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DECREASED SUSCEPTIBILITY TO LIPID PEROXIDATION OF GOTO-KAKIZAKI RATS: RELATIONSHIP TO MITOCHONDRIAL ANTIOXIDANT CAPACITY

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Summary

The respiratory function and the antioxidant capacity of liver mitochondrial preparations isolated from Goto-Kakizaki non-insulin dependent diabetic rats and from Wistar control rats, with the age of 6 months, were compared. It was found that Goto-Kakizaki mitochondrial preparations presented a higher coupling between oxidative and phosphorylative systems, compared to non-diabetic preparations. Goto-Kakizaki mitochondria presented a lower susceptibility to lipid peroxidation induced by ADP/Fe^{2+}, as evaluated by the formation of thiobarbituric acid substances. The decreased susceptibility to peroxidation in diabetic rats was correlated with an increase in mitochondrial vitamin E (α-tocopherol) content and GSH/GSSG ratio. Moreover, the glutathione reductase activity was significantly increased, whereas the glutathione peroxidase was decreased. Superoxide dismutase activity was unchanged in diabetic rats. Fatty acid analyses showed that the content in polyunsaturated fatty acids of Goto-Kakizaki mitochondrial membranes was significantly higher compared to controls. These results indicate that the lower susceptibility to lipid peroxidation of mitochondria from diabetic rats was related to their antioxidant defense systems, and may correspond to an adaptative response of the cells against oxidative stress in the early phase of diabetes.

Type 2 (non-insulin dependent) diabetes mellitus is one of the most common metabolic diseases in man, affecting about 100 million people around the world (about 3% of the human population) (1,2).

Clinically, it is a heterogeneous disease, characterized by a low glycaemic control, due to impairment of the balance between ß-cell secretion of insulin, peripheral insulin action and hepatic glucose production. However, it is still not clear which is the exact mechanism leading to increase of plasma glucose levels (3).

Even being a disease with a high genetic susceptibility, other important risk factors are involved (as increased intake of sugars and lipids and decreased physical exercise), it becomes difficult to anticipate if a given individual will became diabetic. Thus, with the use of animal

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models it became possible to predict the development of diabetes, and to distinguish the pathogenic mechanisms involved in the onset of the disease. Goto-Kakizaki rat (GK rat), a non-obese, spontaneously diabetic animal model (4) produced by repeated selective breeding of Wistar non-diabetic rats, and characterized by Goto and co-workers (5-7), is currently used as an animal model of type 2 diabetes mellitus. Until the age of 6 months GK rats exhibit a moderate but stable fasting hyperglycaemia (evident from 6 weeks of age), which does not progress to a ketotic state. Therefore, at the beginning of the diabetes, GK rats do not present severe complications associated to the disease, thus being an appropriate model to study the events at the onset of diabetes, as compared to genetically obese diabetic rats, which present severe hyperglycaemia and hyperlipidemia (8).

Mitochondria are a major source of reactive oxygen species (ROS) within eucaryotic cells, due to energetic metabolism. These oxygen free radicals can react with cellular components, especially membrane lipids, producing cell damage (9). Under normal conditions, potentially toxic ROS generated by mitochondrial respiratory metabolism are efficiently neutralized by cellular antioxidant defense mechanisms. However, this balance can easily be broken, leading to cellular dysfunction (10-12). This imbalance between pro- and antioxidant systems is very important in many disease processes, including diabetes mellitus, and is probably related to the complications associated to the disease (13).

The mitochondrial respiratory chain activities coupled to oxidative phosphorylation and compartmentation of energy-rich metabolites (ATP, CrP) at sites of cell specific function, are some of the fundamental aspects of glucose metabolism (for review see 14). In fact, it has been shown that in streptozotocin-induced diabetic rats, the mitochondrial dysfunction may contribute to diabetes-associated pathology (15, 16). However, at present little is known about mitochondrial function in non-insulin dependent diabetes mellitus.

In order to clarify the role of the antioxidant systems in type 2 diabetes, we investigated the possible alterations in GK mitochondrial lipid peroxidation, the fatty acid composition and antioxidant systems of liver mitochondria isolated from rats of about 6 months (26 weeks) of age. In addition, we determined also the effect of diabetes on the mitochondrial respiratory control ratio (RCR) and ADP/O. We found that diabetic GK rats present a higher content of unsaturated fatty acids as compared to non-diabetic rats. However, our results indicate that GK rats mitochondrial induced lipid peroxidation is decreased, suggesting the existence of a higher antioxidant capacity in diabetic rats.

Materials and Methods

Animals: Male spontaneously diabetic GK 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.I. Susuki). Control animals were non-diabetic male Wistar rats of similar age, obtained from our local colony (Animal Research Center Laboratory, University Hospitals, Coimbra). Animals were kept under controlled light and humidity conditions and with free access to powdered rodent chow (diet C.R.F. 20, Charles Rivers, France) and water. Glucose tolerance tests were used as a selection index.

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

Preparation of Mitochondria: Wistar and GK rats were maintained *ad libitum* for at least 12 hours, before being sacrificed for cervical displacement, according to a preestablished method (17),

with slight modifications (18).

Protein was determined by the Bradford method, using BSA (bovine serum albumin) as a standard (19, 20).

Blood Glucose and HbA1c Levels Determination: Blood glucose levels were determined, immediately after animals' sacrifice, through the glucose oxidase reaction, using a glucometer (Glucometer Elite, Bayer, Portugal S.A.) and compatible reactive tests. HbA1c levels were determined through ionic exchange chromatographic assay (Abbott IMx Glicohemoglobina, Abbott Laboratories, Portugal).

Determination of Thiobarbituric Acid Reactive Substances: Lipid peroxidation was measured as thiobarbituric acid-reactive products, as described previously (21), with slight modifications. To induce lipid peroxidation, mitochondria (1 mg) were incubated in 1 ml of reaction medium (150 mM KCl and 25 mM Tris-Cl, pH 7.2), in the presence of 1 mM ADP and 0.1 mM Fe^{2+} (22), at 30 °C during 20 min. with continuous stirring. The reaction was stopped by lowering the temperature to 0-4 °C by placing the tubes on ice. To measure the extent of lipid peroxidation, 0.5 ml of cold 40% TCA and 0.67 % TBA was added to 0.5 ml of sample material and the tubes were treated for 15 min. in a boiling water bath. The tubes were allowed to cool at room temperature and centrifuged at 3000 rpm for 10 min.. The supernatant was collected and the absorbance was measured at 530 nm in a Bausch & Lomb Spectronic 21. The amount of malonyldialdehyde (MDA) formed was calculated using a molar extinction coefficient of 1.56×10^5 $cm^{-1}.min^{-1}$ and expressed as nmol MDA/mg protein (23).

Fatty Acid Analysis: 1 ml of homogenate was extracted according to Bligh and Dyer (24) and the chloroform phase was dried under a stream of N_2. The preparation of fatty acid methyl esters (FAME) was performed according to Madeira and Antunes-Madeira (25) using 5 % sulfuric acid in methanol as the derivatizing reagent. The FAME analysis was performed using a Varian Aerograph series 2700 gas chromatograph, adapted for capillary columns, with nitrogen as the carrier gas. The column used was a SGE glass capillary column (25QC5/BPX70 0.5) and the temperature program was the following: 5 min. at 140 °C; 4 °C/min. to 210 °C; 10 min. at the upper temperature. The results were calculated using a Milton Roy CI-4100 integrator.

Determination of Vitamin E Content in Mitochondrial Membranes: The extraction and separation of membrane vitamin E (α-tocopherol) was performed by following the methods described previously (26-28). 1.5 ml SDS 10 mM were added to 2 mg mitochondrial protein, followed by addition of 2 ml of absolute ethanol. Then, 2 ml of *n*-hexane and 50 ml of KCl 3 M was added, and the mixture was vortexed for about 3 min.. The extract was centrifuged at 2000 rpm, for 3 min., to allow for phase separation. One ml of the upper phase containing *n*-hexane (*n*-hexane layer) was recovered and evaporated to dryness under a stream of N_2, and kept at -80 °C, until further use. The extract was redissolved in *n*-hexane and vitamin E content was analyzed by reverse phase HPLC. A 4.6 x 200 mm Spherisorb S10w column was eluted with *n*-hexane modified with 0.9 % methanol, at a flow of 1.5 ml/ min. Detection was performed by a UV detector, at 287 nm.

Reduced (GSH) and Oxidized (GSSG) Glutathione Measurements In Mitochondria: GSH and GSSG levels were determined with fluorescence detection after reaction of the supernatant of the H_3PO_4/ EDTA-NaH_2PO_4 deproteinized mitochondria solution with the reagent *o*-phthalaldeyde, at pH 8.0, according to Hissin and Hilf (29).

Enzymatic Assays: Glutathione peroxidase was assayed by the method of Flohé and Gunzler (30). The activity of the enzyme was measured indirectly by determining the oxidation of NADPH, at

340 nm, induced by the action of glutathione peroxidase. Reaction mixtures contained 50 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 1 mM GSH, 0.24 U glutathione reductase and 0.1 mg of mitochondrial sample. The reaction, performed at 30 °C, was initiated by the addition of 0.15 mM NADPH. At 3 min, 1.2 mM t-butyl hydroperoxide was added and the absorvencies recorded for 5 min. Results are expressed as μmol GSH oxidized.min-[1] per mg protein.

The glutathione reductase was assayed by following the oxidation of NADPH at 340 nm (31). Reaction mixtures contained 100 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.15 mM NADPH and about 0.1 mg protein. The reaction was initiated by the addition of 1 mM GSSG. Results are expressed as μmol NADPH oxidized min-[1] per mg protein.

Superoxide dismutase (MnSOD) was determined by inhibition of the reduction rate of nitroblue tetrazolium (NBT), monitored at 550 mm using xanthine-xanthine oxidase system (32). Reaction mixtures contained 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 100 μM NBT, Triton X-100 (0.025%, v/v), 2 mM KCN and hipoxanthine 100 μM. The reaction was initiated by the addition of xanthine oxidase 0.025 U/ml. One unit of activity is defined as that amount of enzyme which inhibits the reduction rate of NBT by 50%.

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), and additionally, when succinate was used, 2 μM of rotenone were added to the medium. To induce state 3 respiration, 300-400 nmol of ADP were used. The respiratory control ratio (RCR) and ADP/O ratios were calculated according to Chance and Williams (33).

Statistics: The results are presented as mean ± SEM of the indicated number of experiments and statistical significance was determined using paired Student's *t*-test or one-way analysis of variance (ANOVA).

Results

A. Characterization of Animals

At 6 month's, GK rats body weights were significantly lower than those of Wistar rats.

Blood glucose and glycated hemoglobin (HbA$_{1c}$, which gives the mean values of glycaemias of the previous 2 months) are significantly increased as compared to control (Table 1).

### B. Studies of Respiratory Indexes of GK and Wistar Liver Mitochondria

Respiratory control ratios (RCR) were determined in the presence of a FAD-linked substrate (succinate) and a NAD-linked substrate (glutamate + malate). In the presence of both respiratory substrates, GK rats RCR are increased as compared to Wistar rats (Figure 1), since State 3 respiration is increased in GK rats (Table 2).

ADP/O ratios were evaluated in the presence of succinate as an oxidable substrate (Figure 2). The ADP/O ratio represents the coupling between the ADP phosphorylation and oxygen utilization, and therefore can be used to estimate the approximate quantity of oxygen used by the respiratory chain, but not to phosphorylate ADP. We found that ADP/O ratios of GK rats are increased as compared to control rats.

Table 1. Characterization of GK and Wistar rats with 26 weeks of age

Condition	Wistar	GK
Body Weight (g)	523 ± 11.7 (n=6)	369 ± 4.9 * (n=9)
Glycaemia (mg/dl)	103.3 ± 4.53 (n=14)	184.2 ± 15.50 * (n=13)
HbA$_{1C}$ (%)	5.1 ± 0.16 (n=4)	10.4 ± 0.56 ** (n=4)

Data are mean ± SEM of the number of animals indicated on the table. Glycaemia was determined by glucose oxidase reaction (Glucometer Elite, Bayer, Portugal S.A.). HbA$_{1c}$ (%) was determined through ionic change chromatographic assay (Abbott IMx Glicohemoglobina kit). Values statistically different from control: * p < 0.005; ** p< 0.0005.
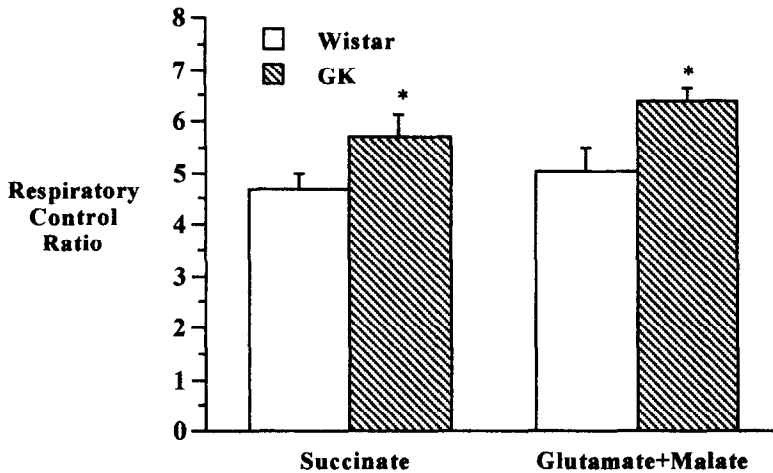
Fig. 1

Respiratory control ratio (RCR) of GK and Wistar rats, determined in the presence of succinate and glutamate + malate as oxidizable substrates. To induce state 3 respiration, 300-400 nmol ADP were used. Data are mean ± SEM of 10-12 independent experiments. Values statistically different from control: * p < 0.05.

C. Fatty Acid Composition of Liver Mitochondria Isolated from GK and Wistar Rats

The fatty acid composition of mitochondrial membranes was evaluated (Figure 3 and Table 3). We observe that GK rats mitochondrial membranes present a higher content of unsaturated long chain fatty acids (namely arachidonic (20:4) and docosohexanoic (22:6) acids) with a marked decrease in saturated fatty acids (palmitic (16:0) and stearic (18:0) acids).

We also observe a marked increase in the ratio between unsaturated and saturated fatty acids (up to 67 %) and in unsaturation index (up to 51 %) in GK mitochondrial preparations. Our results

indicate also a small increase of average chain length (up to 4%) in diabetic rats.

Table 2. GK diabetic and Wistar rats State 4 and State 3 respiration

Condition	Wistar	GK
Glutamate + malate		
State 4 respiration	14.7 ± 1.05	14.5 ± 0.75
(nmol O/min/mg protein)	(n=12)	(n=12)
State 3 respiration	72.7 ± 7.06	91.1 ± 4.54 **
(nmol O/min/mg protein)	(n=12)	(n=12)
Succinate		
State 4 respiration	32.6 ± 2.57	31.3 ± 2.57
(nmol O/min/mg protein)	(n=10)	(n=10)
State 3 respiration	148.3 ± 7.14	175.8 ± 11.35 **
(nmol O/min/mg protein)	(n=10)	(n=10)

State 4 and State 3 respiration determined in the presence of glutamate+malate and succinate as respiratory substrates. To induce state 3 respiration, 300 nmol ADP were used. Data are mean ± SEM of the indicated number of independent experiments. Values statistically different from control: ** $p < 0.005$.
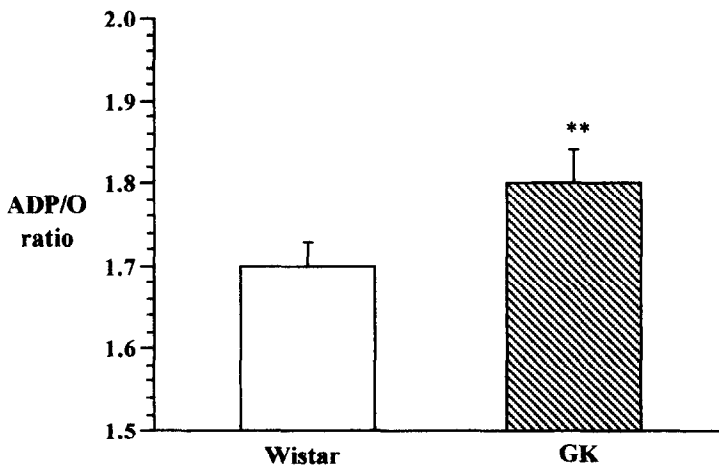
Fig. 2
ADP/O ratio of GK and Wistar rats, determined in the presence of succinate as a respiratory substrate. To induce state 3 respiration, 300 nmol ADP were used. Data are mean ± SEM of 10 independent experiments. Values statistically different from control: ** $p < 0.005$.

D. Susceptibility to Lipid Peroxidation of Mitochondrial Preparations from GK and Wistar Rats

Lipid peroxidation was assessed with thiobarbituric acid-reacting substances (TBARS) assay and

was expressed as nmol MDA/ mg protein.

Table 3. GK diabetic and Wistar rats mitochondrial fatty acid composition determined by GLC analysis

| Property	Wistar	GK
Unsaturation index	119.71 ± 4.67	181.5 ± 14.09
Average chain length	17.69 ± 0.06	18.32 ± 0.14
% saturates	54.76 ± 1.24	42.03 ± 2.49
% unsaturates	45.24 ± 1.24	57.97 ± 2.49
% monounsaturates	12.06 ± 0.14	11.18 ± 0.23
% polyunsaturates	33.19 ± 1.09	47.79 ± 2.72
unsaturates / saturates	0.83 ± 0.04	1.39 ± 0.14

The results are the mean ± SEM for 4 separate analysis. The unsaturation index expresses the number of double bonds per 100 fatty acid molecules. Average chain length is S(chain length x mole %) of individual species (38). All data from GK preparations were significantly different from control (p< 0.05).
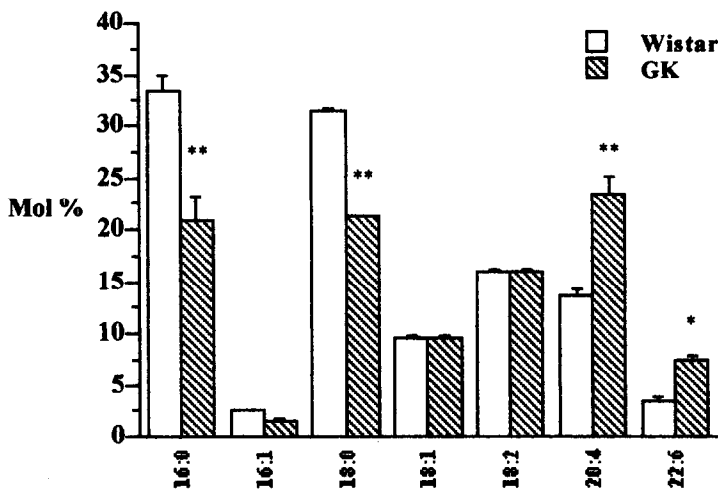
Fig. 3

Fatty acid composition of GK and Wistar mitochondrial membranes, determined by GLC (gas-liquid chromatography) analysis. Data are mean ± SEM of 4 separate analysis (either GK and Wistar rats). The data given for the fatty acid analysis does not include minor components, which constitute less than 6 % of the total fatty acid content. When error bars are absent, SEM is encompassed by the size of the symbols. Values statistically different from control: * p < 0.05; ** p < 0.005.

Initial levels of TBARS (Figure 4) were the same in liver mitochondrial preparations isolated from GK and control rats (0.94 ± 0.148 and 1.09 ± 0.201 nmol MDA/ mg protein, respectively). However, the preparations from liver of diabetic GK rats were less susceptible to induced lipid peroxidation (using ADP + Fe^{2+} as peroxidation inducer), as compared to control (12.1 ± 2.42 and 16.5 ± 1.67 nmol MDA/ mg protein, respectively in GK and Wistar rats).
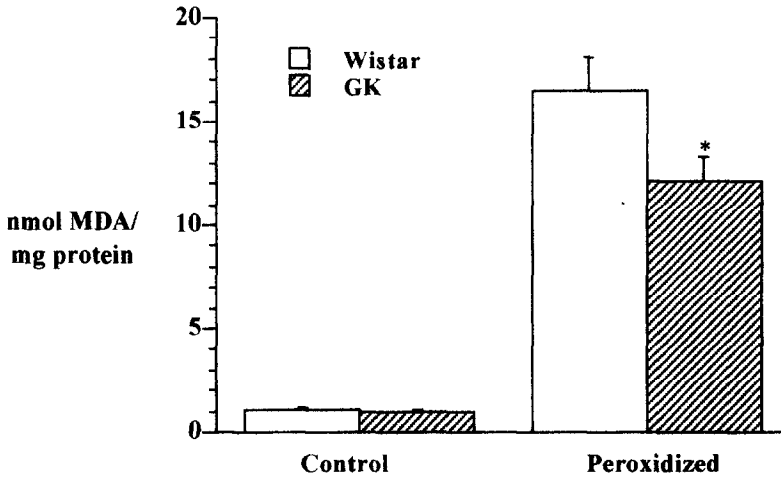
Fig. 4

Lipid peroxidation of GK and Wistar mitochondrial membranes, evaluated from TBARS (thiobarbituric acid-reactive species) production. In the absence of peroxidation inducer (ADP/ Fe^{2+}), TBARS production of Wistar and GK preparations are similar. However, in the presence of (ADP/ Fe^{2+}), lipid peroxidation is lower in GK rats. Data are mean ± SEM of 7 independent experiments. Values statistically different from control: * $p < 0.05$.

These results were unexpected, since the amount of unsaturated fatty acids is largely increased in diabetic GK rats. Thus, it seems acceptable to us that diabetic GK rats antioxidant capacity is enhanced as compared to normal rats.

E. Mitochondrial α-tocopherol Levels

We found that the lower susceptibility to lipid peroxidation of GK liver mitochondrial preparations was correlated with the increased vitamin E (α-tocopherol) contents of diabetic rats (2.28 ± 0.550 versus 4.29 ± 0.858 nmol α-tocopherol/ mg protein, respectively in Wistar and GK rats) (Figure 5).

F. Mitochondrial GSH/GSSG Ratio and Antioxidant Enzyme Activities in GK and Wistar Rats

Since glutathione in the reduced form (GSH) is known to play an important role in protecting cells (and organelles) from oxidative stress, GSH/GSSG ratios were evaluated both in GK and Wistar mitochondrial preparations (Figure 6). We found that GSH/GSSG ratio is higher in kGK diabetic rats (up to 40 %) compared to Wistar animals . Thus, our results point to a higher antioxidant capacity of GK rats as compared to normal rats.

Table 4 shows the activity of the antioxidant enzymes in liver mitochondrial preparations of normal Wistar and diabetic GK rats. As can be observed, MnSOD levels were not altered by
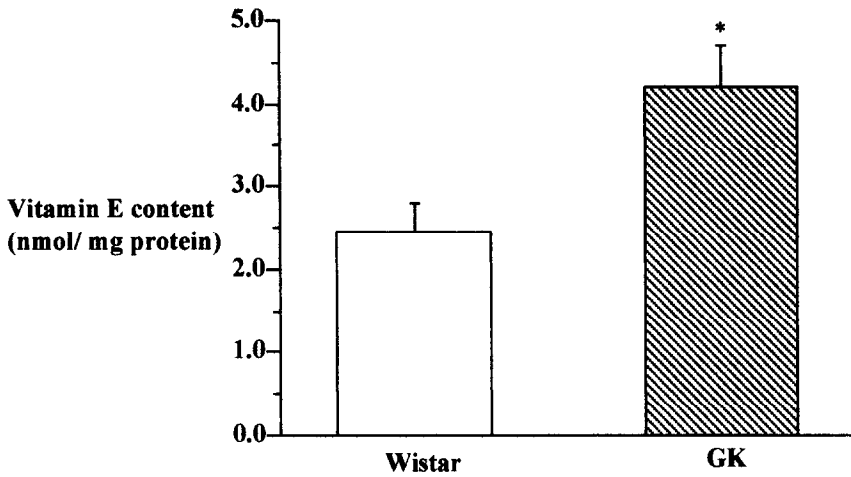
Fig. 5

Vitamin E (α-tocopherol) content of GK and Wistar mitochondrial preparations, evaluated by HPLC (high pressure liquid chromatography) analysis. Data are mean ± SEM of 5 independent experiments. Values statistically different from control: * $p < 0.05$.
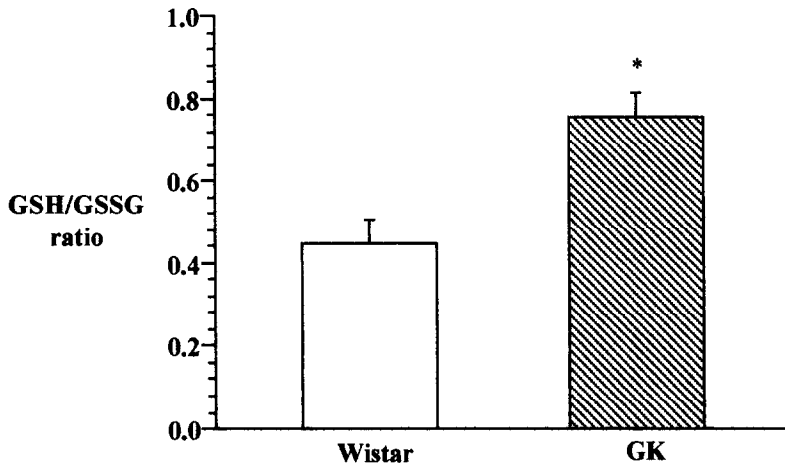

Fig. 6

Glutathione status of GK and Wistar mitochondrial preparations. GSH and GSSG levels were determined with fluorescence detection. GSH levels were 7.9 ± 0.25 and 12.2 ± 0.4 nmol/mg protein, while GSSG levels were 19.75 ± 0.5 and 15.8 ± 0.75 nmol/mg protein, respectively in Wistar and GK preparations. Data are mean ± SEM of 5 independent experiments (Wistar and GK rats). Values statistically different from control: * $p < 0.05$.

diabetes. Glutathione reductase activity increased significantly (p<0.05) in mitochondria of diabetic GK rats (22.61 ± 1.15 μmol NADPH/min/mg protein) as compared with normal animals (17.19 ± 0.46 μmol NADPH/min/mg protein). Glutathione peroxidase activity was significantly decreased (p<0.05) in diabetic animals from 148.95 ± 9.1 to 117.94 ± 7.87 μmol GSH/min/mg protein, respectively in control Wistar and diabetic GK rats.

Table 4. Activities of mitochondrial glutathione reductase, glutathione peroxidase and superoxide dismutase in the liver from GK diabetic and Wistar rats.

Enzyme	Wistar	GK
GSSG reductase	17.19 ± 0.46	22.62 ± 1.15 *
GSH peroxidase	148.95 ± 9.10	117.94 ± 7.87 *
Superoxide dismutase	3.73 ± 0.12	3.84 ± 0.13

The activity of GSSG reductase was expressed in μmol NADPH oxidized.min-1 per mg protein and the activity of GSH peroxidase in μmol GSH oxidized . min-1 per mg protein. Superoxide dismutase activity was expressed in units per mg protein. The results are the mean $\pm$ SEM for 4 different preparations. Values statistically different from control: * $p < 0.05$.

Discussion

Type 2 diabetes mellitus is a heterogeneous disease, characterized by low blood glucose control (intolerance to glucose) and results either from resistance to glucose in peripheral tissues (skeletal muscle and adipocytes) and/or a relative decrease of β-cell activity (34). Depending on several factors (obesity, age of onset, severity of glucose intolerance, and mode of inheritance), clinical features of individuals suffering from type 2 diabetes mellitus are highly variable (35).

Due to constant hyperglycaemic levels, type 2 diabetes mellitus patients are exposed to an increased oxidative stress (12), since the production of several reducing sugars (through glycolysis and polyol pathways) is enhanced. These reducing sugars can easily react with lipids and proteins (nonenzymatic glycation reaction) (3), increasing the production of ROS (36).

In fact, our results indicate a significant increase in glycated hemoglobin (HbA_{1c}) of GK rats. This parameter is extremely useful in clinical trials, since it becomes possible to estimate the mean values of glycaemia during the last two months before the analysis. Thus, our results indicate mean values of glycaemia (extrapolated from HbA_{1c} values) of 92 and 250 mg glucose/ dl blood, respectively in control and in GK diabetic rats. As a result, GK rats were undoubtedly exposed to a marked oxidative stress, as shown by the mean values of glycaemia.

The increased production of ROS could be reflected in mitochondrial membrane fatty acid composition. Nevertheless, analyses of fatty acid composition of liver mitochondria show a higher content of polyunsaturated long chain fatty acids (namely arachidonic and docosohexanoic acids) and a marked decrease in saturated fatty acids (palmitic and stearic acids) in GK diabetic rats as compared to Wistar non-diabetic rats. Consequently, we observed an increase in the unsaturation index (which reflects the average number of unsaturations per 100 C-C bonds), and a marked increase (up to 67 %) in the ratio between the contents of unsaturated/saturated fatty acids in GK rats. Our analyses also indicate an increase in fatty acids average length chain in the GK preparations.

Interestingly, studies of susceptibility of liver mitochondrial preparations to lipid peroxidation *in vitro* (evaluated by TBARS formation), showed that GK mitochondrial preparations were less

susceptible to lipid peroxidation than control preparations. These results are in agreement with the observations of Kristal et al. (15) and Sukalski et al. (37) using mitochondria of streptozotocin-induced diabetic rats, with brief periods of induced diabetes duration. Also, these results could indicate a decrease in susceptibility of induction to lipid peroxidation in vivo in GK rats, since the content in polyunsaturated fatty acids is significantly higher than in Wistar non-diabetic rats.

We found that GK mitochondrial preparations were less susceptible to in vitro oxidation, evaluated both by TBARS formation and O_2 consumption (data not shown). This lower susceptibility of GK mitochondrial preparations to induced oxidation is correlated with an increase in mitochondrial vitamin E (α-tocopherol) content. It is well known that vitamin E is one of the major lipid soluble chain-breaking antioxidants that can prevent the propagation of lipid peroxidation chain reaction, by scavenging lipid peroxyl radicals (38, 39). Therefore, despite the increase in ROS production due to hyperglycemic condition, the increase in α-tocopherol content in liver mitochondrial membranes of diabetic GK rats is consistent with the lower lipid peroxidation levels observed. These results, as the results described by Sukalski et al. (37), point to alterations in metabolism (or storage) of α-tocopherol in GK diabetic rats as compared to Wistar rats.

Our studies indicate that GK liver mitochondria respiratory indexes (RCR and ADP/O) were enhanced, compared to control rats. The observed enhanced phosphorylative efficiency can also be correlated with the larger concentration of α-tocopherol, since vitamin E is reported to interact with the early parts of respiratory chain, protecting the thiol groups of proteins from oxidative stress. Moreover, it seems that α-tocopherol interacts with ubiquinone (Q_{10}) in the reduced form (ubiquinol), a component of mitochondrial redox chain with antioxidant capacity. This antioxidant effect of ubiquinol seems to be due to its capacity of regenerating oxidized α-tocopherol (40). Furthermore, a higher coupling between oxidative and phosphorylative systems generates an increased ROS production (41). Therefore, the increased efficiency of phosphorylative oxidation in GK rats can be correlated with the higher concentration of α-tocopherol in GK membranes.

Studies of glutathione content were also performed in mitochondrial diabetic and control preparations, since intracellular GSH plays an important role in the metabolism of hydroperoxydes and free radicals (42). Moreover, GSH plays a crucial role in mitochondrial function, since mitochondria lack catalase, and therefore peroxides are reduced only by glutathione peroxidase. During this reaction GSH is oxidized and glutathione disulfide (GSSG) is formed. Most of GSSG formed in glutathione peroxidase catalyzed reactions is subsequently reduced by glutathione reductase, an important antioxidant enzyme. So, GSH/GSSG ratio may be regarded as a measure of the antioxidant capacity of biological systems (43, 44). The observation that GSH/GSSG ratio is higher in GK preparations suggest that GSH could protect diabetic mitochondria from damage mediated by free radicals. To clarify the reasons why reduced glutathione is increased in mitochondria isolated from diabetic rats we measured also the activities of glutathione reductase and glutathione peroxidase. Both the enzymes are involved in the redox cycling of glutathione. Glutathione peroxidase by removing hydrogen peroxides and lipid peroxides (30) and glutathione reductase by restoring reduced glutathione maintaining the intracellular GSH concentration (31). In our study, glutathione reductase activity was found to be increased whereas glutathione peroxidase activity was decreased. Our findings related to glutathione are in contradiction with the results published by Sukalski et al. (37) who reported that liver mitochondria isolated from diabetic animals had lower levels of gluthathione reductase, glutathione peroxidase and SOD than mitochondria isolated from control animals. However, it is important to refer that the published values are from mitochondria isolated from streptozotocin-

treated rats, a model of insulin-dependent diabetes mellitus. More recently, Kristal et al. (15) have found that SOD activity did not changed while glutathione peroxidase levels were reduced by diabetes induced by streptozotocin.

The increase in glutathione reductase activity may be the reason why glutathione level increase in diabetic mitochondria and could represent a compensatory mechanism of mitochondria in order to maintain higher levels of glutathione. The increase concentration of mitochondrial glutathione may contribute to mitochondrial protection by decreasing the susceptibility of diabetic mitochondria against reactive oxygen intermediates and free radicals and by increasing antioxidant defenses (45). The decrease in glutathione peroxidase activity may reflect the utilization of this enzyme by oxygen free radicals generated during diabetes.

In conclusion, we found that, at the age of 6 months, diabetic GK mitochondrial preparations present a higher coupling between oxidative and phosphorylative systems as compared to control preparations. Moreover, our results indicate that the lower susceptibility to lipid peroxidation of mitochondria from diabetic rats was related to their antioxidant defense mechanisms.

Acknowledgments

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