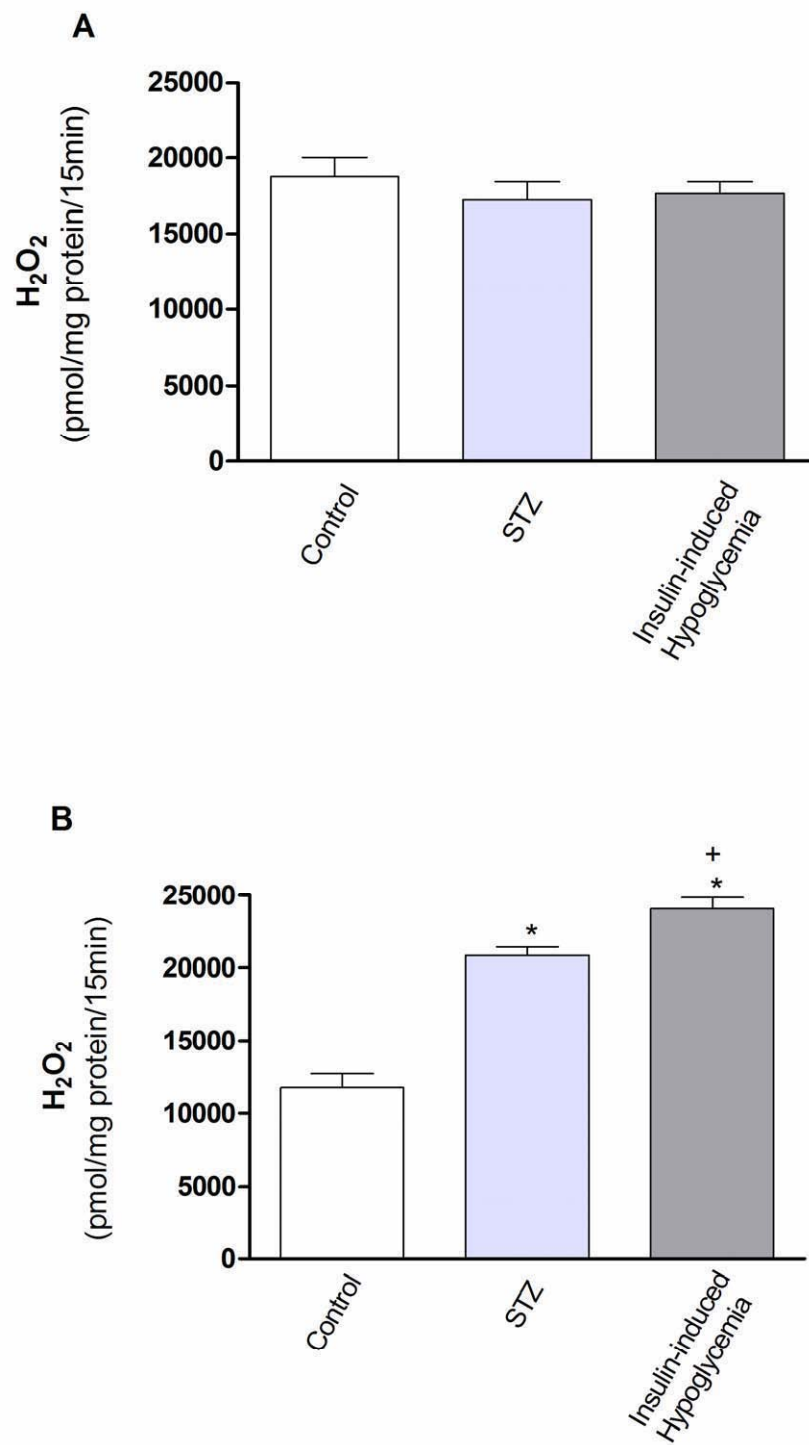


Figure 5

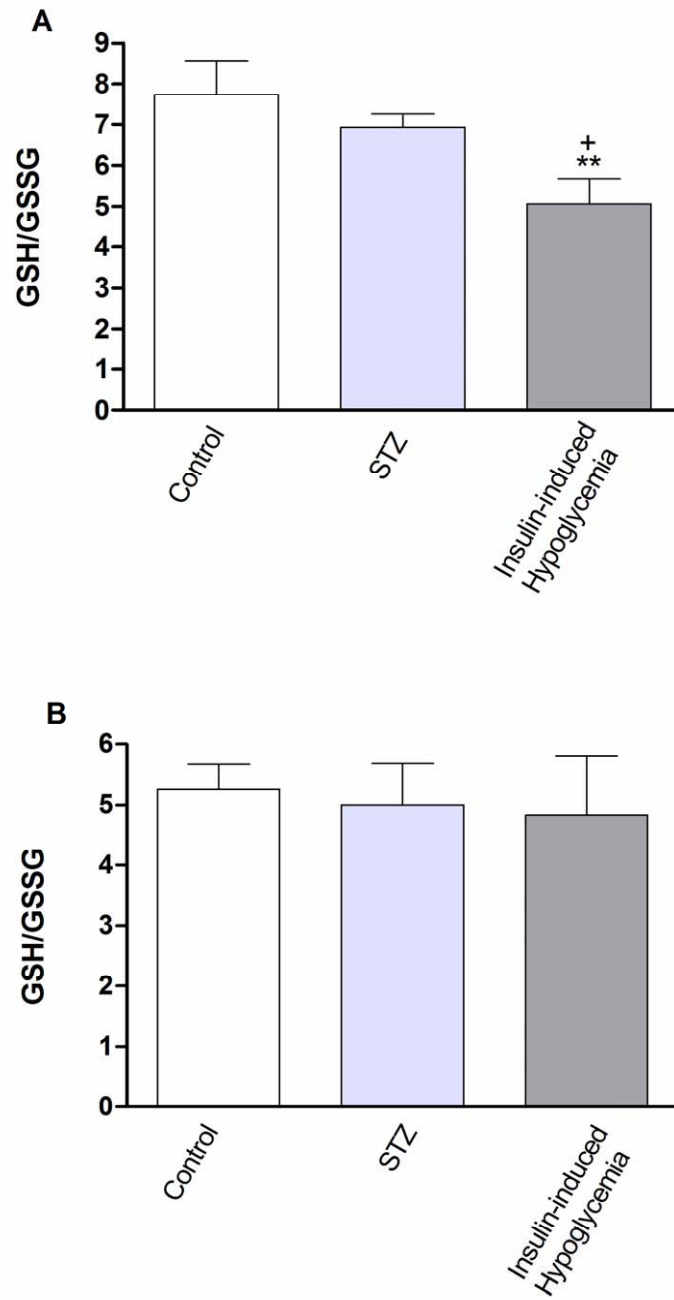


Figure 6

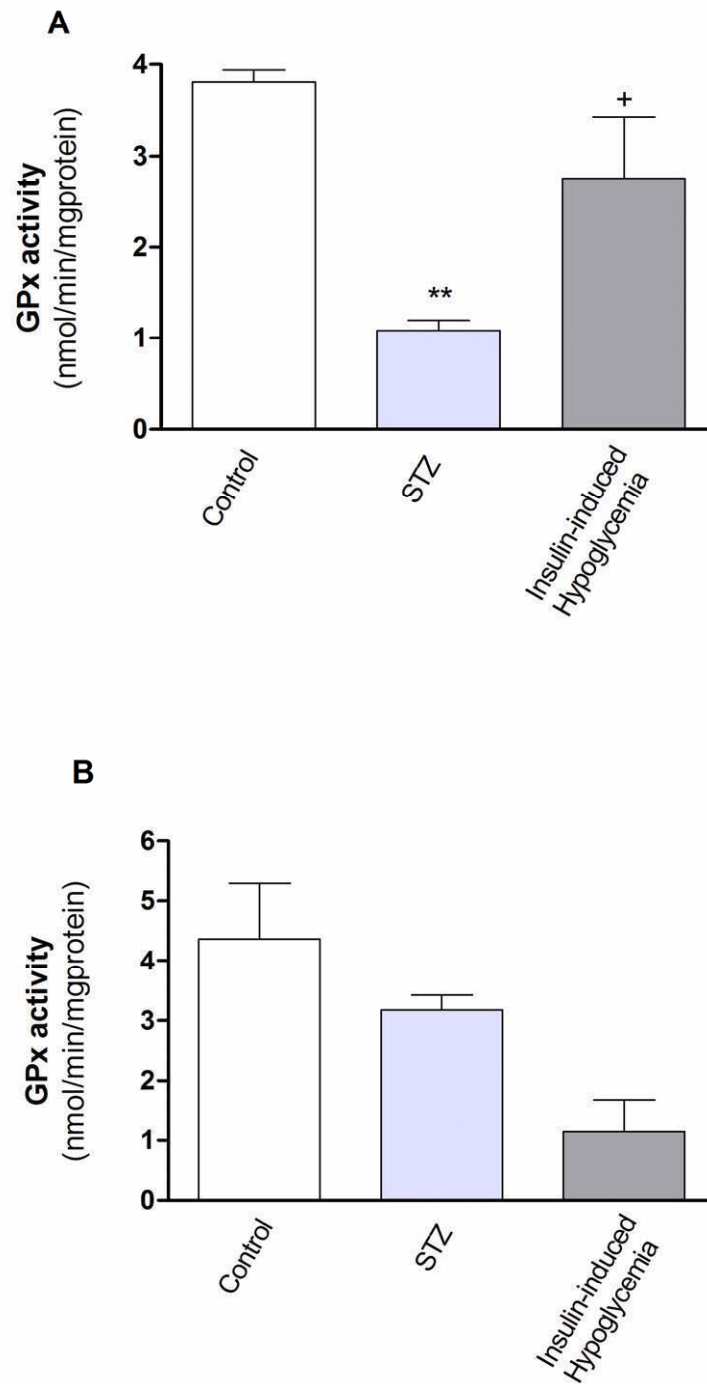


Figure 7

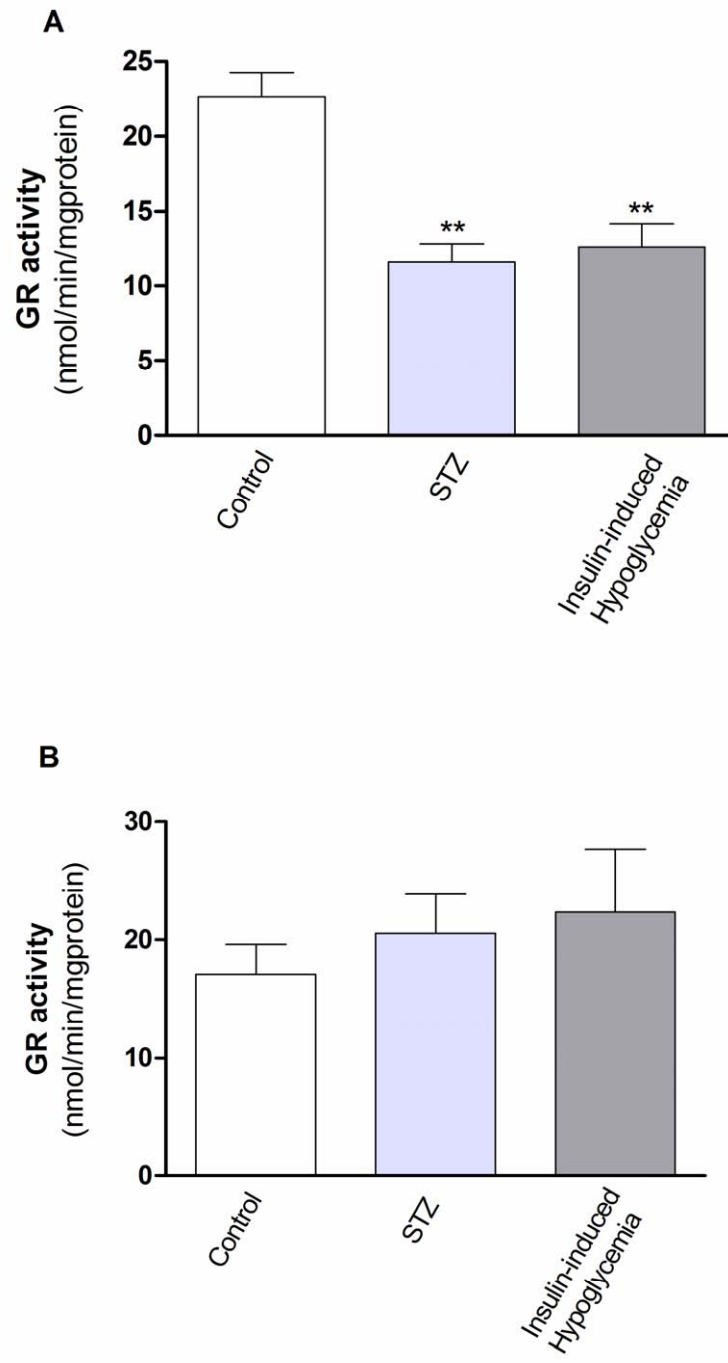


Figure 8

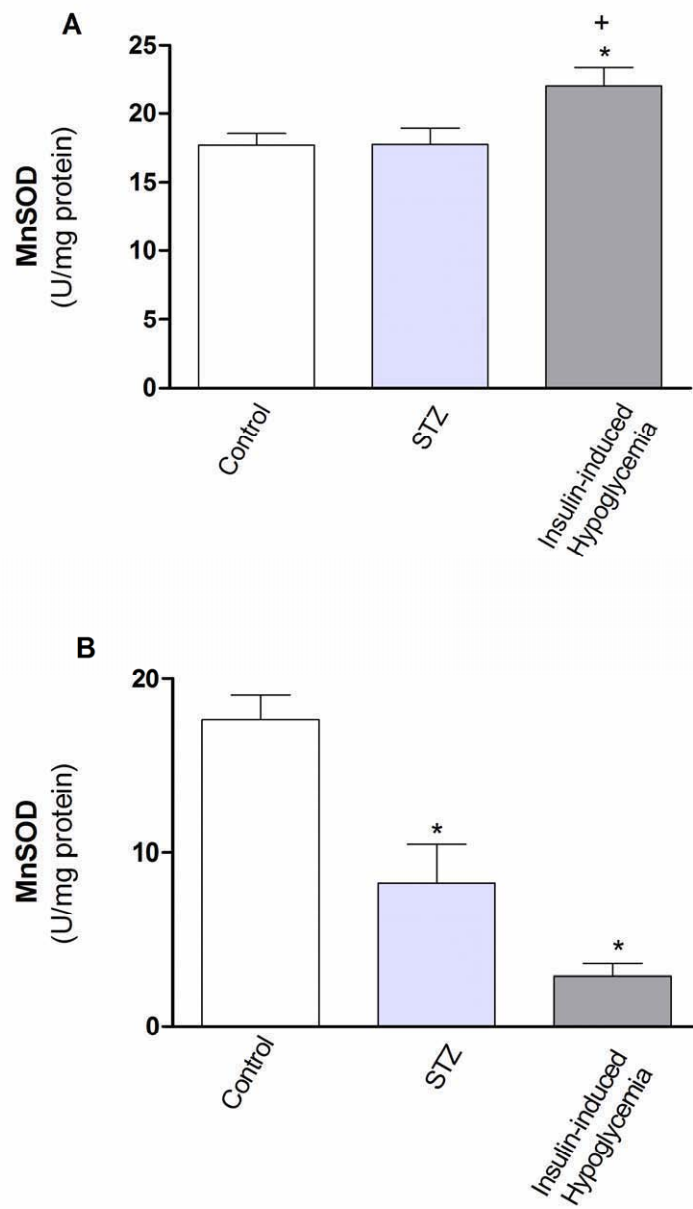


Figure 9

Table 1: Characterization of the experimental animal models

	Control	STZ	Insulin-induced Hypoglycemia
Body weight (g)	354±4	259±11 ^{***}	245±9 ^{***}
Brain weight (g)	2.5±0.2	2.4±0.1	2.4±0.2
Glucose (mg/dl)	89±3	415±26 ^{***}	33±3 ^{***;+++}
Hb_{A1C} (%)	1.9±0.2	7.8±0.4 ^{**}	7.0±0.2 ^{**}

Data are the means ± SEM of 12-14 animals from each condition studied. Statistical significance: *** p<0.001; **p<0.01 when compared with Control rats; +++ p<0.001 when compared with STZ-treated rats. Hb_{A1C}- glycated hemoglobin.

Table 2: Effect of STZ-induced diabetes and insulin-induced hypoglycemia in the cortical mitochondrial oxidative phosphorylation system ($\Delta\Psi_m$, repolarization level and repolarization lag phase).

	Control	STZ	Insulin-induced hypoglycemia
$\Delta\Psi_m$ (-mV)	179 \pm 2	182 \pm 2	179 \pm 3
Repolarization level (-mV)	144 \pm 2	146 \pm 2	145 \pm 2
Repolarization lag phase (min)	2.1 \pm 0.1	2.1 \pm 0.1	2.9 \pm 0.2 ^{**+}

The oxidative phosphorylation parameters were evaluated in freshly isolated cortical mitochondrial fractions (0.8 mg) in 1 ml of the reaction medium supplemented with 3 μ M of TPP⁺ and energized with 5 mM succinate in the presence of 2 μ M rotenone. Data are the mean \pm SEM of 8-10 animals from each condition studied. Statistical significance: **p<0.01 when compared with control cortical mitochondria; ⁺p<0.05 when compared with STZ cortical mitochondria.

Table 3: Effect of STZ-induced diabetes and insulin-induced hypoglycemia in the hippocampal mitochondrial oxidative phosphorylation system ($\Delta\Psi_m$, repolarization level and repolarization lag phase).

	Control	STZ	Insulin-induced hypoglycemia
$\Delta\Psi_m$ (-mV)	173 \pm 3	178 \pm 3	178 \pm 3
Repolarization level (-mV)	145 \pm 5	137 \pm 5	152 \pm 2
Repolarization lag phase (min)	2.7 \pm 0.4	3.1 \pm 0.4	1.92 \pm 0.3

The oxidative phosphorylation parameters were evaluated in freshly isolated hippocampal mitochondrial fractions (0.8 mg) in 1 ml of the reaction medium supplemented with 3 μ M of TPP⁺ and energized with 5 mM succinate in the presence of 2 μ M rotenone. Data are the mean \pm SEM of 6-10 animals from each condition studied.