Alterations of Liver Mitochondrial Bioenergetics in Diabetic Goto-Kakizaki Rats

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Respiratory indexes and the transmembrane electrical potential ($\Delta\Psi$) were evaluated in mitochondrial preparations from 6-month-old Goto-Kakizaki (GK) and Wistar rats in the presence of glutamate + malate and succinate. We found that in diabetic GK mitochondria, flavin adenine dinucleotide (FAD)-linked respiratory indexes (respiratory control ratio [RCR] and adenosine diphosphate [ADP] to oxygen ratio [ADP/O]) are increased and uncoupled respiration is largely enhanced, indicating increased respiratory chain activity in GK rats. $\Delta\Psi$ development in GK mitochondrial preparations, energized using glutamate + malate or succinate as substrates, and the repolarization rate upon phosphorylation of the added ADP were significantly higher in GK mitochondrial preparations. These results indicate an enhanced activity of the phosphorylation system, confirmed by evaluating $\Delta\Psi$ development when the mitochondria are energized by adenosine triphosphate (ATP). Moreover, recovery of the potential upon a phosphorylative cycle is increased in GK mitochondria, reflecting a more efficient coupling between the phosphorylative and oxidative system. Contrasting with results obtained for alloxan- or streptozotocin-induced diabetic rats, this study clearly demonstrates no impairment of mitochondrial bioenergetics in diabetic GK rats. On the contrary, at this age, we observed a higher efficiency of the phosphorylation system as compared with Wistar rats.

TYPE 2 DIABETES MELLITUS is a heterogeneous disease, characterized by a decrease in the insulin response to glucose (preferentially). This decreased physiological response to insulin is a consequence of the interplay between β-cell dysfunction, peripheral insulin resistance, and elevated hepatic glucose production. However, it is still not clear which is the primary abnormality and which abnormalities are secondary to increased plasma glucose levels. These high glucose levels are toxic, since they favor glycosylation of proteins, decreasing their biological activity ("glucose toxicity").[1]

To clarify the sequence of events leading to type 2 diabetes, it is important to analyze individuals before they develop the disease. Even as a disease with high genetic susceptibility, it is difficult to predict if a given individual will develop diabetes, since other risk factors are involved.

The use of animal models in these studies has the advantage that the development of diabetes can be predicted, making it possible to distinguish the pathogenic mechanisms involved in the onset of the disease. Goto-Kakizaki rats (GK rats) are currently used as an animal model of type 2 diabetes mellitus. This is a non-obese spontaneously diabetic rat[2] produced by selective breeding of Wistar rats, which have been progressively characterized by Goto et al.[3-5] GK rats exhibit 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. Moreover, these rats do not show increased insulin secretion in response to glucose, although plasma levels may be normal[6] or elevated,[7] possibly due to defects in β-cell stimulus-secretion coupling.[8,9] Thus, at the onset of diabetes, GK rats do not have severe complications of the disease, and are thus an appropriate model to study events at the onset of type 2 diabetes, as compared with alloxan- or streptozotocin-induced diabetic or genetically obese diabetic rats, which show severe hyperglycemia.[10]

This study was undertaken to evaluate possible dysfunctions in mitochondrial oxidative phosphorylation in GK rats, since this animal model of type 2 diabetes shows different characteristics versus obese or pharmacologically induced models of type 2 diabetes (which show impaired glucose tolerance, obesity, and hyperlipidemia).[10-12]

We investigated the possible alterations in the GK rat mitochondrial redox chain and phosphorylative system in liver mitochondria isolated from rats at about 6 months of age (26 weeks). Although GK rats exhibit mild stable hyperglycemia until this age, the permanent high glucose levels can lead to protein and lipid glycosylation, producing alterations in energy metabolism. Moreover, alterations in adenosine triphosphate (ATP) generation affect the glycolysis ratio, decreasing glucose utilization, since glucokinase (and hexokinase) activity depends on the ATP intracellular pool.[13,14]

MATERIALS AND METHODS

Animals

Male spontaneously diabetic GK rats were obtained from a local breeding colony (Animal Research Center Laboratory, University Hospitals, Coimbra, Portugal) established in 1995 with breeding couples from the colony at Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr K. Suzuki). Control animals were nondiabetic male Wistar rats of similar age obtained from our local colony (Animal Research Center Laboratory, University Hospitals, Coimbra). The animals were kept under conditions of controlled light and humidity with free access to powdered rodent chow (C.R.F. 20; Charles River Laboratories, Villemoisson-sur-Orge, France) and water. Glucose tolerance tests were used as a selection index.

Materials

All reagents and chemicals used were of the highest-grade purity commercially available.

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Preparation of Mitochondria

Wistar and GK rats (6 months) were maintained ad libitum for at least 12 hours, before death by cervical displacement according to a previously established method[15] with slight modifications.[16] Briefly, liver mitochondria were isolated in medium containing 210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES (pH 7.4), 0.2 mmol/L EGTA, 0.1 mmol/L EDTA, and 0.1% fat-free bovine serum albumin. EGTA, EDTA, and bovine serum albumin were omitted from the final washing medium, adjusted at pH 7.2. The mitochondrial pellet was washed twice, suspended in the washing medium, and immediately used. Protein content was determined by the Bradford method, using bovine serum albumin as a standard.[17,18]

Membrane Potential

The mitochondrial transmembrane potential ($\Delta\Psi$) was estimated by calculating the transmembrane distribution of tetraphenylphosphonium (TPP^+) with a TPP^+-selective electrode prepared as previously reported.[16]

[^{3}H]TPP$^+$ Uptake

To determine possible differences in the accumulation of TPP^+ by mitochondria of Wistar and GK rats, [^{3}H]TPP^+ uptake was determined. Mitochondria (1 mg) of Wistar and GK rats were incubated for 3 minutes with [^{3}H]TPP^+ (0.25 μCi) at 25°C with magnetic stirring (at this time, uptake was maximal). The solution was passed through filters, and the filters were washed twice with standard medium. Radioactivity retained in the filters was determined by liquid scintillation spectrometry.

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 substrates (glutamate + malate or succinate) were added to the standard reaction medium (1 mL). Additionally, 2 μmol/L rotenone was added when succinate was used. To induce state 3, 300 to 400 nmol adenosine diphosphate (ADP) was used. The respiratory control ratio (RCR) and ADP to oxygen ratio (ADP/O) were calculated according to the method used by Chance and Williams.[19] Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled respiration was performed by adding 1.5 μmol/L FCCP to mitochondria energized with glutamate + malate or succinate.

Adenine Nucleotides

Adenine nucleotides (ATP, ADP, and adenosine monophosphate [AMP]) were extracted using an acidic extraction procedure and separated by reverse-phase liquid chromatography as previously described.[16]

Statistics

The results are presented as the mean ± SEM. Statistical significance was determined using a paired Student's *t* test.

RESULTS

Characterization of the Animals

GK rats gained weight with age, similar to Wistar rats. However, the body weight of GK rats was significantly lower than that of control rats at 6 months of age (364 ± 4.9 *v* 523 ± 11.7 g, respectively, GK *v* Wistar, $P < .005$). This difference in body weight at this age reflects the initial difference found when GK and Wistar rats are born and is not related to malnutrition or weight loss.

Blood glucose levels were significantly higher in GK rats compared with Wistar rats (121 ± 5.7 *v* 68 ± 2.5 mg/dL, respectively, $P < .005$).

Studies of Respiratory Indexes

To investigate possible alterations in the redox chain and phosphorylative system, FCCP-stimulated (uncoupled) respiration and the RCR were evaluated in the presence of a FAD-linked substrate (succinate). The results are summarized in Table 1.

In the presence of succinate, state 3 respiration was increased in GK rats compared with Wistar rats (respectively, 175.80 ± 11.35 *v* 148.30 ± 7.14 nmol O · mg protein^{-1} · min^{-1}, $P < .05$). As a result, the RCR ratio was increased in GK rats (5.71 ± 0.41 *v* 4.70 ± 0.29, respectively, GK *v* Wistar, $P < .05$), since the rate of substrate oxidation (state 4 respiration) was slightly lower in GK diabetic rats versus controls (respectively, 31.31 ± 1.95 *v* 32.59 ± 2.57 nmol O · mg protein^{-1} · min^{-1}).

To evaluate the coupling between ADP phosphorylation and oxygen utilization,[20] the ADP/O ratio was determined in GK and Wistar nondiabetic rats using succinate as a respiratory substrate. In the presence of the FAD-linked respiratory substrate, GK rats had a higher ADP/O ratio (1.80 ± 0.04 *v* 1.70 ± 0.03, respectively, GK *v* Wistar, $P < .005$). These differences in the ADP/O ratio may reflect alterations in the oxidative system. Indeed, in the presence of succinate, FCCP-stimulated respiration was largely increased in diabetic GK rats compared with Wistar rats (respectively, 155.76 ± 4.72 *v* 68.44 ± 3.05 nmol O · mg protein^{-1} · min^{-1}, $P < .0005$). Thus, in GK rats, the respiratory chain seemed to be largely stimulated as compared with normal Wistar rats.

Studies of Transmembrane Electrical Potential

Transmembrane electrical potential ($\Delta\Psi$) is the main component of the electrochemical gradient (μH^+), accounting for greater than 90% of the total proton motive force.[21] Therefore, the determination of alterations associated with $\Delta\Psi$ is of major importance in studies of mitochondrial oxidative phosphorylation.

Table 1. Respiratory Indexes Determined in the Presence of Succinate as Respiratory Substrate

Rat Group	RCR	FCCP-Stimulated Respiration (nmol O/mg/min)	ADP/O Ratio
Wistar	4.70 ± 0.29 (n = 10)	68.44 ± 3.05 (n = 4)	1.70 ± 0.03 (n = 10)
GK	5.71 ± 0.41* (n = 10)	155.76 ± 4.72‡ (n = 4)	1.80 ± 0.04† (n = 10)

NOTE. Data are the mean ± SEM of the number of experiments indicated.
*$P < .05$; †$P < .005$; ‡$P < .0005$: *v* Wistar rats.

Fluctuations of $\Delta\Psi$ in mitochondrial preparations of GK and Wistar rats were studied in the presence of NAD- and FAD-linked substrates (glutamate + malate and succinate, respectively; Fig 1). The observed discrepancies in $\Delta\Psi$ in diabetic GK and Wistar rats energized with glutamate + malate and succinate could be due to a different accumulation of TPP^+ in both mitochondrial preparations, therefore not reflecting differences in $\Delta\Psi$ development. To evaluate possible changes in TPP^+ accumulation, [^{3}H]TPP^+ uptake in the absence of respiratory substrates was determined and no significant differences were observed (118.7 ± 6.13 *v* 115.8 ± 8.73 nmol TPP^+/mg protein accumulated, respectively, in GK *v* Wistar). Moreover, we also observed that the amount of TPP^+ accumulated before the addition of respiratory substrates was similar in both preparations (data not shown).

The depolarization following ADP addition showed no significant differences in GK and Wistar rats, but the repolarization rate was highly increased in diabetic GK rats. This increase in the repolarization rate may reflect a higher rate of coupling of the F_0F_1-ATPase.[22]

Studies of F_0F_1-ATPase Activity

After the addition of small amounts of ADP (35 to 40 nmol/mg protein), the repolarization rate was highly increased in GK rats compared with nondiabetic Wistar rats in the presence of glutamate + malate or succinate as respiratory substrates (Table 2). This increased repolarization rate indicates that diabetic GK rats had more efficient oxidative phosphorylation coupling. To confirm this involvement, we determined $\Delta\Psi$ development in nonrespiring mitochondria energized by ATP. Apparently, the mitochondrial $\Delta\Psi$ sustained by ATPase activity is higher in GK rats compared with control Wistar rats (Table 3). Moreover, the lower endogenous ATP/ADP ratio (Table 4) found in diabetic GK rat mitochondria (without addition of respiratory substrates or ADP) was highly favorable to the observed enhancement of oxidative phosphorylation coupling.

DISCUSSION

Type 2 diabetes mellitus is a metabolic disease characterized by intolerance to glucose, leading to hyperglycemia. Type 2 diabetes results from glucose resistance in peripheral tissues (skeletal muscle and adipocytes), impaired insulin sensitivity resulting in excessive postabsorptive glucose output, and a relative failure of pancreatic β-cell function.[23] Individuals with type 2 diabetes present with highly variable clinical features depending on the degree of obesity, age of onset, severity of glucose intolerance, and mode of inheritance.[24]

Owing to constant hyperglycemic levels, several complications are associated with diabetes, namely nephropathies, retinopathies, neuropathies, and cardiovascular complications. Therefore, in the search for effective interventional treatment of the disease, diabetic animal models closely resembling each subtype of type 2 diabetes in the human population are of major importance.[25]

In our studies, we used mitochondrial preparations isolated from liver, since it is well known that the liver plays an important role in the control of blood glucose levels and also that the mitochondrion is an intracellular organelle of major

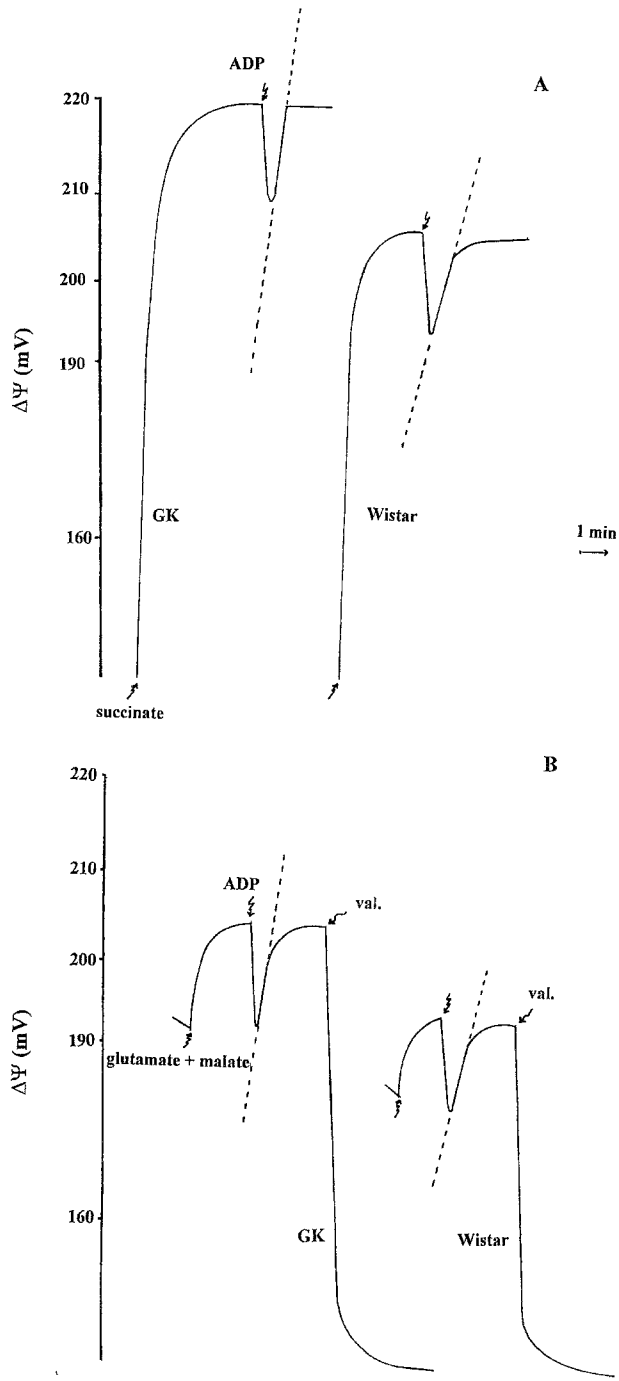

Fig 1. $\Delta\Psi$ development upon addition of succinate (A) or glutamate + malate (B) as respiratory substrates in GK and Wistar mitochondrial preparations. Mitochondria (1 mg protein) were added to 1 mL standard reaction medium supplemented with 3 mmol/L TPP^+. Addition of ADP (30-40 mmol) induced a state 3 condition. When glutamate + malate were used as respiratory substrates, valinomycin (1 mg) was added to determine the baseline. In the presence of either succinate or glutamate + malate, $\Delta\Psi$ development by GK mitochondria was higher. Data represent typical recordings from several experiments.

Table 2. Repolarization Rate Determined in the Presence of Glutamate + Malate and Succinate as Respiratory Substrates

Rat Group	Glutamate + Malate	Succinate
Wistar	100.00 ± 2.88 (n = 6)	100.00 ± 3.94 (n = 9)
GK	179.67 ± 6.44 (n = 6)*	182.34 ± 18.79† (n = 9)

NOTE. Standard reaction medium (1 mL) was supplemented with mitochondria (1 mg) and 3 μmol/L TPP^+ (in the presence of succinate, 2 μmol/L rotenone was added). Mitochondria were energized with glutamate + malate or succinate. To induce phosphorylation, 30-40 nmol ADP was used. Data are the mean ± SEM of the number of experiments indicated.

*$P < .05$, †$P < .005$: *v* Wistar rats.

importance in aerobic energy metabolism and intracellular electrolyte homeostasis. Thus, any perturbation of liver mitochondrial metabolism will have repercussions on blood glucose metabolism. Moreover, the liver is exposed (like pancreatic β cells) to elevated glucose concentrations as compared with other organs and tissues. Therefore, like β cells, the liver is highly exposed to glucose injury (glucose toxicity).

Since insulin exerts a direct effect on carbohydrate oxidation,[26] it was previously reported that mitochondrial dysfunctions were associated with glucose intolerance. These studies[27-30] reported a decrease in liver mitochondrial respiratory indexes, such as the RCR or ADP/O ratio, associated with diabetes. However, in studies to evaluate the effect of type 2 diabetes on mitochondrial functions, animals with pharmacologically induced diabetes with severe hyperglycemia were used. Since GK rats, until the age of 6 months, exhibit moderate and stable hyperglycemia, we expected a different pattern for GK liver mitochondrial functions.

Our data clearly demonstrate that diabetic GK rat mitochondrial bioenergetic functions are not impaired at the age of 6 months. GK mitochondrial preparations energized with a NAD- or FAD-linked substrate developed a higher $\Delta\Psi$. Also, the RCR and repolarization rate for diabetic GK rats evaluated in the presence of both glutamate + malate and succinate were significantly increased, possibly reflecting an enhancement of oxidative phosphorylation coupling. To confirm the involvement of mitochondrial ATPase, we determined $\Delta\Psi$ development upon addition of ATP in nonrespiring mitochondria. Apparently, the mitochondrial $\Delta\Psi$ sustained by ATPase activity is higher in GK rats, in accordance with our previous results

Table 3. $\Delta\Psi$ Development in GK and Wistar Mitochondrial Preparations Energized With ATP

ATP (nmol)	Energy (mV)	
	Wistar	GK
25	122.83 ± 4.19	127.57 ± 5.22
50	121.59 ± 4.84	128.93 ± 5.81
100	129.10 ± 7.10	135.36 ± 4.69

NOTE. Standard reaction medium (1 mL) was supplemented with mitochondria (1 mg), 3 μmol/L TPP^+, and 2 μmol/L rotenone. Mitochondria were energized with the amount of ATP indicated. Addition of oligomycin (1 mg/mg protein) completely abolished the membrane potential developed upon addition of ATP. Data are the mean ± SEM of 3 different experiments.

Table 4. Endogenous Adenine Nucleotide Content and ATP/ADP Ratio of GK and Wistar Mitochondrial Preparations

Parameter	Wistar	GK
ATP (nmol/mg protein)	3.25 ± 0.35 (n = 6)	3.58 ± 0.42 (n = 6)
ADP (nmol/mg protein)	2.08 ± 0.09 (n = 6)	5.34 ± 0.55* (n = 6)
ATP + ADP + AMP (nmol/mg protein)	19.18 ± 1.12 (n = 4)	19.88 ± 1.50 (n = 4)
ATP/ADP ratio	1.57 ± 0.17 (n = 6)	0.74 ± 0.08* (n = 6)

NOTE. Data are the mean ± SEM of the number of experiments indicated.

*$P < .005$ *v* Wistar rats.

showing an increase of GK mitochondrial ATPase activity.[22] In our opinion, this increased activity results from alterations in the F_0F_1-ATPase complex in GK rat liver mitochondria, since titrations of the enzyme complex performed with oligomycin showed that GK mitochondria require a larger amount of oligomycin to completely abolish phosphorylation. Thus, GK mitochondria have a higher content of F_0F_1-ATPase units or the enzyme complex has a different conformation, which enables both a decreased binding of the inhibitor and an enhancement of its biological activity in order to respond rapidly to the energetic demands of the cell.[31,32]

The enhanced mitochondrial oxidative phosphorylation activity is probably related to the decreased endogenous ATP/ADP ratio (without the addition of substrates or ADP) found in GK mitochondria, since mitochondrial oxidative and phosphorylative pathways are controlled by the ATP/ADP ratio.[33-35] It should be noted that the content of endogenous ATP (without addition of ADP) is similar in both mitochondrial preparations. However, GK mitochondria had a substantially higher content of endogenous ADP (5.34 ± 0.55 *v* 2.08 ± 0.09 nmol/mg protein, respectively, GK *v* control, $P < .005$). Therefore, it seems plausible that this higher ADP content may have a regulatory function by decreasing the endogenous ATP/ADP ratio.

Our results clearly demonstrate that the phosphorylative system of GK rats is not the only altered mitochondrial function. Indeed, in the presence of succinate, a FAD-linked substrate, FCCP-stimulated respiration of GK diabetic rats was largely increased compared with control rats. These results clearly indicate an increased activity of the mitochondrial respiratory chain.

We conclude that the higher recovery of $\Delta\Psi$ in GK mitochondria reflects a more efficient coupling between phosphorylative and oxidative systems, and this effect could result from alterations in F_0F_1-ATPase, with a decrease of ΔpH dissipated through the subunits of the enzyme complex and a decrease in electron slippage.[31,36] This lower electron slippage could be responsible for the increased ADP/O ratio determined in the presence of succinate in diabetic GK rats.[20,37] To better elucidate the mechanisms by which diabetic GK rats show this type of mitochondrial bioenergetic behavior, additional studies are currently under way.

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