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Biochimica et Biophysica Acta 1639 (2003) 113–120
BIOCHIMICA ET BIOPHYSICA ACTA
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Diabetes induces metabolic adaptations in rat liver mitochondria: role of coenzyme Q and cardiolipin contents

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Received 6 January 2003; received in revised form 29 July 2003; accepted 12 August 2003

Abstract

Several studies have been carried out to evaluate the alterations in mitochondrial functions of diabetic rats. However, results are sometimes controversial, since experimental conditions diverge, including age and strain of used animals. The purpose of this study was to evaluate the metabolic modifications in liver mitochondria, both in the presence of severe (STZ-treated rats) and mild hyperglycaemia [Goto–Kakizaki (GK) rats], when compared with control animals of similar age. Moreover, metabolic alterations were evaluated also at initial and advanced stages of the disease.

We observed that both models of diabetes (type 1 and type 2) presented a decreased susceptibility of liver mitochondria to the induction of permeability transition (MPT). Apparently, there is a positive correlation between the severity of diabetes mellitus (and duration of the disease) and the decline in the susceptibility to MPT induction. We also found that liver mitochondria isolated from diabetic rats presented some metabolic adaptations, such as an increase in coenzyme Q and cardiolipin contents, that can be responsible for the observed decrease in the susceptibility to multiprotein pore (MPTP) opening.

Keywords: Diabetes mellitus; Type 1 diabetes; Type 2 diabetes; Goto–Kakizaki (GK) rat; Streptozotocin-induced diabetic (STZ) rat; Mitochondrial permeability transition (MPT)

1. Introduction

In higher animals, mitochondria play an essential role in cellular energy metabolism since most of the ATP is produced by mitochondrial oxidative phosphorylation. Therefore, it is essential to have mitochondria intimately involved in metabolic regulation and maintenance of ATP production efficiency [1,2].

The inner mitochondrial membrane (IMM) possesses an intrinsically low permeability to ions and solutes, allowing energy conservation in the form of a proton electrochemical potential difference. The IMM also possesses a set of channels and transporters that regulate ion fluxes across the membrane since this management is essential for both metabolic regulation and energy conservation.

Another important role for mitochondria is the calcium buffering capacity, avoiding excessive cytosolic calcium accumulation. Mitochondrial calcium uptake also controls the activity of Ca^{2+}-sensitive dehydrogenases and other metabolic processes. In the presence of high external Ca^{2+} concentrations, isolated mitochondria can easily undergo an increased permeability to solutes with molecular masses up to 1500 kDa. This event is called the mitochondrial permeability transition (MPT) and is caused by the opening of multiprotein pore (MPTP) formed by several proteins of the outer and IMM, including the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT) and the matrix protein cyclophilin [3,4]. MPTP opening can be induced by increasing amounts of Ca^{2+} and oxidative stress.

Previous studies have focused in abnormalities in the MPT during diabetes [5,6], as well as changes in mitochondrial bioenergetics and antioxidant defences [7–9].

Diabetes mellitus is a common degenerative disease and one of the leading causes of morbidity and mortality in

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0925-4439/$ - see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.bbadis.2003.08.001

developed countries. Clinically, diabetes mellitus is a heterogeneous disease, with a common phenotype of impaired glucose tolerance and, depending on the basis of the management required to control glucose homeostasis, it can be divided into type 1 and type 2 diabetes [10–12].

With the purpose of understanding the physiological and pathological changes in this complex disease, animal models for diabetes are important research tools since they allow for a tight control over experimental conditions, almost impossible to achieve in human populations [13,14]. In the present study, two animal models for diabetes were used in order to study abnormalities in liver MPT. STZ-induced diabetic rats, commonly used as an animal model for type 1 diabetes mellitus, were obtained after selective destruction of β-cells by streptozotocin (STZ), a broad-spectrum antibiotic with diabetogenic effects. STZ-injected rats present many characteristics seen in insulin-dependent diabetic human patients: hypoinsulinemia, hyperglycaemia, ketonuria and hyperlipidaemia [13,15]. Goto–Kakizaki (GK) rats are currently used as a non-obese animal model of type 2 diabetes mellitus [16], obtained by selective breeding of normal Wistar rats, using glucose intolerance as selection index [17–19].

In the present study we investigated the impact of diabetes mellitus on mitochondrial calcium fluxes and on the MPT susceptibility in the presence of Ca^{2+}/P_i both in STZ and GK rats. The study was conducted during the progression of diabetes, with the purpose of assessing the mitochondrial metabolic modifications in liver mitochondria. As much as we know, no reports were available concerning mitochondrial alterations in calcium handling during the progression of the disease in the two described models. Our results showed that in liver mitochondria diabetes decreased the susceptibility to the induction of MPT.

2. Materials and methods

2.1. Materials

Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosurea) 1-D-glucopyranose] was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and prepared prior to use in 100 mM citrate, pH 4.5. Calcium Green 5-N was obtained from Molecular Probes (Eugene, OR, USA). All other reagents and chemicals used were of the highest grade of purity commercially available.

2.2. Animals

Spontaneously diabetic male GK rats, 13 or 20 weeks of age, were obtained from our local breeding colony (Animal Research Center Laboratory, University Hospitals, Coimbra), established in 1995 with breeding couples from the colony at the Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr. K. Susuki). Control animals were non-diabetic male Wistar rats of similar age.

Animals were kept under controlled light (12-h day/night cycle), temperature (22–24 °C) and humidity (50–60%) conditions and with free access to powdered rodent chow (diet URF1, Charles Rivers, France) and water (pH 5.5), except during the fasting periods. In this study, the "Principles of Laboratory Animal Care" (NIH publication 83-25, revised 1985) were followed. During this period glycaemia was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer).

2.3. Induction and characterization of STZ-induced diabetes

Male Wistar rats weighing about 200 g (10 weeks) were randomly divided into two groups of 10 animals each. In order to induce diabetes, one group was injected intraperitoneally with a single injection of streptozotocin (STZ, 50 mg/kg), after a 16-h fasting period. The volume used was always 0.5 ml/200 g body weight. Control animals were injected with the same volume of citrate solution. In the following 24 h, animals were orally feed with glycosilated serum in order to avoid hypoglycaemia resulting from massive destruction of ß-cells and release of intracellular insulin associated with STZ treatment [13]. Animals were kept 3 or 9 weeks before the experiments. In this study, in order to avoid another variability factor than diabetes, we used two groups of animals with similar age: 13 weeks of age in one group (STZ-treated rats with 3 weeks after treatment, GK rats 13 weeks of age and control rats with similar age, Wistar rats with 3 weeks after injection with vehicle and/or 13 weeks of age) and 19–20 weeks of age in the other group (STZ-treated rats with 9 weeks after treatment, GK rats 20 weeks of age and control group with similar age, Wistar with 9 week after injection with vehicle and/or 20 weeks of age. Our results show that intravenous administration of citrate does not modify the parameters studied.

During this period, weight was measured and glycaemia was determined from the tail vein as described before. Values were taken in fasting conditions just before STZ administration and in non-fasting conditions in the weeks after. If feeding blood glucose in the tail vein exceeded 250 mg/dl, animals were used as diabetic.

2.4. Glycaemia and HbA_{1C} evaluation

Blood glucose concentration was determined immediately after animal sacrifice (Glucometer-Elite, Bayer). The glycated hemoglobin (HbA_{1C}) values were determined in blood collected at the time of animals' death through ionic exchange chromatography (Abbott Imx Glicohemoglobin, Abbott Laboratories, Portugal).

2.5. Preparation of liver mitochondria

Mitochondria were isolated from the livers of normal and diabetic rats (maintained ad libitum for at least 12 h before

being sacrificed), according to a previously established method [20], with slight modifications.

Homogenization medium contained 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA and 0.1% defatted bovine serum albumin (BSA). EDTA, EGTA and defatted BSA were omitted from the final washing medium and adjusted to pH 7.2. The mitochondrial pellet was washed twice, suspended in the washing medium and immediately used. Protein was determined by the biuret method, using BSA as a standard [21].

2.6. Membrane potential ($\Delta\Psi$) measurements

The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of tetraphenylphosphonium ion (TPP^+) with a TPP^+-selective electrode, prepared as previously reported [22], using a calomel electrode as a reference. TPP^+ uptake was measured from the decrease in TPP^+ concentration in the medium. The potential difference between the selective and the reference electrodes was measured with an electrometer and continuously recorded. A matrix mitochondrial volume of 1.1 μl/mg was assumed and valinomycin was used to calibrate the baseline. Reactions were carried out at 25 °C in 1 ml of the reaction media (200 mM sucrose, 10 mM Tris–Cl, 10 μM EGTA, 1 mM KH_2PO_4, pH 7.2), supplemented with 3 μM TPP^+, 2 μM rotenone and 0.1 μg oligomycin, 1 mg mitochondria and 5 mM succinate. The calcium accumulation capacity was determined by adding small pulses of $CaCl_2$ (10 nmol/mg protein each); these small amounts of calcium were added until the opening of MPTP was observed (as an irreversible drop in $\Delta\Psi_m$).

2.7. Measurement of mitochondrial Ca^{2+} fluxes

The uptake and following release of Ca^{2+} by isolated mitochondria was evaluated with the hexapotassium salt of the calcium-sensitive fluorescent probe Calcium Green 5-N, according to Oliveira et al. [23]. The reactions were carried out in 2 ml of reaction medium (200 mM sucrose, 10 mM Tris–Cl, 10 μM EGTA, 1 mM KH_2PO_4; pH 7.2), supplemented with 0.6 mg liver mitochondria, 2 μM rotenone, 0.1 μg oligomycin, 5 mM succinate and 100 nM Calcium Green 5-N, and stirred continuously in a water-jacketed cuvette holder at 25 °C. Fluorescence was continuously monitored with a Perkin-Elmer LS-50B spectrofluorometer (excitation 506 nm and emission 531 nm), for 50 s, prior to the addition of Ca^{2+} (33 nmol/mg protein). Excess EGTA was used to stop the reaction and to obtain the baseline. As the addition of Ca^{2+} produced a different peak in control and diabetic rats (probably, due to different cardiolipin contents in mitochondrial inner membrane), the data were 'corrected' based on the ratio of these peaks [(1/mean value of Wistar peaks) × 100]; this correction was performed to each different pair of mitochondrial preparations (diabetic and control). Calcium fluxes were expressed as arbitrary units of fluorescence (AFU), determined 100 and 210 s after the addition of Ca^{2+}.

2.8. Extraction and quantification of coenzyme Q

Aliquots of mitochondria containing 2 mg protein/ml were extracted according to the method described previously [24]. The extract was evaporated to dryness under a stream of N_2, and stored at −80 °C, for HPLC analysis. Liquid chromatography was performed using a Gilson HPLC apparatus with a reverse phase column (RP18; Spherisorb; S5 ODS2), as described by Chung et al. [25]. Samples were eluted from the column with methanol/heptane (10:2 v/v) at a flow rate of 2 ml/min. Detection was performed using a UV detector at 269 nm. Identification and quantification were based on pure standards by their retention times and peak areas, respectively. The levels of coenzyme Q (CoQ_9 and CoQ_{10}) were expressed in pmol/mg protein.

2.9. Quantification of cardiolipin content

Mitochondrial cardiolipin content was quantified using an established spectrophotometric assay using 10-nonyl-acridine orange (NAO) [26]. Briefly, rat liver mitochondria (0.25 mg protein/ml) were suspended in 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.2). Aliquots of this suspension (150 μl) were added to increasing amounts of NAO (1–25 μM) and the final volume adjusted to 1.5 ml with the suspension buffer. The samples were incubated for 5 min at room temperature and then centrifuged at 35,000 × *g* for 5 min, using a Beckman ultracentrifuge (model TL-100 and TL-100 rotor). The pellets were discarded and the amount of unbound NAO in the supernatant was measured spectrophotometrically at 495 nm. A standard curve was generated using NAO (1–25 μM) in the absence of mitochondria. The number of moles of NAO per milligram of protein was calculated by subtracting the sample absorbance (unbound NAO) from the value of absorbance correspondent in standard curve (full amount of NAO) [27]. Cardiolipin content was calculated as the half of this value due to the 2:1 stoichiometric relationship between NAO and cardiolipin [27].

2.10. Citrate synthase assay

Citrate synthase was determined according to Coore et al. [28]. Briefly, freeze-thawed liver mitochondria (100 μg) were incubated with 1 ml of Tris-based media [100 mM Tris, 200 μM Acetyl-CoA and 200 μM DTNB (5,5′ -dithio-bis-2-nitrobenzoic acid)]. The absorbance of the suspension was continuously measured at 412 nm under stirring and at 25 °C. After a basal line setting, 100 μM oxaloacetate was added. In this protocol, we measured the formation rate of a coloured product resulting from the condensation of DTNB and coenzyme A. Our results did not show any significant difference between citrate synthase contents, thus pointing

toward no significant differences in the number of mitochondria per milligram of protein in control and diabetic (GK and STZ) rats.

2.11. Statistic analysis of data

The results are presented as mean ± S.E. of the number of experiments indicated and statistical significance between diabetic rats and their control group was determined using unpaired Student's *t* test. Multiple comparison was performed using one-way ANOVA, with the Student–Newman–Keuls as a post-test. $P < 0.05$ was considered significant.

3. Results

3.1. Characterization of animals

Blood glucose levels, determined immediately after the sacrifice of the animals, were significantly higher in diabetic rats when compared to controls (Table 1). However, GK rats presented mild hyperglycaemia (120–210 mg/dl), while STZ-treated rats presented severe hyperglycaemias (>380 mg/dl). In order to estimate the severity of diabetes, glycated hemoglobin (HbA_{1c}) was also evaluated since HbA_{1c} is a very useful parameter to evaluate the severity of diabetes, indicating the average blood glucose levels presented 2–3 months prior to the analysis. The determined levels of HbA_{1c} (Table 1) confirmed that blood glucose levels were significantly increased in diabetic rats and these levels in STZ-treated rats were also significantly augmented as compared to GK rats.

3.2. Calcium accumulation capacity

Calcium accumulation capacity of liver mitochondria from diabetic and control rats was determined observing the effect of known amounts of calcium ('pulses' of 10 nmol $CaCl_2$ each) in the mitochondrial $\Delta\Psi$ (Fig. 1 and Table 2). Mitochondria possess a finite capacity for accumulating calcium before undergoing the calcium-dependent MPT. Our results showed that liver mitochondria from diabetic rats were able to accumulate a higher amount of added Ca^{2+}. Therefore, liver mitochondria isolated from diabetic rats could handle a larger number of pulses of calcium (5 to 11 pulses of 10 nmol Ca^{2+}/mg protein) compared to Wistar rats (about 4 pulses of 10 nmol Ca^{2+}/mg protein; see Fig. 1 for typical experiment) before the irreversible drop in $\Delta\Psi$, compatible with the MPTP opening. This decline in $\Delta\Psi$ was abolished in the presence of

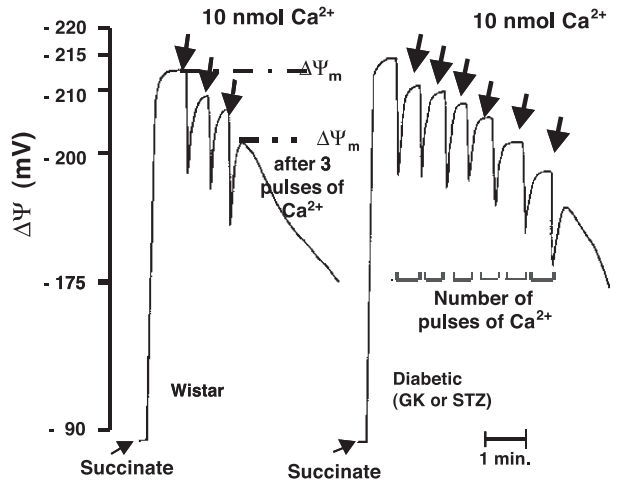

Fig. 1. Calcium accumulation capacity of liver mitochondria isolated from Wistar control and diabetic rats: The Ca^{2+} accumulation capacity, measured with a TPP^{+} selective electrode, was determined by adding several pulses of $CaCl_2$ (10 nmol/mg protein each), in order to induce MPT. The MPTP opening was assessed as a drop in membrane potential. In the presence of cyclosporin A, a specific inhibitor of MPT induction, the number of pulses of calcium needed to induce a similar decrease membrane potential is much higher (both for diabetic and control mitochondrial preparations, not shown). The traces are representative of experiments performed with different mitochondrial preparations.

Table 1
Characterization of animals

Condition	3 weeks after treatment		9 weeks after treatment		13 weeks of age		20 weeks of age	
	Wistar	STZ	Wistar	STZ	Wistar	GK	Wistar	GK
Glycaemia (mg/dl)	115.1 ± 4.5 ($n=6$)	409.3 ± 17.9 ($n=6$)***,#,&&	97.3 ± 5.5 ($n=6$)	468.6 ± 15.6 ($n=6$) ***,&&	101.3 ± 5.0 ($n=8$)	141.2 ± 9.4 ($n=8$) *,###,&	85.7 ± 5.0 ($n=9$)	162.0 ± 28.6 ($n=9$)*,##
HbA_{1C}	5.23 ± 0.21 ($n=6$)	10.25 ± 0.43 ($n=6$)***,#,&&	5.84 ± 0.47 ($n=6$)	11.71 ± 0.39 ($n=6$) ***,&&	5.04 ± 0.12 ($n=6$)	7.32 ± 0.67 ($n=6$)*,##	5.43 ± 0.33 ($n=6$)	8.46 ± 0.89 ($n=6$)**,##

Data are mean ± S.E. obtained from the number of samples indicated, each obtained from a different animal. Glycaemia and HbA_{1C} were determined as described in Materials and methods.

* $P < 0.05$ as compared to controls.
** $P < 0.01$ as compared to controls.
*** $P < 0.001$ as compared to controls.
# $P < 0.05$ compared with STZ (9 weeks after treatment).
$P < 0.01$ compared with STZ (9 weeks after treatment).
$P < 0.001$ compared with STZ (9 weeks after treatment).
& $P < 0.05$ compared with GK (20 weeks of age).
&& $P < 0.01$ compared with GK (20 weeks of age).

Table 2
Effect of diabetes on Ca^{2+} accumulation capacity

Condition	$\Delta\Psi_m$ (−mV)	$\Delta\Psi_m$ (−mV) (after 3 pulses of 10 nmol Ca^{2+})	Pulses of Ca^{2+} (mean value)
Wistar (3 weeks after treatment)	225.0 ± 1.20 (n=6)	219.9 ± 3.13 (n=6)	4.70 ± 0.62 (n=6)$^{\#\#\#}$
STZ (3 weeks after treatment)	228.2 ± 1.61 (n=6)	223.3 ± 0.65 (n=6)	5.50 ± 0.29 (n=6)*,$^{\#\#\#}$
Wistar (9 weeks after treatment)	223.1 ± 2.96 (n=6)	218.2 ± 1.37 (n=6)	4.59 ± 0.22 (n=6)
STZ (9 weeks after treatment)	230.6 ± 2.46 (n=6)*	227.3 ± 2.74 (n=6)*	11.20 ± 0.70 (n=6)***
Wistar (13 weeks of age)	220.6 ± 2.00 (n=7)	215.8 ± 2.95 (n=8)	3.62 ± 0.19 (n=7)$^{\#\#\#}$
GK (13 weeks of age)	225.0 ± 1.12 (n=7)$^{\&\&\&}$	222.2 ± 1.37 (n=7)	5.56 ± 0.45 (n=7)*,$^{\#\#}$
Wistar (20 weeks of age)	222.6 ± 2.45 (n=8)	212.5 ± 2.55 (n=8)	4.00 ± 0.22 (n=8)$^{\#\#\#}$
GK (20 weeks of age)	235.8 ± 1.99 (n=8)***	228.0 ± 1.86 (n=8)***	5.62 ± 0.28 (n=8)*,$^{\#\#\#}$

The mitochondrial electric potential measurements ($\Delta\Psi$) were performed with a TPP^+ selective electrode. $\Delta\Psi$ was evaluated as described in Materials and methods. Data are mean ± S.E. of the number of independent experiments indicated, performed with at least three different mitochondrial preparations.

* $P<0.05$ compared to controls.
*** $P<0.001$ compared to controls.
$^{\#\#}$ $P<0.01$ compared to STZ (9 weeks after treatment).
$^{\#\#\#}$ $P<0.001$ compared to STZ (9 weeks after treatment).
$^{\&\&\&}$ $P<0.001$ compared to GK (20 weeks of age).

cyclosporine A, a specific inhibitor of MPT (data not shown).

Previous results, showing the higher calcium capacity of diabetic mitochondria, were confirmed by determinations of extramitochondrial calcium movements, using the fluorescent calcium-sensitive probe Calcium Green 5-N. The addition of Ca^{2+} leads to an increase of the Ca^{2+} content in the external medium, which is associated to the rapid increase in fluorescence (the first peak in Fig. 2A). As the calcium is accumulated by mitochondria, the fluorescence decreases. The MPTP opening leads to the release of the previous accumulated calcium and consequently to an increase in fluorescence. The release of calcium was completely abolished (both in Wistar and diabetic rat liver mitochondria) by cyclosporine A, indicating that the release was due to MPTP opening (Fig. 2A). We observed that 100 s after the addition of calcium (33 nmol $CaCl_2$/mg protein), the Ca^{2+} loss by diabetic liver mitochondria was significantly lower when compared to control Wistar rats (Fig. 2B). Moreover, 210 s after the addition of Ca^{2+}, the release of calcium was significantly decreased in diabetic rats (except for GK rats with 13 weeks of age) compared to controls (Fig. 2C). Therefore, our results indicated that mitochondrial calcium

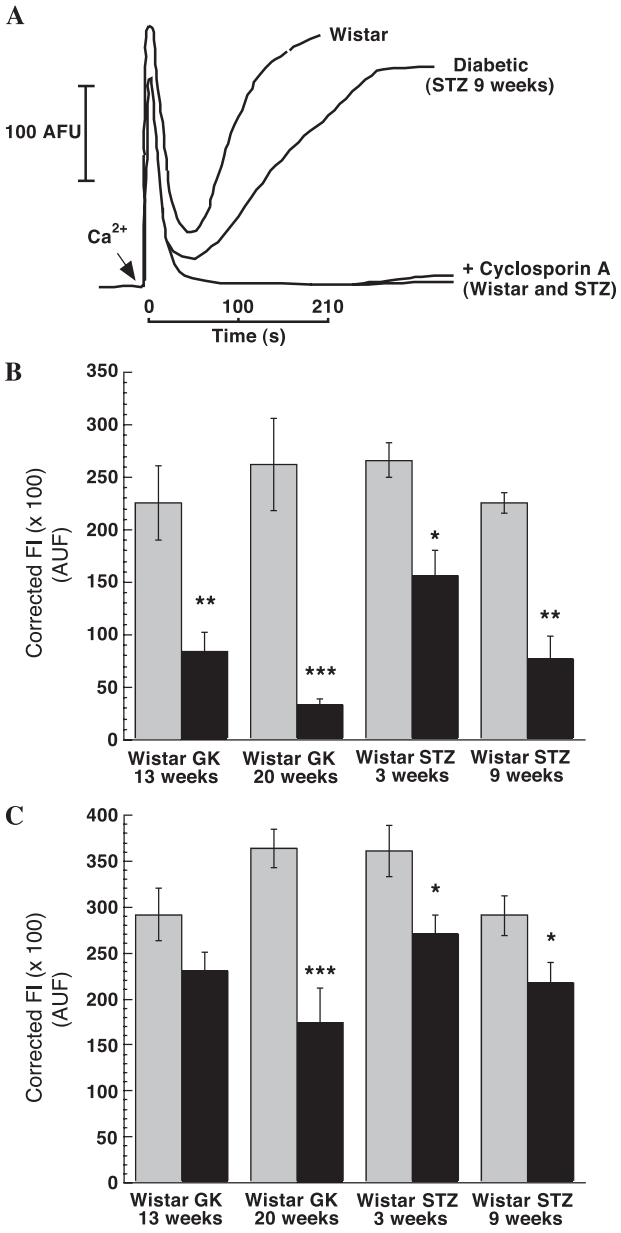

Fig. 2. Mitochondrial Ca^{2+} fluxes in liver mitochondria isolated from diabetic and Wistar control rats: (A) Typical measurements of calcium movements using the fluorescent calcium sensitive probe Calcium Green 5-N, as described in Materials and methods. The reactions were carried out in 2 ml of reaction medium supplemented with 0.6 mg protein, 2 μM rotenone, 0.1 μM oligomycin, 5 mM succinate and 100 nM Calcium Green 5-N. Fluorescence was monitored continuously for 50s, prior to the addition of 33 nmol Ca^{2+}/mg protein, and stopped with excess EGTA to obtain the baseline. The calcium release was inhibited by 0.8 μM cyclosporin A, indicating that the observed release was due to MPTP opening. Calcium fluxes are expressed as arbitrary units of fluorescence (AFU), determined 100 s (B) and 210 s (C) after the addition of 33 nmol Ca^{2+}/mg protein, as described in Materials and methods. Data are mean ± S.E. of four independent experiments performed with different mitochondrial preparations. *$P<0.05$, **$P<0.01$, ***$P<0.001$, as compared to Wistar control rats.

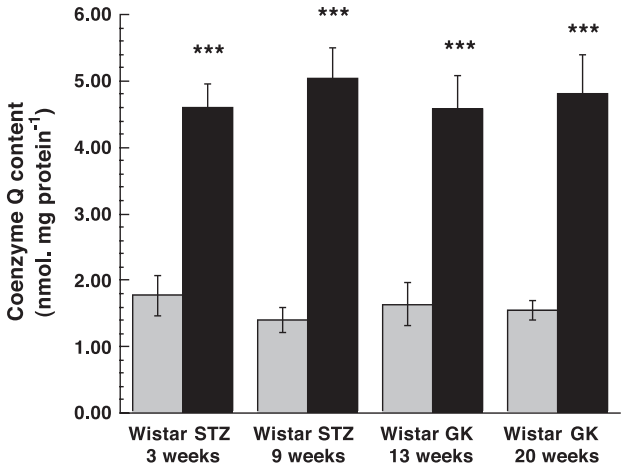

Fig. 3. Coenzyme Q content in liver mitochondria isolated from Wistar control and diabetic rats. The levels of coenzyme Q (CoQ_9 and CoQ_{10}) were measured by HPLC as described in Materials and methods and expressed in pmol/mg protein. Data are mean ± S.E. of five different mitochondrial preparations. ***$P<0.001$ as compared to Wistar control rats.

accumulation capacity increased with the increase in diabetes severity.

3.3. Mitochondrial CoQ content

CoQ in rat mitochondria consists of two main homologues, CoQ_9 and CoQ_{10}. In Wistar non-diabetic rats, CoQ_{10} represented 10–16% of the total amount of mitochondrial CoQ, while in diabetic rats this ratio varied between 8% and 12%. However, the amount of CoQ, particularly CoQ_9, was significantly increased in liver mitochondria isolated from diabetic rats (up to 2.5-fold) (Fig. 3). Results of citrate synthase quantification and analysis by electron microscopy (data not shown) showed that the contamination level of mitochondrial preparations was very low. Therefore, the contents of CoQ determined can be attributed to the IMM.

3.4. Mitochondrial cardiolipin content

In order to help in the understanding of the observed resistance of diabetic mitochondria to MPT induction, cardiolipin (diphosphatidylglycerol) contents were evaluated [26]. Our results showed that liver mitochondria isolated from diabetic rats presented a significantly higher content of cardiolipin, except for the STZ-treated rats, 3 weeks after STZ treatment (Fig. 4).

4. Discussion

Mitochondria are the major ATP producer in eukaryotic mammalian cells and also the main intracellular sources and target of reactive oxygen species (ROS). Moreover, mitochondria play an important role in the regulation of intracellular Ca^{2+} homeostasis. Previous reports describe some abnormalities in MPTP associated with diabetes [5,6]. Since the opening of MPTP can be induced by increasing amounts of Ca^{2+} and by oxidative stress, and considering that diabetes leads to increased oxidative stress, due to constant hyperglycaemia [29–31], it should be expected an increase in Ca^{2+}-dependent MPT induction during diabetes [32]. However, mitochondria isolated from STZ-treated rats liver [5] and from GK rats heart [6] presented a lower susceptibility to MPT induction in the presence of Ca^{2+}. These observations were on the basis of the present study, designed to evaluate differences in MPT susceptibility due to diabetes in liver mitochondria. Our results showed that mitochondria from diabetic rats were less susceptible to the induction of MPTP assessed by Ca^{2+}/P_i. Surprisingly, we noted that the

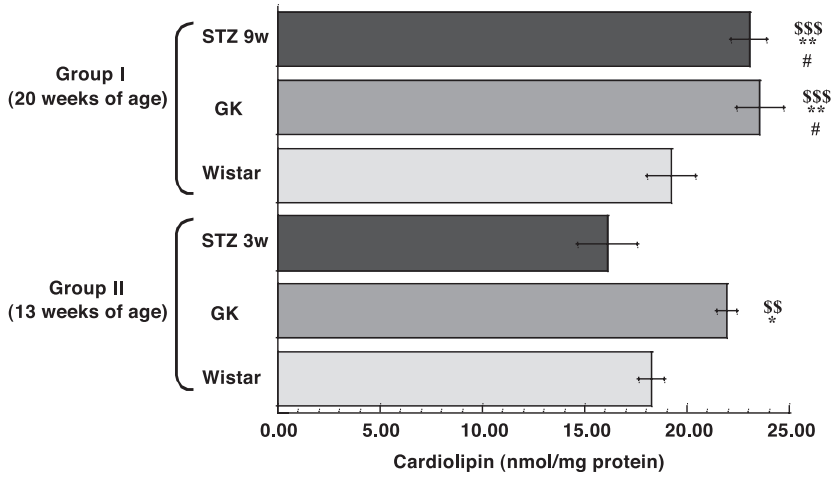

Fig. 4. Cardiolipin content in liver mitochondria isolated from diabetic and Wistar control rats. Mitochondrial cardiolipin content was quantified using an established spectrophotometric assay using 10-NAO, as described in Materials and methods. Data are mean ± S.E. of five different mitochondrial preparations. $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$, compared to STZ 3 weeks after treatment; *$P<0.05$. **$P<0.01$ as compared to Wistar 13 weeks of age (3 weeks after treatment with STZ); $^{\#}P<0.05$ as compared to Wistar 20 weeks of age (9 weeks after treatment with STZ).

Ca^{2+} accumulation capacity increased, with increased diabetes time length and severity, as observed in STZ-treated rats after 9 weeks of treatment and GK rats 20 weeks of age. These observations were even more surprising since the levels of endogenous oxidation products in these mitochondria and in liver mitochondria from STZ-treated rats 3 weeks after treatment were lower when compared to controls [evaluated as thiobarbituric acid reactive substances (TBARS) formation [33]; data not shown].

In order to find some explanations for the observed results, we evaluated the CoQ content in liver mitochondria. It is well known that oxidative stress can trigger the MPT pore opening [32,34]. Coenzyme Q acts as an electron carrier from mitochondrial respiratory complexes I and II to complex III; also, CoQ in its fully reduced form (ubiquinol) is a potent antioxidant [35–38], preventing lipid peroxidation. Furthermore, as ubiquinone-binding site regulates the MPT pore [39], an increase in CoQ contents (CoQ_0 and, probably, also CoQ_9 and CoQ_{10}) may enhance the calcium retention capacity in mitochondria [3]. Our results showing that the amount of CoQ was significantly increased in liver mitochondria from diabetic rats suggest that this increase in CoQ contents can, in part, be responsible for the decreased susceptibility for MPTP opening, observed in diabetic mitochondrial preparations (in addition to the increase in the activity of Complexes II and IV of the respiratory chain [40]). Additionally, the increase in CoQ contents in diabetic rats can be responsible for the higher membrane potential developed upon energization with succinate; we also found an increase in Complex II (and IV) specific activity with increase in diabetes severity [40].

It has also been found that the membrane lipid composition is able to modulate the MPTP opening [26]. Cardiolipin (diphosphatidylglycerol), the only phospholipid synthesized by the mitochondria [26], has been proposed to regulate the induction of MPT due to its ability to bind calcium [41–43], owing to its strongly negatively charged headgroups. The increased pool of negatively charges is thought to nonspecifically bind Ca^{2+}, preventing its action on protein sites that play a role in MPTP opening. Our data indicated that the content in cardiolipin was significantly increased in diabetic mitochondrial preparations (except for STZ-treated rats 3 weeks after treatment), when compared to Wistar mitochondria, suggesting that mitochondria have the capacity to enhance the synthesis of cardiolipin or adjust phospholipid metabolism, in order to decrease the susceptibility to the induction of MPT. The higher content in cardiolipin of the IMM of diabetic rats makes it more impermeable to protons (and other ions), which could explain the higher values of $\Delta\Psi$ observed.

The higher calcium loading capacity in diabetic rats may interfere with the normal calcium pathways of the cell. Inside mitochondria, calcium can stimulate some dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [44,45]) and have a regulatory effect on ATP synthase [46].

Previous reports from our group [6,47,48] on the susceptibility on heart and brain mitochondrial preparations to MPTP induction showed that GK mitochondrial preparations from heart had reduced susceptibility to MPT induction [6], in agreement with the liver mitochondria results reported in this study, while heart mitochondria isolated from STZ-treated rats after 3 weeks of treatment had an increase susceptibility to the induction of MPT [47]. Brain mitochondria isolated from STZ rats after 9 weeks of treatment were less susceptible to MPT induction.

In conclusion, our results suggest that liver mitochondria isolated from diabetic rats presented some metabolic adjustments, namely increase in CoQ and membrane cardiolipin contents that can be responsible for the observed decrease in the susceptibility to MPT induction in the presence of Ca^{2}. These adjustments seem to vary between the tissues studied in our lab (liver, brain and heart). We cannot exclude the possibility that liver mitochondria from control rats present a higher number of spontaneous (but reversible) MPTP openings, compared to diabetic liver mitochondria.

Acknowledgements

This work was supported by Fundação para a Ciência e a Tecnologia (FCT; Portuguese Research Council). Fernanda M. Ferreira and Paulo J. Oliveira are recipients of PhD grants from FCT (SFRH/BD/3247/2000 and PRAXIS XXI/BD/21494/99, respectively).

References

[1] T.E. Gunter, K.K. Gunter, S.S. Sheu, C.E. Gavin, Mitochondrial calcium transport: physiological and pathological relevance, Am. J. Physiol. 267 (1994) C313–C339.
[2] P. Bernardi, V. Petronilli, F. Di Lisa, M. Forte, A mitochondrial perspective on cell death, Trends Biochem. Sci. 26 (2001) 112–117.
[3] L. Walter, V. Nogueira, X. Leverve, M.P. Heitz, P. Bernardi, E. Fontaine, Three classes of ubiquinone analogs regulate the mitochondrial permeability transition pore through a common site, J. Biol. Chem. 275 (2000) 29521–29527.
[4] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (1999) 233–249.
[5] B.S. Kristal, M. Matsuda, B.P. Yu, Abnormalities in the mitochondrial permeability transition in diabetic rats, Biochem. Biophys. Res. Commun. 222 (1996) 519–523.
[6] P.J. Oliveira, A.P. Rolo, R. Seiça, C.M. Palmeira, M.S. Santos, A.J. Moreno, Decreased susceptibility of heart mitochondria from diabetic GK rats to mitochondrial permeability transition induced by calcium phosphate, Biosci. Rep. 21 (2001) 45–53.
[7] Y. Ihara, Y. Yamada, S. Toyokuni, K. Miyawaki, N. Ban, T. Adachi, A. Kuroe, T. Iwakura, A. Kubota, H. Hiai, Y. Seino, Antioxidant α-tocopherol ameliorates glycemic control of GK rats, a model of type 2 diabetes, FEBS Lett. 473 (2000) 24–26.
[8] E.C. Opara, E. Abdel-Rahman, S. Soliman, W.A. Kamel, S. Souka, J.E. Lowe, S. Abdel-Aleem, Depletion of total antioxidant capacity in type 2 diabetes, Metabolism 48 (1999) 1414–1417.
[9] R.W. Rinehart, J. Roberson, D.S. Beattie, The effect of diabetes on

protein synthesis and the respiratory chain of rat skeletal muscle and kidney mitochondria, Arch. Biochem. Biophys. 213 (1982) 341–352.
[10] C.D. Berdanier, Diabetes and nutrition: the mitochondrial part, J. Nutr. 131 (2001) 344S–353S.
[11] M.M. Engelgau, L.S. Geiss, The burden of diabetes mellitus, in: J.L. Leahy, N.G. Clark, W.T. Cefalu (Eds.), Medical Management of Diabetes Mellitus, Mark Dekker, New York, 2000, pp. 1–17.
[12] G. Emilien, J.M. Maloteaux, M. Ponchon, Pharmacological management of diabetes: recent progress and future perspective of daily drug treatment, Pharmacol. Ther. 81 (1999) 37–51.
[13] B. Rodrigues, P. Poucheret, M.L. Battell, J.H. McNeill, Streptozotocin-induced diabetes: Induction, mechanism(s), and dose dependency, in: J.H. McNeill (Ed.), Experimental Models of Diabetes, CRC Press, Boca Raton, FL, 1999, pp. 3–17.
[14] R.S. Surwit, P.G. Williams, Animal models provide insight into psychosomatic factors in diabetes, Psychosom. Med. 58 (1996) 582–589.
[15] W.C. Stanley, G.D. Lopaschuk, K.M. Kivilo, Alterations in myocardial energy metabolism in streptozotocin diabetes, in: J.H. McNeill (Ed.), Experimental Models of Diabetes, CRC Press, Boca Raton, FL, 1999, pp. 19–38.
[16] P. Serradas, M.N. Gangnerau, M.H. Giroix, C. Saulnier, B. Portha, Impaired pancreatic b cell function in the fetal GK rat, J. Clin. Invest. 101 (1998) 899–904.
[17] Y. Goto, K. Susuki, T. Ono, M. Sasaki, T. Toyota, Development of diabetes in the non-obese NIDDM rat (GK) rat, Adv. Exp. Med. Biol. 246 (1988) 29–31.
[18] Y. Goto, M. Kakizaki, N. Masaki, Spontaneous diabetes produced by selective breeding of normal Wistar rats, Proc. Jpn. Acad. 51 (1975) 80–85.
[19] Y. Goto, M. Kakizaki, The spontaneous-diabetes rat: a model of noninsulin dependent diabetes mellitus, Proc. Jpn. Acad. 57 (1981) 381–384.
[20] P. Gazotti, K. Malmstron, M. Crompton, Preparation and assay of animal mitochondria and submitochondrial vesicles, in: E. Carafoli, G. Sememza (Eds.), Membrane Biochemistry: A Laboratory Manual on Transport and Bioenergetics, Springer-Verlag, New York, 1979, pp. 62–69.
[21] A.G. Gornall, C.J. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (1949) 751–766.
[22] N. Kamo, M. Muratsugu, R. Hongoh, Y. Kobatake, Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state, J. Membr. Biol. 49 (1979) 105–121.
[23] P.J. Oliveira, P.M. Coxito, A.P. Rolo, D.L. Santos, C.M. Palmeira, A.J. Moreno, Inhibitory effect of carvedilol in the high-conductance state of the mitochondrial permeability transition pore, Eur. J. Pharmacol. 412 (2001) 231–237.
[24] M. Takada, S. Ikenoya, T. Yuzuriha, K. Katayama, Simultaneous determination of reduced and oxidized ubiquinones, Methods Enzymol. 105 (1984) 147–155.
[25] A.P. Chung, F. Rainey, M.F. Nobre, J. Burghardt, M.S. daCosta, *Meiothermus cerbereus* sp.nov., a new slightly thermophilic species with high levels of ε-hydroxy fatty acids, Int. J. Syst. Bacteriol. 47 (1997) 1225–1230.
[26] M.J. Lieser, J. Park, S. Natori, B.A. Jones, S.F. Bronk, G.J. Gores, Cholestasis confers resistance to the rat liver mitochondrial permeability transition, Gastroenterology 115 (1998) 693–701.
[27] J.M. Petit, A. Maftah, M.H. Ratinaud, R. Julien, 10*N*-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria, Eur. J. Biochem. 209 (1992) 267–273.
[28] H.G. Coore, R.M. Denton, B.R. Martin, P.J. Rande, Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones, Biochem. J. 125 (1971) 115–127.
[29] J.W. Baynes, Role of oxidative stress in development of complications in diabetes, Diabetes 40 (1991) 405–412.
[30] P. Rosen, P.P. Nawroth, G. King, W. Moller, H.J. Tritschler, L. Packer, The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society, Diabetes/Metab. Res. Rev. 17 (2001) 189–212.
[31] P. Rosen, X. Du, G.Z. Sui, Oxidative stress in diabetes: why does hyperglycemia induce the formation of reactive oxygen species? in: L. Packer, P. Rosen, H.J. Tritschler, G.L. King, A. Azzi (Eds.), Antioxidants in Diabetes Management, Marcel Dekker, New York, 1998, pp. 17–31.
[32] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Mitochondrial permeability transition and oxidative stress, FEBS Lett. 495 (2001) 12–15.
[33] M.S. Santos, D.L. Santos, C.M. Palmeira, R. Seiça, A.J. Moreno, C.R. Oliveira, Brain and liver mitochondria isolated from diabetic Goto–Kakizaki rats show different susceptibility to induced oxidative stress, Diabetes/Metab. Res. Rev. 17 (2001) 223–230.
[34] L. Scorrano, V. Petronilli, P. Bernardi, On the voltage dependence of the mitochondrial permeability transition pore. A critical appraisal, J. Biol. Chem. 272 (1997) 12295–12299.
[35] L. Ernster, P. Forsmark-Andrée, Ubiquinol: an endogenous antioxidant in aerobic organisms, Clin. Investig. 71 (1993) S60–S65.
[36] F.M. Rauscher, R.A. Sanders, J.B. Watkins III, Effects of coenzyme Q_{10} treatment on antioxidant pathways in normal and streptozotocin-induced diabetic rats, J. Biochem. Mol. Toxicol. 15 (2001) 41–46.
[37] C.M. Palmeira, D.L. Santos, R. Seiça, A.J. Moreno, M.S. Santos, Enhanced mitochondrial testicular antioxidant capacity in Goto–Kakizaki diabetic rats: role of coenzyme Q, Am. J. Physiol., Cell Physiol. 281 (2001) C1023–C1028.
[38] J.A. Crestanello, N.M. Doliba, N.M. Doliba, A.M. Babsky, K. Niborii, M.D. Osbakken, G.J.R. Whitman, Effect of Coenzyme Q_{10} supplementation on mitochondrial function after myocardial ischemia reperfusion, J. Surg. Res. 102 (2002) 221–228.
[39] E. Fontaine, F. Ichas, P. Bernardi, A ubiquinone-binding site regulates the mitochondrial permeability transition pore, J. Biol. Chem. 273 (1998) 25734–25740.
[40] F.M. Ferreira, C.M. Palmeira, R. Seiça, A.J. Moreno, M.S. Santos, Diabetes and mitochondrial bioenergetics: alterations with age, J. Biochem. Mol. Toxicol. 17 (2003) 214–222.
[41] N. Brustovetsky, M. Klingenberg, Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca^{2+}, Biochemistry 35 (1996) 8483–8488.
[42] A.E. Vercesi, A.J. Kowaltowski, M.T. Grijalba, A.R. Meinicke, R.F. Castilho, The role of reactive oxygen species in mitochondrial permeability transition, Biosci. Rep. 17 (1997) 43–52.
[43] J.M. Petit, O. Huet, P.F. Gallet, A. Maftah, M.H. Ratinaud, R. Julien, Direct analysis and significance of cardiolipin transverse distribution of mitochondrial inner membranes, Eur. J. Biochem. 220 (1994) 871–879.
[44] J.G. McCormack, Characterization of the effects of Ca^{2+} on the intramitochondrial Ca^{2+}-sensitive enzymes from rat liver and within intact rat liver mitochondria, Biochem. J. 231 (1985) 581–595.
[45] J.G. McCormack, A.P. Halestrap, R.M. Denton, Role of calcium ions in regulation of mammalian intramitochondrial metabolism, Physiol. Rev. 70 (1990) 391–425.
[46] D.A. Harris, A.M. Das, Control of mitochondrial ATP synthesis in the heart, Biochem. J. 280 (1991) 561–573.
[47] P.J. Oliveira, Cardiac mitochondria function: studies on the impact of disease conditions and on the protection afforded by carvedilol, PhD Thesis, University of Coimbra (Portugal), 2002, pp. 103–110.
[48] P.I. Moreira, M.S. Santos, A.J. Moreno, R. Seiça, C.R. Oliveira, Increased vulnerability of brain mitochondria in diabetic (Goto–Kakizaki) rats with aging and amyloid-β exposure, Diabetes 52 (2003) 1449–1456.