Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1709 (2005) 231 – 239

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Structural and functional changes in heart mitochondria from sucrose-fed hypertriglyceridemic rats

Karla Carvajal*, Mohammed El Hafidi, Alvaro Marin-Hernández, Rafael Moreno-Sánchez

Department of Biochemistry, Instituto Nacional de Cardiología. Juan Badiano #1, Col. Sección XVI, Tlalpan, México, D.F., Mexico

Received 26 March 2005; received in revised form 26 July 2005; accepted 5 August 2005
Available online 22 August 2005

Abstract

In the heart of sugar-induced hypertriglyceridemic (HTG) rats, cardiac performance is impaired with glucose as fuel, but not with fatty acids. Accordingly, the glycolytic flux and the transfer of energy diminish in the HTG heart, in comparison to control heart. To further explore the biochemical nature of such alteration in the HTG heart, the components of the non-glycolytic energy systems involved were evaluated. Total creatine kinase (CK) activity in the myocardial tissue was depressed by 30% in the HTG heart whereas the activity of the mitochondrial CK (mitCK) isoenzyme fraction that is functionally associated with oxidative phosphorylation decreased in isolated HTG heart mitochondria by 45%. Adenylate kinase (AK) was 20% lower in the HTG heart. In contrast, respiratory rates with 2-oxoglutarate (2-OG) and pyruvate/malate (pyr) were significantly higher in HTG heart mitochondria than in control mitochondria. 2-OG dehydrogenase activity was also higher in HTG mitochondria. Respiration with succinate was similar in both groups. Content of cytochromes b, $c+c_1$ and $a+a_3$, and cytochrome c oxidase activity, were also similar in the two kinds of mitochondria. A larger content of saturated and monounsaturated fatty acids was found in the HTG mitochondrial membranes with no changes in phospholipids composition or cholesterol content. Mitochondrial membranes from HTG hearts were more rigid, which correlated with the generation of higher membrane potentials. As the mitochondrial function was preserved or even enhanced in the HTG heart, these results indicated that deficiency in energy transfer was associated with impairment in mitCK and AK. This situation brought about uncoupling between the site of ATP production and the site of ATP consumption (contractile machinery), in spite of compensatory increase in mitochondrial oxidative capacity and membrane potential generation.

Keywords: Creatine kinase; Energy metabolism; Heart mitochondria; Hypertriglyceridemia; Oxidative phosphorylation

1. Introduction

Myocardial performance depends upon the ATP generated through oxidative phosphorylation (OxPhos) [1]. To ensure the ATP supply from the production to the consuming sites, the cell utilizes the channeling system of creatine kinase (CK) and adenylate kinase [1]. Alterations in the energy producing and transfer systems (decrease in respiratory chain enzymes and CK activities) have been detected in some cardiac diseases, such as heart failure and ventricular hypertrophy [2–5]. The CK system consists of two cytosolic and two mitochondrial (mitCK) isoenzymes. It has been proposed that in the ventricular cardiac muscle, mitochondrial CK isoenzymes are associated with the adenine nucleotide translocase (ANT) [6]. In consequence, the ATP synthesized by OxPhos that emerges from the mitochondrial matrix to the cytosol is taken up by mitCK to generate phosphocreatine (PCr) and ADP, which in turn can be retaken by ANT [6]. Then, the cytosolic CK isoenzyme located in the myofibrils is responsible for ATP generation from PCr.

Cardiovascular diseases are often associated with energy deficit, and in many cases this is also accompanied by lipid disorders such as hyperlipidemias and obesity [7,8]. However, the nature of such deficit is still unclear. Since in heart most of the energy is produced by mitochondria, structural and functional changes derived from or caused by metabolic disorders could compromise the energetic status of the

* Corresponding author. Tel.: +52 55 5573 2911x1422; fax: +52 55 5573 0926.
E-mail address: Karla_ca@yahoo.com (K. Carvajal).

0005-2728/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2005.08.001

organ. In fact, alterations in cellular and mitochondrial membrane composition have been described to affect not only electrical properties of heart, but also energy production [8–10].

Similarly, the insulin resistance syndrome is often associated with the development of cardiovascular disorders [11,12]; the mechanisms leading to alteration in specific energy producing and transfer systems have not been fully studied. Animal models of insulin resistance are difficult to establish, since in most cases, these models also develop other metabolic disorders, such as diabetes and obesity [7,13]. In this regard, sugar-fed rat appears as a suitable alternative to dissect some alterations related to the insulin resistance syndrome, as this model initially develops only hypertriglyceridemia, followed by insulin resistance, mild hypertension and obesity at later stages [14–16].

In the heart of the sugar-induced hypertriglyceridemic rat model, cardiac performance is impaired with glucose or lactate as fuel, but not with fatty acids or ketone bodies [17]. Furthermore, lower glycolytic and oxygen uptake rates are observed in the HTG heart with glucose as fuel. However, the diminution in both glycolysis and OxPhos does not fully account for the severe impairment in cardiac work. Thus, it has been proposed that the hypertriglyceridemic state induces alterations in other sites of the ATP-utilization pathways, which is apparent when ATP production is low (with glucose or lactate as fuel), but no when ATP production is high (with fatty acids as fuel) [17]. Under an ischemic–reperfusion episode, in which HTG hearts are extremely sensitive [15], a low ATP production situation may develop. To explore the nature of such impairment in the HTG heart, the functionality of the components of both the energy transfer system and oxidative phosphorylation were evaluated.

2. Methods

2.1. Animals

Wistar male rats weighing 250 g were used. Hypertriglyceridemia was induced by feeding rats with 30% (w/v) sucrose in their drinking water for 12–15 weeks as previously described [14]. Normotriglyceridemic weight- and age-matched rats were used as controls. Mechanical performance of the isolated heart was evaluated in a Langendorff apparatus as previously described [17]. Serum thyroid hormone was assayed by radioimmunoassay with a commercial kit [18].

2.2. Mitochondrial assays

Rat heart mitochondria were isolated from male normo- and hyper-triglyceridemic (HTG) Wistar rats according to a method previously described using the protease type XXVII (Nagarse, Sigma Chem.) [19]. 2-oxoglutarate dehydrogenase (2-OGDH) activity was assayed in mitochondrial extracts as previously reported [20]. Total CK, mitCK, AK [21], and cytochrome *c* oxidase activities were assayed in extracts from whole heart and isolated heart mitochondria by standard methods.

2.2.1. Oxygen uptake rates and transmembranal electrical potential

Mitochondrial respiratory rates were measured at 30 °C by using a Clark-type O_2 electrode and incubating 0.25 mg protein/ml of fresh mitochondria in an air-saturated KME (120 mM KCl, 20 mM MOPS and 1 mM EGTA at pH 7.2) medium that contained 5 mM Pi and the indicated oxidizable substrate. Mitochondrial CK activity coupled to OxPhos was determined in fresh mitochondria as the stimulation of the oxygen uptake rate by the addition of 2 mM ATP, in the presence of 25 mM creatine and 2 mM Mg^{2+} [22]. The apparent $K_{m\ ADP}$ values of OxPhos were determined by measuring ADP-stimulated oxygen uptake at different concentration of ADP in the absence and presence of 25 mM creatine and 2 mM Mg^{2+}; the ADP stock was calibrated by enzymatic assay.

Response coefficients of the rate of OxPhos towards creatine (R_{Cr}^{OxPhos}) were determined in freshly prepared mitochondria from control and HTG hearts by measuring state 3 (ADP-stimulated) respiration at different concentrations of creatine. Flux control coefficients (C_{CK}^{OxPhos}) were calculated from the following equation [23] that correlates the elasticity coefficient of mitCK towards creatine (ε_{Cr}^{CK}) with response and flux control coefficients:

$$R_{Cr}^{OxPhos} = C_{CK}^{OxPhos}\varepsilon_{Cr}^{CK}$$

The membrane potential was quantitatively determined by measuring the ^{3}H-tetraphenylphosphonium (^{3}H-TPP$^+$) distribution across the inner mitochondrial membrane; 2 mg of mitochondrial protein were incubated in 0.5 ml of KME medium, 0.8 μ ^{3}H-TPP$^+$ (0.06–0.07 μCi/nmol), 5 mM Pi and 10 mM succinate or 2-oxoglutarate with or without 2 mM ADP as described elsewhere [24].

2.2.2. Heme cytochrome content

The concentration of mitochondrial heme $C+C_1$; B, and A_1+A_3 was determined from the absorbance difference spectra of reduced minus oxidized heme groups of the different cytochromes in mitochondrial samples. Two mg of mitochondrial protein was added to 2 ml of a 1:1 mixture of KME: glycerol. Ammonium persulfate and dithionite were used to achieve full oxidation and reduction, respectively. To estimate the concentration of cytochromes the following extinction coefficients were used: 19 mM^{-1} cm^{-1} (550–540 nm) for $c+c_1$ [25]; 16 mM^{-1} cm^{-1} (605–630 nm) for $a+a_3$ [26]; and 25 mM^{-1} cm^{-1} (563–578 nm) for b [27].

2.2.3. Lipid composition analysis

The composition of fatty acids in the mitochondrial membranes was determined by gas chromatography in a

Table 1
Physiological parameters of rats

	Control	HTG
Body weight (g)	433±42	411±32
Triglycerides (mmol/L)	0.8±0.3	1.7±0.9*
Arterial blood pressure (mm Hg)	114±14	149±10**
Insulin (mU/mL)	7.6±0.6	11.5±1.9*
Work performance (RPP) (beats/min/mm Hg)	20323±1258	9264±1248**

Values are mean±S.D. ($n=6$). ***P, 0.001. **P, 0.01. *P, 0.05. RPP, rate pressure product, using 10 mM glucose as fuel.

Carlo Erba chromatograph model 2300, fitted with a 25 m × 0.25 mm i.d. fused-silica capillary column, coated with CP-Sil 88 at 195 °C (oven temperature). Total fatty acids were extracted from 2 mg of mitochondrial protein with a 1:2 organic mixture of methanol/chloroform in the presence of 0.002% butyl-hydroxy-toluene (BHT) and using 100 μg margaric acid as internal standard. Derivation of the extracted fatty acids into their corresponding fatty acid methyl esters was made in the presence of 2% H_2SO_4 and methanol. After this, lipids were extracted with hexane and concentrated by evaporating under a N_2 atmosphere. Lipids were dissolved with hexane; an aliquot of 10 μl from this last solution was then injected into the gas chromatograph. [16]. For determination of total cholesterol in mitochondrial membranes (10 mg protein), total lipids were extracted with methanol/chloroform/BHT as described above, in the presence of 50 μg stigmasterol as internal standard. The lipid extracts were then incubated overnight at room temperature with hexamethyldisilazane and trimethylchlorsilane in dry pyridine. This procedure converts free cholesterol and stigmasterol to their corresponding trimethyl silyl ethers. Cholesterol was quantified by gas chromatography using a gas chromatograph CarloErba 2003 equipped with a flame ionization detector and a split-less inlet system, and a fused silica non-polar capillary column SE 54 (30 m, 0.35 mm ID). The injector and detector temperatures were 275 °C and 270 °C, respectively. The oven temperature was 270 °C throughout the analysis. Helium was used as carrier gas at a flow rate of 5 ml/min.

Phospholipids were separated by HPLC as follows. Total fatty acids were extracted as described above and dissolved in hexane/2-propranol (3:1 v/v). This solution was filtered through a 0.5-μm pore diameter membrane; an aliquot of 10 μl was then applied to the HPLC apparatus (Waters), which was equipped with a 5-μm Bondesil NH_4 column (150 × 4.6 mm), a μ-Bondapak NH_2 column (15 × 3.9 mm) as guard column and an UV absorbance detector system from Varian [28]. The mobile phase was acetonitrile/methanol/0.2% triethylamine pH 4.0 (67:22:11, v/v) at a flow rate of 1 ml/min.

2.2.4. Membrane fluidity

The fluorescence polarization of 1, 6-diphenylhexatriene (DPH) was analyzed to determine the fluidity of the mitochondrial membranes. Mitochondria (2 mg protein) were incubated at temperatures ranging from 20 to 45 °C for 30 min in 2 ml of a medium that contained 0.25 M sucrose adjusted to pH 7.3 with Tris base and 1 mM DPH solubilized in dimethylformamide. Each sample was subjected to polarization analysis in an Aminco Bowman fluorometer by measuring fluorescence at 340 nm excitation and 417 nm emission [29].

2.3. Protein determination

Protein concentration was determined by the Lowry method in samples used in enzymatic assays and by Biuret for isolated mitochondria.

2.4. Statistical analysis

Two tailed Student's t-test for non-paired samples was used to establish significant differences between the groups. Data are expressed as mean±standard deviation (S.D.). Differences were considered significant when a $P<0.05$ was obtained. Apparent K_m values for ADP were estimated by fitting experimental data to a non-linear regression (Michealis–Menten model) equation. The statistical tests, fitting analysis and graphs were carried out with the statistical package Prism 3.0 from GraphPad Software, Inc.

3. Results

3.1. Physiological characteristics of animals

Body weight data and serum triglycerides (TGs) levels are depicted in Table 1. As previously reported [14,17],

Table 2
Enzymatic activities in heart mitochondria

Enzyme	Control	HTG
Creatine-mediated state 3 respiration (CK, polarographic method)	1334±115	734±86*
nmol ADP/min/mg protein	$n=7$	$n=7$
CK (spectrophotometric method)	3200±100	3000±600
nmol ADP/min/mg protein	$n=6$	$n=5$
CK (spectophotometric method)	100±5	90±5
DTT-omitted (% of +DTT value)	$n=4$	$n=5$
2-OGDH	180±21	230±22*
nmol NADH/min/mg protein	$n=8$	$n=8$
AK	229±7	170±14*
nmol ADP/min/mg protein	$n=5$	$n=5$
COX	1158±405	1391±668
ng atoms oxygen/min/mg protein	$n=4$	$n=4$

* Denotes significant difference at $P<0.05$. The rate of state 3 respiration in fresh isolated heart mitochondria was determined with 10 mM 2-OG as oxidizable substrate. The rate of ADP formation was calculated by using the ADP/O ratio obtained with the same preparation oxidizing the same substrate and in the presence of 800 nmol ADP. ADP/O ratio values for both groups were similar and ranged between 2.5 and 2.8. 2-OGDH, 2-oxoglutarate dehydrogenase. AK, adenylate kinase. COX, cytochrome c oxidase. Activities were assayed under maximal rate conditions, and initial rates were taken.

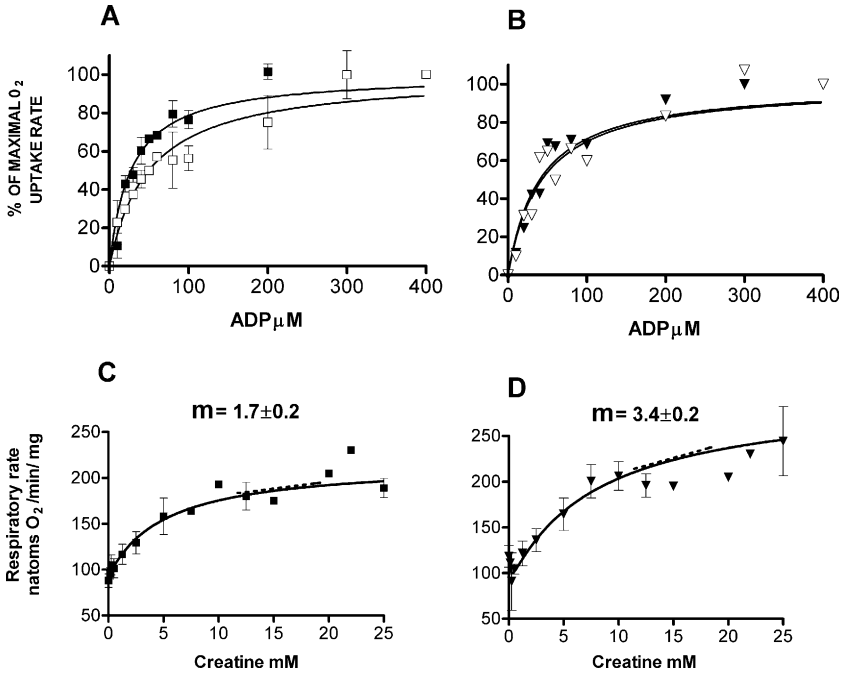

Fig. 1. Apparent affinity of mitochondrial respiration for ADP and creatine in heart mitochondria. Mitochondria isolated from control (A) and HTG (B) hearts were incubated in KME medium with the indicated ADP concentrations in the presence (open symbols) or in the absence (close symbols) of 25 mM creatine (Cr) as described under Methods. Control (C) and HTG mitochondria (D) were incubated with the indicated creatine concentrations in the presence of 2 mM ATP and 2 mM Mg^{2+}. The solid lines represent the best fit to the Michaelis–Menten equation, which was made by the computer program Prism 3.0. Absolute values for maximal oxygen uptake are depicted in Table 3. Data represent the mean±S.D. of 5 different mitochondrial preparations.

sucrose feeding by 15 weeks induced an increase in circulating TGs concentration and a slight, but significant higher arterial pressure (Table 1); no other physiological alterations such as central obesity, insulin resistance or hyperinsulinemia were apparent at this stage. In agreement with previous work [17], cardiac performance with glucose as fuel was significantly lower in HTG hearts (Table 1). With palmitate or octanoate, no differences in cardiac work are found between control and HTG hearts [17].

Table 3
Rat heart mitochondrial respiratory state 3 rates (nanogram atoms oxygen/min/mg protein)

Substrate	Control	HTG
10 mM 2-OG	291±77	376±85*
	$n=7$	$n=7$
5 mM glu/mal	253±67	303±79
	$n=6$	$n=6$
5 mM pyr/mal	289±157	415±103*
	$n=3$	$n=3$
10 mM succinate	322±63	459.5±78*
	$n=8$	$n=8$
0.15 μM CCCP	324±40	420±20*
10 mM 2-OG	$n=3$	$n=3$

Values are mean±S.D. of n different mitochondrial preparations. Values for state 3 were determined by adding 0.4 mM ADP to 0.5 mg of mitochondrial protein with the indicated substrate. Abbreviations: 2-OG: 2-oxoglutarate; glu/mal: glutamate/malate; pyr/mal: pyruvate/malate. * Denotes significant difference versus control, $P<0.05$. The respiratory control ratios were 8.2±2 and 6.1±3 for HTG and control heart mitochondria, respectively, with 2-OG; and 5±2 and 4±2 with succinate.

3.2. CK and AK activities

The activity of mitCK that is functionally coupled to OxPhos severely diminished in mitochondria isolated from HTG hearts, when measured by the stimulation of oxygen uptake induced by ATP in the presence of creatine (Table 2). However, when the mitCK activity was determined by a spectrophotometric method, no difference was observed between control and HTG mitochondria. Omission of DTT from the reaction medium did not affect mitCK activity in control mitochondria, whereas a significant 10% diminution was observed in HTG mitochondria (Table 2). AK activity

Table 4
Cytochrome *c* oxidase activity and cytochrome content in rat heart mitochondria

	Control	HTG
TMPD OXIDASE	6500±2500	8000±2900
(ng atoms oxygen/min/mg protein)	$n=6$	$n=6$
CYTOCHROME *b*	0.13±0.04	0.14±0.03
(nmol/mg protein)	$n=3$	$n=3$
CYTOCHROMES $c+c_1$	0.25±0.07	0.26±0.04
(nmol/mg protein)	$n=3$	$n=3$
CYTOCHROMES a_1+a_3	0.21±0.08	0.21±0.04
(nmol/mg protein)	$n=3$	$n=3$

Values are mean±S.D. of n different mitochondrial preparations.

was slightly but significantly lower (25%) in whole HTG hearts in comparison to control hearts (Table 2). Our control values closely corresponded to those reported by others under similar conditions [21].

To further explore the structural and functional coupling of mitCK with OxPhos, the affinity for extra mitochondrial ADP was evaluated in intact mitochondria (Fig. 1 A and B). Apparent $K_{m\ ADP}$ values without Cr were 40±9 and 43±13 μM, and with Cr were 27±4 and 50±13 μM for control (Fig. 1A) and HTG (Fig. 1B) mitochondria, respectively. Thus, the affinity for ADP in control mitochondria increased significantly ($P<0.05$) when creatine was in the reaction mixture, as expected from a functional coupling between mitCK and the adenine nucleotide translocase [30]. In contrast, in HTG mitochondria no significant change in the $K_{m\ ADP}$ value was observed in the presence of 25 mM Cr.

A response coefficient of the OxPhos+CK pathway (J) towards Cr can be estimated from the slope to the tangent of the curve of J versus Cr, which is scaled to give a dimensionless measure (Fig. 1C, D). The R^{J}_{Cr} values were 0.117 and 0.275 for control and HTG mitochondria, respectively, at the physiological concentration of 15 mM creatine [31]. By considering the kinetic parameters of the isolated mitCK [32], a ε^{CK}_{Cr} value of 0.5 was estimated. Assuming a similar elasticity for creatine in both types of mitochondria, the C^{OxPhos}_{CK} values for control and HTG mitochondria were 0.23 and 0.55, respectively.

Alteration of components of OxPhos by the hypertriglyceridemic status might affect the transfer of energy from mitochondrial ATP to PCr. Therefore, OxPhos was evaluated in mitochondria incubated with different oxidizable substrates. Surprisingly, the rate of state 3 (ADP-stimulated) respiration with NAD-linked substrate was significantly higher in HTG mitochondria than in control mitochondria. Moreover, state 3 respiration supported by succinate, as well as uncoupled respiration, was also higher in HTG mitochondria than in control mitochondria (Table 3). The higher state 3 respiratory rates in HTG mitochondria were not due to uncoupling between the respiratory chain and ATP synthesis, since their respiratory control values (see legend to Table 3) were similar or even higher than in control mitochondria. Similarly, 2-OGDH activity was significantly higher in HTG mitochondria than in control mitochondria (Table 2). In contrast, the cytochrome *c* oxidase activity, and the content of cytochromes were similar in both types of mitochondria (Table 4). Respiratory rates and COX activities were similar to those reported by others [33].

Table 5
Membrane potential (mV) in rat heart mitochondria

Substrate	Control		HTG	
	State 3	State 4	State 3	State 4
10 mM	131±6	162±5	140±6*	160±6
2-OXOGLUTARATE	$n=5$	$n=5$	$n=5$	$n=5$
10 mM SUCCINATE	126±4	160±7	131±3	154±4
	$n=3$	$n=4$	$n=4$	$n=4$

Values are mean±S.D. of n different mitochondrial preparations. * Denotes significant difference versus control, $P<0.05$.

Table 6
Fatty acid composition in rat heart mitochondria

	Control $n=5$	HTG $n=5$
Palmitic C16:0	12.9±0.4	18.1±0.9**
Palmitoleic C16:1n-7	1.5±0.3	4.3±2.1
Oleic C18:1n-9	10.8±1.2	14.6±0.3**
Stearic C18:0	18.7±0.9	17.4±0.8
Linoleic C18:2n-6	16.8±0.5	14.7±1.9
γ-Linolenic C18:3n-6	1.2±0.6	0.6±0.2
Dihomo γ-Linolenic C20:3n-6	0.5±0.1	0.6±0.1
Arachidonic C20:4n-6	15.2±0.8	11.7±0.4*
Eicosapentaenoic C20:5n-3	0.4±0.1	0.6±0.1
Docosapentaenoic C22:5n-3	2.3±0.3	1.7±0.5
Docosahexaenoic C22:6n-3	12.8±1	10.8±1.2
SFA	31.5±0.7	35.4±0.8**
MUFA	13±1.2	27.6±1.9**
PUFA (n-6)	33.7±1.6	27.6±1.9*
PUFA (n-3)	19.1±1.8	15.9±1.6

Values are the mean±S.D. of n different preparations. SFA: saturated fatty acids (FA); MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Data represent the weight of each individual FA/weight of total FA as percentage. The absolute values for the content of total fatty acids were 65.5±7.6 ($n=5$) and 85±11 μg/ mg protein ($n=5$) in control and HTG mitochondria, respectively; these values were not significantly different. * Denotes significant difference versus control, $P<0.05$ and **$P<0.001$.

3.3. Membrane potential and lipid composition of mitochondrial membranes

In accordance with the respiratory rate measurements, the membrane potential in state 3 was 9 mV higher with 2-OG, and 5 mV higher with succinate in HTG mitochondria (Table 5), in comparison to control mitochondria. Under state 4 (basal) conditions, the membrane potential was similar for both types of mitochondria.

The heart mitochondrial fatty acid and phospholipid composition was determined to examine whether hypertriglyceridemia induces changes on the mitochondrial membranes fluidity. HTG mitochondria showed an important rise in the content of palmitic and oleic acids, a saturated and a monounsaturated acid, respectively (Table 6). A decrease in arachidonic acid was also found, which is a polyunsaturated fatty acid that is precursor of prostaglandins. The significant

Table 7
Phospholipid and cholesterol composition of heart mitochondrial membranes

Phospholipids	Control	HTG
PC	42±4	40±6
PE	0.3±0.3	0.6±0.2
PS	55±4	58±5
PI	1.9±0.7	1.1±0.7
SM	1.1±0.5	0.5±0.5
Cholesterol (nmol/mg prot)	5.4±0.5	4.5±0.9

Data represent the mean of the percentage of each phospholipid (mean of %±S.D., $n=5$). PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS phosphatidylserine, PI: posphatidylinositol, SM: Sphingomyelin.

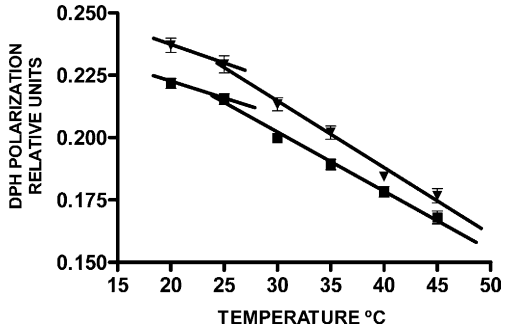

Fig. 2. Fluorescence polarization of DPH in mitochondrial membranes. Polarization of 1,6-diphenylhexatriene (DPH) was significantly different ($P<0.05$) in HTG membranes (▼) in comparison with their matched controls (■).

increase in total saturated (SFA) and monounsaturated fatty acids (MUFA) and decrease in total polyunsaturated fatty acids (PUFA) suggested a change in the HTG mitochondrial membrane fluidity. Phospholipid composition of mitochondrial membranes from HTG hearts did not show significant changes (Table 7), indicating that the main effect on lipid structure lied on the saturation level of the fatty acids. Similar values on fatty acid composition have been reported for normal heart mitochondria [10]; also alterations in fatty acid composition induced by age and diabetes have been shown to influence membrane fluidity [10,12].

3.4. Membrane fluidity

To further study the effect of sucrose feeding on membrane fluidity, the fluorescence polarization of DPH mitochondrial membranes at several temperatures was evaluated (Fig. 2). Polarization of DPH in HTG membranes was significantly higher over the range of tested temperatures, indicating a major order in the lipid arrangement of the bilayer, which confers higher rigidity [10].

4. Discussion

4.1. Mitochondrial CK

Mitochondrial CK is located in the inter-membranal space at the contact sites between the inner and outer membranes [1]. MitCK may associate with the adenine nucleotide translocase; hence, it may directly take up the ATP generated in the mitochondrial matrix to synthesize PCr [1]. The adequate function of such system depends on two components: the producing and the consuming ATP/PCr sites. Thus, when one of these components is hindered, the complete "energy circuit" may be impaired. In the HTG hearts, total CK activity diminished (cf. Table 2), from which approximately 30% [34,35] corresponds to the mitochondrial isoform.

The CK-mediated ADP-stimulated respiration decreased by 45% in HTG mitochondria. However, when the mitCK was spectrophotometrically assayed, the values of activity were similar in both types of mitochondria. These results suggested that there was a disturbance in the functional association between mitCK and OxPhos in the intact HTG heart mitochondria, which was not related to a decrease in the amount of active enzyme. It has been established that mitCK is functionally coupled to OxPhos by channeling the ADP generated by the forward reaction directly to the ATP/ADP translocase [30]. This functional coupling can be experimentally assessed by determining the apparent $K_{m\ ADP}$ of OxPhos when mitCK is working, i.e., in the presence of creatine. In contrast to control mitochondria, no significant change in the $K_{m\ ADP}$ value in the presence of Cr was observed in HTG mitochondria, thus suggesting a loss of the functional coupling between mitCK and OxPhos.

As CK isoforms are highly susceptible to oxidative damage [36,37], it is possible that in the HTG heart, which is subjected to high oxidative stress [15], the overall CK activity might also be affected. In this regard, there was a decline of about 10% in the mitCK activity when DTT was omitted from the assay reaction; thereby oxidation of essential thiol groups seemed involved in the inactivation of the enzyme in situ. The AK activity also significantly diminished, which very likely contributed to the severe energy deficit in the HTG heart.

It has been documented that the mitCK octamer is directly coupled to the adenine nucleotide translocase, whereas the dimer does not interact with the translocase [38,39]. Changes in the mitCK oligomeric state occur during oxidative stress, such as that developed during ischemia and reperfusion, in which there is a shift from the octameric to the dimeric and less functional form [40]. Thus, the lowering in the direct coupling of mitCK with OxPhos in HTG mitochondria suggests diminution in the octamer/dimer ratio of the mitCK isoform. As the amount of active enzyme remained constant, then it is conceivable that the structural and oligomeric state of the mitCK has been altered [38,39]. Therefore, the diminished functional association of mitCK with OxPhos in the HTG heart mitochondria might be related to changes in the oligomeric proportions of mitCK evoked by the increased oxidative damage present in these animals [15,16].

Caution should be taken to extrapolate the last conclusion to the whole organ, since in the intact heart, there are other factors that might influence mitochondrial function and the contractile performance such as myofibrillar protein composition, substrate availability and calcium handling mechanisms, which could also be affected by the hypertriglyceridemic state. In addition, it was found that mitCK exerts significant flux control of OxPhos, mainly in HTG mitochondria. This observation indicated that the rate of respiration attained in the presence of ADP (or ATP) and creatine does not completely depend on the mitCK activity and hence it should not be taken as a direct measurement of its activity. Others steps, mainly the

ATP/ADP translocase, also contribute to the control of flux [41].

4.2. Mitochondrial respiration

Heart mitochondrial respiratory rates of HTG rats were similar or higher than those of control hearts. This observation was in agreement with our previous report that in the intact HTG heart oxidation of non-glycolytic substrates is not impaired [17]. The oxidation of pyruvate by HTG mitochondria slightly increased, suggesting that the content of active PDH did not diminish in the HTG heart. In fact, in the whole HTG heart, the content of active PDH complex was identical to that of control hearts [17]. The observed diminution in the oxidation of low concentrations of Pyr in the whole HTG heart can be explained in terms of a diminution in the active form of the PDH complex, whereas the total enzyme remained constant [17]. In isolated mitochondria, the level of active PDH may be re-established since the inhibitory high levels of acetyl CoA and fatty acids diminish during the isolation procedure of mitochondria; in addition, the use of saturating concentration of pyruvate induces activation of PDH in intact mitochondria [42]. Moreover, the increase in state 3 respiration in HTG heart mitochondria when 2-OG was oxidized also correlated with the slight increase in respiration of the whole HTG heart when fatty acids were the source of energy [17].

It has been documented that sugar-induced hypertriglyceridemia is often linked to mild hyperthyroidism [43], which could lead to stimulation of protein synthesis. Indeed, high levels of thyroid hormones increase expression of enzymes of the respiratory chain such as bc_1 complex, some ATP synthase subunits and cytochrome *c* oxidase [44]. Thus, the increase in respiratory rates in HTG heart mitochondria could be explained by an increase in OxPhos enzymes. However, we determined that the levels of circulating thyroid hormone were similar between HTG and control rats (2.9 ± 0.5 for HTG rats versus 3.1 ± 0.6 μg/100 ml for control rats, $n = 10$).

Moreover, data from cytochrome content and cytochrome *c* oxidase activity in total heart homogenates (Table 2) and isolated mitochondria (Table 4) revealed the absence of overexpression of respiratory enzymes or different mitochondrial yield induced by the hypertriglyceridemic state. Only an enhanced activity of the 2-OGDH complex associated with the HTG state was found, suggesting that the increased OxPhos with NAD-linked substrate was due at least partially to stimulation of the Krebs cycle flux. In turn, the increase in 2-OGDH activity does not fully explain the increase found in respiratory rates of HTG heart mitochondria, since oxygen uptake with 2-OG or Pyr increased by 30 and 40% respectively, whereas 2-OGDH activity only increased by <20%. Thus, the increase in the rate of succinate oxidation in HTG mitochondria indicated that other factors such as altered membrane fluidity and increased membrane potential, may also be involved in improving respiratory performance.

4.3. Lipid composition of mitochondrial membranes

In the HTG rat, changes in liver desaturases enzymes have been reported, which may lead to variations in lipid composition in the different cellular membranes [29]. Changes in the lipid profile of HTG heart cellular structures have not been yet reported. Indeed, significant changes in the SFA and MUFA content of HTG mitochondrial membranes were found; these changes very likely altered membrane fluidity by increasing rigidity, as determined by fluorescence polarization. A decreased fluidity confers better coupling between ATP synthesis and electron transfer, since proton leak is prevented and the membrane potential is enhanced [45,46]. This observation may readily explain the higher membrane potentials found in HTG heart mitochondria.

Furthermore, the membrane fluidity is also determined by the phospholipid composition, which may also modify the activity of some respiratory enzymes such as bc_1 complex and the ATP/ADP translocase [45–48]. However, negligible changes in phospholipid and cholesterol composition were detected in mitochondrial membranes from HTG hearts, indicating that the increased level of saturated fatty acids is indeed responsible for the decrease in membrane fluidity. On the other hand, the decrease in the content of polyunsaturated fatty acids in HTG mitochondria may have induced compensatory mechanisms leading to increases in respiratory rates, as judged by the enhanced uncoupled respiration, and respiratory efficiency, mainly due to an improvement in utilization of the H^+ motive force [49].

In conclusion, a high carbohydrate diet may promote a functional uncoupling between energy producing and consuming sites in the HTG heart, which is evident when glucose becomes the only oxidizable substrate (anoxic episodes) and hence ATP production is compromised. The functional uncoupling consists primarily of an alteration in the ATP transfer system, involving the mitCK and AK. Hypertriglyceridemia also induces an increase in the mitochondrial oxidative pathways such as Krebs cycle and respiratory chain as well as structural alterations in the mitochondrial membrane physical state. Whether these other alterations are part of a compensatory mechanism or merely the result of metabolic response to an excess of substrate (free fatty acids) deserves further exploration.

Acknowledgements

We thank Dr. Raúl Covián and Eduardo Martínez for technical assistance. The present work was partially supported by CONACyT-México grant No. 43811-Q.

References

[1] T. Walliman, Dissecting the role of creatine kinase. The phenotype of "gene knockout" mice deficient in a creatine kinase isoform sheds

new light on the physiological function of the phosphocreatine circuit, Curr. Biol. 1 (1994) 42–46.
[2] K. Carvajal, R. Moreno-Sánchez, Cardiac metabolic disturbances in cardiovascular diseases, Arch. Med. Res. 34 (2003) 89–99.
[3] W. Kurt, M. Spindler, R. Tian, J.S. Ingwall, Impaired cardiac energetics in mice lacking muscle-specific isoenzymes of creatine kinase, Circ. Res. 82 (1998) 898–907.
[4] P.P. Dzeja, K.T. Vitkevicius, M.M. Redfield, J.C. Burnett, A. Terzic, Adenylate kinase-catalyzed phosphotransfer in the myocardium. Increased contribution to heart failure, Circ. Res. 84 (1999) 1137–1143.
[5] M. Van Bilsen, P.J.H. Smeets, A.J. Gilde, G.J. van der Vusse, Metabolic remodelling of the failing heart: the cardiac burn-out syndrome? Cardiovasc. Res. 61 (2004) 218–226.
[6] V.A. Saks, V.V. Kupriyanov, G.V. Elizarova, W.E. Jacobus, Studies of energy transport in heart cells. The importance of creatine kinase localization for the coupling of mitochondrial phosphorylcreatine production to oxidative phosphorylation, J. Biol. Chem. 255 (1980) 755–763.
[7] C. Christoffersen, E. Bollano, M.L.S. Lindergaard, E.D. Bartels, J.P. Goetze, C.B. Andersen, L.B. Nielsen, Cardiac lipid accumulation associated with diastolic dysfunction in obese mice, Endocrinology 144 (2003) 3483–3490.
[8] J. Marín-García, M.J. Goldenthal, Fatty acid metabolism in cardiac failure: biochemical, genetic and cellular analysis, Cardiovasc. Res. 54 (2002) 516–527.
[9] S. Pepe, Mitochondrial function in ischemia and reperfusion of the ageing heart, Proceedings of the Australian Physiological and Pharmacological Society Symposium: Ischaemia and the Heart, Clin. Exp. Pharmacol. Physiol., 27, 2000, pp. 745–750.
[10] S. Pepe, P.L. McLennan, Mitochondrial function. Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and post-ischemic recovery of contractile function, Circulation 105 (2002) 2303–2308.
[11] L. Atkinson, R. Kozak, S.E. Kelly, A. Onay-Besickci, J.C. Russell, G.D. Lopaschuk, Potential mechanisms and consequences of cardiac triacylglycerol accumulation in insulin-resistant rats, Am. J. Physiol.: Endocrinol. Metab. 284 (2003) E923–E930.
[12] S. Ovide-Bordeaux, A. Grynberg, Docosahexaenoic acid affects insulin deficiency- and insulin resistance-induced alterations in cardiac mitochondria, Am. J. Physiol., Regul. Integr. Comp. Physiol. 286 (2004) R519–R527.
[13] H.N. Ginsberg, Insulin resistance and cardiovascular disease, J. Clin. Invest. 106 (2000) 453–458.
[14] G. Baños, K. Carvajal, G. Cardoso, J. Zamora, M. Franco, Vascular reactivity and effect of serum in a rat model of hypertriglyceridemia and hypertension, Am. J. Hypertens. 10 (1997) 379–388.
[15] K. Carvajal, M. El Hafidi, G. Baños, Myocardial damage due to ischemia and reperfusion in hypertriglyceridemic and hypertensive rats: participation of free radicals and calcium overload, J. Hypertens. 17 (1999) 1607–1616.
[16] M. El Hafidi, G. Baños, In vivo plasma lipid oxidation in sugar-induced rat hypertriglyceridemia and hypertension, Hypertension 30 (1997) 624–628.
[17] K. Carvajal, G. Baños, R. Moreno Sánchez, Impairment of glucose metabolism and energy transfer in the hypertriglyceridemic rat heart, Mol. Cell. Biochem. 249 (2003) 157–165.
[18] S.G. Robles, M. Franco, C. Zazueta, N. Garcia, F. Correa, G. Garcia, E. Chavez, Thyroid hormone may induce changes in the concentration of the mitochondrial calcium uniporter, Comp. Biochem. Physiol. 135 (2003) 177–182.
[19] R. Moreno-Sánchez, R. Hansford, Relation between cytosolic free calcium and respiratory rates in cardiac myocytes, Biochem. J. 256 (1988) 403–412.
[20] J. Rodríguez-Zavala, J.P. Pardo, R. Moreno-Sánchez, Modulation of 2-oxoglutarate dehydrogenase complex by inorganic phosphate, Mg^{2+} and other effectors, Arch. Biochem. Biophys. 379 (2000) 78–84.
[21] E. De Sousa, V. Veksler, X. Bigard, P. Mateo, B. Serrurier, R. Ventura-Clapier, Dual influence of disease and increased load on diaphragm muscle in heart failure, J. Mol. Cell. Cardiol. 33 (2000) 699–710.
[22] V.S. Saks, Z.A. Khucua, A.V. Kuznetsov, V. Veksler, V.G. Sharov, Heart mitochondria in physiological salt solutions: not ionic strength but salt composition is important for association of creatine kinase with the inner membrane surface, Biochem. Biophys. Res. Commun. 139 (1986) 1262–1271.
[23] D. Fell, Understanding the Control of Metabolism, Portland Press, London, 1997, (300 p.).
[24] H. Rottenberg, Membrane potential and surface potential in mitochondria: uptake and binding of lipophilic cations, J. Membr. Biol. 81 (1984) 127–138.
[25] M.D. Esposti, G. Lenaz, The kinetic mechanism of ubiquinol:cytochrome *c* reductase at steady state, Arch. Biochem. Biophys. 289 (1991) 303–312.
[26] K.A. Gray, M. Grooms, H. Myllykallio, C. Moomaw, G. Salughter, F. Daldal, *Rhodobacter capsulatus* contains a novel cb-type cytochrome c oxidase without a Cu_A center, Biochemistry 33 (1994) 3120–3127.
[27] J.W. Priest, J.W. Hajduk, Cytochrome c reductase purified from *Crithidia fasciculata* contains an atypical cytochrome c1, J. Biol. Chem. 267 (1992) 20188–20195.
[28] S. Mawatari, K. Murakami, Analysis of membrane phospholipid peroxidation by isocratic high-performance liquid chromatography with ultraviolet detection, Anal. Biochem. 264 (1998) 118–123.
[29] M. El Hafidi, A. Cuéllar, J. Ramírez, G. Baños, Effect of sucrose addition to drinking water, that induces hypertension in the rats, on liver microsomal Δ9 and Δ5-desaturase activities, J. Nutr. Biochem. 12 (2001) 396–403.
[30] F.N. Gellerich, M. Kapischke, W. Kunz, W. Neumann, A. Kuznetsov, D. Brdiczka, K. Nicolay, The influence of the cytosolic oncotic pressure on the permeability of the mitochondrial outer membrane for ADP: implications for the kinetic properties of mitochondrial creatine kinase and for ADP channeling into the intermembrane space, Mol. Cell. Biochem. 133–134 (1994) 85–104.
[31] V.A. Saks, R. Ventura-Clapier, X. Leverve, A. Rossi, M. Rigoulet, Bioenergetics of the Cell: Quantitative Aspects, in Developments in Molecular and Cellular Biochemistry, vol. 25, Kluwer Academic Publishers, USA, 1998 (460 p.).
[32] P. Kaldis, T. Wallimann, Functional differences between dimeric and octameric mitochondrial creatine kinase, Biochem. J. 308 (1995) 623–627.
[33] E.J. Lesnesfsky, B. Tandler, J. Ye, T.J. Slabe, J. Turkaly, C.L. Hoppel, Myocardial ischemia decrease oxidative phosphorylation through cytochrome oxidase in subsarcolemmal mitochondria, Am. J. Physiol. 273 (1997) H1544–H1554.
[34] T. Walliman, M. Dolder, U. Schuttner, M. Eder, T. Hornemann, E. OGorman, A. Rück, D. Brdiczka, Some new aspects of creatine kinase compartmentation, structure, function and regulation of cellular and mitochondrial bioenergetics and physiology, Biofactors 8 (1998) 229–234.
[35] M. Wyss, J. Smeitink, R.A. Wevers, T. Walliman, Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism, Biochim. Biophys. Acta 1102 (1992) 119–166.
[36] H. Mekhfi, V. Veksler, P. Mateo, V. Maupoil, L. Rochette, R. Ventura-Clapier, Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils, Circ. Res. 78 (1996) 1016–1027.
[37] W. Kabsch, K. Fritz-Wolf, Mitochondrial creatine kinase—A square protein, Curr. Opin. Struct. Biol. 7 (1997) 811–818.
[38] M. Forstner, M. Kriechbaum, P. Laggner, T. Walliman, Structural changes of creatine kinase upon substrate binding, Biophys. J. 75 (1998) 1016–1023.
[39] Schlattner, T. Wallimann, Octamers of mitochondrial creatine kinase isoenzymes differ in stability and membrane binding, J. Biol. Chem. 275 (2000) 17314–17320.
[40] S. Soboll, D. Brdiczka, D. Jahnke, A. Schmidt, U. Schlattner, S.

Wendt, M. Wyss, T. Walliman, Octamer–dimer transitions of mitochondrial creatine kinase in heart disease, J. Mol. Cell. Cardiol. 31 (1999) 857–866.
[41] F.N. Gellerich, S. Trumbeckaite, T. Müller, M. Deschauer, Y. Chen, Z. Gizatullina, S. Zierz, Energetic depression caused by mitochondrial dysfunction, Mol. Cell. Biochem. 256/257 (2004) 391–405.
[42] R.G. Hansford, L. Cohen, Relative importance of pyruvate dehydrogenase interconversion and feed-back inhibition in the effect of fatty acids on pyruvate oxidation by rat heart mitochondria, Arch. Biochem. Biophys. 191 (1978) 65–81.
[43] E. Shafrir, The plurimetabolic syndrome in animals: models for experimentation, their implications and relation to NIDDIM, in: G. Crepaldi, A. Tiengo, E. Manzato (Eds.), Diabetes. Obesity and Hyperlipidemias V: the Plurimetabolic Syndrome, Excerpta Medica, 1993, pp. 45–55 (Amsterdam).
[44] M.T. Pillar, H.J. Seitz, Thyroid hormone and gene expression in the regulation of mitochondrial respiratory function, Eur. J. Endocrinol. 136 (1997) 231–239.
[45] D.J. Pehowich, Thyroid hormone status and membrane n-3 fatty acid content influence mitochondrial proton leak, Biochim. Biophys. Acta 1411 (1999) 192–200.
[46] F.L. Hoch, Lipids and thyroid hormones, Prog. Lipid Res. 27 (1988) 199–207.
[47] V. Bobyleva, M. Bellei, T.L. Pazienza, U. Muscatello, Effect of cardiolipin on functional properties of isolated rat liver, Biochem. Mol. Biol. Int. 41 (1997) 469–480.
[48] M. Schlame, D. Rua, M.L. Greenberg, The biosynthesis and functional role of cardiolipin, Prog. Lipid Res. 39 (2000) 257–288.
[49] V. Nogueira, M. Piquet, A. Devin, C. Fiore, E. Fontaine, G. Brandolin, M. Rigoulet, X.M. Leverve, Mitochondrial adaptation to in vivo polyunsaturated fatty acid deficiency: increase in phosphorylation efficiency, J. Bioenerg. Biomemb. 33 (2001) 53–61.