J Bioenerg Biomembr (2010) 42:245–253
DOI 10.1007/s10863-010-9284-9

Effects of a high fat diet on liver mitochondria: increased ATP-sensitive K^+ channel activity and reactive oxygen species generation

Ariel R. Cardoso · João Victor Cabral-Costa · Alicia J. Kowaltowski

Received: 23 February 2010 / Accepted: 22 March 2010 / Published online: 7 April 2010

Abstract High fat diets are extensively associated with health complications within the spectrum of the metabolic syndrome. Some of the most prevalent of these pathologies, often observed early in the development of high-fat dietary complications, are non-alcoholic fatty liver diseases. Mitochondrial bioenergetics and redox state changes are also widely associated with alterations within the metabolic syndrome. We investigated the mitochondrial effects of a high fat diet leading to non-alcoholic fatty liver disease in mice. We found that the diet does not substantially alter respiratory rates, ADP/O ratios or membrane potentials of isolated liver mitochondria. However, H_2O_2 release using different substrates and ATP-sensitive K^+ transport activities are increased in mitochondria from animals on high fat diets. The increase in H_2O_2 release rates was observed with different respiratory substrates and was not altered by modulators of mitochondrial ATP-sensitive K^+ channels, indicating it was not related to an observed increase in K^+ transport. Altogether, we demonstrate that mitochondria from animals with diet-induced steatosis do not present significant bioenergetic changes, but display altered ion transport and increased oxidant generation. This is the first evidence, to our knowledge, that ATP-sensitive K^+ transport in mitochondria can be modulated by diet.

A. R. Cardoso · J. V. Cabral-Costa · A. J. Kowaltowski
Departamento de Bioquímica, Instituto de Química,
Universidade de São Paulo,
São Paulo, Brazil

A. J. Kowaltowski (✉)
Cidade Universitária,
Av. Prof. Lineu Prestes, 748,
05508-900 São Paulo, SP, Brazil
e-mail: alicia@iq.usp.br

Keywords Steatosis · Respiration · Mitochondrial bioenergetics · Mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$) · Reactive oxygen species (ROS)

Abbreviations

ATP	Adenosine triphosphate
DZX	Diazoxide
5-HD	5-hydroxydecanoate
MS	Metabolic syndrome
mitoK$_{ATP}$	Mitochondrial ATP-sensitive potassium channels
MPT	Mitochondrial permeability transition
NAFLD	Non-alcoholic fatty liver diseases
ROS	Non-alcoholic steatohepatitis (NASH), reactive oxygen species

Introduction

Obesity is an increasing health concern, particularly in developed countries (Begriche et al. 2006; Nicolson 2007; Nisoli et al. 2007) in which energy expenditure is low and diets are rich in fats and simple sugars (Bray et al. 2004; Jew et al. 2009). The obese phenotype is related to pathological states, which include insulin resistance, type II diabetes, dyslipidemia, pro-inflammatory and pro-thrombotic states, hypertension and non-alcoholic fatty liver diseases (NAFLD). The concerted manifestation of these pathologies is termed metabolic syndrome (MS), although the criteria for this diagnosis is still debated (Federspil et al. 2006).

One of the most prevalent pathologies observed in obesity and the MS is NAFLD, which affects up to 70% of obese and diabetic individuals. NAFLD is a wide term that includes steatosis, characterized by lipids vacuoles in

the cytosol leading to hepatocyte ballooning; non-alcoholic steatohepatitis (NASH), characterized by necroinflammation and fibrosis, and cirrhosis in the absence of alcohol abuse (Fromenty et al. 2004; Begriche et al. 2006). Steatosis in isolation is not associated with overt limitations in liver functions, but it can progress into NASH, which can evolve into cirrhosis and hepatocellular carcinoma (Begriche et al. 2006; Pessayre 2007; Hashimoto et al. 2009).

A well-established animal model of steatosis is induced by diet in mice (Anstee and Goldin 2006). Using a mouse model, Day and James (1998) proposed the two hit theory for NASH development: the first hit, steatosis, sensitizes the liver to the induction of inflammation by a second pathogenic insult that promotes oxidative stress and, hence, steatohepatitis. Steatosis is promoted by the accumulation of long chain fatty acids (LCFA), while NASH is caused by oxidative stress, inflammation and/or infection (Day and James 1998). Despite this mechanistic suggestion, specific molecular pathways involved in this pathology are not yet clearly understood.

Mitochondria, as the central coordinators of energy metabolism, have been extensively shown to be involved in the metabolic syndrome (Begriche et al. 2006; Nicolson 2007). In humans, mitochondria in NASH present morphological alterations with paracrystalline inclusion bodies and are frequently swollen. Alterations in lipid oxidation are observed as well (Sanyal et al. 2001; Le et al. 2004). Ultrastructural modifications in liver mitochondria were observed in high-fat fed rats. They presented enlarged organelles, forming megamitochondria (Altunkaynak and Ozbek 2009). However, few studies have explored mitochondrial function under conditions of steatosis and NASH. Considering the central role of these organelles in lipid metabolism, we decided to study basic mitochondrial bioenergetic functions under conditions of early and prolonged NAFLD in mice.

Furthermore, mitochondria are the most quantitatively relevant source of reactive oxygen species (ROS) production in most cells (Boveris and Chance 1973; Kowaltowski et al. 2009). Liver mitochondrial ROS generation in rats has specific characteristics. Relative to other tissues, liver produces more hydrogen peroxide in state 3, and there is an elevated basal production of ROS, mainly from lipid-derived substrates (Tahara et al. 2009). Changes in redox state promoted by alterations in mitochondrial ROS production can alter the activity of JNK enzymes and disturb insulin signaling, leading to insulin resistance (Nishikawa et al. 2007). As a result, we also investigated the redox results of NAFLD by measuring mitochondrial oxidant production.

Another consequence of mitochondrial ROS release is the enhancement of potassium transport across the inner mitochondrial membrane due to the activity of ATP-sensitive potassium channels (Zhang et al. 2001; Facundo et al. 2007). The result of this transport is mild mitochondrial uncoupling and prevention of ROS formation (Ferranti et al. 2003; Facundo et al. 2006; Facundo et al. 2007; Fornazari et al. 2008). Alberici et al. (2006) demonstrated that these channels present increased activity in hypertriglyceridemic mice, suggesting that they may participate in energy metabolism and redox regulation in metabolic disorders (Alberici et al. 2009). As a result, we also verified if NASH promoted by a high-fat diet could alter mitochondrial K^+ transport.

Materials and methods

Animals

Swiss mice were obtained from the *Biotério do Conjunto das Químicas* (*Universidade de São Paulo*), an internationally accredited animal facility. All studies were conducted in accordance with guidelines established by the NIH Guide for the Care and Use of Laboratory Animals and the *Colégio Braslieiro de Experimentação Animal* and were approved by the institutional *Comissão de Ética em Cuidados e Uso Animal*. Mice had access to standard laboratory rodent chow (Rhoster, Brazil) and water ad libitum and were housed at 22 °C on a 12-h light-dark cycle. In the diet group, animals received commercial soy oil supplementation (Liza®, Brazil, 30% *v*/*v*) plus 9 g/L sodium stearoyl-lactilate (Purac®, Brazil), an emulsifier, in the drinking water (modified from Warwick et al. 2000). Animals were offered high fat diets starting at the 6th week of life, and maintained on the diet for either 2 or 10 months. The diet results in 55% fat consumption and induces obesity without altering plasmatic lipid or glucose levels. General metabolic parameters such as plasmatic glucose, total cholesterol and triglycerides were measured using commercial enzymatic colorimetric assays (Doles®, Brazil).

Isolation of mouse liver mitochondria

Mitochondria were isolated by conventional differential centrifugation at 4 °C. A liver homogenate was prepared in 300 mM sucrose, 2 mM ethylene glycol-bis (aminoethyl ether)-N,N,N′,N′ tetraacetic acid (EGTA), 10 mM HEPES buffer (pH 7.2, KOH), and 1 g/L bovine serum albumin and centrifuged at 800 g for 5 min. The supernatant was recentrifuged at 12,000 g for 10 min. This cycle was repeated to remove contaminant blood. The pellet was washed in the same medium. Protein quantification was measured using Lowry's method (Lowry et al. 1951) and bovine serum albumin as the protein standard (modified from Castilho et

al. 1995; Cancherini et al. 2003; Alberici et al. 2006). Isolated mitochondria were kept over ice and used within 90 min of preparation to ensure mitoK$_{ATP}$ activity. Mitochondria isolated in this manner lose matrix K^+ and contract due to low levels of this ion in the isolation buffer and recover K^+ when suspended in K^+-rich buffers.

Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in light scattering of the suspension measured at 520 nm in suspensions incubated in experimental buffer (150 mM KCl, 10 mM Hepes, 2 mM inorganic phosphate, 2 mM $MgCl_2$, adjusted to pH 7.4 with KOH), at 37 °C, with continuous stirring, using a temperature-controlled Hitachi 4500 fluorimeter. Swelling rates of freshly isolated mitochondria were measured soon after their addition to K^+-rich buffers. Total mitoK$_{ATP}$ activity was measured as the difference in swelling under control conditions and experiments in which mitoK$_{ATP}$ was inhibited by ATP (Beavis et al. 1993; Kowaltowski et al. 2001; Facundo et al. 2007). Protein quantification was performed as described in (Lowry et al. 1951).

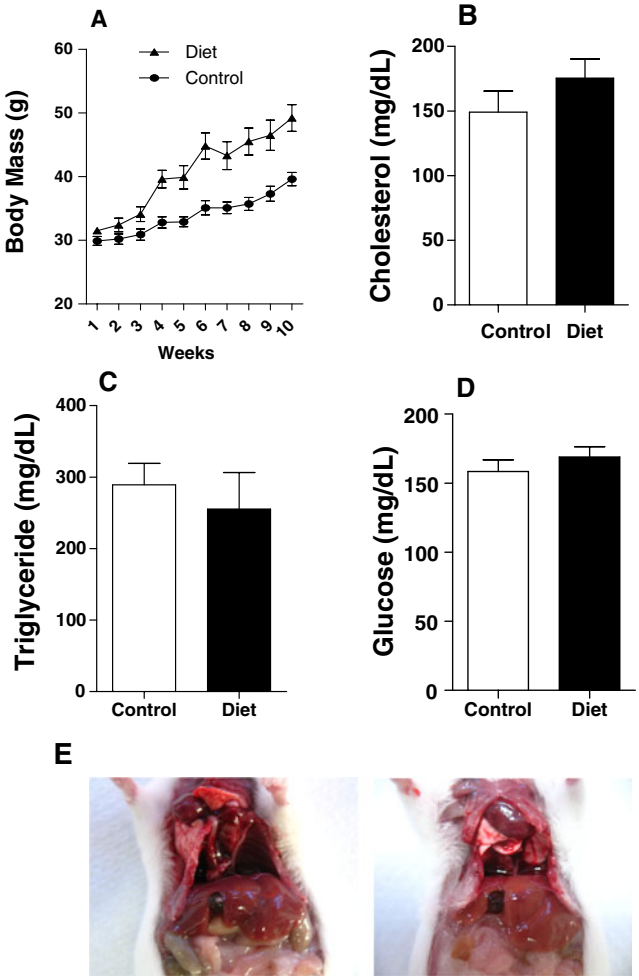

Fig. 1 Body mass, plasmatic cholesterol, triglyceride and glucose levels in female mice subjected to 2 months of hyperlipidic diet, versus control. **a** Body mass over time, n=10 animals per group, p< 0.05 from week 4. **b** Total plasmatic cholesterol, n=5 per group. **c** Plasmatic triglycerides, n=5 per group. **d** Plasmatic glucose, n=9 animals per group. **e** Typical macroscopic aspect of the livers from control (*left*) and high-fat diet (*right*) animals

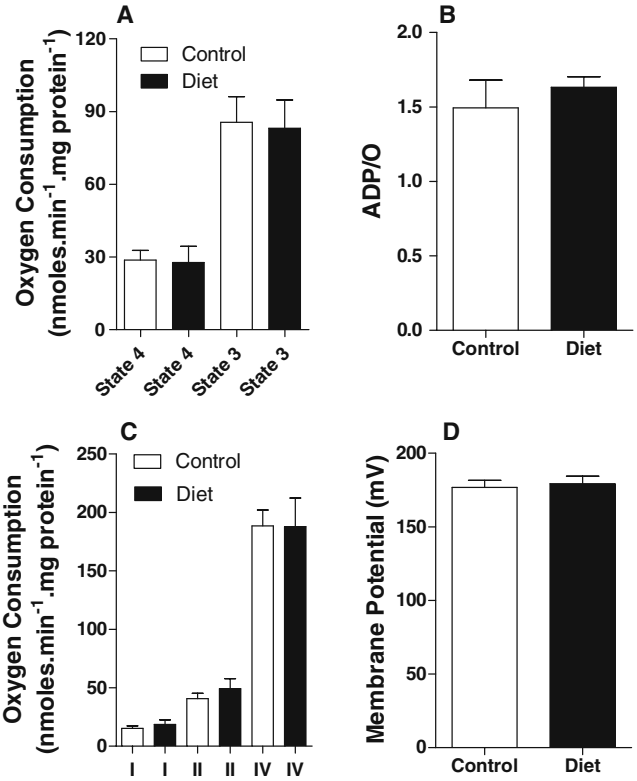

Fig. 2 Hyperlipidic diets do not change oxygen consumption and membrane potentials in liver mitochondria. **a** Mitochondria were incubated in buffer containing 150 mM KCl, 10 mM Hepes, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM succinate with 1 µg/mL oligomycin for state 4 or 1 mM ADP for state 3 measurements, pH 7.4, at 37 °C with magnetic stirring, n=4 per group, p>0.05. **b** Mitochondria were energized with 2 mM malate/glutamate (I); 2 mM succinate with 0.8 µM rotenone (II); 0.8 µM rotenone, 0.15 ng/ml antimycin A and 2 mM TMPD/ascorbate (IV), n=4, p>0.05. **c** For ADP/O ratio measurements 50 nmoles ADP were added to the suspension, n=4, p> 0.05. **d** Mitochondrial membrane potentials were measured fluorimetrically in the same buffer as (**a**), with 1 µg/mL oligomycin, n=5, p>0.05

Oxygen consumption and ADP/O ratios

Oxygen consumption was measured in mitochondrial suspensions incubated in experimental buffer (150 mM KCl, 10 mM Hepes, 2 mM inorganic phosphate, 2 mM $MgCl_2$, adjusted to pH 7.4 with KOH), at 37 °C, with continuous stirring, using a computer-interfaced Clark-type oxygen electrode from Hansatech Instruments Ltd. (Alberici et al. 2006; Tahara et al. 2009). ADP/O ratios were

calculated by measuring the oxygen consumption (in nmoles) needed to phosphorylate a known concentration of added ADP (50 μmoles). Protein quantification was performed as described in (Lowry et al. 1951).

Mitochondrial H_2O_2 release

H_2O_2 release was measured in mitochondrial suspensions incubated in experimental buffer (150 mM KCl, 10 mM Hepes, 2 mM inorganic phosphate, 2 mM $MgCl_2$, adjusted to pH 7.4 with KOH), at 37 °C, with continuous stirring. Amplex Red (5 μM) oxidation was followed in the presence of 1 U/mL horseradish peroxidase, using malate/glutamate, succinate, (1 mM of each), or palmitoyl carnitine (50 μM) as substrates. In most experiments (excluding those conducted in State 3 promoted by the addition of 1 mM ADP), 1 μg/mL oligomycin was present. Amplex Red is oxidized in the presence of extramitochondrial horseradish peroxidase bound to H_2O_2, generating resorufin, which can be detected fluorimetrically at 563 nm excitation and 587 nm emission (Zhou et al. 1997; Chen et al. 2003; Muller et al. 2004; Facundo et al. 2007; Tahara et al. 2009). Controls conducted in the absence of mitochondria or in the absence of peroxidase indicate that nonspecific probe oxidation is negligible. Protein quantification was performed as described in Lowry et al. (1951).

Inner mitochondrial membrane potentials

Mitochondrial inner membrane potential ($\Delta\Psi$) measurements were conducted in experimental buffer (150 mM KCl, 10 mM Hepes, 2 mM inorganic phosphate, 2 mM $MgCl_2$, adjusted to pH 7.4, with KOH), at 37 °C, with continuous stirring. $\Delta\Psi$ was estimated through fluorescence changes of 5 μM safranin O at excitation and emission wavelengths of 485 and 586 nm, respectively. Data obtained were calibrated using a K^+ gradient. The $\Delta\Psi$ value obtained for each K^+ concentration was determined using the Nernst equation, assuming intramitochondrial K^+ to be 150 mM, and plotted against measured fluorescence values to generate a calibration curve for each experiment. It should be noted that errors in the estimated concentrations of intramitochondrial K^+ do not substantially alter calculated $\Delta\Psi$ values (Akerman and Wikström 1976; Kowaltowski et al. 2002). Maximal Ca^{2+} accumulation was calculated (as μmoles/mg protein) by following the effect of Ca^{2+} additions on $\Delta\Psi$ (Ichas et al. 1997). Protein quantification was performed as described in Lowry et al. (1951).

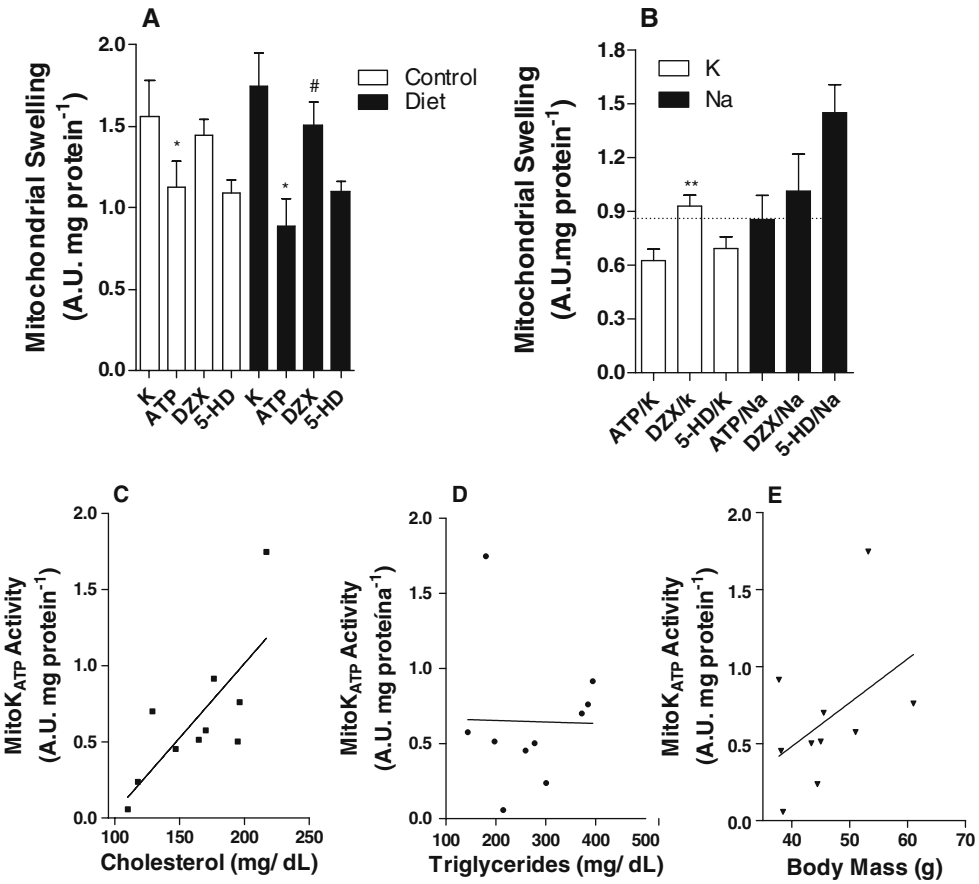

Fig. 3 $MitoK_{ATP}$ activity is increased in animals subjected to hyperlipidic diets and correlates with cholesterol levels. Mitochondria were incubated in buffer containing 150 mM KCl, 10 mM Hepes, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM succinate and 1 μg/mL oligomycin at 37 °C, pH 7.4, with magnetic stirring. **a** Mitochondrial swelling was measured under different conditions to calculate $mitoK_{ATP}$ activity; without channel modulators in K; 1 mM ATP (ATP); ATP plus 30 μM diazoxide (DZX); ATP plus DZX plus 60 μM 5-hydroxydecanoate (5-HD), $n=5$ experiments per group, *, $p<0.05$ in relation to K of each group, #, $p<0.05$ in relation to ATP of diet group. **b** K^+ salts were substituted with Na^+, $n=10$, **, $p<0.01$. The *dotted line* represents swelling rates in the absence of ATP. **c**–**e** Correlations between $mitoK_{ATP}$ activity and plasmatic lipids or body mass; in (**c**) $p<0.05$, $R^2=0.5794$

Data analysis

Data are representative or averages ± SEM of at least 3 repetitions using different preparations. Statistical analysis was performed using one-way analysis of variance comparisons and analysis of variance (for more than two variables) and Student *t*-tests (for two variables) conducted using Origin 7.0 software (OriginLab) and Prism 5 (GraphPad). $p<0.05$ was considered significant.

Results

Our aim in this study was to characterize mitochondrial bioenergetic changes found in steatosis promoted by a high fat diet. The diet adopted consisted in supplementation with soy oil, and induced body mass increases (Fig. 1a), which were significant from the 4th week of treatment on. No alterations in general metabolic parameters such as plasmatic glucose, total cholesterol and triglycerides were observed (Fig. 1b–d), although significant steatosis developed (Fig. 1e). Hence, our model represents a condition of diet-induced obesity with steatosis, but not associated with dyslipidemia.

We next investigated aspects of liver mitochondrial physiology in the animals treated for 2 months with this diet versus controls (Fig. 2). No differences were noted in respiratory control ratios (RCR), calculated as the ratio between oxygen consumption in state 3 (in the presence of 1 mM ADP) and state 4 (in the presence of oligomycin to inhibit oxidative phosphorylation, Fig. 2a). ADP/O ratios were calculated to determine the coupling between the respiratory chain and ATP-synthase and show no differences between groups (Fig. 2b). Maximum oxygen consumption promoted by substrates of the different respiratory complexes also showed similar values for diet and control groups (Fig. 2c). Finally, $\Delta\Psi$, estimated by following safranin O fluorescence, was equal between groups as well (Fig. 2d). Overall, these results indicate that no significant changes in mitochondrial bioenergetic function accompany diet-induced steatosis.

We investigated next if the diet changes the mitochondrial ability to accumulate Ca^{2+} ions. 1 nmol additions of $CaCl_2$ were made sequentially to the suspension, and $\Delta\Psi$ was followed (results not shown). A decrease in $\Delta\Psi$ promoted by Ca^{2+} was observed in both groups, and could be attributed to the mitochondrial permeability transition, since it was prevented by cyclosporin A (Lemasters et al. 1998). No differences were observed between the experimental groups. Hence, our results indicate that obesity and steatosis do not change mitochondrial ability to accumulate Ca^{2+} or trigger the permeability transition.

We evaluated next if K^+ transport through mitoK_{ATP} channels was altered in diet-induced obesity and steatosis

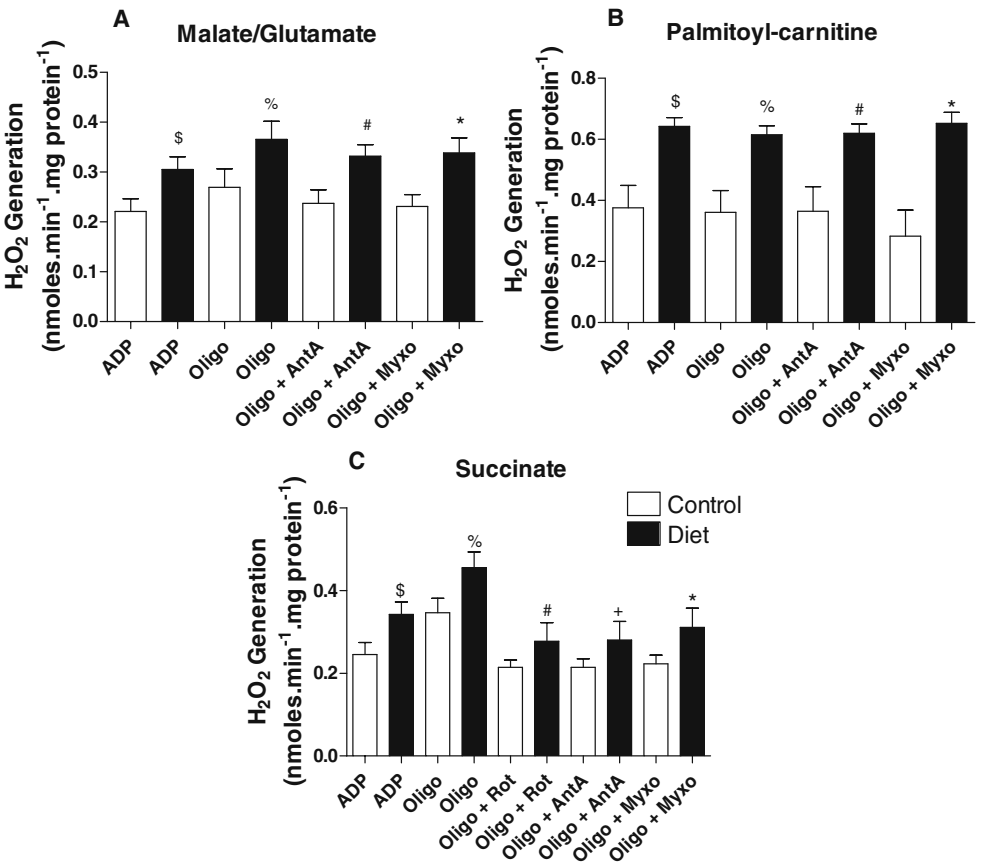

Fig. 4 Mitochondrial hydrogen peroxide generation is increased by high fat diets, Mitochondria were incubated in buffer containing 150 mM KCl, 10 mM Hepes, 2 mM KH_2PO_4, 2 mM $MgCl_2$, and, where indicated, 1 mM ADP, 1 μg/ml oligomycin, 0.8 μM rotenone, 0.150 ng antimycin A, 0.250 ng myxothiazol at 37 °C, pH 7.4, with magnetic stirring. **a** 2 mM malate plus glutamate were used as substrates. **b** 0.1 mM palmitoylcarnitine was used. **c** 2 mM succinate was employed, $n=9$ experiments per group in all panels. Symbols represent $p<0.01$ in relation to control groups

(see Fig. 3). Alberici et al. (2006) demonstrated that transgenic dyslipidemic mice present enhanced channel activity, and hypothesized this may be a compensatory mechanism to promote the oxidation of excess lipids (Vercesi et al. 2007). Channel activity was measured using the swelling assay (Beavis et al. 1993; Kowaltowski et al. 2001). We found (Fig. 3a) that mitochondrial swelling inhibition by ATP and the sensitivity to diazoxide (a channel activator) was greater in the diet group, suggesting that mitoK$_{ATP}$ activity was higher. Controls using sodium salts instead potassium demonstrate that these effects are specific for K^+ transport (Fig. 3b). We then evaluated if the activity of the channel (measured by the difference between the opened state, without ATP, and closed state, with ATP) correlated with the animal's metabolic parameters and body mass. MitoK$_{ATP}$ activity correlated significantly with cholesterol levels (Fig. 3c), but not body mass or triglyceride levels (Fig. 3d and e).

Since mitoK$_{ATP}$ regulates mitochondrial ROS release in cardiac, cerebral and liver tissues (Alberici et al. 2009; Facundo et al. 2006; Facundo et al. 2007; Ferranti et al. 2003; Fornazari et al. 2008), we asked if ROS release under our conditions was modulated by channel activity. We observed that the activation of mitoK$_{ATP}$ did not alter H_2O_2 release, respiratory rates or $\Delta\Psi$ in state 4 mitochondria (results not shown). However, the generation of H_2O_2 was larger in the diet group, using different respiratory substrates (Fig. 4). To evaluate possible sites of ROS overproduction, we used different respiratory inhibitors (Fig. 4, see Tahara et al. 2009). Myxothiazol and Antimycin A were used to inhibit different sites within complex III. Rotenone was employed to inhibit complex I and prevent reverse electron transport in mitochondria energized by succinate. No differences were found between groups using inhibitors. Indeed, we found that palmitoyl-carnitine was the substrate that generated most ROS, suggesting that lipid oxidative metabolism in liver mitochondria is an important source of oxidants (Fig. 4b) (Tahara et al. 2009). The generation of H_2O_2 was not strongly altered by different respiratory states or respiratory inhibitors, as observed previously for rat liver mitochondria (Tahara et al. 2009).

Since 2 months on a high-fat diet lead to changes in mitochondrial K^+ transport rates and ROS release, but does not compromise oxidative phosphorylation, we asked next if the continuous use of this diet could compromise mitochondrial function. We treated animals for a 10 months and observed a dramatic increase in body mass (Fig. 5a), although plasmatic glucose (5b), cholesterol (5c) and triglycerides (5d) remained unaltered.

ADP/O ratios were measured and showed a decrease in the diet-treated animals (Fig. 6a). $\Delta\Psi$ and RCRs were unchanged (Fig. 6b and c). MitoK$_{ATP}$ activity (Fig. 7a) was no longer enhanced after this prolonged exposure to the high-fat diet and O_2 consumption remind unaltered (Fig. 7b). Finally, we measured H_2O_2 generation, supported by succinate, and noted it was strongly enhanced in the high-fat diet group (Fig. 7c).

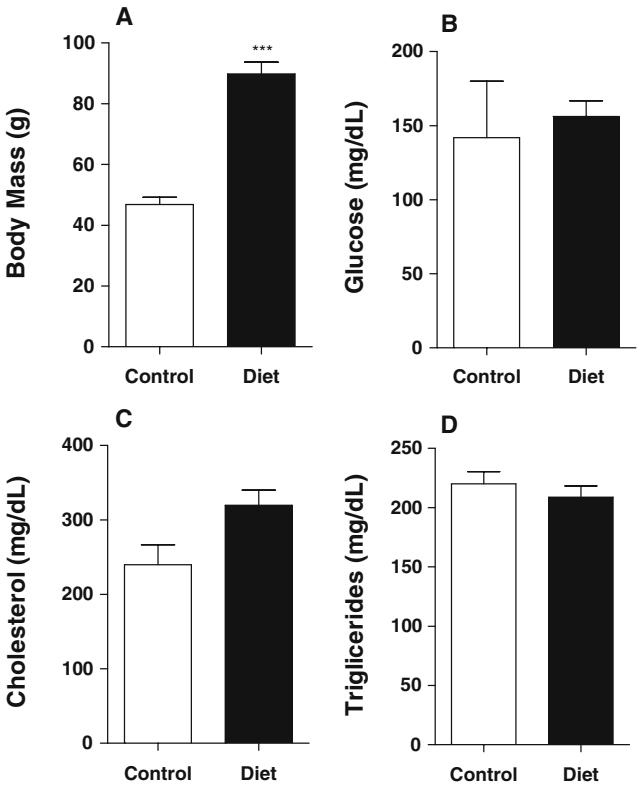

Fig. 5 Body mass, plasmatic cholesterol, triglycerides and glucose in female mice subjected to 10 months of hyperlipidic diet versus control. **a** Body mass, n=6 animals per group, p<0.001. **b** Plasmatic glucose, n=6 animals per group, p>0.05. **c** Total plasmatic cholesterol, n=6 per group, p>0.05. **d** Plasmatic triglycerides, n=5 per group, p>0.05

Discussion

This manuscript examines the effects of dietary-induced steatosis on mitochondrial bioenergetics, ion transport and oxidant generation. Dietary-induced models of metabolic disease are of interest since many studies focus exclusively on genetic models, in which metabolic alterations are very large and dissimilar to those frequently observed in humans (Anstee and Goldin 2006). Swiss mice, commonly used in investigations pertaining to the metabolic syndrome due to their tendency to develop obesity (Plum et al. 2002; Gault et al. 2007; Romanatto et al. 2009), were maintained on a soy oil-supplemented diet that promoted obesity and steatosis (Fig. 1), but did not lead to hyperglycemia or hypertriglyceridemia, often observed in diets rich in saturated fatty acids (Grubb et al. 2004; Wang et al. 2006; Kim et al. 2007; Tsukumo et al. 2007).

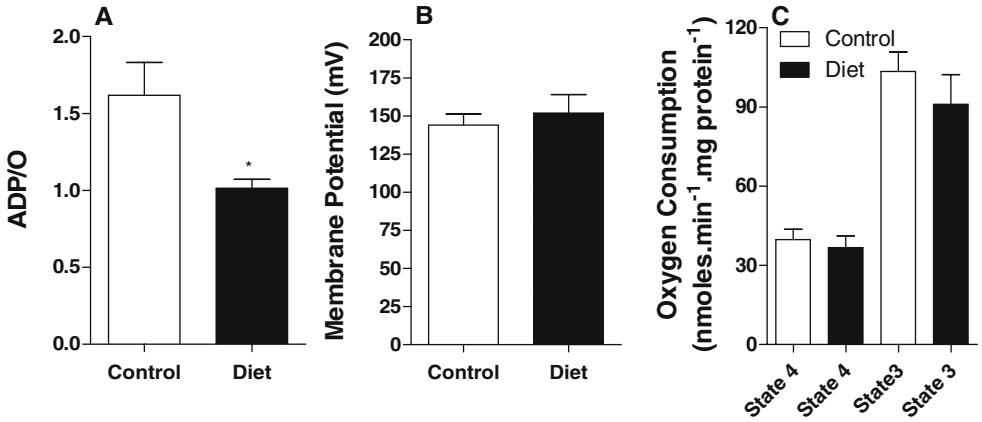

Fig. 6 Hyperlipidic diet effects on mitochondria after 10 months. Mitochondria were incubated in buffer containing 150 mM KCl, 10 mM Hepes, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM succinate, pH 7.4, at 37 °C, with magnetic stirring; **a** For ADP/O ratio measurements, 50 nmoles ADP were added to the suspension, n=4, p>0.05. **b** Mitochondrial membrane potentials were measured fluorimetrically in the same buffer as (**a**) with 1 μg/ml oligomycin, n=5, p>0.05. **c** 1 μg/ml oligomycin for state 4 or 1 mM ADP for state 3 measurements were present as indicated, n=5 per group, p>0.05

Interestingly, although mitochondrial bioenergetic alterations are widely shown to be associated (Mantena et al. 2009) and even causative (Simoneau et al. 1999; Kelley et al. 2002; Caraceni et al. 2004) of metabolic diseases, we found no overt changes in mitochondrial bioenergetics in the livers of the obese, steatotic, animals offered the diet for 2 months (Fig. 2). After long-term exposure to the diet (Fig. 6), respiration supported by complex IV substrate TMPD tends toward lower values, as well as ADP/O ratios. Obviously, these bioenergetic effects, in addition to being quite discreet, are not causative of the steatotic phenotype, observed even after short-term dietary intervention.

On the other hand, mitochondria isolated from the high-fat diet animals consistently presented increased ROS release rates, both after 2 and 10 months on the diet (Figs. 4 and 7). This is consistent with other studies involving obesity and steatosis, which uncovered increased levels of ROS biomarkers in the liver (Sanyal et al. 2001; Seki et al. 2002; Alberici et al. 2009; Mantena et al. 2009). Since maximal respiratory rates in the presence of different substrates (Fig. 3) were equal in control and steatotic groups, the difference in ROS formation is probably not related to partial respiratory inhibition observed in some metabolic disease models (Mantena et al. 2009; Nakamura et al. 2009), although a non-rate-limiting inhibition cannot be excluded. Increased ROS release may be related, at least in part, to enhanced electron leakage during β-oxidation, since it is magnified when fatty acids are used as substrates (Fig. 4).

Because these changes in ROS release are observed in the initial phases of steatosis, they could be causative of the illness and may act as signaling molecules changing the

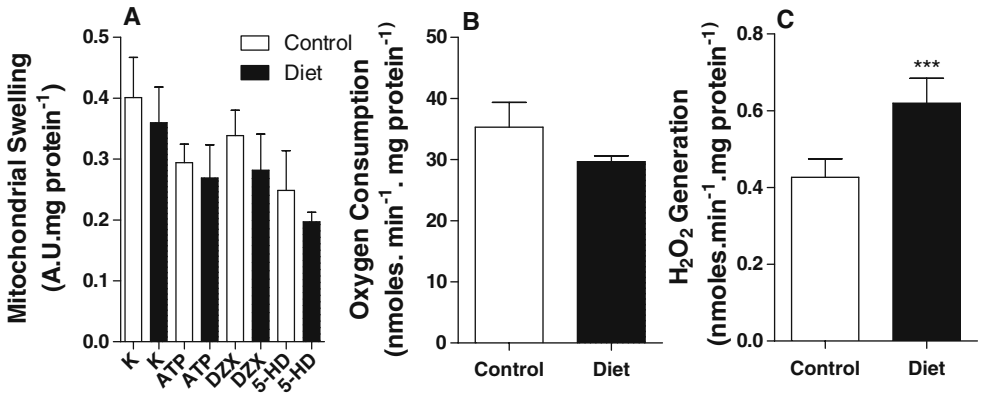

Fig. 7 MitoK_{ATP} activity, O_2 consumption and H_2O_2 release after 10 months of hyperlipidic diet. Mitochondria were incubated in buffer containing 150 mM KCl, 10 mM Hepes, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM succinate and 1 μg/ml oligomycin at 37 °C, pH 7.4, with magnetic stirring. **a** Mitochondrial swelling was measured under different conditions to calculate mitoK_{ATP} activity. Without channel modulators in K; 1 mM ATP (ATP); ATP plus 30 μM diazoxide (DZX); ATP plus DZX plus 60 μM 5-hydroxydecanoate (5-HD), n=5 experiments per group, p>0.05. **b** Mitochondria were incubated in the same medium as (**a**), and respiratory activity was measured, n=4, p>0.05. **c** Mitochondria were incubated in the same media as (**a**), with added Amplex Red/HRP as described in Fig. 4

expression and activity of mitochondrial proteins (Ip et al. 2003; Furukawa et al. 2004; Tattoli et al. 2008). UCP2 and mitoK$_{ATP}$ are activated by ROS (Zhang et al. 2001; Echtay et al. 2002; Jezek and Hlavatá 2005; Facundo et al. 2006; Facundo et al. 2007; Fornazari et al. 2008) and modulate ROS generation by promoting mild uncoupling (Jezek et al. 2004; Facundo et al. 2007; Fornazari et al. 2008). We investigated if our high-fat diet induced mild uncoupling pathways, focusing mainly on mitoK$_{ATP}$.

We observed increased activity of a K^+-selective, ATP sensitive transport pathway in mitochondria isolated from animals on the high-fat diet, suggesting enhanced mitoK$_{ATP}$ activity. Since K^+ transport was measured indirectly through mitochondrial swelling, it could be argued that the changes were not related to K^+ transport activity. However, we found no changes in H^+ or Na^+ transport, respiratory rates and $\Delta\Psi$ between the groups, indicating that these changes are, indeed, related to mitoK$_{ATP}$ activity.

The increased channel activity promoted by a high-fat diet suggests that mitoK$_{ATP}$ may have an important role regulating energy metabolism in the liver. This is further supported by the strict correlation found between mitoK$_{ATP}$ activity and cholesterol levels (Fig. 3). Although we do not know why this correlation occurred, it is possible it may relate to changes in redox state, since cholesterol synthesis requires large amounts of NADPH (Vercesi et al. 2007). The channel activity is also determined by the balance between levels of intracellular inhibitors (such as adenine nucleotides and acyl-CoA groups) and activators (guanosine nucleotides) (Paucek et al. 1996) and phosphorylation, at least in cardiac and brain tissues (Hassouna et al. 2004; Raval et al. 2007). It is possible these are also altered by diet. In experiments with rats, Samuel et al. demonstrated that PKC epsilon is overactivated by a high-fat diet (Samuel et al. 2004), which could alter mitoK$_{ATP}$ activity (Hassouna et al. 2004; Raval et al. 2007).

Alberici et al. (2006) found that transgenic animals that over-expressed ApoCIII and present high levels of triglycerides (~500 mg/dL) show increases in liver mitochondrial mitoK$_{ATP}$ activity and hypothesized that mild uncoupling promoted by channel activity may be an adaptation to oxidize the excess lipid and attenuate ROS production. Indeed, they demonstrated that mitoK$_{ATP}$ could down-regulate ROS generation in transgenic mice (Alberici et al. 2009). Interestingly, while mitoK$_{ATP}$ was important to regulate mitochondrial redox state in genetically-induced trygliceridemia (Alberici et al. 2009), in our model, ROS release from mitochondria was not significantly altered by the activity of this channel (data not shown). This may be due to the poor response of liver mitochondrial ROS release to uncoupling (Tahara et al. 2009).

Overall, we find that diet-induced steatosis is not accompanied by overt changes in mitochondrial bioenergetics, but involves significant changes in K^+ transport and ROS generation. The finding that mitoK$_{ATP}$ channels in the liver are activated by diet provides evidence that these channels may play an important role in the regulation of energy metabolism. This could be an intriguing new function for these channels, which to date have mostly been associated with tissue protection against ischemic damage (Facundo et al. 2007; Costa and Garlid 2008).

Acknowledgements We thank Professors Anibal Vercesi, Helena de Oliveira and Nadja S. Pinto for discussions. We thank Professors Marilia Seelander and Mario Saad for help establishing the high-fat diet animal model and the *Biotério do Conjunto das Químicas* for the excellent support with animal care. We thank Camille Caldeira for technical aid in experiments. Finally, we thank CNPq, CAPES, FAPESP and INCT Redoxoma for financial support.

Disclosures The authors declare no conflict of interests.

References

Akerman KE, Wikström MK (1976) FEBS Lett 68:191–197
Alberici LC, Oliveira HC, Patrício PR, Kowaltowski AJ, Vercesi AE (2006) Gastroenterology 131:1228–1234
Alberici LC, Oliveira HC, Paim BA, Mantello CC, Augusto AC, Zecchin KG, Gurgueira SA, Kowaltowski AJ, Vercesi AE (2009) Free Radic Biol Med 47:1432–1439
Altunkaynak BZ, Ozbek E (2009) Turk J Gastroenterol 20:93–103
Anstee QM, Goldin RD (2006) Int J Exp Pathol 87:1–16
Beavis AD, Lu Y, Garlid KD (1993) J Biol Chem 268:997–1004
Begriche K, Igoudjil A, Pessayre D, Fromenty B (2006) Mitochondrion 6:1–28
Boveris A, Chance B (1973) Biochem J 134:707–716
Bray GA, Nielsen SJ, Popkin BM (2004) Am J Clin Nutr 79:537–543
Cancherini DV, Trabuco LG, Rebouças NA, Kowaltowski AJ (2003) Am J Physiol Renal Physiol 285:F1291–F1296
Caraceni P, Bianchi C, Domenicali M, Maria Pertosa A, Maiolini E, Parenti Castelli G, Nardo B, Trevisani F, Lenaz G, Bernardi M (2004) J Hepatol 41:82–88
Castilho RF, Kowaltowski AJ, Meinicke AR, Bechara EJ, Vercesi AE (1995) Free Radic Biol Med 18:479–486
Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ (2003) J Biol Chem 278:36027–36031
Costa AD, Garlid KD (2008) Am J Physiol Heart Circ Physiol 295:H874–H882
Day CP, James OF (1998) Gastroenterology 114:842–845
Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD (2002) Nature 415:96–99
Facundo HT, Fornazari M, Kowaltowski AJ (2006) Biochim Biophys Acta 1762:202–212
Facundo HT, de Paula JG, Kowaltowski AJ (2007) Free Radic Biol Med 42:1039–1048
Federspil G, Nisoli E, Vettor R (2006) Pharmacol Res 53:449–456
Ferranti R, da Silva MM, Kowaltowski AJ (2003) FEBS Lett 536:51–55
Fornazari M, de Paula JG, Castilho RF, Kowaltowski AJ (2008) J Neurosci Res 86:1548–1556
Fromenty B, Robin MA, Igoudjil A, Mansouri A, Pessayre D (2004) Diabetes Metab 30:121–138

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I (2004) J Clin Invest 114:1752–1761
Gault VA, McClean PL, Cassidy RS, Irwin N, Flatt PR (2007) Diabetologia 50:1752–1762
Grubb SC, Churchill GA, Bogue MA (2004) Bioinformatics 20:2857–2859
Hashimoto E, Yatsuji S, Tobari M, Taniai M, Torii N, Tokushige K, Shiratori K (2009) J Gastroenterol 19:89–95
Hassouna A, Matata BM, Galiñanes M (2004) Am J Physiol Cell Physiol 287:C1418–C1425
Ichas F, Jouaville LS, Mazat JP (1997) Cell 89:1145–1153
Ip E, Farrell GC, Robertson G, Hall P, Kirsch R, Leclercq I (2003) Hepatology 38:123–132
Jew S, AbuMweis SS, Jones PJ (2009) J Med Food 12:925–934
Jezek P, Hlavatá L (2005) Int J Biochem Cell Biol 37:2478–2503
Jezek P, Zácková M, Růzicka M, Skobisová E, Jabůrek M (2004) Physiol Res 53:S199–S211
Kelley DE, He J, Menshikova EV, Ritov VB (2002) Diabetes 51:2944–2950
Kim F, Pham M, Luttrell I, Bannerman DD, Tupper J, Thaler J, Hawn TR, Raines EW, Schwartz MW (2007) Circ Res 100:1589–1596
Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD (2001) Am J Physiol Heart Circ Physiol 280:H649–H657
Kowaltowski AJ, Cosso RG, Campos CB, Fiskum G (2002) J Biol Chem 277:42802–42807
Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, Vercesi AE (2009) Free Radic Biol Med 47:333–343
Le TH, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, Iezzoni JC, Hespenheide EE, Al-Osaimi A, Peterson TC (2004) Hepatology 39:1423–1429
Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B (1998) Biochim Biophys Acta 1366:177–196
Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265–275
Mantena SK, Vaughn DP, Andringa KK, Eccleston HB, King AL, Abrams GA, Doeller JE, Kraus DW, Darley-Usmar VM, Bailey SM (2009) Biochem J 417:183–193
Muller FL, Liu Y, Van Remmen H (2004) J Biol Chem 279:49064–49073
Nakamura S, Takamura T, Matsuzawa-Nagata N, Takayama H, Misu H, Noda H, Nabemoto S, Kurita S, Ota T, Ando H, Miyamoto K, Kaneko S (2009) J Biol Chem 284:14809–14018
Nicolson GL (2007) J Cell Biochem 100:1352–1369
Nishikawa T, Kukidome D, Sonoda K, Fujisawa K, Matsuhisa T, Motoshima H, Matsumura T, Araki E (2007) Diabetes Res Clin Pract 1:S161–S164
Nisoli E, Clementi E, Carruba MO, Moncada S (2007) Circ Res 6:795–806
Paucek P, Yarov-Yarovoy V, Sun X, Garlid KD (1996) J Biol Chem 271:32084–32088
Pessayre D (2007) J Gastroenterol Hepatol 1:S20–S27
Plum L, Giesen K, Kluge R, Junger E, Linnartz K, Schürmann A, Becker W, Joost HG (2002) Diabetologia 45:823–830
Raval AP, Dave KR, DeFazio RA, Perez-Pinzon MA (2007) Brain Res 1184:345–353
Romanatto T, Roman EA, Arruda AP, Denis RG, Solon C, Milanski M, Moraes JC, Bonfleur ML, Degasperi GR, Picardi PK, Hirabara S, Boschero AC, Curi R, Velloso LA (2009) J Biol Chem 284:36213–36222
Samuel VT, Liu ZX, Qu X, Elder BD, Bilz S, Befroy D, Romanelli AJ, Shulman GI (2004) J Biol Chem 279:32345–32353
Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN (2001) Gastroenterology 120:1183–1192
Seki S, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa KJ (2002) Hepatol 37:56–62
Simoneau JA, Veerkamp JH, Turcotte LP, Kelley DE (1999) FASEB J 13:2051–2060
Tahara EB, Navarete FD, Kowaltowski AJ (2009) Free Radic Biol Med 46:1283–1297
Tattoli I, Carneiro LA, Jéhanno M, Magalhaes JG, Shu Y, Philpott DJ, Arnoult D, Girardin SE (2008) EMBO Rep 9:293–300
Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araújo EP, Vassallo J, Curi R, Velloso LA, Saad MJ (2007) Diabetes 56:1986–1998
Vercesi AE, Castilho RF, Kowaltowski AJ, Oliveira HC (2007) IUBMB Life 59:263–268
Wang Y, Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasam B, Nair MG, Peters JM, Busik JV, Olson LK, Jump DB (2006) J Lipid Res 47:2028–2041
Warwick ZS, McGuire CM, Bowen KJ, Synowski SJ (2000) Am J Physiol Regul Integr Comp Physiol 278:R196–R200
Zhang DX, Chen YF, Campbell WB, Zou AP, Gross GJ, Li PL (2001) Circ Res 89:1177–1183
Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP (1997) Anal Biochem 253:162–168