Mitochondrial dysfunction in the liver of type 2 diabetic Goto–Kakizaki rats: improvement by a combination of nutrients

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Abstract
Treatment with a combination of four nutrients, i.e. $\alpha$-lipoic acid, acetyl-l-carnitine, nicotinamide and biotin, just as with pioglitazone, significantly improves glucose tolerance, insulin release, plasma NEFA, skeletal muscle mitochondrial biogenesis and oxidative stress in Goto–Kakizaki (GK) rats. However, it is not known whether treatment with these nutrients can improve mitochondrial function and reduce oxidative stress in GK rats. The effects of a combination of these four nutrients on mitochondrial function, oxidative stress and apoptosis in GK rat liver were investigated. Livers of untreated GK rats showed (1) abnormal changes in the activities of mitochondrial complexes (decreases in I, III and IV and increases in II and V), (2) increases in protein oxidation, (3) decreases in antioxidant enzymes (superoxide dismutase, glutathione S-transferase, NADH-quinone oxidoreductase-1), (4) a decrease in total antioxidant capacity but increases in reduced glutathione level and glyceraldehyde 3-phosphate dehydrogenase expression and (5) significant increases in apoptosis biomarkers, including expression of p21 and p53. A 3-month treatment with the four nutrients significantly improved most of these abnormalities in GK rats, and the effects of the nutrient combination were greater than those of pioglitazone for most of these indices. These results suggest that dietary supplementation with nutrients that are thought to influence mitochondrial function may be an effective strategy for improving liver dysfunction in GK diabetic rats.

Key words: $\alpha$-Lipoic acid: Acetyl-l-carnitine: Nicotinamide: Biotin: Oxidative damage: Phase II enzymes: Apoptosis

Type 2 (non-insulin-dependent) diabetes mellitus is one of the most common metabolic diseases in humans, affecting about 3% of the human population. Clinically, it is rather a group of related diseases, characterised by chronic hyperglycaemic levels, due to an impairment in the balance among the three processes of $\beta$-cell secretion of insulin, peripheral resistance to insulin and hepatic glucose production$^{1,2}$. Depending on several factors, such as obesity, age of onset, mode of inheritance and severity of glucose intolerance, clinical features of individuals suffering from type 2 diabetes are highly variable.

Despite being one of the most common non-communicable diseases in the world, the exact mechanism (or mechanisms) leading to glucose intolerance is still not clear$^{1,3}$. Recently, mitochondrial dysfunction due to oxidative damage has become known as a major contributor to ageing, to degenerative diseases such as cancer, and to the metabolic syndrome, obesity and type 2 diabetes$^{4,5}$. A group of antioxidants/metabolites which can act to improve mitochondrial function and protect mitochondria from oxidative damage, when supplemented in the diet, have been defined as mitochondrial nutrients$^{6,7}$. 

Abbreviations: CoQ, coenzyme quinone; CPT-1, carnitine palmitoyltransferase I; LA, lipoic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GK, Goto–Kakizaki; GSH, reduced glutathione.

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One good example of a mitochondrial nutrient is R-α-lipoic acid (LA)\(^7\sim 9\). Here, the terms nutrients or mitochondrial nutrients denote the combination of LA, acetyl-l-carnitine, nicotinamide and biotin.

The Goto–Kakizaki (GK) rat is known as a model of type 2 diabetes. The pathogenesis of diabetes in GK rats involves insulin resistance, an abnormal glucose metabolism as well as an impaired ontogenetic development of pancreatic islet cells\(^1\sim 11\) and mitochondrial dysfunction in the liver\(^1\sim 12\). This model is particularly relevant to the understanding of human type 2 diabetes because mild hyperglycaemia has been demonstrated as early as 2–4 weeks after birth in these animals. Until 12 months of age, GK rats exhibit a moderate and stable fasting hyperglycaemia, which does not progress to a ketotic state. Therefore, at an early stage of diabetes, the GK rat does not present the severe complications associated with this condition. It is thus an appropriate model to study events occurring at the onset of diabetes, compared with obese diabetic rats which present severe hyperglycaemia and hyperlipidaemia\(^1\sim 5\).

Treatment of GK rats with mitochondrial nutrients significantly improves glucose tolerance, insulin release, plasma NEFA levels, skeletal muscle mitochondrial biogenesis\(^1\sim 4\) and immune function\(^5\). The rationale for the selection and dosage levels of these nutrients, as detailed earlier\(^1\sim 4,5\), targets substances that influence mitochondrial processes either synergistically or by mechanisms as widely disparate as possible. Briefly, acetyl-l-carnitine and LA were selected for the ability of each to ameliorate mitochondrial dysfunction: acetyl-l-carnitine by its ability to facilitate the transport of long-chain fatty acids across the mitochondrial membrane and LA by its ability to improve insulin sensitivity by adenosine monophosphate-activated protein kinase (AMPK) activation. Both nutrients were included because the combination is more effective than either nutrient administered alone. Niacin was added for its ability to lower plasma glucose, plasma NEFA and hepatic glucose production, and for its enhancement of insulin-stimulated glucose uptake in streptozotocin-treated rats. Finally, biotin was selected based on its role as a cofactor for four mitochondrial carboxylases, activities of which are thought to be normalised by a combination of biotin and LA.

The liver is one of the major organs affecting and being affected by diabetes and hyperglycaemia. Excessive gluconeogenesis in the liver contributes to hyperglycaemia\(^1\), and chronically elevated blood glucose has a significant impact on the liver itself. Diabetic patients have been found to have excessive glycogen deposition in the liver\(^17\) and excessive build-up of fat in the liver as steatosis\(^18\). More recently, Almon et al.\(^19\) compared gene expression patterns in the liver of Wistar and GK rats and found that 311 genes are differentially expressed in the liver of the two strains. A functional analysis of these genes indicated that disruption of lipid metabolism in the liver is a major consequence of chronic hyperglycaemia in the GK strain and that chronic inflammation contributes significantly to the development of diabetes in GK rats. In the present study, the effects of treatment with mitochondrial nutrients on mitochondrial function, oxidative damage and apoptosis in the liver of GK rats are presented, and compared with those of pioglitazone treatment.

### Materials and methods

#### Animals and treatments

Male diabetic GK rats, 4 weeks old, together with age-matched male non-diabetic Wistar rats were purchased from SLAC Laboratory Animal Company Limited (Shanghai, China). All animals were housed at 23 ± 2°C under 12 h light–12 h dark cycles, and allowed access to food and water ad libitum. National Institutes of Health\(^20\) principles of laboratory animal care were strictly followed, and all experimental procedures were approved by the Institutional Animal Use and Care of the Institute for Nutritional Science, Shanghai Institutes of Biological Sciences.

Rats were divided into four groups (each group, \(n = 10\)): (1) untreated Wistar and (2) GK control groups, (3) a pioglitazone group that received pioglitazone (20 mg/kg per d) by oral administration, (4) a nutrient group that received a combination of R-α-LA (50 mg/kg per d), acetyl-l-carnitine (100 mg/kg per d), biotin (0.1 mg/kg per d) and nicotinamide (15 mg/kg per d) by oral administration. The control groups received the same oral administration volume of PBS alone. The treatments started at 6 weeks of age, and continued for 3 months. At approximately 18 weeks of age, animals were anaesthetised with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and killed in order to obtain the liver.

#### Isolation of liver mitochondria and liver tissue protein

Liver mitochondria were isolated as described previously\(^21\) with a slight modification\(^22,23\). Mitochondrial protein concentration was determined using a Pierce BCA™ Protein Assay kit (product no. 23225; Thermo Scientific, Rockford, IL, USA), with bovine serum albumin as a standard. Mitochondria were stored at −80°C until enzyme analysis.

For tissue protein collection, approximately 100 mg of liver samples were homogenised in ice-cold solubilisation buffer containing 65 ml-Tris (pH 7–4), 150 mm-NaCl, 5 mm-EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, aprotonin (1 μg/ml), leupeptin (1 μg/ml), 10 mm-sodium fluoride, 1 mm-Na₂VO₄ and 1 mm-phenylmethylsulfonyl fluoride. Protein concentrations were determined as described earlier.

#### Assays of complexes I, II, III, IV and V activities in mitochondria

The activities of the mitochondrial complexes NADH–coenzyme quinone (CoQ) oxidoreductase (complex I),
succinate–CoQ oxidoreductase (complex II), CoQ–cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V) were measured spectrophotometrically as described previously (24–26).

Assays for activities of total antioxidant capacity, superoxide dismutase, glutathione S-transferase, NAD(P)H:quinone oxidoreductase 1 and reduced glutathione in mitochondria

Total antioxidant capacity was assayed with a commercially available assay kit (Jiancheng Biochemical, Inc., Nanjing, China). The principle of the test is to measure the change in colour upon conversion of Fe$^{3+}$ to Fe$^{2+}$ by the reducing components in the samples. Optical density was measured at 520 nm with a microplate reader. Total superoxide dismutase activity was assayed using a commercially available SOD Detection Kit (Jiancheng Biochemical, Inc.). The assay is based on the superoxide generated by the xanthine/xanthine oxidase system. Optical density at 550 nm was measured with a microplate reader. Glutathione S-transferase activity was measured with 2 μg protein in a final 200 μl volume of 1 mM-reduced glutathione (GSH), 1 mM-chloro-2,4-dinitrobenzene, and bovine serum albumin (3 mg/ml) in 10 mM-sodium phosphate buffer. The mixture was scanned at 340 nm for 5 min at 25°C as described previously (27).

NAD(P)H:quinone oxidoreductase 1 activity was measured with 10 μg protein in a final 200 μl volume of 50 mM-Tris–HCl (pH 7·4), 80 μM-dichlorophenolindophenol (DCPIP), bovine serum albumin (1 mg/ml), 0·005% Tween-20 (v/v), and 0 or 10 μM-dicoumarol; the reaction was initiated with 180 μM-NADPH. The reduction of DCPIP was scanned at 600 nm at 25°C with a microplate reader. NAD(P)H:quinone oxidoreductase 1 activity was considered to be the dicoumarol-inhibitable part of the DCPIP reduction.

GSH content was determined in the liver with a commercially available GSH Detection Kit (Jiancheng Biochemical, Inc.) that utilises a thiol-specific reagent, dithionitrobenzoic acid. The adduct was measured spectrophotometrically at 412 nm.

Western blot of glyceraldehyde 3-phosphate dehydrogenase in mitochondria

Liver mitochondrial protein samples (10 μg) were subjected to 10% SDS-PAGE; proteins were then transferred to nitrocellulose membranes and blocked with 5% non-fat milk/TBST (Tris-buffered saline Tween 20) for 1 h at room temperature. Membranes were incubated with primary monoclonal antibodies directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000) overnight at 4°C. Western blots were developed using enhanced chemiluminescence (Roche) and quantified by scanning densitometry (Bio-Rad Laboratories, Hercules, CA, USA) after incubation with horseradish peroxidase-conjugated secondary antibody.

Detection of mitochondrial protein carbonyls

Protein carbonyls in liver mitochondria were assayed with the Oxyblot protein oxidation detection kit (Chemicon, Temecula, CA, USA) (24,28).

Western blot analysis of p53 and p21

Liver tissue proteins were subjected to 10% SDS-PAGE and detected with primary antibodies against p53 (1:1000, Mouse Ab no. 12 506; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (1:1000, Mouse Ab no. 2946; Cell Signaling Technology, Danvers, MA, USA) or α-tubulin (1:5000). Western blots were developed using enhanced chemiluminescence (Roche) and quantified by scanning densitometry (Bio-Rad Laboratories, Hercules, CA, USA) after incubation with horseradish peroxidase-conjugated secondary antibody.

Determination of NEFA contents of Goto–Kakizaki liver tissue

NEFA contents of supernatants were estimated by a commercially available NEFA kit (Jiancheng Biochemical, Inc.). The test is based on the reaction of NEFA with Cu$^{2+}$ to form Cu salts, which are detected photometrically at 440 nm.

RNA isolation and RT-PCR for PPARα and carnitine palmitoyltransferase I

Total RNA was isolated using the single-step TRI-reagent (TRIZol), and 1 μg of isolated RNA was then reverse-transcribed into complementary DNA. Primer sequences used for quantification of mRNA by real-time quantitative PCR for PPARα, carnitine palmitoyltransferase I (CPT-1), and 18S ribosomal RNA and mRNA have been described previously (29).

Statistical analysis

Data are presented as means with their standard errors. Statistical significance was calculated with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) using one-way ANOVA. P values <0.05 were considered significant.

Results

Activities of liver mitochondrial complexes

As shown in Fig. 1, all five complexes composing the respiratory chain were assayed photometrically.
Compared with Wistar controls, activities of complexes I, III and IV distinctly decreased in the liver mitochondria of GK rats ($P_{0.01}$). Conversely, the activities of complexes II and V distinctly increased in GK rats. The nutrient treatment significantly prevented the decreases in complexes I, III and IV ($P_{0.05}$); in contrast, treatment with pioglitazone produced no significant improvement. As for complexes II and V, nutrient treatment showed no significant changes compared with GK untreated rats, while pioglitazone treatment significantly increased the activity of complex V.

Liver mitochondrial protein carbonyl levels

As shown in Fig. 2, protein carbonyl levels were higher in the liver mitochondria of GK rats compared with those of control rats ($P_{0.01}$). Nutrient treatment significantly decreased the carbonyls in liver mitochondria compared with GK untreated rats, while pioglitazone treatment significantly increased the activity of complex V.

Protein expression of glyceraldehyde 3-phosphate dehydrogenase in liver mitochondria

GAPDH protein expression was significantly increased in the liver mitochondria of untreated GK rats compared with the expression in Wistar rat mitochondria ($P_{0.05}$); the nutrient and pioglitazone groups demonstrated significant normalisation of protein expression compared with the GK group ($P_{0.05}$), as shown in Fig. 4.

Protein expression of the apoptosis-related factors, p53 and p21 in liver tissue

p53 protein expression was significantly increased in the liver tissue of GK rats (Fig. 5) compared with that in Wistar rats, and the increase in p53 was accompanied by an
increased expression of p21 protein. Nutrient treatment tended to inhibit the increase in p53 and p21, while treatment with pioglitazone did not produce a significant difference.

**NEFA content in the liver**

As shown in Fig. 6, NEFA content was higher in the liver of diabetic GK rats than that in Wistar rats; treatment with nutrients significantly decreased the NEFA level ($P<0.05$).

**PPARα and CPT-1 gene expression changes in liver tissue**

Levels of PPARα mRNA were lower in GK rats, although the difference was not statistically significant. Administration of nutrients to GK rats significantly increased the expression of PPARα levels (Fig. 7). In contrast, levels of CPT-1 mRNA were significantly lower in GK rats than in Wistar rats ($P<0.05$). Administration of either nutrients or pioglitazone to GK rats significantly increased the levels of CPT-1 in the GK rat liver (Fig. 7).

**Discussion**

As with β-cells, the liver is highly exposed to the possibility of glucose injury (glucose toxicity). Emerging evidence supports the hypothesis that insulin responsiveness of the liver in type 2 diabetes is caused by mitochondrial dysfunction$^{(5)}$. It has been shown that liver mitochondrial function appears to be more efficient and that the liver has higher levels of antioxidants such as vitamin E, GSH and CoQ in 6-month-old GK rats than in non-diabetic control rats$^{(30–33)}$. It is difficult to understand why GK rat liver mitochondria function at high efficiency while operating under the stressful conditions of diabetic hyperglycaemia. One possible explanation for over-reactive mitochondria and high antioxidant response is a diabetes-induced metabolic maladaptation$^{(33)}$.

In the present study, liver mitochondrial function is significantly altered in GK rats, as indicated by the decreases in complex I, III and IV activities and increases in complex II and V activities. Treatment with the combination of mitochondrial nutrients, more significantly than with pioglitazone, normalised the activity of liver mitochondrial complexes in GK rats towards that in non-diabetic Wistar rats$^{(31)}$. The changes in complexes I and IV are consistent with a previous report by Ferreira et al.$^{(5)}$, but the changes in complexes I and IV are different. Ferreira et al.$^{(5)}$ showed that complex I and complexes II and III did not change while complex IV increased. The lack of change of complexes II and III in Ferreira et al.’s work may be due to an increase in...
complex II combined with a decrease in complex III, but the reasons for the differences in complexes I and IV between the present study and Ferreira et al.’s are unknown. The only difference between the two studies is that in the present study, nutrients were administered by oral administration, whereas Ferreira et al. used animals without any treatment. Whether stress induced by oral administration caused a decrease in the activity of complexes I and IV warrants further study.

GSH, CoQ and α-tocopherol are important endogenous antioxidants, and are generally decreased by oxidative insult. GK rat liver mitochondria have increases in mitochondrial CoQ(33), vitamin E (α-tocopherol) contents and reduced:oxidised glutathione ratios (30), possibly in response to increased oxidative stress. In agreement with these results, liver GSH levels in GK rats were significantly increased, compared with those in Wistar rats. Treatments with nutrients or pioglitazone reversed the GSH increase by a similar amount, suggesting that they may play a role in relieving hyperglycaemia-induced oxidative stress in the liver. However, it should be pointed out that the lack of the measurement of oxidised glutathione is a limitation in the present study.

GAPDH expression was prominently higher in GK rats, compared with that in Wistar rats (Fig. 4). GAPDH was first characterised as a glycolytic dehydrogenase, but it has been shown since to exert several functions as diverse as apoptosis induction, transfer RNA export, DNA repair or as a receptor-associated kinase(35). It is a pleiotropic enzyme that is overexpressed during apoptosis and in several human chronic pathologies. This protein accumulates in mitochondria during apoptosis, and regulates pro-apoptotic mitochondrial membrane permeabilisation, a decisive event in the intrinsic pathway of apoptosis. This latter functionality provided motivation to investigate protein expression of the apoptosis-regulating factors p53 and p21. Consistent with the elevation of GAPDH and mitochondrial dysfunction, levels of both p53 and p21 proteins were elevated – the first report of increased expression of these factors in the GK rat liver. Treatment with either the nutrient combination or pioglitazone significantly decreased GAPDH expression (Fig. 4). These treatments also produced a non-significant decrease in p53 and p21 (Fig. 5), consonant with the statistically significant reduction in these two proteins seen in GK rat thymocytes reported earlier(15).

As evidence indicating a possible increase in oxidative stress and consequent cell death/tissue damage in GK rat mitochondria, amounts of oxidised proteins were clearly elevated in GK rats, and total antioxidant capacity, total superoxide dismutase, glutathione S-transferase and
NAD(P)H:quinone oxidoreductase 1 activities were substantially decreased. These results suggest that hyperglycaemia-induced metabolic maladaptation will consequently cause oxidative damage in GK rats. Treatment of GK rats with mitochondrial nutrients efficiently prevented oxidative damage in liver. These results are in line with the improvement of impaired glucose tolerance and hyperinsulinaemia\(^{14}\), and closely parallel similar ameliorative effects of these nutrients on the same oxidative parameters in GK rat thymocytes\(^{15}\).

NEFA content in diabetic GK rat plasma is substantially elevated\(^{35}\), although GK rats demonstrate neither severe dyslipidaemia nor fatty liver. In the present study, NEFA content in the liver tissue of GK animals increased significantly compared with that of Wistar animals, and nutrient treatment significantly ameliorated this increase (Fig. 6). This pattern parallels previously reported results in plasma\(^{13}\). Nutrient treatment also affected the expression of hepatic genes related to lipid oxidation pathways that are altered in GK animals. Expression of PPAR\(\alpha\) and CPT-1 gene expression decreased in the GK rat liver. PPAR\(\alpha\) and CPT-1 regulate the mitochondrial and peroxisomal fatty acid \(\beta\)-oxidation systems as well as the microsomal \(\omega\)-oxidation system. Defects in these factors have been reported to lead to defects in the utilisation of hepatic fat as an energy source and consequently to hypoketonaemia that has a prominent impact on the pathogenesis of fatty liver disease\(^{37,38}\). In the GK model, NEFA accumulation was suppressed by mitochondrial nutrient treatment along with significant up-regulation of PPAR\(\alpha\) and CPT-1 mRNA transcription, indicating that mitochondrial nutrients decreased NEFA contents by up-regulating lipid oxidation pathways. In GK rat muscle, comparable decreases in PPAR\(\alpha\) and CPT-1 gene expression levels that were restored to wild-type levels by nutrient treatment suggest that similar metabolic alterations may occur in the latter tissue as well\(^{13}\).

In summary, GK rat livers differ from Wistar controls by displaying abnormal changes in the activities of mitochondrial complexes, an increase in oxidative stress, a decrease in antioxidant defences and increases in NEFA levels and apoptosis. Furthermore, a 3-month treatment with a combination of four nutrients ameliorated these abnormalities to the same degree as did treatment with the antidiabetic drug pioglitazone. These results suggest that dietary supplementation with these nutrients may be an effective strategy to improve liver dysfunction in GK diabetic rats by improving mitochondrial function, inhibiting oxidative damage and reducing apoptosis.

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