Insulin Attenuates Diabetes-Related Mitochondrial Alterations: A Comparative Study

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Abstract: This study evaluated and compared the effect of insulin treatment on the status of brain, heart and kidney mitochondria isolated from 12-week streptozotocin (STZ)-induced diabetic rats versus STZ-diabetic animals treated with insulin during a period of 4 weeks. Mitochondria isolated from 12-week citrate (vehicle)-treated rats were used as control. Several mitochondrial parameters were evaluated: respiratory indexes (state 3 and 4 of respiration, respiratory control and ADP/O ratios), transmembrane potential, depolarization and repolarization levels, ATP, glutathione and coenzyme Q contents, production of hydrogen peroxide, superoxide dismutase, glutathione peroxidase and glutathione reductase activities and the ability of mitochondria to accumulate calcium. We observed that diabetes promoted a significant decrease in kidney and brain mitochondrial coenzyme Q9 content while this parameter was increased in heart mitochondria. Furthermore, diabetes induced a significant increase in hydrogen peroxide production in kidney mitochondria this effect being accompanied by a significant increase in glutathione peroxidase and reductase activities. Furthermore, brain mitochondria isolated from diabetic animals presented a lower ATP content and ability to accumulate calcium. In contrast, heart and kidney mitochondria presented a slight higher capacity to accumulate calcium. Insulin treatment normalized the levels of coenzyme Q9 and glutathione peroxidase and reductase activities and increased ATP content and the ability to accumulate calcium. Altogether these results suggest that insulin treatment attenuates diabetes-induced mitochondrial alterations protecting against the increase in oxidative stress and improving oxidative phosphorylation efficiency. In this line, insulin therapy, besides its well-known importance in the maintenance of glycemic control, may help to protect against mitochondrial dysfunction associated to several age-related disorders such as diabetes.

Key Words: Brain, diabetes, heart, insulin, kidney, mitochondria, oxidative stress, streptozotocin.

INTRODUCTION

In type 1 diabetes, pancreatic β-cells are destroyed leading to a final absolute insulin deficiency. Without insulin to move glucose into cells, blood sugar levels become excessively high, a condition known as hyperglycemia. Data from the literature point to the association between several cardiovascular [1], renal [2] and nervous system [3] complications and type 1 diabetes.

Oxidative damage is widely considered to be a cause of diabetic complications [4], and markers of oxidative damage are found in diabetic patients [5]. Recently, it has been proposed that overproduction of reactive oxygen species (ROS) may be the initiating event leading to long-term development of diabetic complications [6]. ROS, such as the superoxide radical, the hydroxyl radical, and hydrogen peroxide, are continuously produced in most cells under physiological conditions, and their levels are regulated by a number of enzymes and physiological antioxidants. The principal enzymes that detoxify ROS include superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRd) and catalase. When the production of ROS exceeds the ability of the cell to detoxify them, oxidative stress develops affecting the integrity of biological tissues.

The fact that mitochondria are the major generators and direct targets of ROS has led several investigators to foster the idea that oxidative stress and damage in mitochondria are contributory factors to several pathological conditions including diabetes. In this respect, it has been shown that defects in mitochondrial performance could contribute to the development of insulin resistance [7] and mitochondrial oxidative capacity has been considered a good predictor of insulin sensitivity [8]. Insulin is the key postprandial hormone involved in fuel metabolism [9]. Recent reports using combinations of isotopic dilution techniques, protein separation methods and muscles biopsies indicated that *in vivo* mitochondrial protein synthesis and enzyme activity might be stimulated by insulin [10,11].

Previous studies from our laboratory indicate that streptozotocin (STZ)-induced diabetes interferes with liver [12] and heart [13] mitochondrial function. Although we observed that brain mitochondria isolated from STZ-diabetic rats are not significantly affected under basal conditions, these organelles show a higher susceptibility in the presence of the neurotoxic agent amyloid β-peptide suggesting an intrinsic vulnerability of diabetic mitochondria [14]. The present study was aimed to evaluate and compare the effect of insulin treatment on the status of mitochondria isolated

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from brain, heart and kidney of STZ-induced diabetic rats. For this purpose we used mitochondria isolated from STZ-diabetic rats, diabetic rats treated with insulin and control animals. Several mitochondrial parameters were analyzed: respiratory indexes (states 3 and 4 of respiration, respiratory control ratio and ADP/O ratio), transmembrane potential ($\Delta\Psi m$), depolarization and repolarization levels, ATP, glutathione and CoQ contents, antioxidant enzymes activities (SOD, GPx, GRd), production of H_2O_2 and the ability of mitochondria to accumulate Ca^{2+}.

RESULTS

Insulin Attenuates the Alterations in Glycemia Levels and in Body/Organ Weight Promoted by STZ-Induced Diabetes

STZ rats showed a significant increase in blood glucose and HbA_{1C} levels and a significant decrease in body weight when compared with control rats (Table **1**). Furthermore, STZ-induced diabetes promoted a significant decrease in brain and heart weight and a significant increase in the kidney/body weight ratio. These data confirm the state of diabetes mellitus in STZ-treated rats. However, insulin treatment attenuated the alterations promoted by STZ-induced diabetes. Diabetic animals treated with insulin had a significant decrease in glycemia and HbA_{1C} levels, a significant increase in body and brain and heart weight and a significant decrease in the kidney/body weight ratio when compared with diabetic animals without insulin treatment (Table **1**).

Insulin Reverses the Decrease in State 3 and 4 of Respiration in Brain Mitochondria Promoted by STZ-Induced Diabetes

We observed that only state 3 (consumption of oxygen in the presence of substrate and ADP) and state 4 (consumption of oxygen after ADP has been consumed) of respiration of brain mitochondria were significantly decreased by diabetes (Table **2**) this effect being reversed by insulin treatment. The respiratory control ratio (RCR) and ADP/O ratio remained statistically unchanged (Table **2**). RCR is the ratio between mitochondrial respiration state 3 and state 4. ADP/O ratio, an indicator of oxidative phosphorylation efficiency, is expressed by the ratio between the amount of ADP added and the oxygen consumed during the state 3 respiration. Furthermore, diabetes and/or insulin treatment did not induce any significant alteration in the above parameters in heart and kidney mitochondria (Table **2**).

Insulin Avoids the Decrease in Brain Oxidative Phosphorylation Efficiency Promoted by STZ-Induced Diabetes

As shown in Table **3**, diabetes and insulin treatment did not induce a significant change on $\Delta\Psi m$, repolarization (the capacity of mitochondria to reestablish $\Delta\Psi m$, after ADP phosphorylation) and depolarization potential (corresponding to ADP phosphorylation) in all mitochondrial preparations studied. However, brain mitochondria isolated from diabetic animals showed a significant decrease in ATP content (Table **3**) when compared with control and diabetic rats treated with insulin. Kidney and heart mitochondria did not show any significant change concerning ATP content (Table **3**). Insulin treatment was capable of increase brain mitochondrial ATP content to a level similar to that of control animals (Table **3**).

Insulin Counteracts Oxidative Stress Promoted by STZ-Induced Diabetes

We observed that brain and kidney mitochondrial CoQ9 content was significantly decreased in STZ diabetic animals while heart mitochondrial CoQ9 content was significantly higher in those rats when compared with control animals. These alterations were normalized by insulin treatment (Table **4**). However, we did not observe any significant alteration promoted by STZ-induced diabetes and/or insulin treatment on glutathione content (Table **5**).

Table 1. Characterization of Control, STZ, STZ + INS Rats

	12-week control	12-week STZ diabetic	8-week STZ diabetic/ 4 week insulin treated
Body weight (g)	517.8 ± 8.1	331.8 ± 21.6***	401.9 ± 6.4***,&&&
Brain weight (g) Brain weight/body weight	1.98 ± 0.06 $3.82x10^{-3}$ ± 0.09	1.30 ± 0.32* $4.21x10^{-3}$ ± 1.37	2.03 ± 0.09& $5.10x10^{-3}$ ± 0.18
Heart weight (g) Heart weight/body weight	1.44 ± 0.07 $2.78x10^{-3}$ ± 0.18	1.0 ± 0.06** $3.06x10^{-3}$ ± 0.28	1.22 ± 0.06*,&& $3.07x10^{-3}$ ± 0.14
Left + right kidney weight (g) Kidney weight/body weight	3.11 ± 0.13 $6.01x10^{-3}$ ± 0.28	3.34 ± 0.07 $10.19x10^{-3}$ ± 0.51***	3.48 ± 0.12 $8.88x10^{-3}$ ± 0.34***,&&
Plasma glucose (mg/ml)	92.8 ± 3.8	527.4 ± 24.9***	381.3 ± 53.32***,&
HbA_{1c} (% of total hemoglobin)	5.3 ± 0.4	11.6 ± 0.6***	8.5 ± 0.4***,&&&

Values are mean ± SEM from five animals for each condition studied. ***p<0.001; **p<0.01; *p<0.05 compared with control rats at the same age. &&&p<0.001; &&p<0.01; $p<0.05 compared to diabetic rats.

Table. 2. Respiratory Indexes of Brain, Heart and Kidney Mitochondria Isolated from Control, STZ and STZ+INS Rats

	12-week control	12-week STZ diabetic	8-week STZ diabetic/ 4 week insulin treated
Brain			
State 4	47.46 ± 2.63	41.65 ± 2.95*	44.57 ± 4.28
State 3	98.82 ± 5.32	93.32 ± 4.89*	95.58 ± 2.99
RCR	2.28 ± 0.09	2.24 ± 0.11	2.14 ± 0.12
ADP/O	1.27 ± 0.08	1.34 ± 0.07	1.32 ± 0.07
Heart			
State 4	61.72 ± 6.65	57.67 ± 7.73	57.42 ± 19.37
State 3	145.95 ± 3.56	157.03 ± 8.71	141.72 ± 19.83
RCR	3.38 ± 0.09	2.74 ± 0.05	2.50 ± 0.11
ADP/O	1.22 ± 0.03	1.31 ± 0.07	1.18 ± 0.17
Kidney			
State 4	60.51 ± 12.37	53.28 ± 7.63	58.28 ± 10.98
State 3	125.47 ± 13.89	136.10 ± 10.07	115.63 ± 10.61
RCR	2.23 ± 0.28	2.67 ± 0.29	2.50 ± 0.11
ADP/O	1.05 ± 0.12	1.13 ± 0.08	0.96 ± 0.09

The values for state 4 and state 3 are in natoms O/min/mg protein, and for ADP/O in nmol ADP/natoms O. Values are the mean ± SEM from four animals for each condition studied. *p<0.05 when compared with control rats.

GPx and GRd are two antioxidant enzymes involved in the detoxification of ROS. We observed that only kidney mitochondria obtained from STZ rats presented a significant higher activity of both GPx and GRd, this effect being reversed by insulin treatment (Fig. **1**).

The production of H_2O_2 by mitochondria gives an indication of the propensity of mitochondria to originate and/or exacerbate oxidative stress. We observed that only kidney mitochondria obtained from STZ rats showed a significant higher production of H_2O_2, this effect being reversed by insulin treatment (Fig. **2**).

Insulin Normalizes the Capacity of Brain Mitochondria to Accumulate Ca^{2+}

Mitochondrial transmembrane potential ($\Delta\Psi m$) drop is a typical phenomenon that follows the induction of mitochondrial permeability transition pore (MPTP). As shown in

Table 3. Effect of STZ-Induced Diabetes and Insulin Treatment on the Oxidative Phosphorylation Efficiency of Brain, Heart and Kidney Mitochondria Isolated from Control, STZ and STZ+INS Rats

	ΔΨm (- mV)	Depolarization (- mV)	Repolarization (- mV)	ATP (nmol/mg prot)
BRAIN				
Control	177.3 ± 2.20	163.3 ± 1.00	178.3 ± 1.25	153.04 ± 3.05
STZ	172.4 ± 2.83	164.3 ± 2.20	169.3 ± 2.22	128.22 ± 2.29**
STZ + INS	175.1 ± 1.40	168.3 ± 1.10	180.3 ± 1.45	142.84 ± 4.90#
HEART				
Control	239.89 ± 0.79	196.70 ± 0.58	242.13 ± 0.92	31.51 ± 2.57
STZ	241.68 ± 0.77	188.64 ± 2.45	244.24 ± 0.97	26.32 ± 3.01
STZ + INS	239.34 ± 1.79	190.22 ± 1.42	242.23 ± 1.67	31.90 ± 5.63
KIDNEY				
Control	213.09 ± 1.09	202.35 ± 0.76	213.13 ± 1.95	3.97 ± 0.58
STZ	207.87 ± 1.27	185.69 ± 2.45	207.42 ± 0.87	4.16 ± 0.54
STZ + INS	213.39 ± 1.26	190.76 ± 1.65	214.32 ± 0.96	5.41 ± 0.46

Freshly isolated mitochondria in 1 ml of the reaction medium supplemented with 3 μM TPP^+ and 2 μM rotenone were energized with 5 mM succinate. **p<0.01, when compared with control rats. #*p*<0.05 when compared with STZ rats. Data shown represent mean ± S.E.M. from 5 animals for each condition studied.

Table 4. Coenzyme Q Content in Brain, Heart and Kidney Mitochondria of Control, STZ and STZ + INS Rats

	12-week control	12-week STZ diabetic	8-week STZ diabetic/ 4 week insulin treated
Brain			
CoQ_9	2.17 ± 0.18	1.59 ± 0.06***	1.93 ± 0.07*, ^{SS}
CoQ_{10}	0.47 ± 0.03	0.35 ± 0.04	0.36 ± 0.03
Heart			
CoQ_9	12.42 ± 0.29	14.00 ± 0.55***	12.91 ± 0.39^{SS}
CoQ_{10}	0.47 ± 0.07	0.53 ± 0.02	0.54 ± 0.01
Kidney			
CoQ_9	7.20 ± 0.44	6.04 ± 0.36*	6.90 ± 0.53^{S}
CoQ_{10}	0.69 ± 0.06	0.56 ± 0.07	0.68 ± 0.07

Values are the mean ± SEM from five animals for each condition studied. ***P<0.001; *P<0.05 compared with control rats. ^{SS}p<0.01; ^{S}p<0.05 compared with STZ diabetic rats.

figure **3**, heart mitochondria possess the highest capacity to accumulate calcium when compared with the other mitochondrial preparations studied. In control conditions, the addition of 5 mM succinate produced an ΔΨm (negative inside mitochondria), corresponding to the respiratory state 4. Then, the first pulse of Ca^{2+} led to a rapid depolarization (decrease of ΔΨm) followed by a repolarization (recover of ΔΨm). The depolarization was due to the entry of Ca^{2+} into the electronegative mitochondrial matrix, followed by the efflux of H^+ in an attempt to restore the ΔΨm. However, a second and/or third pulse of Ca^{2+} led to a total depolarization of mitochondria. Mitochondria can tolerate some amount of Ca^{2+}, but ultimately their capacity to adapt to Ca^{2+} loads is overwhelmed and mitochondria depolarize completely due to a profound change in the inner membrane permeability. Brain mitochondria isolated from STZ diabetic animals presented a slight decreased capacity to accumulate Ca^{2+} (Fig. **3A**) Surprisingly kidney and heart mitochondria isolated from diabetic rats possess a slightly higher capacity to accumulate Ca^{2+} as compared with control animals (Figs. **3B**, **3C**). However, mitochondria isolated from diabetic rats treated with insulin had a similar behavior to that of control rats. Pre-incubation of mitochondria with 0.85 μM CsA (specific inhibitor of the MPTP) or 1 mM ADP plus 1 μg/ml oligomycin increased significantly the capacity of mitochondria to accumulate Ca^{2+} (data not shown).

DISCUSSION

In this study we analyzed and compared the effect of insulin treatment on brain, heart and kidney mitochondria isolated from STZ-induced diabetic rats. We observed that diabetes induces tissue-specific mitochondrial alterations with heart mitochondria being the less affected by this pathologic condition, which is probably due to the development of adaptive mechanisms such as the increase in antioxidant levels (CoQ9). Recently, Mootha and collaborators [15] performed a proteomic survey of mitochondria from mouse brain, heart, kidney and liver mitochondria and observed the existence of tissue-specific differences in organelle composition, which could be one reason for the observed tissue-specific differences in mitochondrial status

Table 5. Glutathione Content in Brain, Heart and Kidney Mitochondria Isolated from Control, STZ and STZ + INS Rats

	12-week control	12-week STZ diabetic	8-week STZ diabetic/ 4 week insulin treated
Brain			
GSH	8.30 ± 0.68	7.75 ± 0.26	7.72 ± 0.13
GSSG	3.88 ± 0.13	3.55 ± 0.07	3.48 ± 0.26
GSH/GSSG	2.43 ± 0.23	2.18 ± 0.10	2.24 ± 0.15
Heart			
GSH	17.37 ± 3.01	16.63 ± 1.84	16.09 ± 2.40
GSSG	4.9 ± 0.91	4.55 + 0.66	4.93 ± 0.74
GSH/GSSG	3.60 ± 0.16	3.73 ± 0.17	3.32 ± 0.25
Kidney			
GSH	8.12 ± 1.14	9.21 + 0.79	7.09 ± 1.40
GSSG	2.29 ± 0.23	2.58 ± 0.15	2.11 ± 0.39
GSH/GSSG	3.50 ± 0.24	3.56 ± 0.15	3.29 ± 0.23

Values are mean ± SEM from five animals for each condition studied.

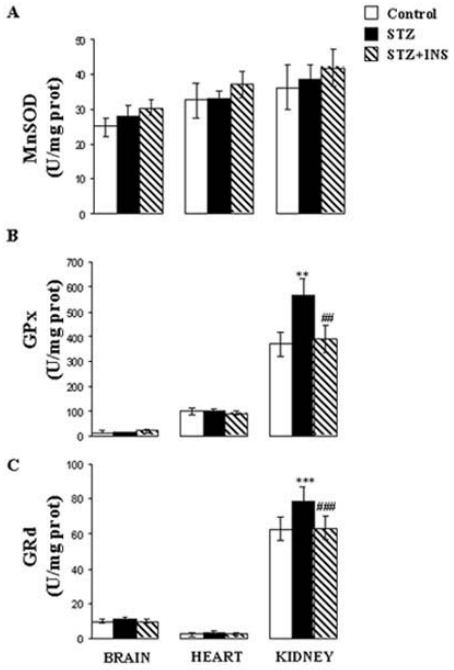

Fig. (1). Effect of STZ-induced diabetes and insulin treatment on manganese superoxide dismutase, glutathione peroxidase and reductase activities. Freshly isolated mitochondria were incubated under standard conditions as described in Materials and Methods. ***p<0.001; **p<0.01 statistically significant when compared to kidney mitochondria isolated from control rats. ###p<0.001; ##p<0.01 statistically significant when compared to kidney mitochondria isolated from STZ diabetic rats. Data shown represent mean ± S.E.M. from 5 animals for each condition studied.

[16]. However, insulin treatment attenuates diabetes-induced mitochondrial alterations suggesting that insulin has a mitochondrial protective function.

For the characterization of the animals, blood glucose and glycated hemoglobin (HbA_{1C}) levels were determined. We observed that STZ-diabetic animals have a significant increase in glycemia and glycated hemoglobin levels as well as a significant decrease in body/organ weight, except the observed increase in kidney/body weight ratio (Table **1**). These data demonstrate that diabetes mellitus was successfully induced in rats injected with STZ. When we compared diabetic animals treated with insulin with those without treatment, we observed that insulin treatment promotes a significant increase of body, brain and heart weight and a significant decrease in kidney/body weight ratio and in the levels of blood glucose and HbA_{1C} (Table **1**). These results are in accordance with human-based studies [17] indicating that insulin treatment improves metabolic control.

Evidence from the literature indicates that there is an increase in oxidative stress in human type 1 diabetes [18] and experimental diabetes [19] and a decrease in the antioxidant capacity [20]. Furthermore, mitochondria are a key element involved in diabetic-related oxidative stress

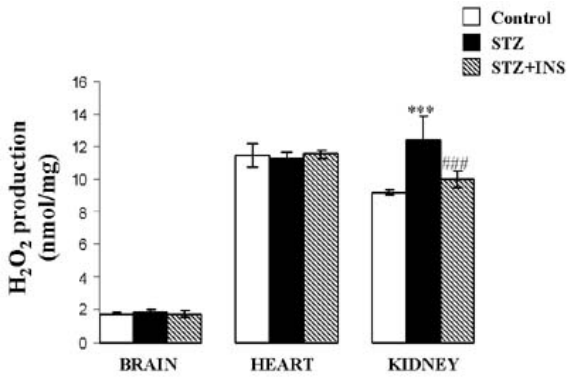

Fig. (2). Effect of STZ-induced diabetes and insulin treatment on mitochondrial H_2O_2 production. Freshly isolated mitochondria were incubated under standard conditions as described in Materials and Methods. ***p<0.001, statistically significant when compared to kidney mitochondria isolated from control rats. ###p<0.001, statistically significant when compared to kidney mitochondria isolated from STZ diabetic rats. Data shown represent mean ± S.E.M. from 5 animals for each condition studied.

because they are the major source of ROS [6]. Surprisingly, we observed that only diabetic kidney mitochondria present a significant increase in H_2O_2 production when compared with control animals (Fig. **2**). Similarly, Anjaneyulu and Chopra [2] reported that diabetic rats exhibit renal dysfunction as a result of a marked increase in oxidative stress. Rosca and collaborators [21] reported that glycation of mitochondrial proteins from diabetic rat kidney is associated with excess superoxide formation. The increase in H_2O_2 (Fig. **2**) is associated with an increase in the activity of GPx (Fig. **1B**) this increase being a potential protective mechanism to neutralize the formation of H_2O_2. It has been reported that the activity of GPx is increased in pancreas [22] and kidney [23] of diabetic rats. GPx, along with catalase, are the primary routes involved in the reduction of intracellular H_2O_2 formation. Because catalase does not reduce organic peroxides, GPx is the primary element involved in the reduction of organic hydroperoxides, including lipid hydroperoxides produced by the reduction of oxygen radicals with phospholipids in cell membranes [24]. Since GPx requires GSH as substrate for its activity, the levels of GSH should be, at least, maintained for the appropriate function of the enzyme. Indeed, our results show that glutathione levels are not statistically altered (Table **5**), which could result from the interplay between the increased activity of both GPx and GRd (Figs. **1B**, **1C**). Furthermore, the maintenance of $\Delta\Psi m$ in all the mitochondrial preparations studied (Table **3**) can also be correlated with the unchanged content of GSH (Table **5**). $\Delta\Psi m$, which normally accounts for 80% of the protonmotive force, contributes for the high degree of reduction of the matrix $NADPH/NADP^+$ pool and, in turn, this pool helps to maintain the matrix glutathione pool in the reduced state. GSH is abundant in mitochondria and is a first-line defence in the cellular antioxidant system. In agreement with our results, Baydas *et al*. [25] observed that although STZ diabetic rats presented higher levels of lipid peroxidation in hippocampus, cortex and cerebellum as compared to control rats, no significant

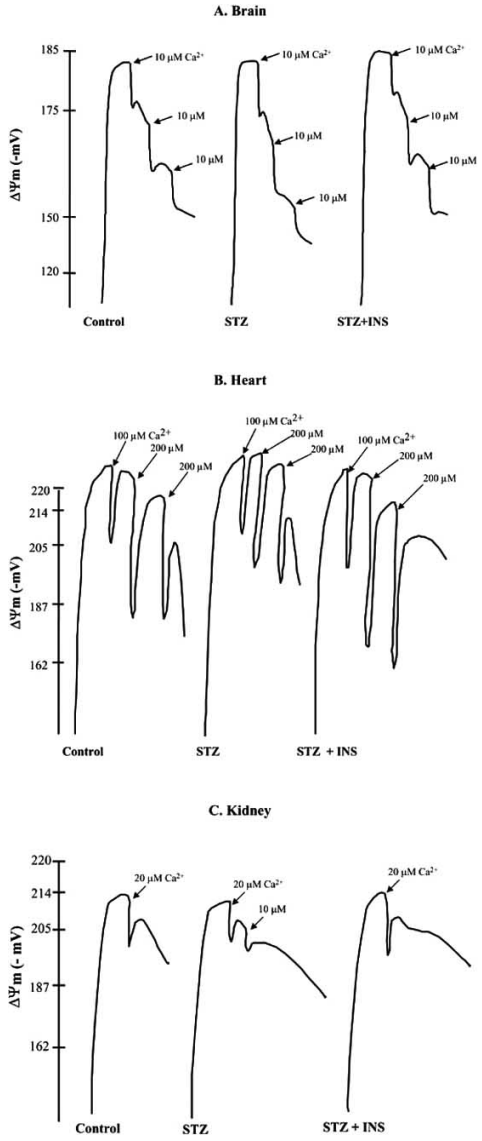

Fig. (3). Effect of STZ-induced diabetes and insulin treatment on the ability of mitochondria to accumulate Ca^{2+}. Freshly isolated mitochondria in 1 ml of the standard medium supplemented with 3 μM TPP^{+} and 2 μM rotenone were energized with 5 mM succinate. **A.** Brain mitochondria; **B.** Heart mitochondria; **C.** Kidney mitochondria. Ca^{2+} was added 1.5 min after mitochondria energization with succinate. The traces are typical of three experiments.

alterations were found in GSH levels in the same brain regions. Similarly, McLennan *et al*. [26] observed that the level of hepatic glutathione was maintained in diabetic animals.

Our results also show that diabetic brain and kidney mitochondria possess a lower content on CoQ9 (Table **4**) indicating a deficit in antioxidant defenses rendering these organelles more prone to oxidative stress-related damage. In contrast, heart diabetic mitochondria possess a higher content of CoQ9 when compared with control animals (Table **4**). The reduced form of CoQ may function as an antioxidant, protecting membrane phospholipids and serum low-density lipoprotein from lipid peroxidation by quenching lipid radicals or lipid peroxidation initiating species and, as recent data indicate, it also protects mitochondrial membrane proteins and DNA from free radical-induced oxidative damage [27,28]. Furthermore, CoQ regulates the mitochondrial permeability transition pore (MPTP) [29]. The MPTP is a phenomenon that is characterized by the opening of pores in the inner mitochondrial membranes and by its sensitivity to a very low concentration of cyclosporin A (CsA). Ca^{2+} and oxidative stress have long been known to favor the mitochondrial permeability transition [30]. An increase or decrease in CoQ9 contents may reduce or enhance, respectively, the induction of MPTP (Figs. **3A**, **3B**). Indeed, we observed that diabetic brain mitochondria present a lower content of CoQ9, which could be related with the decreased capacity to accumulate calcium (Fig. **3A**). Previous results from our laboratory also show that brain mitochondria isolated from type 2 diabetes show a lower ability to accumulate Ca^{2+} [31]. Accordingly, Kostyuk *et al*. [32] observed that primary sensory neurons of STZ-diabetic rats presented a considerable prolongation of residual elevation of cytosolic calcium after termination of membrane depolarisation. Previously, we reported that both models of diabetes (type 1 and type 2) show a decreased susceptibility of heart [33] and liver [12] mitochondria to the induction of MPTP. In addition, we also found that liver mitochondria isolated from diabetic rats show some metabolic adaptations, such as an increase in coenzyme Q that can be responsible for the observed decrease in the susceptibility to multiprotein pore opening [12]. In this study we observed that both diabetic kidney and heart mitochondria possess a slight higher capacity to accumulate Ca^{2+} (Figs. **3B**, **3C**), which could be related with the increase in CoQ9 content (Table **4**).

Furthermore, we observed that although diabetes decreases brain state 3 and 4 of respiration, RCR is maintained (Table **2**), which could be related with the maintenance of ΔΨm (Table **3**). The maintenance of oxidative phosphorylation capacity is extremely important in brain since about 90% of the ATP required for the normal functioning of neurons is provided by mitochondria. However, we also observed that brain mitochondria isolated from diabetic animals present a decrease in ATP contents (Table **3**) that is related with a decrease in ATPase/synthase activity [14]. Previously, we reported that this decrease can be a protective mechanism developed by diabetic rats to avoid a drastic decline in energetic levels. As the activity of ATP synthase enzyme is not down regulated to zero, the electro-chemical gradient across the inner mitochondrial membrane can be maintained or is only slightly reduced by hydrolysis of a small amount of ATP, which could presumably preserve intracellular homeostasis. The membrane potential prevents uncontrolled influx of ATP into the mitochondrial matrix space *via* the electrogenic ATP/ADP translocase thus limiting ATP hydrolysis [14].

Recent findings [34] indicate that insulin is a major regulating factor of mitochondrial oxidative phosphorylation in human skeletal muscle. It has been shown that insulin

selectively stimulates mitochondrial protein synthesis in skeletal muscle and activates mitochondrial enzyme activity [11]. Recently, Katyare and Satav [35] reported that the increase in ATPase activity promoted by STZ-induced diabetes is reversed by insulin. Our results with brain mitochondria show that insulin treatment increases mitochondrial oxidative phosphorylation efficiency by increasing the levels of ATP (Table **3**). Furthermore, insulin was able to normalize mitochondrial antioxidant defenses (CoQ9 content and GPx and GRd activities) that had been altered by diabetes (Table **4**, Figs. **1B**, **1C**). Growing evidence suggests the importance of insulin and insulin growth factors (IGFs) in intracellular antioxidant status by playing a pivotal role in protein kinase B-mediated expression of Bcl_2 protein that prevents the escape of ROS by opposing the oxidative-stress-induced pro-apoptotic action of Bax [36]. Other studies showed that pre-treatment of cells with IGF-1 suppress H_2O_2–induced apoptosis by subsequent inhibition of Bax expression [36]. Recently, Gustafsson *et al*. [37] reported that IGF-1 protect from hyperglycemia-induced oxidative stress and neuronal injuries by regulating the mitochondrial inner membrane potential. Furthermore, IGF-1 plays a role in the regulation of myocardial structure and function [38].

We also observed that insulin is able to increase the ability of mitochondria to accumulate Ca^{2+} (Fig. **3A**) suggesting a role of insulin in Ca^{2+} homeostasis. Kostyuk *et al*. [32] reported that the prolonged residual elevation of cytosolic calcium characteristic of neurons of diabetic animals was partly reversed by insulin treatment. Previous results from our laboratory indicate that insulin treatment is capable of preserving brain mitochondrial function when exposed to the neurotoxic agent, amyloid β-peptide [14]. Insulin affects several brain functions including cognition and memory, and several clinical studies have established links between insulin resistance, diabetes mellitus and Alzheimer's disease [39]. It has also been reported that insulin has a neuroprotective function under oxidizing and/or diabetic conditions by modulating synaptosomal GABA and/or glutamate transport [40,41].

Altogether our results indicate that diabetes induces specific-tissue mitochondrial alterations, heart mitochondria being the most resistant to this pathological condition. However, insulin treatment is capable to protect against diabetic-induced mitochondrial alterations counteracting the increase in oxidative stress and improving oxidative phosphorylation efficiency. Insulin therapy, besides its well-known importance in the maintenance of glycemic control, may play an important role against mitochondrial dysfunction associated to several age-related disorders such as diabetes.

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MATERIALS AND METHODS

Materials

Streptozotocin (STZ) and protease (Subtilisin, Carlsberg) type VIII were obtained from Sigma (Germany). Insulin (Mixtard 30 Novolet) was obtained from Novonardisk A/S (Dinamark). Digitonin was obtained from Calbiochem. All the other chemicals were of the highest grade of purity commercially available.

Animals

Male Wistar and STZ rats (12 weeks) were housed in our animal colony (Laboratory Research Center, University Hospital, Coimbra, Portugal). They were maintained under controlled light (12 hours day/night cycle) and humidity with free access to water (except in the fasting period) and powdered rodent chow (AO4 Panlab). Glucose tolerance tests were used to select diabetic rats for study. Adhering to procedures approved by the Institutional Animal Care and Use Committee, the animals were sacrificed by cervical displacement and decapitation.

Induction, Characterization of STZ-Induced Diabetes and Insulin Treatment

Rats were divided randomly into two groups of animals each. Diabetes was induced in one group of animals after a 16 hour fasting period with a single intraperitoneal injection of STZ, 50 mg/Kg, freshly dissolved in citrate 100 mM, pH 4.5. The volume used was always 0.5 ml/200 g animal weight. Control animals were injected with the same volume of citrate (vehicle) solution. After 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 [42]. After 9 weeks of STZ-induced diabetes, diabetic animals were divided into two groups and one group was injected daily with insulin (5-20 UN/Kg weight). The other group of diabetic rats remained without treatment. Animals were sacrificed after 4 weeks of insulin treatment. During that period, their weight was measured and blood glucose concentration was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer, Portugal). Values were taken during fasting and in non-fasting conditions just before or after STZ and/or insulin administration, respectively. If blood glucose in the tail vein exceeded 250 mg/dl, animals were used as diabetic. Hemoglobin A_{1C} (HbA_{1c}) levels were determined by ion exchange chromatography (Abbott Imx Glicohemoglobin, Abbott Laboratories, Portugal).

Isolation of Brain Mitochondria

Brain mitochondria were isolated by the method of Rosenthal *et al*. [43], with slight modifications, using 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, one rat was decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenised at 4ºC in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.4) containing 5 mg of the bacterial protease. Single brain homogenates were brought to 30 ml and then centrifuged at 2500 rpm for 3 min. The pellet, including the fluffy synapto-somal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 10,000 rpm for 8 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended in 10 ml of medium and recentrifuged at 10,000 rpm for 10 min. The mitochondrial

pellet was resuspended in 300 µl of resuspension medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4). Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin [44].

Isolation of Heart Mitochondria

Heart mitochondria were prepared using a conventional procedure [45]. Briefly, the rats were sacrificed by cervical dislocation followed by decapitation. The heart was immediately excised and finely minced in an ice-cold isolation medium containing 250 mM sucrose. 1 mM EGTA, 10 mM Hepes-KOH (pH 7.4) and 0.1% defatted BSA. The minced blood-free tissue was then ressuspended in 40 ml of isolation medium containing 0.4 mg protease type VIII per g of tissue and homogenized. Exposure to concentrated protease was limited to 2-3 min in order to minimize loss of mitochondrial membrane integrity. The suspension was incubated for 1 min (4ºC) and then re-homogenized. The homogenate was then centrifuged at 9,000 rpm for 10 min 4ºC. The supernatant fluid was decanted and the pellet, essentially devoid of protease, was gently homogenized to its original volume with a loose-fitting homogenizer. The suspension was centrifuged at 200 rpm for 10 min and the resulting supernatant was centrifuged at 10,000 rpm for 10 min with a final washing medium. The pellet was ressuspendend using a paintbrush and repelleted twice at 8,000 rpm for 10 min. EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin [44].

Isolation of Kidney Mitochondria

Kidney mitochondria were prepared using a conventional procedure [46]. After removing the capsule and the renal medulla, the kidneys' cortex was minced finely in an ice-cold isolation medium containing 250 mM sucrose, 10 mM HEPES-KOH, 1 mM EGTA and 0.1% BSA lipid free, pH 7.4. Minced blood-free tissue was homogenized and then centrifuged at 2,500 rpm for 10 min at 4ºC. The supernatant fluid was retained and centrifuged at 10,000 rpm for 10 min. The pellet was resuspended and repelleted twice at 10,000 rpm for 10 min in washing medium containing 250 mM sucrose and 10mM HEPES. Following the final wash, mitochondria were resuspended in 1 ml of the washing medium. Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin [44].

Respiration Measurements

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode [47] connected to a suitable recorder in a 1 ml thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 ºC in 1 ml of the reaction medium with 0.6 mg protein.

Membrane Potential (ΔΨm), Depolarization and Repolarization Potential Measurements

The mitochondrial transmembrane potential was monitored by evaluating the transmembrane distribution of tetraphenylphosphonium (TPP^+) with a TPP^+-selective electrode prepared according to Kamo *et al*. [48] using an $Ag/AgCl_2$ electrode as reference. Reactions were carried out in a chamber with magnetic stirring in 1 ml of reaction medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes, 10 µM EGTA, pH 7.4) supplemented with 3 µM TPP^+. The experiments were started by adding 5 mM succinate to mitochondria in suspension at 0.6 mg protein/ml. After a steady-state distribution of TPP^+ had been reached (2 min of recording), Ca^{2+} was added and ΔΨm recorded. Membrane potential was estimated from the decrease of TPP^+ concentration in the reaction medium as described elsewhere [49]. The isolates were incubated 2 min with 0.85 µM CsA and 2 µg/ml oligomycin plus 1 mM ADP prior mitochondria energization. In the MPTP experiments, after a steady-state distribution of TPP^+ had been reached (2 min of recording), Ca^{2+} was added and ΔΨm recorded.

Analysis of ATP Content

At the end of the ΔΨm experiments, an aliquot of 250 µl of each mitochondrial suspension was rapidly centrifuged at 14,000 rpm for 6 min with 1.2 M perchloric acid. The supernatants were neutralised with 3 M KOH in 1.5 M Tris and centrifuged at 14,000 rpm for 5 min. The resulting supernatants were assayed for ATP by separation in a reverse-phase high performance liquid chromatography. The chromatography apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18 (5 µm) from Merck. An isocratic elution with 100 mM phosphate buffer (KH_2PO_4) pH 6.5 and 1.0% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 6 min.

Measurement of Glutathione Content

Reduced (GSH) and oxidized (GSSG) glutathione were determined with fluorescence detection after reaction of the supernatant of the H_3PO_4 /EDTA -NaH_2PO_4 or H_3PO_4/NaOH deproteinized mitochondria solution, respectively, with *o*-phthalaldehyde (OPT), pH 8.0, according to Hissin and Hilf [50]. In brief, at the end of the ΔΨm experiments, 500 µl of each mitochondrial suspension were rapidly centrifuged at 50,000 rpm (Beckman, TL-100 Ultracentrifuge) for 30 min with 1.5 ml phosphate buffer (100 mM NaH_2PO_4, 5 mM EDTA, pH 8.0) and 500 µl H_3PO_4 2.5%. For GSH determination 100 µl of the supernatant were added to 1.8 ml phosphate 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. For GSSG determination 250 µl of the supernatant were added to 100 µl of *N*-ethylmaleymide 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 (slits 5, 5). The GSH and GSSG contents were determined from comparisons with a linear reduced or oxidized glutathione standard curve, respectively.

Measurement of CoQ Content

CoQ were extracted from aliquots of mitochondria containing 1 mg protein/ml according to the previously

described method [51]. The extract was evaporated to dryness under a stream of N_2, and suspended in ethanol for HPLC analysis. Liquid chromatography was performed using a Gilson high-performance liquid chromatography using a reverse phase column (RP18; Spherisorb; S5 ODS_2) as earlier described [52]. Samples were eluted from the column with methanol:heptane (10:2), by volume), at a flow of 2 ml/min. Detection was performed with a UV detector, at 269 nm. Identification and quantification were based in pure standards by their retention times and peak areas, respectively. CoQ levels (CoQ_9 and CoQ_{10}) in mitochondrial membranes were expressed in pmol/mg protein.

Measurement of Manganese-Superoxide Dismutase (MnSOD) Activity

The activity of manganese-superoxide dismutase (MnSOD) was evaluated using a spectrophotometrical assay [53]. After the incubation of 100 µg of protein in 1.4 ml of phosphate buffer (50 mM K_2HPO_4 and 100 µM EDTA, pH 7.8), 200 µl 0.025 mM hypoxanthine, 66.7 µl Triton X-100, 66.7 µl 0.1 mM nitrobluetetrazolium (NBT) and 13.3 µl 1.33 mM KCN, the reaction started with the addition of 2 µl 0.025 U/ml xanthine oxidase, and the reaction allowed to continue for 3 min, at 550 nm and 25ºC, 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.

Analysis of Glutathione Peroxidase (GPx) and Glutathione Reductase (GRd) Activities

GRd and GPx activities were determined spectrophotometrically at 340 nm, through the analysis of NADPH oxidation [54,55]. Briefly, the activity of GPx was measured upon a 5 min incubation, in the dark, of 10 µl of each sample with 100 µl phosphate buffer (containing 0.25 M KH_2PO_4, 0.25 M K_2HPO_4 and 0.5 mM EDTA, pH 7.0), 100 µl 10 mM GSH (freshly made and protected from light), 100 µl 1 unit GRd and 480 µl H_2O. The quantification occurred after the addition of 100 µl 2.5 mM NADPH and 100 µl 12 mM *tert*-butylhydroperoxide, at 340 nm, and 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. For the activity of GRd, 200 µl of each sample were incubated for 30 s with 1 ml phosphate buffer (containing 0.2 M K_2HPO_4 and 2 mM EDTA, pH 7.0), 100 µl 2 mM NADPH and 700 µl H_2O. The measurements were initiated with the addition of 20 mM GSSG, at 340 nm, (at 30ºC, with continuous magnetic stirring) for 3 min, against blanks prepared in the absence of GSSG, using a Jasco V560 UV/VIS Spectrophotometer.

Measurement of H_2O_2 Production

The rate of hydrogen peroxide (H_2O_2) production was measured fluorimetrically using a modification of the method described by Barja [56]. Briefly, mitochondria were incubated at 37ºC with 10 mM succinate in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCl, 30 mM Hepes, 0,1 mM homovalinic acid and 6U/ml horseradish peroxidase. The incubation was stopped at 15 min with 0,5 ml of cold 2 M glycine buffer containing 25 mM EDTA and NaOH, pH 12. The fluorescence of supernatants was measured at 312 nm as excitation wavelength and 420 nm as emission wavelength. The rate of peroxide production was calculated using a standard curve of H_2O_2.

Statistical Analysis

Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the student *t*-test and one-way ANOVA test for multiple comparisons, followed by the posthoc Tukey-Kramer test.

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