

Diabetes and Mitochondrial Bioenergetics: Alterations with Age

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ABSTRACT: Several studies have been carried out to evaluate the alterations in mitochondrial functions of diabetic rats. However, some of the results reported are controversial, since experimental conditions, such as aging, and/or strain of animals used were different. The purpose of this study was to evaluate the metabolic changes in liver mitochondria, both in the presence of severe hyperglycaemia (STZ-treated rats) and mild hyperglycaemia (Goto-Kakizaki (GK) rats). Moreover, metabolic alterations were evaluated both at initial and at advanced states of the disease.

We observed that both models of type 1 and type 2 diabetes presented alterations on respiratory chain activity. Because of continual severe hyperglycaemia, 9 weeks after the induction of diabetes, the respiratory function declined in STZ-treated rats, as observed by membrane potential and respiratory ratios (RCR, P/O, and FCCP-stimulated respiration) assessment. In contrast, GK rats of 6 months age presented increased respiratory ratios.

To localize which respiratory complexes are affected by diabetes, enzymatic respiratory chain activities were evaluated. We observed that succinate dehydrogenase and cytochrome *c* oxidase activities were significantly augmented both in STZ-treated rats and GK rats of 6 months age. Moreover, H⁺-ATPase activity was also significantly increased in STZ-treated rats with 3 weeks of diabetes and in GK rats of 6 months age as compared to controls. Therefore, these results clearly suggest that both animal models of diabetes present some metabolic adjustments in order to circumvent the deleterious effects promoted by the high glucose levels typical of the disease. © 2003 Wiley Periodicals, Inc. *J Biochem Mol Toxicol* 17:214–222, 2003; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.10081

KEYWORDS: Diabetes Mellitus; Type 1 Diabetes; Type 2 Diabetes; Goto-Kakizaki (GK) Rat; Streptozotocin-Induced Rat; Respiratory Chain

INTRODUCTION

Diabetes mellitus is a common degenerative disease and one of the leading causes of morbidity and mortality in developed countries. Diabetes is a major cause of serious micro-(retinopathy, nephropathy, and neuropathy) and macrovascular diseases (cardiovascular diseases and nontraumatic lower extremity amputations), affecting, therefore, nearly every organ in the body.

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 in type 1 and type 2 diabetes.

Type 1 diabetes occurs mainly in childhood and puberty and is characterized by an absolute insulin deficiency, requiring daily insulin replacement therapy, in addition to diet and physical activity. Type 2 diabetes usually develops in adults over age 40, accounts for 90–95% of all diagnosed cases and is characterized by insulin resistance and/or inadequate compensatory insulin secretion response [1].

To understand the physiological and pathological changes of this complex disease, animal models of diabetes are important research tools, since animal studies allow for tight control over experimental conditions, which is almost impossible to achieve in human populations [2,3].

In the present study, we used STZ-induced rats as type 1 and Goto-Kakizaki (GK) rats as type 2 diabetes mellitus models. STZ-induced diabetic rats, commonly used as an animal model of type 1 diabetes mellitus, are obtained after selective destruction of β -cell

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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 [2]. Therefore, this model is of great use to evaluate the changes promoted by uncontrolled type 1 diabetes.

Goto-Kakizaki (GK) rats are currently used as an animal model of type 2 diabetes. This animal is a nonobese, spontaneously diabetic rat [3], produced by selective breeding of Wistar rats, using glucose intolerance as selection index [4–7]. GK rats exhibit a moderate but stable fasting hyperglycemia (until the age of 6 months), evident from 6 weeks of age, which does not progress to a ketotic state. Furthermore, GK rat is one of the best-characterized animal models of spontaneous non-obese type 2 diabetes mellitus, since it exhibits similar metabolic, hormonal, and vascular disorders to the human disease [3]. Therefore, in the initial stages of diabetes, GK rats do not exhibit severe complications associated with the disease, being an important model to study the initial events of diabetes.

While insulin release decreases in diabetes, glucagon release increases, promoting an enlarged glycogen degradation rate. So, liver cells are first surrounded by higher glucose levels compared to other body cells. These permanent high glucose levels can promote nonenzymatic protein and lipid glycosylation and lead to energy metabolism alterations. Moreover, alterations in adenosine triphosphate (ATP) generation also affect glycolysis ratio, decreasing glucose utilization, since glucokinase (and hexokinase) activity depends on the intracellular pool of ATP [8,9]. Therefore, in the present paper, we compare the possible alterations in liver mitochondrial respiratory system in STZ and GK rats, during the progression of diabetes, with the purpose of evaluating the metabolic modifications in liver mitochondria.

MATERIALS AND METHODS

Materials

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

Animals

Male spontaneously diabetic GK rats with 3 or 6 months of age were used. These rats were obtained from a 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 nondiabetic 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 in the fasting periods. In this study the “Principles of Laboratory Animal Care” (NIH publication 83-25, revised 1985) were followed. During this period, weights were measured and glycaemia was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer).

Induction and Characterization of STZ-Induced Diabetes

Male Wistar rats of about 200 g (3 months) were divided randomly into two groups of 10 animals each. 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 fed with glycolated serum in order to avoid hypoglycemia resulting from massive destruction of β -cells and release of intracellular insulin associated with STZ treatment [2]. Animals were kept 3 or 9 weeks before the experiments. During this period, weights were measured and glycemia was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer). Values were taken in fasting conditions just before STZ administration and in nonfasting conditions in the weeks after. If feeding blood glucose in the tail vein exceeded 250 mg/dL, animals were used as diabetic.

Glycemia and HbA_{1C} Evaluation

Blood glucose concentration was determined immediately after 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).

Preparation of Mitochondria

Mitochondria were isolated from the liver of normal and diabetic rats, maintained *ad libitum* for at least 12 h before being sacrificed, according to a previously established method [10], with slight modifications.

Homogenization medium contained 250 mM sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA, and 0.1% defatted BSA (bovine serum albumin). 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 [11].

Membrane Potential ($\Delta\Psi$) Measurements

The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of TPP⁺ (tetraphenylphosphonium ion) with a TPP⁺ selective electrode prepared, as previously reported [12], 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 volume of 1.1 $\mu\text{L}/\text{mg}$ was assumed and valinomycin was used to calibrate the baseline. Reactions were carried out at 25°C in 1 mL of the reaction media (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 5 mM HEPES, pH 7.2), supplemented with 3 μM TPP⁺, 2 μM rotenone, 1 mg mitochondria, and 5 mM succinate. To induce state 3 (ΔADP), 200 nmol ADP were used. Oxygen consumption was measured simultaneously, as described below.

Mitochondrial Respiration

Oxygen consumption of isolated mitochondria was determined polarographically at 25°C with a Clark oxygen electrode, connected to a suitable recorder in a closed chamber with magnetic stirring. Mitochondria (1 mg) and respiratory substrate (succinate) and rotenone (2 μM) were added to the standard reaction medium (1 mL). To induce state 3, 200 nmol ADP were used. The respiratory control ratio (RCR) and ADP/O ratios were calculated according to Chance and Williams [13]. FCCP-uncoupled respiration was performed by adding 1 μM of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) to mitochondria energized with succinate.

Enzymatic Activities

NADH:Q oxidoreductase (Complex I) activity was evaluated spectrophotometrically at 25°C by observing the NADH absorbance decrease at 340 nm, as previously described [14]. The assays were carried out at 25°C in 1 mL of the standard reaction medium supple-

mented with 60 μM decylubiquinone, 0.1 μg antimycin A, 1 mM KCN, and 0.5 mg protein of broken mitochondria, with three cycles of freezing and thawing. The reaction was initiated with 100 μM NADH. Since only the oxidation of NADH sensitive to rotenone inhibition leads to ATP synthesis, 2 min after the addition of NADH, 2 μM rotenone was added. The activity of the enzyme was determined as the difference of absorbance in the absence and in the presence of rotenone. *Succinate dehydrogenase* (Complex II) activity was determined polarographically [15] at 25°C in 1 mL of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 0.1 μg antimycin A, 1 mM KCN, and 0.5 mg protein of broken mitochondria. The reaction was initiated with 1 mM PMS (phenazine methasulphate), used as an artificial electron acceptor. *Succinate cytochrome c reductase* (Complexes II-III) activity was evaluated spectrophotometrically [16], by following the reduction of oxidized cytochrome *c* by the increase in absorbance at 550 nm. The reaction was initiated by the addition of 5 mM succinate to 2.0 mL of the standard reaction medium supplemented with 2 μM rotenone, 1 mM KCN, 60 μM cytochrome *c*, and 0.3 mg protein of broken mitochondria. *Cytochrome c oxidase* (Complex IV) activity was determined polarographically [17], at 25°C in 1 mL of the standard reaction medium supplemented with 2 μM rotenone, 0.1 μg antimycin A, 10 μM cytochrome *c*, and 0.5 mg protein of broken mitochondria. The reaction was initiated by the addition of 5 mM ascorbate plus 0.25 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamide). *F₁-ATPase* (Complex V) activity was determined by following the amount of protons released upon ATP hydrolysis, through a potentiometric method, as described previously [18,19]. The reaction was carried out at 25°C in 2 mL of reaction medium (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 0.5 mM HEPES, 2 μM rotenone, pH 7.2).

Statistical Analyses

The results are presented as mean \pm SEM 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 were performed using one-way ANOVA, using Student–Newman–Keuls test. For both analysis *p* < 0.05 was considered significant.

RESULTS

Characterization of Animals

Blood glucose levels, determined immediately after each animal's death, were significantly higher in diabetic rats when compared to controls (Table 1).

TABLE 1. Characterization of Animals

Condition	Weight (% of Initial Weight)	Glycaemia (mg/dL)	HbA _{1c}
3 Weeks			
Wistar	123.6 ± 10.68 (n = 6)	115.1 ± 4.48 (n = 6)	5.23 ± 0.209 (n = 6)
STZ	89.7 ± 3.71 (n = 6) ^{***}	409.3 ± 17.92 (n = 6) ^{***,†††}	10.25 ± 0.429 (n = 6) ^{***,†,††}
9 Weeks			
Wistar	190.5 ± 9.00 (n = 6) [¶]	97.3 ± 5.49 (n = 6)	5.84 ± 0.472 (n = 6)
STZ	87.8 ± 5.61 (n = 6) ^{****}	468.6 ± 15.56 (n = 6) ^{***,††}	11.71 ± 0.391 (n = 6) ^{***,††}
3 Months			
Wistar	100.0 ± 3.69 (n = 8)	101.3 ± 5.04 (n = 8)	5.04 ± 0.115 (n = 6)
GK	81.3 ± 1.20 (n = 8)	141.2 ± 9.38 (n = 8) ^{*,†††,†}	7.32 ± 0.670 (n = 6) ^{*,††}
6 Months			
Wistar	173.6 ± 4.30 (n = 9)	85.7 ± 5.00 (n = 9)	5.43 ± 0.327 (n = 6)
GK	112.8 ± 1.49 (n = 9)	162.0 ± 28.58 (n = 9) ^{*,††}	8.46 ± 0.889 (n = 6) ^{*,††}

Values are given as mean ± SEM of the number of independent experiments indicated. Initial weight was considered the weight at the time of injection with STZ or vehicle, in STZ-treated rats and its controls, or the mean weight of Wistar rats of 3 months age, in GK rats, and control group of 6 months age. Blood glucose concentration was determined immediately after animals sacrifice using a commercial glucometer (Glucometer-Elite, Bayer). The glycated hemoglobin (HbA_{1c}) values were determined at the time of animals' death through ionic exchange chromatography (Abbott Imx Glico-hemoglobin, Abbott Laboratories, Portugal).

p* < 0.05, *p* < 0.01, ****p* < 0.001, *****p* < 0.0001 compared to controls; †*p* < 0.05, ††*p* < 0.01 compared to GK (6 months); ‡*p* < 0.05, ‡‡*p* < 0.01, ‡‡‡*p* < 0.001, compared to STZ (9 weeks); ¶*p* < 0.0001, compared to Wistar (3 months/3 weeks).

However, GK rats presented mild hyperglycaemias (120–210 mg/dL), while STZ-treated rats presented severe hyperglycaemias (>380 mg/dL). To estimate the severity of diabetes, glycated hemoglobin (HbA_{1c}) was also evaluated. HbA_{1c} is a very useful parameter to evaluate the severity of diabetes, since it indicates the average blood glucose levels 2–3 months prior to the analysis. The determined levels of HbA_{1c} (see Table 1) confirm that in diabetic rats blood glucose was significantly increased and these levels in STZ-treated rats were also significantly augmented as compared to GK rats. The weight was also determined and results are presented as percentage of initial weight, in order to exclude inaccuracies due to initial body weight variations. We observed that STZ-treated rats lost weight after treatment with this diabetogenic drug, while control

groups gained weight as usual. We also observed that GK rats gained weight slowly than did their control groups.

Studies of Respiratory Indexes

Respiratory indexes (RCR: respiratory control ratio, ADP/O, and FCCP-stimulated respiration) were evaluated in GK, STZ-induced diabetic rats, and respective controls in the presence of succinate as respiratory substrate (Table 2).

We observed that RCR ratio was significantly increased both in GK rats of 3 and 6 months age and in STZ-treated rats, 3 weeks after the treatment, when compared to controls while STZ-treated rats 9 weeks after the treatment showed a slightly decrease. Moreover,

TABLE 2. Effect of Diabetes on Respiratory Indexes

Condition	RCR	ADP/O (nmol ADP/nmol O)	FCCP-Stimulated Respiration (nmol O/mg protein/min)
3 Weeks			
Wistar	3.28 ± 0.119 (n = 6)	1.73 ± 0.074 (n = 6)	239.2 ± 13.09 (n = 5)
STZ	3.78 ± 0.236 (n = 6) ^{*,†††,†}	1.97 ± 0.061 (n = 6) [*]	293.5 ± 10.43 (n = 5) ^{*,†††}
9 Weeks			
Wistar	3.36 ± 0.244 (n = 6)	1.68 ± 0.069 (n = 6)	224.2 ± 12.10 (n = 5)
STZ	3.02 ± 0.122 (n = 6) ^{†††}	1.74 ± 0.074 (n = 6)	196.4 ± 5.17 (n = 5)
3 Months			
Wistar	3.21 ± 0.084 (n = 8)	1.72 ± 0.044 (n = 8)	259.4 ± 12.10 (n = 6)
GK	3.67 ± 0.218 (n = 8) ^{*,†††,†}	1.88 ± 0.033 (n = 8) ^{**}	287.4 ± 7.77 (n = 6) ^{*,†††}
6 Months			
Wistar	3.12 ± 0.298 (n = 9)	1.69 ± 0.021 (n = 9)	250.8 ± 12.62 (n = 7)
GK	4.59 ± 0.124 (n = 9) ^{***,†††}	1.94 ± 0.065 (n = 9) ^{***}	285.4 ± 3.87 (n = 7) ^{*,†††}

Mitochondria (1 mg protein) were incubated in 1 mL of the respiratory standard medium. RCR and ADP/O were determined accordingly to Chance and Williams [13]. FCCP-stimulated respiration was determined upon the addition of 1.5 μM of FCCP to mitochondria energized with succinate. Values are given as mean ± SEM of the number of independent experiments indicated, performed with at least three different mitochondrial preparations.

p* < 0.05, *p* < 0.01, ****p* < 0.001 compared to controls; †*p* < 0.05, ††*p* < 0.001, compared to GK (6 months); ‡*p* < 0.05, ‡‡*p* < 0.001, compared to STZ (9 weeks).

we found that ADP/O ratio was also significantly increased both in GK rats of 3 and 6 months and in STZ-treated rats 3 weeks following the treatment when compared to their respective control group, whereas 9 weeks after the treatment, ADP/O ratio in STZ-treated rats decrease to the same level of non-diabetic rats. Additionally, we observed that FCCP-stimulated respiration is also considerably enhanced in GK rats of 3 and 6 months and in STZ-treated rats 3 weeks after the injection of STZ, compared to controls. However, 9 weeks after the treatment, STZ-treated rats presented a decreased stimulated respiration, indicating an inhibition of its respiratory chain activity.

These results were confirmed by membrane potential evaluation (Table 3). In fact, membrane potential developed after addition of succinate is significantly enlarged in GK rats (with 3 and 6 months of age) and in STZ-treated rats after 3 weeks of treatment, suggesting that their respiratory chain is stimulated. Furthermore, repolarization rate, an evaluation of phosphorylation activity, is also increased in these diabetic rats, indicating that the phosphorylative system is also enhanced as compared to controls. However, 9 weeks after the development of severe diabetes, in STZ-treated rats the respiratory chain and the phosphorylative system activity decreased to the same level as that of the non-diabetic rats. Furthermore, lag phase, another indicative parameter of phosphorylative system activity, is inversely proportional to repolarization rate, corroborating these results.

No significant differences between diabetic and respective controls were observed in the membrane

potential decrease due to the addition of ADP (data not shown).

Evaluation of Enzymatic Activities

Studies of enzymatic activities of respiratory complexes were performed in order to localize which respiratory complexes are affected by diabetes. We observed that NADH:Q oxidoreductase (Complex I) and succinate cytochrome *c* reductase (Complexes II-III) activities were not affected in diabetic rats, when compared to controls (Figure 1A and 1C). However, succinate dehydrogenase (Complex II) activity was significantly increased in STZ-treated rats and in GK rats at the age of 6 months (Figure 1B). Moreover, cytochrome *c* oxidase (Complex IV) is significantly augmented in diabetic rats (Figure 1D), particularly in STZ-treated rats after 9 weeks of treatment and GK rats of 6 months. However, their control groups also present a decreased activity, suggesting that age also diminishes cytochrome *c* oxidase activity.

Mitochondrial H⁺-ATPase activity (Figure 1E) and repolarization rate (Table 3) were significantly enhanced in STZ-treated rats after 3 weeks of treatment and in GK rats of 6 months, while lag phase decreased. These results suggest that the phosphorylative system is stimulated in these diabetic rats. However, we found that STZ-treated rats after 9 weeks of treatment showed a decreased activity of H⁺-ATPase and that this is reinforced by repolarization rate results.

Our results clearly suggest that diabetic rats present some metabolic adjustments, in order to increase ATP

TABLE 3. Effect of Diabetes on Membrane Potential

Condition	Energization (mV)	Repolarization (mV)	Repolarization Rate (% of Mean Control)	Lag Phase (s)
3 Weeks				
Wistar	207.0 ± 1.86 (n = 6)	205.7 ± 1.84 (n = 6)	99.0 ± 6.98 (n = 6)	55.2 ± 3.37 (n = 6)
STZ	217.4 ± 1.78 (n = 6)**,‡	215.3 ± 1.76 (n = 6)***,‡	146.4 ± 6.00 (n = 6)***,‡	39.0 ± 4.35 (n = 6)*
9 Weeks				
Wistar	210.4 ± 2.10 (n = 6)	209.4 ± 2.51 (n = 6)	93.7 ± 6.98 (n = 6)	53.6 ± 6.01 (n = 6)
STZ	209.6 ± 0.86 (n = 6)*†	206.3 ± 0.69 (n = 6)*††	94.8 ± 6.02 (n = 6)*†	52.7 ± 6.80 (n = 6)
3 Months				
Wistar	209.0 ± 1.46 (n = 7)	205.3 ± 1.76 (n = 7)	105.0 ± 12.12 (n = 7)	56.3 ± 4.27 (n = 7)
GK	215.8 ± 2.32 (n = 7)*	213.1 ± 2.25 (n = 7)*	107.6 ± 13.45 (n = 7)†,‡	46.3 ± 3.96 (n = 7)
6 Months				
Wistar	207.1 ± 2.59 (n = 8)	205.8 ± 2.30 (n = 8)	101.4 ± 10.32 (n = 8)	55.9 ± 2.84 (n = 8)
GK	219.2 ± 1.25 (n = 8)***	218.6 ± 1.14 (n = 8)***	142.2 ± 20.17 (n = 8)**	42.9 ± 3.39 (n = 8)*

Values are given as mean ± SEM of the number of independent experiments indicated, performed with at least three different mitochondrial preparations. The membrane potential was measured after the addition of 5 mM succinate, as the respiratory substrate ("energization") and after the phosphorylation of ADP 200 nmol ADP ("repolarization"), corresponding to the recovery of membrane potential. Repolarization rate was determined as the time required to the complete phosphorylation of ADP added; data are presented as the ratio between the values obtained and the mean of nondiabetic rats repolarization rate. Lag phase reflects the time required to phosphorylate the ADP added. In all experiments, the line basement was determined by addition of 10 ng/mL of valinomycin at the end of experiments, in order to completely abolish membrane potential.

p* < 0.05, *p* < 0.01, ****p* < 0.001 compared to controls; †*p* < 0.05, compared to STZ (3 weeks); ‡*p* < 0.05, compared to STZ (9 weeks); §*p* < 0.05, ¶*p* < 0.01, ††*p* < 0.001, compared to GK (6 months).

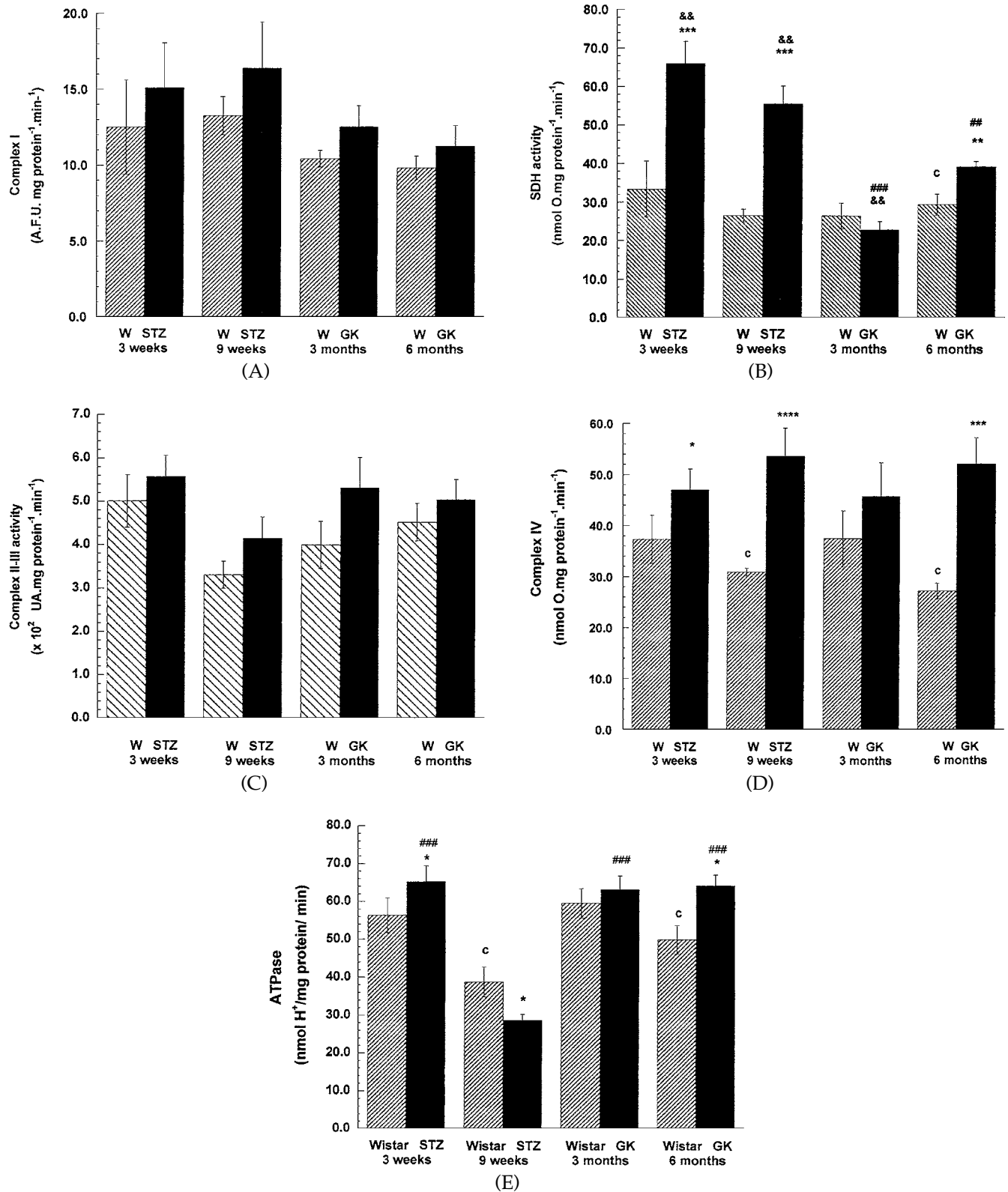


FIGURE 1. Respiratory chain enzymatic activities. (A) NADH:Q oxidoreductase (Complex I), (B) succinate dehydrogenase (Complex II), (C) succinate cytochrome *c* reductase (Complexes II-III), (D) cytochrome *c* oxidase (Complex IV), (E) F₁-ATPase (Complex V); activities were determined as described in Materials and Methods section. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 compared to controls; &&*p* < 0.01 compared to GK (6 months); ##*p* < 0.01, ###*p* < 0.001, compared to STZ (9 weeks); ^c*p* < 0.05, compared to Wistar (3 months/3 weeks).

production and compensate the impairments promoted by the high glucose levels typical of the disease.

DISCUSSION

In response to changes in physiological functions, body energy requirements undergo modifications either during development [20], or disease [21,22]. Several studies have been carried out to evaluate the alterations in mitochondrial functions of diabetic rats [19,23–27], particularly in STZ-treated rats. However, the studies concerning STZ-treated rats have been performed with animals of different strains (Sprague-Dawley, Fisher 344, Long-Evans, Wistar) and, also, diabetes was induced with different amounts of streptozotocin. Moreover, these studies were performed at least 8 weeks after the induction of diabetes. Additionally, in some experiments, insulin is also administered to the animals.

To circumvent all these disparities, we compared the possible alterations in the liver mitochondrial respiratory system, using two animal models of type 1 and type 2 diabetes mellitus: STZ-induced and GK rats, respectively. Moreover, we observed the adjustments to diabetes at the onset of the disease (STZ-induced rats 3-weeks after the treatment and GK rats 3-months old) and as diabetes progresses (STZ-induced rats 9 weeks after the treatment and GK rats 6-months old). These ages were selected for two main reasons: first, the age of the animals from this two groups is similar and secondly, 9 weeks after the treatment with streptozotocin, STZ-treated rats present serious disorders due to their severe hyperglycaemias.

We observed that GK rats, particularly GK rats 6 months-old, presented an improved respiratory activity, since RCR, ADP/O, membrane potential, and FCCP-stimulated respiration ratios are enhanced, as compared to non-diabetic rats, used as controls. When examined in detail, we observed that, as pointed out by some previous results [24,25], succinate dehydrogenase activity was stimulated in GK rats with 6 months, while no differences in respiratory complex II activity were found in GK rats 3-months old. Moreover, we observed that cytochrome *c* oxidase activity and H⁺-ATPase [19] were also increased in diabetic GK rats and that these metabolic adjustments were more evident in GK rats with 6 months. No significant changes were found in Complexes I and II-III. These results suggest that until the age of 6 months, liver mitochondrial oxidative phosphorylation is enhanced in this model of type 2 diabetes.

Since GK rats are important models to determine initial metabolic events of diabetes, we can suggest that stimulation of liver respiratory activity may be an

initial consequence of diabetes. Additionally, we observed similar adjustments in STZ-treated rats, 3 weeks after the induction of diabetes, reinforcing our previous statement.

A decline in ATP generation affects glycolysis ratio, decreasing glucose, fructose, and several aminoacids utilization [8,9], leading to severe energetic impairment. Therefore, improving respiratory activity in liver mitochondria seems to be a metabolic adjustment to circumvent injury in hepatocytes due to a decrease in ATP synthesis. Moreover, (nuclear and) mtDNA transcription is highly coupled to ATP levels [27]. It has also been described that cytochrome *c* oxidase regulates respiratory rates and, therefore, energy metabolism [26], and this regulation is linked to ATP/ADP ratios. Thus, high ATP/ADP ratios decrease respiratory ratios and $\Delta\Psi_m$, due adenine nucleotide binding to cytochrome *c* oxidase. We previously reported that liver mitochondria from GK rats presented a decreased ATP/ADP ratio and an GK rats presented an enlarged ADP content than Wistar rats [24,25]. Moreover, we found that liver mitochondrial ATP/ADP ratio was also significantly decreased in GK rats with 2, 3, and 12 months, as compared to respective controls [24,25]. Therefore, it seems plausible that similar decreases in ATP/ADP ratio occur in STZ-treated rats (particularly, until 3 weeks after streptozotocin administration), and the enhanced activity of respiratory chain of diabetic rats (namely STZ-induced rats 3 weeks after treatment and GK rats of 6 months) can be a consequence of these decreased ratios.

In STZ-treated rats 9 weeks after the induction of diabetes, initial increase in respiratory parameters ends up, owing to continuous severe hyperglycaemia. Furthermore, initial increase in respiratory parameters ends also, despite succinate dehydrogenase (Complex II) and cytochrome *c* oxidase (Complex IV) specific activities still being increased. The most affected component of respiratory chain, undoubtedly is H⁺-ATPase, and this inhibition is reflected in the respiratory ratio (RCR). However, hepatocytes, as well as pancreatic β -cells, are exposed to elevated glucose concentrations, being highly exposed to glucose injury ("glucose toxicity"). Changes in membrane composition, particularly, a reduction in fatty acids unsaturation index and decreased function of membranar enzymes have already been described [23,28]. The decrease in uncoupled respiration, observed in STZ-induced rats 9 weeks after the diabetogenic treatment, can therefore reflect an alteration in respiratory complexes assembly due to membrane variations, rather than a decrease of their specific activity, while a decrease in H⁺-ATPase, in these experimental conditions, can indicate an alteration between F₀F₁-ATP synthase and adenine nucleotide translocator (ANT) coupling due to membranar alterations or a direct inhibition at (one of) these components.

Alterations in respiratory chain activity in non-diabetic rats with age have also been found [29–31]. A slightly decrease in cytochrome *c* oxidase (complex IV) and F1-ATPase (complex V) activities from 9 weeks STZ-induced rat control group and GK rats of 6 months control group was found, comparatively with younger control groups. Moreover, RCR and ADP/O are slightly decreased. These two groups of control animals have similar ages (19 for 9 weeks STZ and 22 for GK rats of 6 months; 14 weeks for 3 weeks STZ and GK of 3 months). Therefore, these results confirm that rat liver mitochondria respiratory chain activity from non-diabetic rats is decreased during age.

These results allow us to conclude that diabetic rats present some metabolic adjustments, in order to increase ATP production, to compensate for the impairments promoted by the high glucose levels typical of the disease. These adjustments are evident in GK rats with 6 months age and in STZ-treated rats 3 weeks after administration of this diabetogenic drug, while GK rats with 3 months age present an intermediate situation. Conversely, phosphorylative and oxidative systems in STZ-treated rats 9 weeks after the treatment show decreased activities, compared to their control group.

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