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Rapeseed oil-rich diet alters hepatic mitochondrial membrane lipid composition and disrupts bioenergetics

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Abstract Diet is directly related with physiological alterations occurring at a cell and subcellular level. However, the role of diet manipulation on mitochondrial physiology is still largely unexplored. Aiming at correlating diet with alterations of mitochondrial membrane composition and bioenergetics, Wistar-Han male rats were fed for 11, 22 and 33 days with a rapeseed oil-based diet and mitochondrial bioenergetics, and membrane composition were compared at each time point with a standard diet group. Considerable differences were noticed in mitochondrial membrane lipid composition, namely in terms of fatty acyl chains and relative proportions of phospholipid classes, the modified diet inducing a decrease in the saturated to unsaturated molar ratio and an increase in the phosphatidylcholine to phosphatidylethanolamine molar ratio. Mass spectrometry lipid analysis showed significant differences in the major species of cardiolipin, with an apparent increased incorporation of oleic acid as a result of exposure to the modified diet. Rats fed the modified diet during 22 days showed decreased hepatic mitochondrial state 3 respiration and were more susceptible to Ca^{2+}-induced transition pore opening. Rapeseed oil-enriched diet also appeared to promote a decrease in hydroperoxide production by the respiratory chain, although a simultaneous decrease in vitamin E content was detected. In conclusion, our data indicate that the rapeseed oil diet causes negative alterations on hepatic mitochondrial bioenergetics, which may result from membrane remodeling. Such alterations may have an impact not only on energy supply to the cell, but also on drug-induced hepatic mitochondrial liabilities.

Keywords Rapeseed oil · Diet · Liver mitochondria · Wistar rat · Toxicity · Mitochondrial membrane

Amália S. Jurado and Paulo J. Oliveira share senior authorship.

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Introduction

Mitochondria are now perceived as having an intricate physiological role, which surpasses that in ATP production. In fact, besides influencing virtually all energy-dependent cell activity, mitochondria represent the point of convergence of relevant processes, such as generation of reactive oxygen species (ROS), Ca^{2+} homeostasis, apoptosis and intermediate metabolism (Kass 2006). As a consequence of this pivotal role in cell physiology, mitochondria are

predictably very susceptible to pharmacologic interactions. Mitochondrial membrane lipids have shown to be involved in a number of processes associated with these organelles as diverse as protein biogenesis, energy production, membrane fusion and apoptosis (Osman et al. 2011). Several lipids have been proposed as being part of the quaternary structure of mitochondrial membrane proteins, hence, playing a crucial role in their enzymatic activity (Gonzalvez and Gottlieb 2007). The lipid composition and its spatial distribution also affect mitochondrial events as fusion or fission, energy production and the topology of membrane proteins (Osman et al. 2011).

Studies have shown that dietary fat manipulations can alter the activities of mitochondrial electron transport chain complexes III (Barzanti et al. 1994), IV (Barzanti et al. 1994; Yamaoka et al. 1988), and V (Barzanti et al. 1994; Clandinin et al. 1985), as well as the amount of coenzyme Q (CoQ) in mitochondrial membranes (Huertas et al. 1991; Mataix et al. 1997). Mitochondrial production of ROS (Ramsey et al. 2005) and Ca^{2+}-induced permeability transition (O'Shea et al. 2009) have also been reported to be influenced by diet.

The objective of the present work is to perform a thorough study regarding the effects of a specific dietary lipid manipulation on liver mitochondrial physiology, focusing on two different aspects: (a) alterations of membrane lipid composition and (b) effects on mitochondrial bioenergetics, oxidative stress and calcium tolerance. The hypothesis is that direct manipulation of mitochondrial lipid composition by diet will result in multi-target alterations with consequences for mitochondrial physiology. Since the liver plays a crucial role in lipid metabolism (Reddy and Sambasiva Rao 2006), the present work focused on liver mitochondria, whose lipid composition is presumably susceptible to changes induced by a lipid-enriched diet, as previously reported (Chanadiri et al. 2006).

In order to investigate diet-induced effects on mitochondrial membrane physiology and bioenergetics, a rapeseed oil-rich diet was chosen, taking into account the previous knowledge available from a number of earlier studies. The major part of the rapeseed oil production is consumed domestically by the producing countries. The relevance of this oil on human nutrition has increased remarkably in the last decades, representing 15 % of world vegetable oil production in 2008, just behind palm oil and soybean oil (Rosillo-Calle et al. 2009). The interest on the use of rapeseed oil in this context arose from the observation that heart pathological lesions took place after longtime dietary exposure to that oil (Abdellatif and Vles 1970). Bioenergetic studies revealed that the efficiency of rat heart mitochondria to oxidize substrates was substantially lowered when rapeseed oil was incorporated in the diet (Houtsmuller et al. 1970). Decreased rates of ATP synthesis and ADP/O ratios were also associated with cardiac mitochondria isolated from animal-fed diets containing rapeseed oils (Clandinin 1978; Renner et al. 1979). Later, it was described that rats fed with high erucic acid rapeseed oil showed higher mitochondrial phosphatidylcholine (PC) levels than rats fed with soybean oil, and rats fed with low erucic acid rapeseed oil had an increased content in cardiac mitochondrial cardiolipin (CL) (Innis and Clandinin 1981b). Based on the reported effects on heart mitochondria, rapeseed oil was considered as a suitable diet component aiming to promote compositional and functional alterations in hepatic mitochondria, seeking the establishment of membrane structure–activity relationships at the mitochondrial level, which has deserved little attention so far.

Methods

Experimental design and ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Facilities of the Center for Neuroscience and Cell Biology (CNC) of the University of Coimbra. All animal handlers, including the senior author, are accredited by the Federation for Laboratory Animal Science Associations, and the animal facilities at the CNC are accredited by the National Office of Veterinary ("Direcção Geral de Veterinária", DGV).

Two-month-old male Wistar-Han rats were maintained on a reverse 12 H light–dark cycle with food and water ad libitum. The control group had access to a standard rodent diet, while the treated group was fed with a modified diet, which had the same composition as the control diet but containing 20 % rapeseed oil (supplementary online material Table S1) calorically adjusted for high fat. Both diets were purchased from Dyets Inc. (Bethlehem, PA) (Table S2). Animal weight and diet consumption were followed on a weekly basis. After 11, 22 or 33 days of diet consumption, rats were killed by cervical dislocation followed by decapitation, and organs (liver, heart and kidneys) were harvested and weighted, with a portion of the liver stored at −80 °C for posterior processing. Blood from rats killed after 33 days of diet consumption was collected after decapitation to sterile tubes without additives. After blood clot formation, serum was separated by centrifugation at 1,600*g* for 10 min at 4 °C (Sigma 3-16K, 1,333 rotor). The supernatant was then transferred to microcentrifuge vials and centrifuged at 16,000*g* for 20 min at 4 °C (Eppendorf 5415 R, FL062 rotor). Serum samples were maintained for a short time at 4 °C for analysis by an external certified laboratory. Due to the multitude of endpoints investigated

and experimental protocol setup, we were not able to use a similar number of animals/independent samples per experiment. The total number of animals used in each of the two diet groups (control vs. modified) was as follows: 7 (11 days), 8 (22 days) and 15 (33 days). Control and modified diet animals were killed daily in a paired basis.

Liver mitochondria isolation and purification

Mitochondria were isolated from the livers of the male Wistar-Han rats according to a protocol established in our laboratory (Pereira et al. 2007). Homogenization medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 0.5 mM EGTA and 0.1 % fat-free bovine serum albumin. EGTA and bovine serum albumin were omitted from the final washing medium, adjusted at pH 7.4. Protein content was determined by the biuret method (Gornall et al. 1949) calibrated with bovine serum albumin. For mitochondrial lipid content analysis and with the concern of making sure that analyzed lipid was exclusively of mitochondrial origin, mitochondrial suspensions were further purified by Percoll fractionation (Neuburger et al. 1982), as described in detail in the supplementary online material.

Mitochondrial lipid and protein composition

Mitochondrial lipids were extracted by the method of Bligh and Dyer (1959) and quantified by measuring the amount of inorganic phosphate (Bartlett 1959), after hydrolysis of the extracts in 70 % $HClO_4$, at 180 °C, for 120 min (Bottcher et al. 1961). Fatty acid composition was determined by gas–liquid chromatography after trans-methylation, as previously described (Rosa et al. 2000). Cardiolipin extracts were analyzed by electrospray ionization (ESI) mass spectrometry (MS), as previously described (Maciel et al. 2011), using a LXQ linear ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA) operating in negative mode. More details are found in the supplementary online material.

Changes in selected mitochondrial protein content were assessed by Western blotting. Small pieces of liver sample were homogenized in RIPA buffer and protein content in the supernatants was quantified with the Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL). A more detailed description of the method is available in the supplementary online material.

Mitochondrial oxygen consumption and transmembrane electric potential

Oxygen consumption of isolated mitochondria was determined polarographically with a Clark-type oxygen electrode (Estabrook 1967) connected to a suitable recorder, in a 1-ml thermostated water-jacketed chamber with magnetic stirring, at 30 °C. The standard respiratory medium consisted of 125 mM sucrose, 65 mM KCl, 2.5 mM $MgCl_2$, 5 mM KH_2PO_4 and 5 mM HEPES (pH 7.4). Mitochondria (1 mg/mL) and substrates (10 mM glutamate/5 mM malate or 10 mM succinate plus 3 μM rotenone) were added to the respiration medium, and state 3 respiration was initiated by the addition of 125 nmol ADP. Addition of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 μg/ml) resulted in uncoupled respiration (FCCP state).

Mitochondrial transmembrane electric potential ($\Delta\Psi$) was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP^+) according to previously established methods (Kamo et al. 1979) and by using an $Ag/AgCl_2$ electrode as reference. Mitochondria (1 mg/mL) were suspended with constant stirring, at 30 °C, in 1 ml of the standard respiratory medium supplemented with 3 μM TPP^+ and were energized by adding 10 mM glutamate/5 mM malate or 10 mM succinate plus 3 μM rotenone. The distribution of TPP^+ was allowed to reach a new equilibrium (2 min) before further additions. ADP (125 nmol) was added to initiate phosphorylation. The electrode was calibrated with TPP^+ assuming Nernstian distribution of the ion across the synthetic membrane and $\Delta\Psi$ is expressed in mV. A matrix volume of 1.1 μl/mg protein was assumed.

Calcium-induced mitochondrial depolarization resulting from mitochondrial permeability transition (MPT) induction was also determined with a TPP^+ electrode. The assays were performed in 1 ml of the swelling media (200 mM sucrose, 10 mM Tris–MOPS, pH 7.4, 10 μM EGTA, 1 mM KH_2PO_4 and 2.5 mM glutamate plus 1.25 mM malate or 2.5 mM succinate plus 3 μM rotenone). $CaCl_2$ (100 nmol/mg protein) was added to initiate MPT, which was followed as the progressive decrease in the membrane potential observed after Ca^{2+} addition.

Calcium-induced mitochondrial swelling was also determined to follow MPT induction. In this case, mitochondrial osmotic volume changes were followed as the decrease of absorbance at 540 nm using a Jasco V-560 UV/VIS Spectrophotometer (Jasco, Tokyo, Japan). Mitochondrial suspension (1.5 mg of protein) was added to swelling buffer under constant stirring and temperature (30 °C). Calcium (66.7 nmol/mg protein) was added to the preparation 1 min after starting the experiment. To confirm that the swelling observed in these experiments resulted from MPT induction, assays were performed where cyclosporin A (1 μM) was added to the mitochondrial preparation before the addition of calcium.

Citrate synthase activity

Citrate synthase activity was assayed for means of assessing the integrity of mitochondrial preparations. The activity

was measured spectrophotometrically at 412 nm and 30 °C. Buffer (0.1 M Tris/HCl, pH 8.1) was pre-incubated at 30 °C for 5–10 min and measurements were conducted at 30 °C with constant magnetic stirring in a Jasco V-560 spectrophotometer. To start activity determinations, portions of mitochondrial preparations (200 μg protein) were added to the buffer containing 0.1 mM 5,5-dithio-bis-(2-nitrobenzoic) acid (DTNB), 0.5 mM oxaloacetate and 0.31 mM acetyl CoA (Kuznetsov et al. 2002). Total activity of the enzyme was determined upon addition of Triton X-100 (final concentration ca. 0.25 %).

Oxidative stress markers

The rate of hydroperoxide production by isolated mitochondria was determined fluorimetrically using a modification of the method described by Barja (1999). Briefly, mitochondria were incubated at 30 °C with 5 mM glutamate/2.5 mM malate or 5 mM succinate in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCl, 30 mM Hepes, 0.1 mM homovanillic acid and 6 U/ml horseradish peroxidase. The incubation was stopped at 15 min with 0.5 ml of cold 2 M glycine buffer containing 25 mM EDTA and NaOH (pH 12). The mitochondrial suspensions were then centrifuged at 850*g* for 10 min. The fluorescence of the supernatants (resulting from the dimer produced by hydrogen peroxide oxidation of homovanillic acid) was measured at 420 nm with excitation at 312 nm and the rate of peroxide production was calculated using a standard curve of H_2O_2.

Levels of lipid peroxidation in samples of liver tissue homogenates (prepared as for western blot analysis and diluted in phosphate buffer saline solution, PBS, to assure 1 mg/mL protein concentration) were assessed by the fluorimetric determination of malondialdehyde (MDA) adducts separated by high-performance liquid chromatography (HPLC) using the ClinRep® complete kit (RECIPE, Munich, Germany). Extraction and separation of vitamin E (α-tocopherol) from liver tissue homogenate (1 mg/mL protein) were performed by following a method previously proposed by Vatassery and Younoszai (1978) and described in the supplementary online material. Protein reactive carbonyls were analyzed in samples according to the method of Robinson et al. (1999) with slight modifications, as described in the supplementary material.

Statistical analysis

Results were expressed as mean ± standard error of the mean for a number of independent experiments, specified for each case. Multiple comparisons were performed using one-way ANOVA with the Student–Newman–Keuls as a posttest and pairing was made for observations normal versus modified diet rats sacrificed and processed in the same day. A value of $p < 0.05$ was considered significant.

Results

Animal data

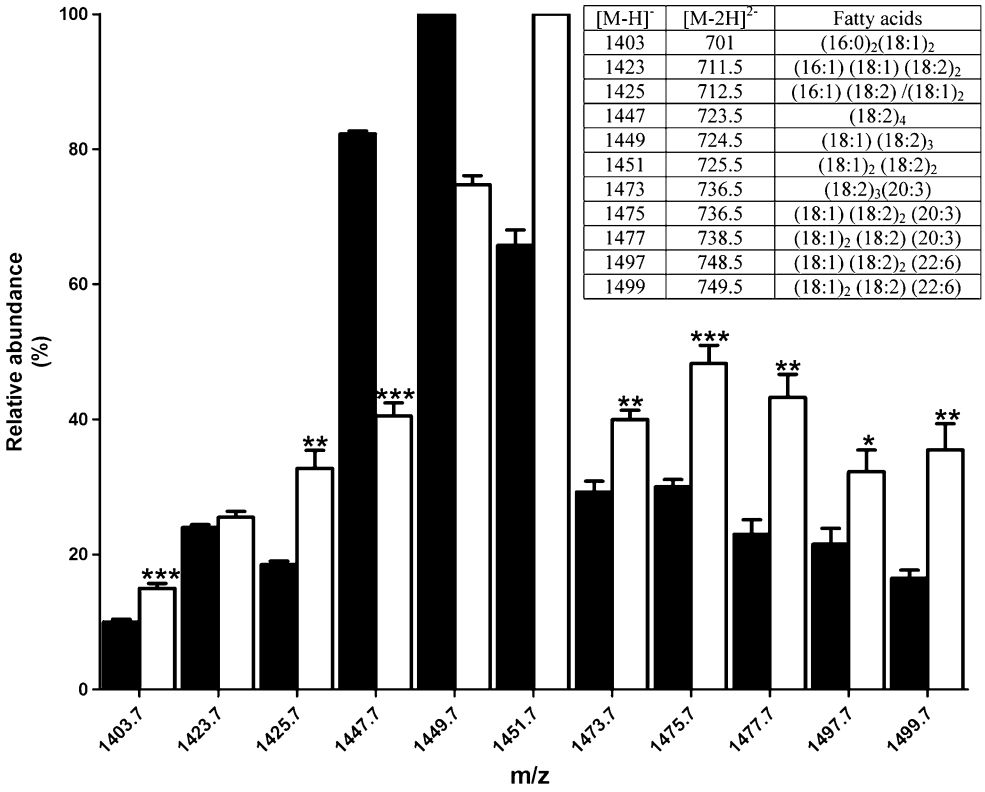
To evaluate potential physiological alterations in the experimental animals fed the modified diet, plasma analyses were performed by an independent specialized laboratory. Plasma aliquots were collected from rats fed the normal and the modified diet, at the three experimental time points, to determine the content in urea, cholesterol, creatinine, triglycerides, uric acid, glucose and total protein, the ureic nitrogen/creatinine ratio and the activities of aspartate aminotransferase, alanine aminotransferase, creatine kinase and lactate dehydrogenase. The only significant alteration observed in rats submitted to the 20 % rapeseed oil diet was a decrease in the triglyceride content (56 ± 7.1 mg/dL as compared to 137 ± 29.5 mg/dL for control rats) after 33 days of treatment.

Rats assigned to the modified diet showed a slight delay in growth (Fig. S1, left), probably related to a lower food intake (Fig. S1, right). Besides the decrease in total weight, rats eating the modified diet showed an increase in heart weight (in relation to total body weight) after 22 days of treatment (Table S3). No apparent macroscopic lesions were found in the organs of the animals of both groups.

Mitochondrial membrane lipid composition

Comparing the mitochondrial membrane lipid composition of rats fed the modified diet with that of control rats, the most striking difference in terms of major phospholipid classes regarded phosphatidylcholine (PC) and phosphatidylethanolamine (PE) contents (Table S4). PC increased and PE decreased in liver mitochondria from rats fed the alternative diet, such that PC/PE molar ratio was 1.27 for control rats and 1.82 for rats fed the rapeseed oil-enriched diet after 33 days of treatment. For the same treatment time (33 days), a small but statistically significant decrease in cardiolipin (CL) content was found. Interestingly, after 11 days of modified diet treatment, the differences in the three phospholipid content were already noticed.

In terms of fatty acyl chains, relevant differences occur concerning particular fatty acids, which were absent in the control group (normal diet) but appeared in mitochondria from rats fed the modified diet, including n-eicosanoic (C20:0), eicosenoic (C20:1), eicosapentaenoic (C20:5), docosanoic (C22:0), erucic (or docosenoic, C22:1) and tetracosanoic (C24:0) acids. Also, significant alterations in fatty acid relative concentrations (expressed in molar



$[M-H]^-$	$[M-2H]^{2-}$	Fatty acids
1403	701	$(16:0)_2(18:1)_2$
1423	711.5	(16:1) (18:1) $(18:2)_2$
1425	712.5	(16:1) (18:2) /$(18:1)_2$
1447	723.5	$(18:2)_4$
1449	724.5	(18:1) $(18:2)_3$
1451	725.5	$(18:1)_2$ $(18:2)_2$
1473	736.5	$(18:2)_3$(20:3)
1475	736.5	(18:1) $(18:2)_2$ (20:3)
1477	738.5	$(18:1)_2$ (18:2) (20:3)
1497	748.5	(18:1) $(18:2)_2$ (22:6)
1499	749.5	$(18:1)_2$ (18:2) (22:6)

Fig. 1 Relative abundance of $[M–H]^-$ ions of cardiolipin species observed in the ESI–MS spectra for cardiolipin extracts from rats fed a control diet (*black bars*) and for extracts from rats fed the diet containing 20 % rapeseed oil (*white bars*). In *inset*, the CL species corresponding to each $[M–H]^-$ ion are presented. Values depicted are mean ± standard error of mean for 4 animals, 33 days after the beginning of the treatment. Comparisons were performed using one-way ANOVA, with the Student–Newman–Keuls as a posttest for control diet rats versus rats fed the modified diet. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

percentages) occurred in liver mitochondria from rats fed the modified diet, namely with regard to estearic (C18:0) and linoleic acids (C18:2), which decreased, and oleic acid (C18:1), which increased (Table S4). Overall, a progressive increase in hepatic mitochondrial content in unsaturated fatty acids was detected in rats fed the modified diet, at the expense of saturated fatty acids, (Fig. S2A), resulting in a decrease in the saturated to unsaturated ratio (Fig. S2B). More specifically, the content in monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) increased after 33 days of treatment with the modified diet (Table S4).

Alterations of the mitochondrial content in different CL molecular species as a consequence of diet manipulation were also evaluated (Fig. 1a), given the increasing relevance of this particular phospholipid on mitochondrial physiology. While the ion $[M–H]^-$ at *m/z* 1,449.7, attributed to the CL (C18:1) $(C18:2)_3$ corresponds to the most abundant species in mitochondria from control rats, for rat fed the rapeseed oil diet, the most abundant CL ion was observed at *m/z* 1,451.7, corresponding to CL $(C18:1)_2$ $(C18:2)_2$. Significant alterations were observed in the relative abundances of the other $[M–H]^-$ ions in CL MS spectra, being possible to foresee an increase in the relative abundance of ions corresponding to CL species containing the longest fatty acyl chains (with 20 and 22 carbon atoms) for rats fed the modified diet, showing that the fatty acyl pattern of hepatic mitochondrial CL is rather susceptible to diet.

Mitochondrial protein composition

The expression of several mitochondrial proteins with important functions in energy production was investigated by Western blotting. In terms of the mitochondrial proteins involved in oxidative phosphorylation, the influence of diet in protein content was limited during the time period studied. The modified diet group showed a decrease in complex III subunit core 2, complex II SDHA and Complex IV MTCO1, but only for 22 days (Fig. 2). Among proteins that constitute or regulate the MPT pore, only the adenine nucleotide translocator (ANT) showed a decrease after 11 days of treatment with the alternative diet (Fig. 2). Other proteins for which no differences were found between groups included uncoupling protein 2 (UCP-2), which has been described as participating in fatty acid translocation (Cioffi et al. 2009), cytochrome *c*, pyruvate dehydrogenase and tafazzin, an enzyme involved in CL (and PC) maturation/remodeling (Xu et al. 2006), as shown in Fig. S3.

Mitochondrial bioenergetics

The effects of dietary changes on mitochondrial respiration were also investigated. Alterations were evident after only

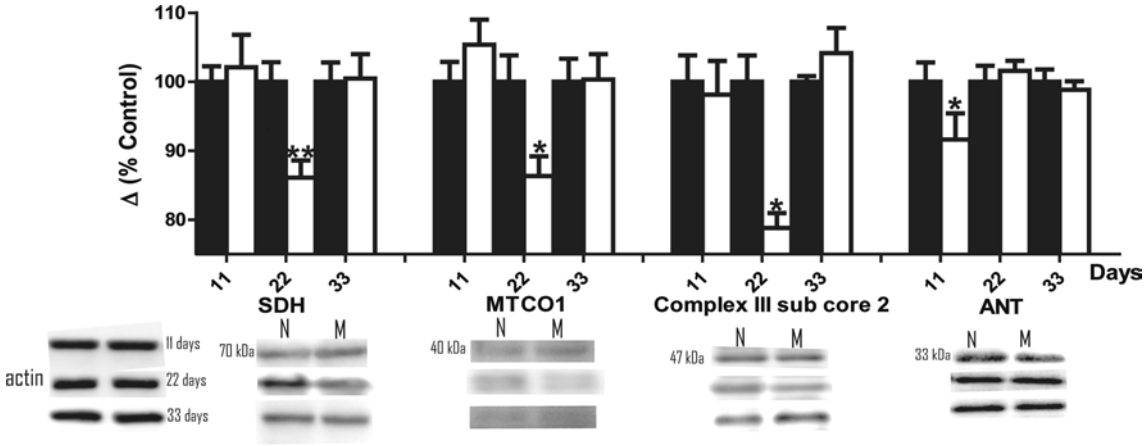


Fig. 2 Relative contents of mitochondrial proteins that underwent diet-induced changes, taking as 100 % the contents of the respective proteins in the normal diet fed rat group (control). Abbreviations are used for succinate dehydrogenase subunit A (SDH); and mitochondria marker, cytochrome C oxidase subunit I (MTCOI). Representative blots for the three different experimental time points are presented below the bars of the respective proteins. Each well was loaded with liver tissue samples (corresponding to 25 μg protein) from rats fed a standard rodent diet (N; *black bars*) or rats fed a modified diet containing 20 % rapeseed oil (M; *white bars*). Actin was used as a protein loading control. Values presented are mean ± standard error of mean for at least 5 samples from different animals and presented as percentage of control. Comparisons were performed using one-way ANOVA, with the Student–Newman–Keuls as a post-test for control diet rats versus rats fed the modified diet. $^{**}P < 0.01$; $^{*}P < 0.05$

11 days of treatment with 20 % rapeseed oil diet (Fig. 3 and S4A). Mitochondria from rats fed this diet displayed decreased respiratory state 3 (Fig. 3a, f), increased respiratory state 4 (Fig. 3b, g) and decreased FCCP-uncoupled respiration (Fig. 3c, h), as compared to the normal diet-fed group. As expected, the RCI was lower in the diet-modified group (Fig. 3d, i) as well as the ADP/O ratio (Fig. 3e, j). Similar differences between groups were observed regardless of complex I (glutamate–malate) or complex II (succinate) substrates were used.

Differences were also observed in the mitochondrial transmembrane electric potential between the two groups (Fig. 4 and S4B). Mitochondria from rats fed the modified diet developed a lower transmembrane electric potential upon substrate addition, which was significantly different from the control group after 22 days of treatment (Fig. 4a, e). The extent of ADP-induced depolarization also decreased in this group (Fig. 4b, f), and, in this case, significant differences were noticed at an early time point (11 days). Significant differences regarding mitochondrial repolarization were detected after 22 or 33 days of treatment, with mitochondria from rats fed the rapeseed oil-enriched diet repolarizing to a lower membrane potential (Fig. 4c, g). Liver mitochondria from diet-modified animals took more time to phosphorylate ADP, which is in accordance with the decreased state 3 respiration. In this case, the effect was already apparent after 11 days of treatment (Fig. 4d, h). The observed effects on respiration were clearly substrate-independent.

Alterations induced by the modified diet on MPT induction were also found, although the high variability of results prevented the detection of robust statistical differences (Fig. 5). When calcium-induced mitochondrial depolarization was followed, liver mitochondria from diet-modified animals showed a higher rate of calcium-induced depolarization when compared to the control group (Fig. 5a–c). When the same phenomenon was measured by following calcium-induced mitochondrial swelling (Fig. 5d, e), differences were only visible after 33 days of treatment, with mitochondria from diet-modified animals consistently showing higher calcium susceptibility as compared to the control group. Calcium-induced mitochondrial swelling was inhibited in both groups in the presence of cyclosporin A, the classic MPT pore inhibitor (Broekemeier and Pfeiffer 1989).

In order to reject the possibility that the alterations detected in mitochondrial function were due to mitochondrial disruption upon isolation or to a lower isolation yield, citrate synthase activity was measured as a common quantitative marker enzyme for mitochondrial integrity (Scaini et al. 2011). No statistical differences were found in the initial citrate synthase activity (Fig. S5A). When analyzing total citrate synthase activity upon detergent addition, no differences were found for the two experimental groups, at least for 33 days of treatment (Fig. S5B), which means that daily protein quantifications were accurate.

Oxidative stress

Dietary effects on specific oxidative stress-related endpoints were also investigated. Formation of hydroperoxide by the respiratory chain was determined in liver

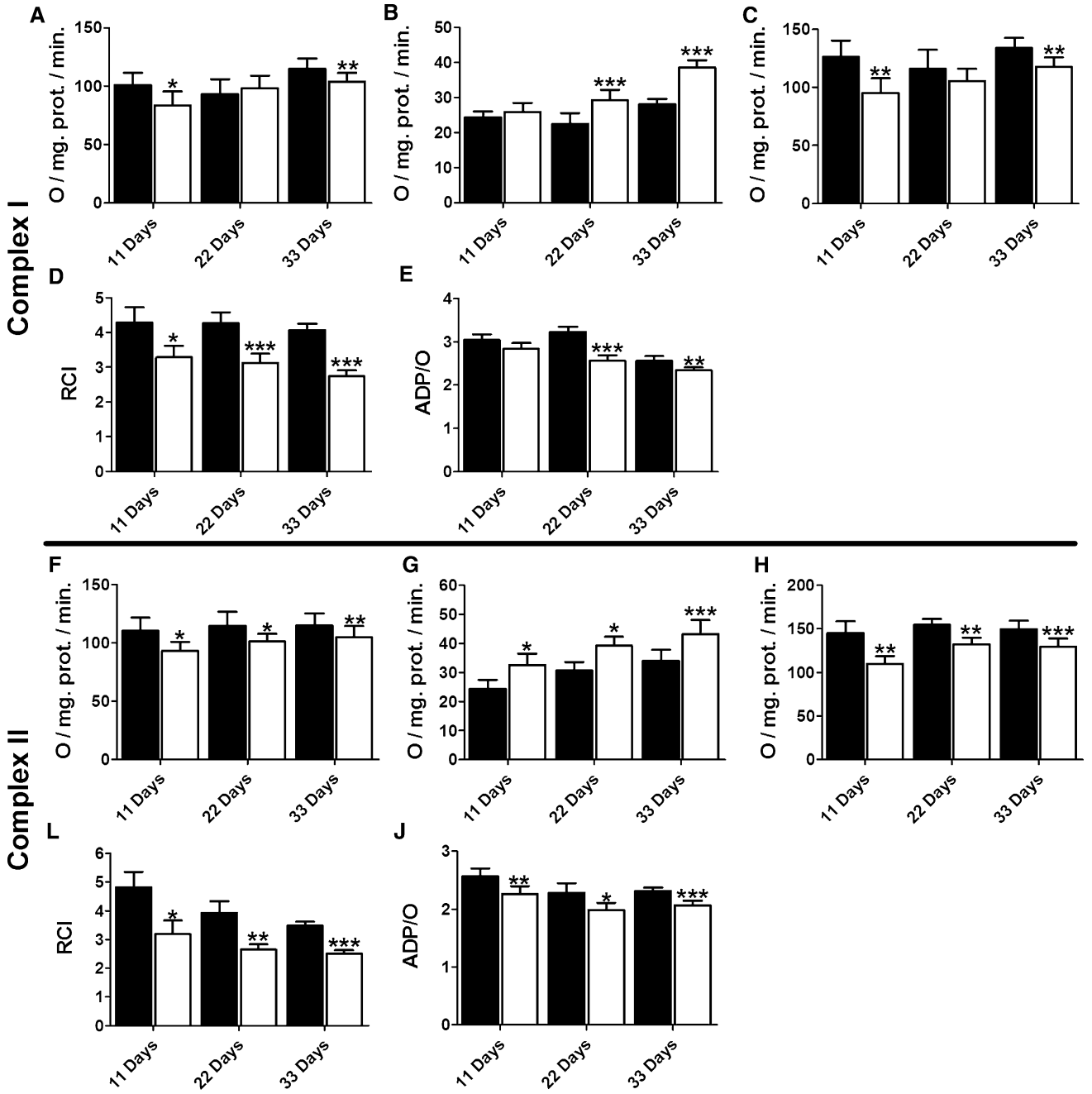


Fig. 3 Oxygen consumption rates determined with liver mitochondria from rats fed a normal diet (*black bars*) or a diet containing 20 % rapeseed oil (*white bars*). A mixture of glutamate plus malate (Complex I) and succinate (Complex II) were used as respiratory substrates. The parameters presented are respiratory states 3 (**a** and **f**) and 4 (**b** and **g**), uncoupled respiration (**c** and **h**), RCI (**d** and **i**) and the ADP/O ratio (**e** and **j**). Values depicted are mean ± standard error of mean for 7 animals (11 days), 8 animals (22 days) and 15 animals (33 days). Comparisons were performed using one-way ANOVA, with the Student–Newman–Keuls as a posttest for control diet rats versus rats fed the modified diet. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

mitochondria from both dietary groups after 33 days of treatment. The generation of H_2O_2 by mitochondria in the basal state (with no substrate) was significantly lower for rats fed the diet containing 20 % rapeseed oil (Fig. 6a, b). Glutamate/malate-respiring mitochondria (Fig. 6a) showed significant differences between groups when the respiration inhibitors rotenone (complex I) and antimycin A (complex III) were added to promote hydroperoxide production, the mitochondria from rats fed the alternative diet presenting lower H_2O_2 production. The generation of H_2O_2 by succinate-respiring mitochondria (Fig. 6b) was significantly lower for rats fed the modified diet as compared to

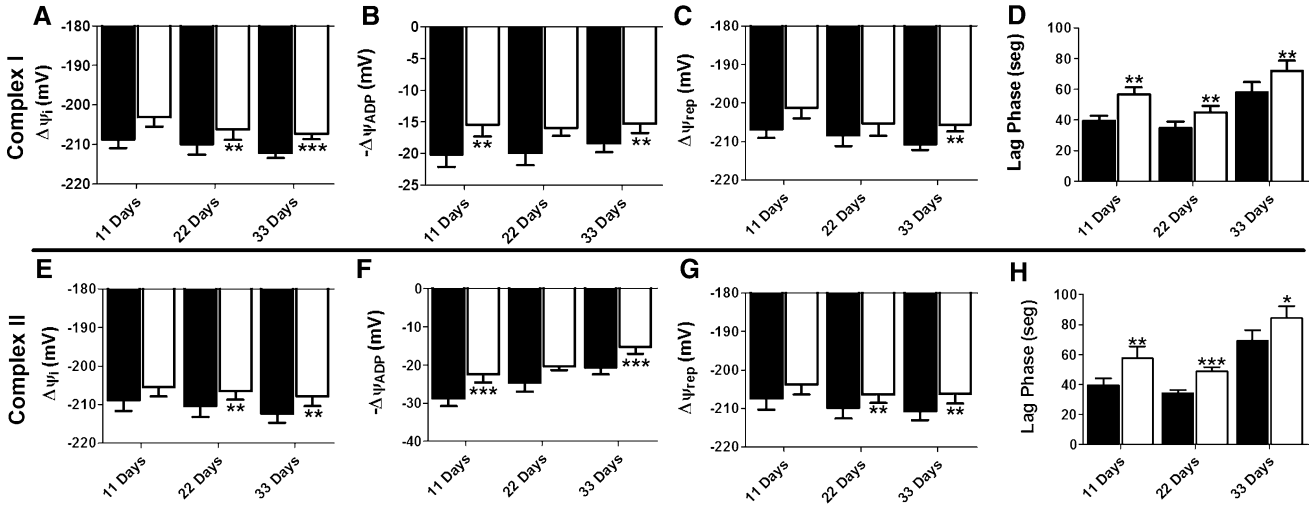


Fig. 4 Mitochondrial transmembrane electric potential studies with liver mitochondria from rats fed a control diet (*black bars*) or a diet containing 20 % rapeseed oil (*white bars*). Substrates used for the respiratory studies were a mixture of glutamate plus malate (Complex I) or succinate (Complex II). The parameters presented are initial potential developed upon substrate addition (**a** and **e**), depolarization induced by ADP (**b** and **f**), potential after repolarization (**c** and **g**) and the lag phase of the repolarization (**d** and **h**). Values depicted are mean ± standard error of mean for 7 animals (11 days), 8 animals (22 days) and 15 animals (33 days). Comparisons were performed using one-way ANOVA, with the Student–Newman–Keuls as a posttest for control diet rats versus rats fed the modified diet. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

the control group, but no significant differences between groups were found upon addition of the aforementioned respiratory inhibitors. Total hepatic tissue vitamin E content was lower in the diet-manipulated group (Fig. 6c), although no differences between groups were found for total tissue MDA, a marker of lipid peroxidation (Fig. S6A) and for protein carbonyl groups (Fig. S6B and C), regardless the treatment time.

Discussion

The present study is a step ahead in the current knowledge concerning the mitochondrial effects of a rapeseed oil-based diet in terms of modification of mitochondrial physiology. Our choice of a rapeseed oil-containing diet was based on the fact that this diet, which has an important and significant role in human diet, was already demonstrated to promote changes in terms of lipid composition in heart mitochondria (Innis and Clandinin 1981b). Many studies regarding the effects of rapeseed oil-enriched diets on membrane composition and function (Dewailly et al. 1977; Innis and Clandinin 1981a, b; Renner et al. 1979) were triggered by the correlation between longtime exposure to these diets and rat heart lesions, including necrotic foci, aggregations of mononuclear cells and an increase in the connective tissue elements. These adverse effects were attributed to erucic acid, a major constituent in rapeseed oil (Abdellatif and Vles 1970). Interestingly, other studies point exactly in the opposite direction. Animals fed a rapeseed oil-enriched diet displayed reduced mortality rate and infarct size and an increased probability of spontaneous reperfusion in the postischemic period (Nguemeni et al. 2010). In this case, the content in α-linolenic acid would be responsible for most of the protective effects. Another study reported that regular intake of optimized rapeseed oils may help improving lipid status and prevent oxidative stress (Attorri et al. 2010). These studies underline the potential cardioprotective action of rapeseed oils as a functional food supplement, although the conflicting results require a further look into this subject. Specific composition of rapeseed oil and general differences that may arise from the refining techniques used percentage of oil in the diet and feeding protocols may, at least partly, account for such dissonant reports.

In the present work, Wistar-Han rats fed a 20 % rapeseed oil diet for 33 days did not show altered biochemical plasma markers or internal lesions, in agreement with previous studies (Ohara et al. 2008). Extra attention was given to the hearts of these animals, which showed increased weight in relation to total animal weight (Table S3). In fact, the increase in heart-to-total body weight seems to be a consequence of the lower body weight reported for the animals, rather than reflecting an increase in the organ weight itself. The difference in animal weight gain during the treatment is probably related to the decrease in the consumption of the modified diet. As a result of the calorie adjustment, the absence of sucrose in the modified diet probably

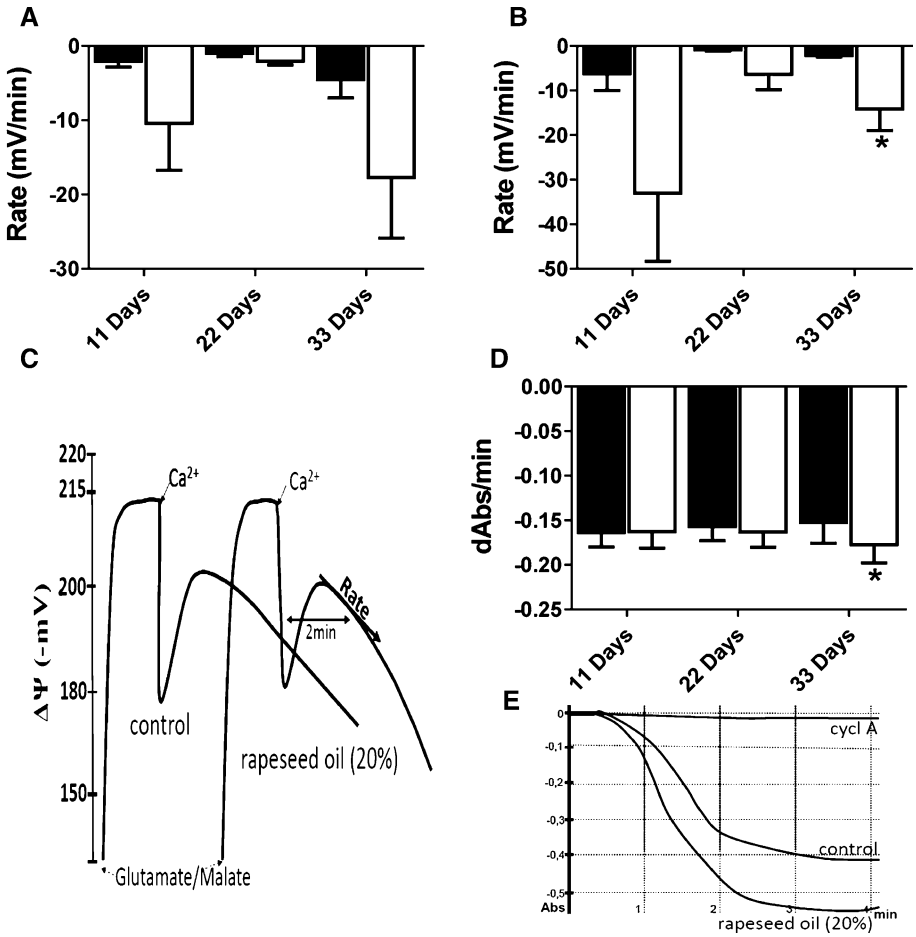


Fig. 5 Calcium-induced mitochondrial permeability transition determined in liver mitochondria from rats fed a normal diet (*black bars*) or a diet containing 20 % rapeseed oil (*white bars*). Substrates used were glutamate plus malate (**a** and **c**) or succinate (**b**, **d** and **e**). In **a** and **b**, MPT induction was assessed from the rate of the depolarization induced by Ca^{2+}. Typical traces for Ca^{2+}-induced mitochondrial permeability transition, including information about how the rate of depolarization was determined, as followed with a TPP^{+} electrode, are presented in C (for 33 days, where the most significant differences occur). The rates of absorbance decrease (dAbs/dt) as a result of mitochondrial swelling are shown in **d**; **e** depicts typical absorbance traces for mitochondrial swelling experiments performed after 33 days of dietary treatment (point at which the most significant changes are found). Values depicted are mean ± standard error of mean for 7 animals per parameter/trace shown. Comparisons were performed using one-way ANOVA, with the Student–Newman–Keuls as a posttest for control diet rats versus rats fed the modified diet. $***P < 0.001$; $**P < 0.01$; $*P < 0.05$

makes the rats less avid to this particular diet. It was also reported that Wistar rats fed a high-content fat diet for up to 2 months were able to counteract obesity development by parallel increase in energy expenditure (Iossa et al. 2000). Therefore, it was unlikely that the high-fat modified diet used in the present study, which was calorically adjusted to the control one, would result in weight gain.

Phospholipid composition found for the control group is coherent with previous reports for liver mitochondria from male Wistar rats (de Kroon et al. 1997; Osman et al. 2011). The most noticeable diet-induced alteration in mitochondrial membrane lipid composition was a significant shift in the PC/PE ratio, described previously for heart homogenates and heart mitochondria as a consequence of oils that included a significant amount of erucic acid, including rapeseed oil (Blomstrand and Svensson 1975). The main differences found in terms of fatty acid composition after 33 days of diet consumption were a decrease in stearic and linoleic acid content and an increase in oleic acid content, besides the appearance of previously undetected fatty acids (Table S4). Our results seem to be in agreement with studies (Innis and Clandinin 1981a, b; Ramsey et al. 2005) showing diet-induced alterations of the mitochondrial membrane lipid composition in a manner that loosely reflects the composition of the dietary fat source. Alterations in membrane phospholipid classes could result from an adaptation mechanism to counteract changes in the pool of fatty acids available for phospholipid de novo synthesis, in order to preserve adequate membrane physical properties (Innis and Clandinin 1981b). Accordingly, the aforementioned

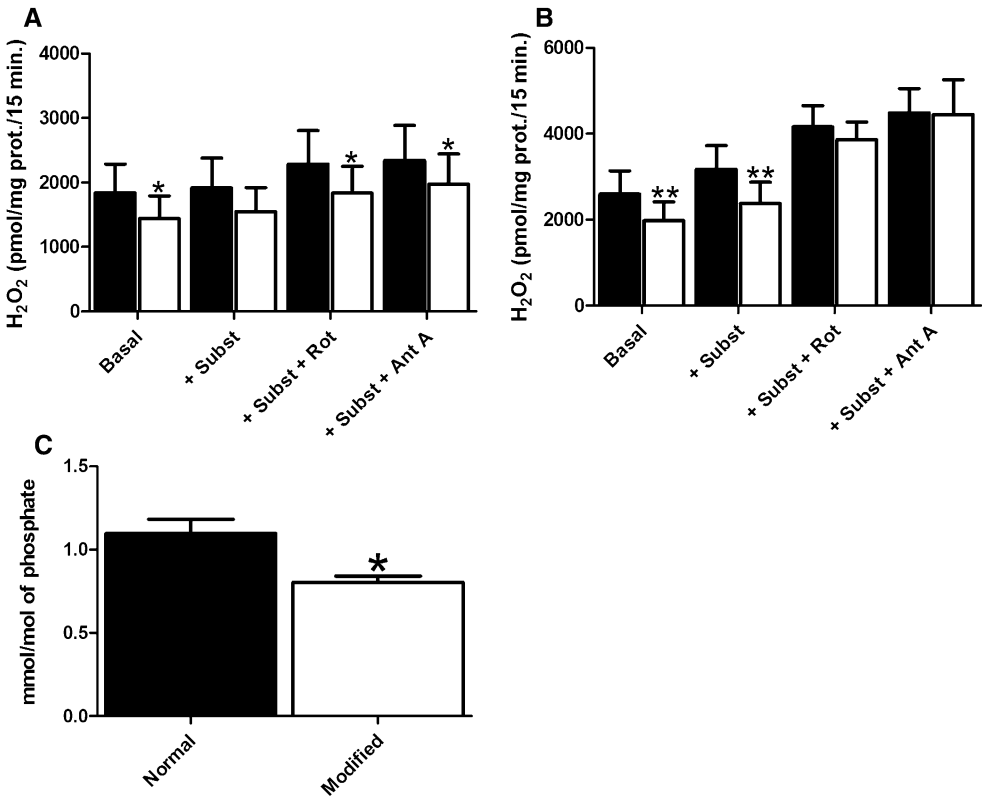


Fig. 6 Determination of oxidative stress markers: hydroperoxide generation in liver mitochondria (**a** and **b**) and Vitamin E content in hepatic tissue (**c**) from rats fed a control diet (*black bars*) or a diet containing 20 % rapeseed oil (*white bars*). Hydroperoxide production was determined in the absence (Basal) or presence (Subst) of respiratory substrate (glutamate/malate mixture—**a**, or succinate—**b**) and in the presence of substrate plus a respiratory inhibitor, rotenone (Rot) or antimycin A (Ant A). Values depicted are mean ± standard error of mean for 7 animals sacrificed 33 days after the onset of the treatment. Comparisons were performed using one-way ANOVA, with the Student–Newman–Keuls as a posttest for control diet rats versus rats fed the modified diet. $^{**}P < 0.01$; $^{*}P < 0.05$

enrichment in PC at the expense of PE observed in mitochondrial membranes from rats fed the modified diet (Table S4) may represent a compensation for the increase in the unsaturated/saturated fatty acid ratio (Fig. S2), tending to counteract possible membrane curvature stress promoted by an increase in unsaturated PE species (Gruner 1985; Israelachvili et al. 1980). Alterations in CL fatty acid composition in rat-fed diets containing rapeseed oil have been previously reported (Blomstrand and Svensson 1975; Dewailly et al. 1977). In our work, $(C18:1)_2$ $(C18:2)_2$ CL species was the most abundant in mitochondria from rats fed the modified diet, whereas the (C18:1) $(C18:2)_3$ CL species predominated in control rats. The lower incorporation of linoleic acid (and higher of oleic acid) in the major CL species found in liver mitochondria from modified diet-fed rats (as compared to control rats) should reflect the decrease in this fatty acid (accompanied by an increase in oleic acid) detected in the lipid extracts from those mitochondria. On the other hand, this change in the major CL species should lead to a decrease in the content of mitochondria oxidizable CL for rats fed the modified diet. Such alterations may impact mitochondrial functions, including signaling to apoptosis (Kagan et al. 2009) or regulation of oxidative phosphorylation fluxes (Claypool 2009).

Putative diet effects on the expression of mitochondrial proteins were then investigated (Fig. 2 and S3). A decrease in the content in some relevant proteins involved in ATP production, such as ANT (Fig. 2), was found in rats fed the modified diet. It is possible that the decreased protein content contributed, at least in part, for alterations in mitochondrial oxygen consumption and transmembrane electric potential (Figs. 3, 4). Dietary effects on mitochondrial bioenergetics (Fig. 3) were similar to those previously described for cardiac mitochondria isolated from chick-fed diets containing rapeseed oils, including a reduction in the respiratory efficiency and decreased ADP/O ratio (Clandinin 1978; Renner et al. 1979). Other possible cause for altered mitochondrial oxidative phosphorylation may reside in CL content changes. Although not required for oxidative phosphorylation, CL contributes to the efficiency of this process under optimal conditions by stabilizing respiratory supercomplexes (Claypool 2009). Integrating all data concerning CL, and given its pivotal importance for mitochondrial physiology, it is reasonable to predict that the diet-induced small decrease in CL content accompanied by reconfiguration of cardiolipin molecules (Table S4 and Fig. 1a) might induce disturbances reflected in bioenergetic alterations. Since similar alterations were induced by the diet in mitochondrial respiration regardless of the respiratory substrates used (glutamate + malate or succinate), the primary cause of those alteration seems to be lipidic in nature, although an effect downstream from complex II could be an alternative explanation.

Liver mitochondria from rats fed the 20 % rapeseed oil diet also displayed higher sensitivity to calcium-induced permeability transition (Fig. 5). Previous reports have shown that dietary-induced manipulation of membrane phospholipids and polyunsaturated fatty acids can alter the flux of Ca^{2+} across the mitochondrial membrane (Pepe et al. 1999), and variation of unsaturated/saturated fatty acid ratio in rat liver mitochondria lipid composition can modulate swelling properties (Cavalcanti et al. 1996; Haeffner and Privett 1975). Accordingly, we observed a decrease in the saturated/unsaturated ratio (Fig. S2) in parallel with an increased susceptibility to calcium-induced swelling (Fig. 5) in mitochondria from rats fed the modified diet. From all MPT pore involved proteins analyzed, only ANT showed to have been altered for the early time point, although no significant alterations in MPT induction were found at that time point. Thus, it is unlikely that alterations in ANT (Fig. 2) or cyclophilin D (Fig. S3) have been responsible for alterations in MPT induction.

Alterations of mitochondrial oxidative stress markers (Fig. 6) surprisingly indicated a decreased production of peroxides by the respiratory chain, regardless the absence or presence of respiratory inhibitors, whose mechanism is presently unknown. The small, but significant decrease in tissue vitamin E content (Fig. 6) may be related to diminished food consumption in the diet-modified group. Previous works refer high-fat diets to have a similar effect to that reported in our study, decreasing mitochondrial H_2O_2 production in liver mitochondria from mice (Català-Niell et al. 2008). Fish oils were reported to also decrease hydroperoxide production in rat liver mitochondria in opposition to corn oil (Ramsey et al. 2005). However, since our results also point to an increase in fatty acid unsaturation, potential positive effects of rapeseed oil in animal life span resulting from decreased ROS might be counterbalanced by the increased susceptibility to lipid peroxidation (Faulks et al. 2006).

The present study shows that dietary-induced changes in mitochondrial membrane composition take place quite rapidly and alter bioenergetics. Since time points longer than 33 days were not studied, it remains to be determined whether a longer-term treatment would contribute to a progressive alteration in the mitochondrial protein content counteracting lipid-induced alterations in mitochondrial activity.

A pertinent question is whether the alterations observed in mitochondrial bioenergetics are a secondary adaptation or a direct consequence of mitochondrial membrane alterations. This is clearly not easy to distinguish, but we anticipate that mitochondrial membrane alterations may directly result in altered enzyme activity and in a secondary remodeling of several energy-producing systems to guarantee the same production of ATP.

One limitation of the present study is the different calorie content in both diets. The control diet had a kcal/kg ratio of about 4,040, while the modified diet presented 4,375 kcal/kg (Table S2), according to the vendor. Taking into account the rat daily diet intake is much less than 1 kg, we believe that differences in daily calorie intake are diluted. A simple empirical exercise shows us that at the last week of treatment (when more experiments were conducted and more data acquired), diet consumption in the modified diet group was 93.3 % of the control group (Fig. S1B). Considering that the modified diet has 108.3 % the calorie content of the control diet, we can consider that the lower diet consumption in the modified diet group actually counteracted the slightly higher calorie density in that diet. Therefore, we strongly argue that differences in the calorie intake were not the primary cause of the alterations found in rats fed the modified diet.

In conclusion, the present study shows that a rapeseed oil-based diet results in alterations in mitochondrial membrane composition and bioenergetics. If, on one hand, this warns that caution is needed in the use of this oil as a potential cardiovascular protector, on the other hand, diet-induced alterations, including those at the membrane level, open new doors to research toward the use of this and other diets to antagonize/synergize drug-induced mitochondrial pharmacological/toxicological effects. This fact may also serve as one of many explanations for the nature of idiosyncratic drug-induced liver injury.

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