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Diabetes and mitochondrial oxidative stress: A study using heart mitochondria from the diabetic Goto-Kakizaki rat

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Abstract

Increasing evidence shows that the overproduction of reactive oxygen species, induced by diabetic hyperglycemia, contributes to the development of several cardiopathologies. The susceptibility of diabetic hearts to oxidative stress, induced *in vitro* by ADP-Fe^{2+} in mitochondria, was studied in 12-month-old Goto-Kakizaki rats, a model of non-insulin dependent diabetes mellitus, and normal (non-diabetic) Wistar rats. In terms of lipid peroxidation the oxidative damage was evaluated on heart mitochondria by measuring both the O_2 consumption and the concentrations of thiobarbituric acid reactive substances. Diabetic rats display a more intense formation of thiobarbituric acid reactive substances and a higher O_2 consumption than non-diabetic rats. The oxidative damage, assessed by electron microscopy, was followed by an extensive effect on the volume of diabetic heart mitochondria, as compared with control heart mitochondria. An increase in the susceptibility of diabetic heart mitochondria to oxidative stress can be explained by reduced levels of endogenous antioxidants, so we proceeded in determining α-tocopherol, GSH and coenzyme Q content. Although no difference of α-tocopherol levels was found in diabetic rats as compared with control rat mitochondria, a significant reduction in GSH (21.5% reduction in diabetic rats) and coenzyme Q levels of diabetic rats was observed. The data suggest that a significant decrease of coenzyme Q_9, a potent antioxidant involved in the elimination of mitochondria-generated reactive oxygen species, may be responsible for an increased susceptibility of diabetic heart mitochondria to oxidative damage. (Mol Cell Biochem **246**: 163–170, 2003)

Key words: heart mitochondria, diabetes, reactive oxygen species, coenzyme Q, α-tocopherol, Goto-Kakizaki rat

Introduction

Diabetes mellitus Type 2 or non-insulin-dependent diabetes mellitus (NIDDM) is a metabolic disorder characterised by hyperglycemia due to a decrease in the response of peripheral tissues (e.g. liver, skeletal muscle, adipose tissue, pancreatic β-cells) to insulin [1]. It has been well established that patients with diabetes mellitus, exhibit a high incidence of cardiac dysfunction and mortality [2]. Clinical studies suggested that diabetic patients have a significantly greater incidence and severity of several cardiopathies (e.g. angina, acute myocardial failure, atherosclerosis) [3, 4], and approximately 80% of all patients with diabetes die of cardiovascular disease [3].

Mitochondria play an important role in the development of NIDDM, which agrees with an age-related decline in the capacity for oxidative phosphorylation, and consequently contributes to its pathophysiology [5, 6]. In several animal models, a relationship has been established between diabetes and some dysfunctions in mitochondrial oxidative events [7, 8], suggesting the occurrence of bioenergetic alterations at the mitochondrial level [7, 9]. Mitochondria which play

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an important role in cell function, appear to constitute one of the main sources of reactive oxygen species (ROS) [10, 11]. It has been estimated that up to 2% of the total mitochondrial oxygen consumption results in ROS generation [9, 11]. Mitochondria are also the cellular component most extensively affected by increased concentrations of ROS. The inner mitochondrial membrane is believed to be particularly susceptible to oxidative damage even under physiological conditions because it is a major site of ROS production and has a high content in polyunsaturated fatty acids (PUFA) [9, 10]. ROS generated in the electron transport chain of mitochondria may react with PUFA which cause lipid peroxidation and alterations in the structure integrity of mitochondrial membranes, causing irreversible swelling and disruption, which are a cause of perturbation of mitochondrial functions [12–14]. Oxidative damage is suspected to be involved in the pathogenesis of several diseases (for review see [9]) particularly in myocardial injury and several reports indicate that oxidative stress could be responsible for the susceptibility of diabetic heart to cardiopathies [6, 7, 15].

Vitamin E (α-tocopherol), an unsaturated lipid found in the heart mitochondrial membrane, was reported to display an higher antioxidant activity, by inhibiting lipid peroxidation [16]. In the presence of lipoperoxides α-tocopherol (TOH) can donate one electron, and create a tocopheroxyl radical ($TO^{\bullet}$) which can be reduced back to TOH by ascorbate or coenzyme Q (CoQ) [17]. The abundant occurrence of CoQ in mitochondria, due to is role in mitochondrial electron transport, indicates CoQ as a preferential regenerator of α-tocopherol [17]. However, research from several groups reported that the antioxidant effects of CoQ are independent of α-tocopherol recycling [17, 18]. The emergence of CoQ as an antioxidant has a special meaning since it is the only lipid-soluble antioxidant known, so far, that is manufactured by the human body [18, 19].

The Goto-Kakizaki (GK) rat has been raised as a non-obese animal model of NIDDM. This animal is spontaneously diabetic, is obtained by selective inbreeding of normal Wistar rats with the highest glucose values during oral glucose tolerance tests [20]. The GK rat exhibits a moderate but stable fasting hyperglycemia, as early as 2–4 weeks after birth, which does not progress to the ketotic state. Our work evaluates the susceptibility of heart mitochondria from Goto-Kakizaki rats and normal Wistar rats to oxidative stress injury induced by the oxidant pair ADP/Fe^{2+}, as well as the contribution of the antioxidant defenses.

Materials and methods

This study conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Chemicals

Ubiquinone 10 (CoQ_{10}) was obtained from Fluka (Germany), ubiquinone 9 (CoQ_9) and α-tocopherol were obtained from Sigma Chemicals (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Animals and blood glucose determination

Male spontaneously diabetic GK rats (46–54 weeks of age; body wt. 384.7 ± 2.4 g; non-fasting blood glucose levels 198.6 ± 13.5 mg/dl) were obtained from a local breeding colony of 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 (44–53 weeks of age; body wt. 672.5 ± 60.5 g; non-fasting blood glucose levels 93.4 ± 2.9 mg/dl) obtained from our local colony. Animals were kept under controlled light and humidity conditions and with free access to powdered rodent chow (diet CRF 20; Charles Rivers, France) and water. Glucose tolerance test was used as a selection index. Blood glucose levels were determined, immediately after animal's sacrifice, through the glucose oxidase reaction, using a glucometer (Glucometer Elite, Bayer, Portugal).

Isolation of rat heart mitochondria

Mitochondria were isolated by the conventional differential centrifugation method from the hearts of Wistar and GK rats [21], with some modifications. The animals were killed by decapitation and the heart was immediately excised and finely minced with a pair of sharp scissors in an ice-cold isolation medium containing 250 mM sucrose, 1 mM EGTA, and 5 mM HEPES–KOH, pH 7.4. The minced blood-free tissue was then resuspended in 5 ml of isolation medium and transferred to a 50-ml glass homogenizer vessel and homogenized with a tightly fitted Potter–Elvehjen homogenizer and a Teflon pestle driven by a motor, during 30 sec. Protease (1 mg subtilisine prepared in 1 ml of isolation medium) was added and the suspension was re-homogenized for an additional 30 sec. Finally 30 ml medium was added, followed by a last 30-sec period homogenization. The homogenate was equally divided into two 50 ml centrifuge tubes and each aliquot was made up to 40 ml with isolation medium and centrifuged at 11,000 × g for 10 min. The supernatant fluid was completely decanted and the pellet, devoid of protease, was gently homogenized to its original volume with isolation medium using a loose-fitting homogenizer. The suspension was centrifuged at 900 × g during 10 min and the obtained

low-speed supernatant was transferred through two layers of cheesecloth to different centrifuge tubes and submitted to centrifugation at 9000 × g for 10 min. The high-speed supernatant was discarded and the pellet was resuspended using a paintbrush and repelleted at 9000 × g for 10 min. EGTA was omitted from the final washing medium. Mitochondria were resuspended in isolation medium (without EGTA) in a final concentration of 20–40 mg/ml.

All the isolation procedure was performed at 0–4°C and the mitochondrial suspension was maintained on ice (0–4°C) throughout the assays. The concentration of mitochondrial membrane proteins was estimated according to the biuret method [22], using bovine serum albumin as standard.

Induction and assay of lipid peroxidation

Lipid peroxidation in rat heart mitochondria was measured polarographically by monitoring the rates of oxygen consumption with an YSI 5300 oxygen monitor (Yellow Spring Instruments) equipped with a Clark oxygen electrode, as previously described [23]. Reaction was run at 30°C in a 1-mL reaction chamber equipped with magnetic stirring and containing 1 mL medium (175 mM KCl, 10 mM Tris-HCl, pH 7.4), supplemented with 3 μM rotenone and 1 mg mitochondrial protein. After 1-min pre-incubation, peroxidation was started by adding 1 mM ADP and 0.1 mM $FeSO_4$. The oxygen solubility at 30°C was considered 232 μM when the medium was air-equilibrated at 760 Torr.

Lipid peroxidation was also measured by determining the amount of lipid peroxides formed during incubation as the amount of thiobarbituric acid reactive species (TBARS) formed, according to a modified procedure described by Ernster and Nordenbrand [24]. Mitochondrial protein (1 mg) was incubated for 45 min, at 30°C, in 1 ml of a medium consisting of 175 mM KCl, 10 mM Tris-HCl, pH 7.4, supplemented with 3 μM rotenone. Membrane lipid peroxidation was started by adding simultaneously ADP/$FeSO_4$ (1 mM/0.1 mM). Lipoperoxidation was interrupted by placing the tubes in ice, which lowered the temperature to 0–4°C. To measure lipid peroxidation, 0.5 mL of cold 40% trichloroacetic acid and 2 mL of 0.67% TBA containing 6.8 mM 2,6-diterbutyl-4-methylphenol (BHT) were added to 0.5 mL of the test material. The mixture was heated at 100°C during 10 min, and was allowed to cool in ice before centrifugation in a bench centrifuge (1500 × g, 5 min). The supernatant was collected and lipid peroxidation was estimated by the appearance of TBARS spectrophotometrically quantified at 535 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol TBARS/mg protein [25].

Extraction and quantification of coenzyme Q and α-tocopherol

CoQ were extracted from aliquots of mitochondria containing 1 mg protein/ml according to the previously described method [26]. The extract was evaporated to dryness under a stream of N_2, and suspended in ethanol for HPLC analysis. Liquid chromatography was performed using a Gilson high-performance liquid chromatography apparatus with a reverse phase column (RP18; Spherisorb; S5 ODS_2) as earlier described [27]. Samples were eluted from the column with methanol:heptane (10:2, by volume), at a flow of 2 ml/min. Detection was performed with a UV detector, at 269 nm. Identification and quantification were based in pure standards by their retention times and peak areas, respectively. CoQ levels (CoQ_9 and CoQ_{10}) in mitochondrial membranes were expressed in pmol/mg protein.

The extraction and separation of α-tocopherol was performed following the method described by Vatassery *et al.* [28]. The extract, evaporated to dryness under a stream of N_2 and kept at –80ºC, was dissolved in n-hexane and the α-tocopherol contents was analyzed by reverse phase HPLC. A 4.6 × 200 mm Spherisob S10w column was eluted with n-hexane modified with 0.9% methanol, at a flow of 1.5 ml/min. Detection was performed with a UV detector, at 287 nm. The levels of α-tocopherol in mitochondrial membranes were expressed in pmol/mg protein.

Assay for GSH contents in mitochondria

Determination of GSH in samples were carried out with fluorescence detection after reaction of the supernatant of the H_3PO_4/EDTA-NaH_2PO_4 deproteinized mitochondria solution with the reagent *o*-phthaldeyde, at pH 8.0, according to Hissin and Hilf [29].

Electron microscopy

Mitochondria were fixed for electron microscopy by adding 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, and were incubated during 2 h at 4°C. After centrifugation (15,000 × g, 3 min), pellets were washed with 0.1 M sodium cacodylate buffer (pH 7.3). The fixed and washed pellet was resuspended in 1% OsO_4 buffered with sodium cacodylate 0.1 M, pH 7.3. After a 2 h incubation, membranes were washed with cacodylate buffer pH 7.3 (without osmium tetroxide). Following pre-inclusion in 1% agar, the samples were dehydrated in grade ethanol and were embedded in Spurr. The ultrathin sections were obtained with a LKB ultra-microtome Ultrotome III and stained with methanolic uranyl acetate followed by lead citrate and examined with a Jeol Jem-100SX

electron microscope operated at 80 kV. For quantitative studies, ultrathin sections from 4 blocks were obtained and 5 micrographs were taken at random on them. So, 20 micrographs with the final magnification of ×20,000 from peroxidized mitochondria and the same number from control mitochondria were analyzed. The relative volumes of mitochondria were calculated according with the Weibel method [30], for which a test area 14 × 13.5 cm was used. This grid contains 112 lines of 1 cm doing 224 points and the total line length of 112 cm.

Data analysis and statistics

Data are presented as mean ± S.E.M. of the indicated number of experiments, each one obtained from a different animal. Statistical evaluation was performed using the paired Student's *t*-test and by using the one way ANOVA Student–Newmann–Keuls post-test for multiple comparisons. Values of $p < 0.05$ were considered significant.

Results

Mitochondrial lipid peroxidation

Heart mitochondria isolated from diabetic GK rats show a higher susceptibility to lipid peroxidation induced by ADP/Fe^{2+}, as assessed by the oxygen consumption (Fig. 1) and the TBARS production (Fig. 2), when compared to Wistar control rats. The time-dependent change in peroxidation related to oxygen concentrations of heart mitochondria was different in preparations isolated from diabetic rats compared to control rats. In both mitochondrial preparations a slow oxygen consumption occurred for several minutes after ADP-Fe^{2+} addition, after which oxygen consumption drastically increased. When mitochondria begun to peroxidize, it occurred at the same rate as control mitochondria. As shown on Fig. 2, there was no variation in the absolute mean of TBARS levels in non-oxidized mitochondria from Wistar and GK rats (0.78 ± 0.69 and 0.97 ± 0.69 nmol/mg protein, respectively). In the presence of ADP/Fe^{2+} both mitochondrial fractions showed a marked increase in TBARS, from 0.78 ± 0.69 to 27.49 ± 2.37 nmol/mg protein in Wistar mitochondria and from 0.97 ± 0.69 to 45.15 ± 2.05 nmol/mg protein in GK mitochondria, indicating the occurrence of oxidative damage to mitochondrial lipids induced by the pro-oxidant pair. The analysis of the TBARS production after treatment of mitochondria with ADP/Fe^{2+} showed a higher value in mitochondria from diabetic rats compared with the controls (45.15 ± 2.05 and 27.49 ± 2.37 nmol/mg protein, respectively). A statistically significant difference ($p < 0.05$) was determined between both preparations, indicating a higher susceptibil-

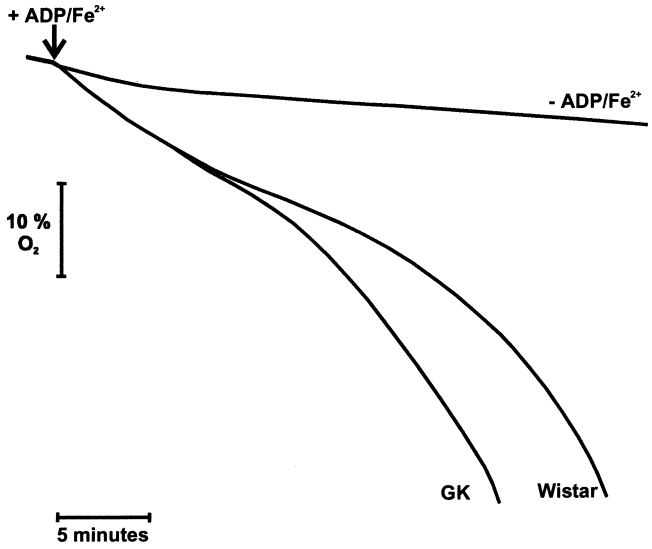

Fig. 1. O_2 consumption due to lipid peroxidation induced by ADP/Fe^{2+} in rat heart mitochondrial membranes isolated from Wistar and GK rats. Rat heart mitochondria (1 mg) were incubated at 30°C, for 30 min, in 1 ml of medium consisting of 175 mM KCl, 10 mM Tris (pH 7.4) supplemented with 3 μM rotenone. Peroxidation was started by adding 1 mM ADP and 0.1 mM $FeSO_4$. The lines represent typical recordings from seven different experiments.

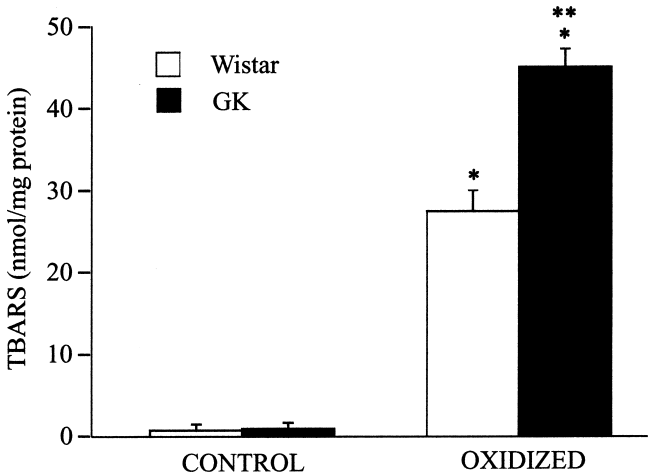

Fig. 2. TBARS formed *in vitro* during lipid peroxidation induced by ADP/Fe^{2+} in rat heart mitochondria of control (Wistar) and diabetic (GK) rats induced by ADP/Fe^{2+} (1 mM/0.1 mM). Rat heart mitochondria (1 mg) were incubated in 1 ml of medium consisting of 175 mM KCl, 10 mM Tris (pH 7.4) supplemented with 3 μM rotenone, at 30°C, for 2 min. Peroxidation was started by adding 1 mM ADP and 0.1 mM $FeSO_4$ and incubated for 45 min. TBARS were determined at the end of each incubation period as described in Materials and methods, and were expressed as nmol TBARS/mg protein. Data are expressed as mean ± S.E.M. of seven independent experiments. Statistical significance: *$p < 0.05$ compared with control group; **$p < 0.05$ compared with Wistar rats.

ity to induced oxidative stress in diabetic heart mitochondria.

Electron microscopy of mitochondria after oxidative stress

Heart mitochondria isolated from Wistar and GK rats were exposed to oxidative attack by ADP-Fe^{2+} and structural-morphological changes were examined by electron microscopy. Mitochondria incubated in the absence of ADP-Fe^{2+} at 30°C maintained their inner envelope membrane intact but, in the presence of ADP-Fe^{2+}, membrane discontinuities and volume fluctuations were observed, as compared to control mitochondria (not shown). In both mitochondrial fractions, swelling was followed by vesiculation of the inner membrane, and crenellation of the outer membrane coupled to its dissociation from the inner membrane. Based on several electron micrographs, the volume of rat heart mitochondria from Wistar and GK rats were measured, before and after induction of oxidative stress (Fig. 3). Control mitochondria from Wistar rats displayed a statistically different average volume from diabetic rat mitochondria (1.73 ± 0.21 and 1.17 ± 0.14 μm^3, respectively). Wistar mitochondria incubated with ADP-Fe^{2+} (oxidized mitochondria) presented a significantly larger volume (2.84 ± 0.31 μm^3), compared to control non-oxidized mitochondria (1.73 ± 0.21 μm^3). A drastic increase in the volume can be seen when comparing GK heart oxidized mitochondria to non-oxidized GK heart mitochondria (3.1 ± 0.59 and 1.17 ± 0.14 μm^3, respectively). Additionally, no statistical difference was found when we compared oxidized Wistar to oxidized GK heart mitochondria (2.84 ± 0.31 and 3.1 ± 0.59 μm^3, respectively).

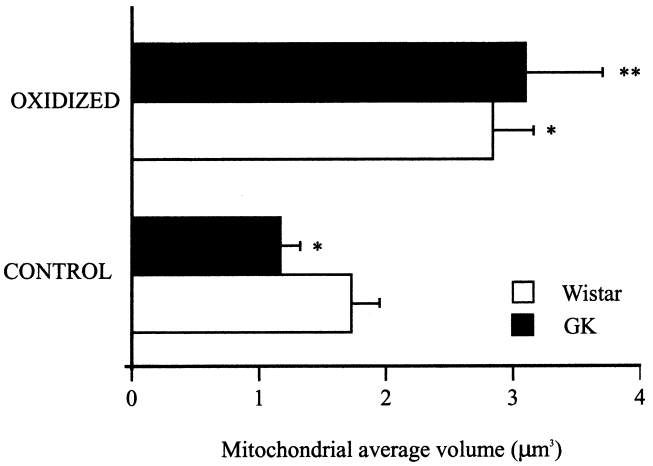

Fig. 3. Average volume of heart mitochondria isolated from Wistar and GK rats, before (control mitochondria) and after (oxidized mitochondria) induction of oxidative damage by ADP/Fe^{2+} (1 mM/0.1 mM). Data are expressed as mean ± S.E.M. of seven independent experiments. Statistical significance: *$p < 0.05$ compared with Wistar-control group; **$p < 0.05$ compared with GK-control rats.

Mitochondrial non-enzymatic antioxidants

Mitochondrial non-enzymatic contents in both preparations were determined: CoQ (Fig. 4), vitamin E (Fig. 5) and GSH (data not shown). Vitamin E (α-tocopherol), GSH and CoQ, as assessed by the two main homologues CoQ_9 and CoQ_{10}, are effective inhibitors of membrane lipid peroxidation. In heart mitochondria of Wistar and GK rats, CoQ_9 represents 96% while CoQ_{10} represents only 4% of the total pool of mitochondrial CoQ (7.21 ± 0.354 and 5.76 ± 0.64 nmol/mg protein, respectively). The content of α-tocopherol in mitochondria from control and diabetic rats display no significant differences (1051.0 ± 193.5 and 1051.7 ± 132.1 pmol/mg protein, respectively), but the GSH contents were significantly ($p < 0.05$) lowered, by 21.5%, in GK rat mitochondria compared to Wistar rat mitochondria (1.42 ± 0.12 and 1.86 ± 0.22 pmol/mg protein, respectively). Additionally, the CoQ_{10} contents have shown no statistical differences between both preparations (0.29 ± 0.039 and 0.23 ± 0.048 nmol/mg, respectively). However, CoQ_9 and consequently, total CoQ ($CoQ_9 + CoQ_{10}$) contents was significantly different ($p < 0.05$) in both mitochondrial preparations (Fig. 4).

Discussion

NIDDM has been studied in several animal models including the GK rat, which was selected for this study because it represents a good model of hereditary non-obese NIDDM that develops into a moderate and stable diabetic state with age [20, 31]. Several reports showed that increased levels of glucose found in NIDDM patients are responsible not only

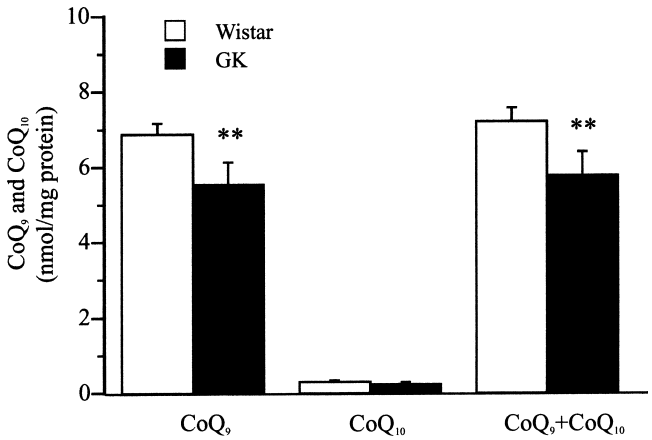

Fig. 4. Coenzyme Q_9 (CoQ_9) and Q_{10} (CoQ_{10}) levels in heart mitochondria isolated from Wistar and GK rats. Coenzyme Q levels were measured by HPLC as described in Materials and methods. Data are the mean ± S.E.M. for seven different samples, each obtained from a different rat. Statistical significance: **$p < 0.05$ as compared to control Wistar rats.

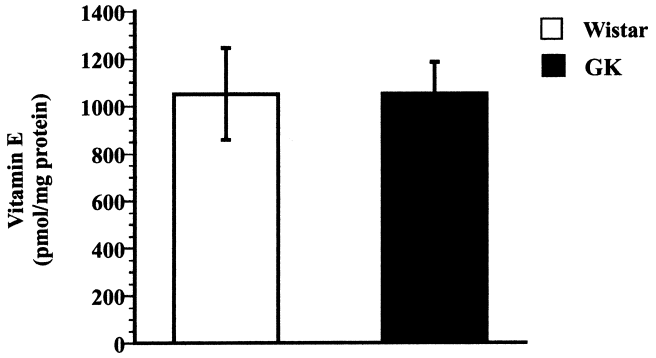

Fig. 5. Vitamin E levels of GK and control heart mitochondria preparations. Vitamin E levels were measured by HPLC as described in Materials and methods. Data are the mean ± S.E.M. for seven different samples, each obtained from a different rat.

for oxygen free radical generation due to non-enzymatic protein glycation, autooxidation of glycation products, ascorbic acid and glucose, but also for changes in tissue content and activity of antioxidant defense systems [32–38]. Elevated levels of glycated products induce an increase of lipid/protein oxidation, that in addition to different levels of antioxidant defences in tissues have been correlated with the extent of tissue damage at the molecular level [33, 36] and with the development of chronic complications of diabetes mellitus [33–35, 38]. Heart tissue has several features that makes it vulnerable to damage from free radicals such as, abundant mitochondria which may leak activated species, and a higher percentage of polyunsaturated fatty acids in mitochondrial and plasma membranes which are susceptible to lipid peroxidation [10, 39–41]. Oxidative stress due to high levels of glucose may disrupt the efficiency of mitochondrial electron transport, reduce ATP availability to cellular function [7, 8, 42] and cause the failure of heart activity as well as myocardial diseases [5, 7].

In this study we found that heart mitochondria isolated from 12-month-old GK rats and Wistar rats display different susceptibilities to oxidative stress induced by ferrous ion in the form of ADP-Fe^{2+}. We observed that oxidative damage induced in heart mitochondria of diabetic rats showed a significant increase of lipid peroxidation (measured by O_2 consumption and TBARS formation). The slow O_2 consumption could be result of mitochondrial non-enzymatic antioxidants consumption or can be ascribed to the formation of an ADP-Fe^{3+}-O_2^- (perferryl) complex [23], and presented a similar rate on both Wistar and GK mitochondria assays. However, the time required for the rapid O_2 uptake (lag time) and the time required to produce an amount of perferryl complex to induce lipid peroxidation [23] is different when diabetic and non-diabetic mitochondrial fractions were tested. A short lag time on GK mitochondrial suspensions indicated a higher susceptibility to lipid peroxidation. The analysis by electron microscopy of morphological changes showed that oxidative stress, induced by ADP-Fe^{2+}, was followed by considerable number of alterations in heart mitochondria with swelling more pronounced in diabetic heart mitochondria. This increase susceptibility is correlated with a decrease in mitochondrial GSH and CoQ_9. The observed decrease in lag phase and the increase in oxygen consumption and TBARS formation, are probably due to the lower contents of CoQ and GSH in GK heart mitochondria. GSH plays a crucial role in the metabolism of hydroperoxides and free radicals [9, 43]. A decrease of GSH content in GK heart mitochondria suggest that diabetic mitochondria could be more susceptible to free radicals damage.

Recently, it became clear that CoQ in the reduced form plays an important role as an antioxidant, and that this might explain its broad distribution in subcellular membranes other than mitochondria [18, 19, 44, 45]. The study of CoQ content is very relevant when studying lipoperoxidation because such a liposoluble antioxidant serves as the first line of defense against lipid peroxidation [18, 46, 47]. The antioxidant function of CoQ, by directly scavenging radicals [46] or by indirectly regenerating vitamin E [45, 47, 48] from its oxidized form, may be correlated with the high susceptibility of diabetic heart mitochondria to oxidative stress. In this study, the concentration of CoQ_9, on a mole base, was estimated to be more than 5–6 times higher than that of heart mitochondria's α-tocopherol; in addition CoQ_9 levels are statistically lower on diabetic mitochondria. A decrease of CoQ levels found in diabetic mitochondria causes a reduction in the antioxidant potential of these membranes and a concomitant increase in the susceptibility to oxidative damage. Vitamin E (α-tocopherol) is suggested to be the most important endogenous antioxidant [47] scavenger of radicals generated in the presence of Fe^{2+} by preventing the propagation of lipid peroxidation chain reactions [42, 43]. The decreased susceptibility to lipid peroxidation in diabetic rats was not correlated to α-tocopherol levels, because diabetic and non-diabetic heart mitochondria did not present statistically different levels.

In conclusion, the present study shows that heart mitochondria from diabetic rats differ significantly from non-diabetic rats in their susceptibility to undergo lipid peroxidation when oxidative damage is induced *in vitro*. A reduced antioxidant potential is responsible for the greater susceptibility of diabetic heart mitochondria to oxidative damage as supported from the lower CoQ and GSH contents of such mitochondria, despite diabetic mitochondria don't have different levels of α-tocopherol compared to non-diabetic mitochondria. Recently, Kucharska *et al.* [49] reported that a deficit of coenzyme Q in heart mitochondria of rats with streptozotocin-induced diabetes mellitus was also associated with an increased lipoperoxidation. Previous reports from our group [50] on the susceptibility of brain and liver mitochondrial

preparations to lipid peroxidation *in vitro*, showed that GK mitochondrial preparations from liver had reduced susceptibility to lipoperoxidation while brain had an increase susceptibility to lipid oxidation, in agreement with the heart mitochondria results reported in this study. Considering the high energy requirements of the heart, possible damage to heart mitochondria induced by oxidative stress could cause a collapse on the cardiac pool of energetic compounds and be responsible for the reported impairment of heart function in diabetes.

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References

1. Groop LC: The molecular genetics of non-insulin-dependent diabetes mellitus. J Intern Med 241: 95–101, 1997
2. Ren J, Davidoff AJ: Diabetes rapidly induces contractile dysfunctions in isolated ventricular myocytes. Am J Physiol 272: H148–H158, 1997
3. Feuvray D, Lopaschuk GD: Controversies on the sensitivity of diabetic heart to ischemic injury: The sensitivity of the diabetic heart to ischemic injury is decreased. Cardiovasc Res 34: 113–120, 1997
4. Paulson DJ: The diabetic heart is more sensitive to ischemic injury. Cardiovasc Res 34: 104–112, 1997
5. Luft R: The development of mitochondrial medicine. Biochim Biophys Acta 1271: 1–6, 1995
6. Momiyama Y, Atsumi Y, Ohsuzu F, Ui S, Morinaga S, Matsuoka, Kimura M: Rapid progression of cardiomyopathy in mitochondrial diabetes. Jpn Circ J 63: 130–132, 1999
7. Schmidt AM, Stern D: A radical approach to the pathogenesis of diabetic complications. Trends Pharmacol Sci 21: 367–369, 2000
8. Palmeira CM, Ferreira FM, Santos DL, Ceiça R, Suzuki K, Santos MS: Higher efficiency of the liver phosphorylative system in diabetic Goto-Kakizaki (GK) rats. FEBS Lett 458: 103–106: 1999
9. Halliwell B, Gutteridge JMC: Free Radicals in Biology and Medicine, 3rd Edit. Oxford University Press, Oxford, 1999
10. Kowaltowski AJ, Vercesi AE: Mitochondrial damage induced by conditions of oxidative stress. Free Radic Biol Med 26: 463–471, 1999
11. Chance B, Sies H, Boveris A: Hydroperoxide metabolism in mammalian organs. Physiol Rev 59: 527–605, 1979
12. Reinheckel T, Wiswedel I, Noack H, Augustin W: Electrophoretic evidence for the impairment of complexes of the respiratory chain during iron/ascorbate induced peroxidation in isolated rat liver mitochondria. Biochim Biophys Acta 1239: 45–50, 1995
13. Marshansky VN, Novgorodov SA, Yaguzhinsky LS: The role of lipid peroxidation in the induction of cation transport in rat liver mitochondria. The antioxidant effect of oligomycin and dicyclohexylcarbodiimide. FEBS Lett 158: 27–30, 1983
14. Carbonera D, Azzone GF: Permeability of inner mitochondrial membrane and oxidative stress. Biochim Biophys Acta 943: 245–255, 1988
15. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: A new perspective on an old paradigm. Diabetes 48: 1–9, 1999
16. Scholz RW, Minicucci LA, Reddy CC: Effects of vitamin E and selenium on antioxidant defense in rat heart. Biochem Mol Biol Int 42: 997–1006, 1997
17. Kagan VE, Serbinova E, Packer L: Antioxidant effects of ubiquinone in microsomes and mitochondria are mediated by tocopherol recycling. Biochem Biophys Res Commun 169: 851–857, 1990
18. Ernster L, Dallner G: Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta 1271: 195–204, 1995
19. Rauchova H, Lenaz G: Coenzyme Q and its therapeutics used. Cezka Slov Farm 50: 78–82, 2001
20. Goto Y, Kakizaki M: The spontaneous diabetic rat: A model of non-insulin dependent diabetes mellitus. Proc Jpn Acad 57: 381–384, 1981
21. Cain K, Skilleter, DN: Preparation and use of mitochondria in toxicological research. In: K. Snell, B. Mullock (eds). Biochemical Toxicology – A Practical Approach. IRL Press, Washington, DC, 1987, pp 217–254
22. Gornall AG, Bardawill CJ, David MM: Determination of serum proteins by means of the biuret reaction. J Biol Chem 177: 751–766, 1949
23. Sassa H, Takaishi Y, Terada H: The triterpene celastrol as a very potent inhibitor of lipid peroxidation in mitochondria. Biochem Biophys Res Commun 172: 890–897, 1990
24. Ernster L, Nordenbrand K: Microsomal lipid peroxidation. Meth Enzymol 10: 574–580, 1967
25. Buege JA, Aust S: Microsomal lipid peroxidation. Meth Enzymol 52: 302–310, 1978
26. Takada M, Ikenoya S, Yuzuriha T, Katayama K: Simultaneous determination of reduced and oxidized ubiquinones. Meth Enzymol 105: 147–155, 1984
27. Chung AP, Rainey F, Nobre MF, Burghardt J, Costa MS: *Meiothermus cerbereus sp.nov.*, a new slightly thermophilic species with high levels of 3-hydroxy fatty acids. Int J System Bacter 47: 1225–1230, 1997
28. Vatassery GT, Morley JE, Kuskowski MA: Vitamin E in plasma and platelets of human diabetic patients and control subjects. Am J Clin Nutr 37: 641–644, 1983
29. Hissin PJ, Hilf R: A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74: 214–226, 1976
30. Weibel ER: In: M.A. Hayat (ed). Principles and Techniques of Electron Microscopy: Biological Applications. Van Nostrand Reinhold, New York, 1973
31. Agardh CD, Agardh E, Hultberg B, Qian Y, Ostenson CG: The glutathione levels are reduced in Goto-Kakizaki rat retina, but are not influenced by aminoguanidine treatment. Curr Eye Res 17: 251–256, 1998
32. Wolff SP, Dean RT: Glucose autoxidation and protein modification: The potential role of autoxidative glycosylation in diabetes. Biochem J 245: 243–250, 1987
33. Oberley LW: Free radicals and diabetes. Free Radic Biol Med 5: 113–124, 1988
34. Baynes JW: Role of oxidative stress in development of complications in diabetes. Diabetes 40: 405–412, 1991
35. Mullarkey CJ, Edelstein D, Brownlee M: Free radical generation by early glycation products: A mechanism for accelerated atherogenesis in diabetes. Biochem Biophys Res Commun 173: 932–939, 1990
36. Sukalski KA, Pinto KA, Berntson JL: Decreased susceptibility of liver mitochondria from diabetic rats to oxidative damage and associated increase in alpha-tocopherol. Free Radic Biol Med 14: 57–65, 1993
37. Jain SK, Palmer M: The effect of oxygen radicals metabolites and vitamin E on glycosylation of proteins. Free Radic Biol Med 22: 593–596, 1997

38. Aguirre F, Martin I, Grinspon D, Ruiz M, Hager A, De Paoli T, Ihlo J, Farach HA, Poole CP Jr: Oxidative damage, plasma antioxidant capacity, and glucemic control in elderly NIDDM patients. Free Radic Biol Med 24: 580–585, 1998
39. Daum G: Lipids of mitochondria. Biochim Biophys Acta 822: 1–42, 1985
40. Werns SW, Lucchesi BR: Free radicals and ischemic tissue injury. Trends Pharmacol Sci 11: 161–166, 1990
41. Wallace KB: Free radical-mediated chemical cardiomiopathies. In: K. Wallace (ed). Free Radical Toxicology. Taylor and Francis, Washington, DC, 1997, pp 205–219
42. Ferreira FM, Palmeira CM, Matos MJ, Seiça R, Santos MS: Decreased susceptibility to lipid peroxidation of Goto-Kakizaki rats: Relationship to mitochondrial antioxidant capacity. Life Sci 65: 1013–1025, 1999
43. Choliparambil KP, Subramanian M, Devasagayam PA, Singh BB: Study on lipid peroxidation potential in different tissues induced by ascorbate-Fe^{2+}: Possible factors involved in their differential susceptibility. Mol Cell Biochem 178: 197–202, 1998
44. Beyer RE: The participation of coenzyme Q in free radical production and antioxidation. Free Radic Biol Med 8: 545–565, 1990
45. Lass A, Sohal RS: Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes. Arch Biochem Biophys 352: 229–236, 1998
46. Forsmark P, Aberg F, Norling B, Nordenbrand K, Dallner G, Ernster L: Inhibition of lipid peroxidation by ubiquinol in submitochondrial particles in the absence of vitamin E. FEBS Lett 285: 39–43, 1991
47. Kagan VE, Packer L: Antioxidants: Function of vitamin E and ubiquinols. In: Methods in Toxicology, Vol. 2. Academic Press, New York, 1993, pp 277–285
48. Stoyanovsky DA, Osipov AN, Quinn PJ, Kagan VE: Ubiquinone-dependent recycling of vitamin E radicals by superoxide. Arch Biochem Biophys 323: 343–351, 1995
49. Kucharska J, Braunova Z, Ulicna O, Zlatos L, Gvozdjakova A: Deficit of coenzyme Q in heart and liver mitochondria of rats with streptozotocin-induced diabetes. Physiol Res 49: 411–418, 2000
50. Santos MS, Santos DL, Palmeira CM, Seiça R, Moreno AJ, Oliveira CR: Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. Diabetes Metab Res Rev 17: 223–230, 2001