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Protective effects of dietary avocado oil on impaired electron transport chain function and exacerbated oxidative stress in liver mitochondria from diabetic rats

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Abstract Electron transport chain (ETC) dysfunction, excessive ROS generation and lipid peroxidation are hallmarks of mitochondrial injury in the diabetic liver, with these alterations also playing a role in the development of non-alcoholic fatty liver disease (NAFLD). Enhanced mitochondrial sensitivity to lipid peroxidation during diabetes has been also associated to augmented content of C22:6 in membrane phospholipids. Thus, we aimed to test whether avocado oil, a rich source of C18:1 and antioxidants, attenuates the deleterious effects of diabetes on oxidative status of liver mitochondria by decreasing unsaturation of acyl chains of membrane lipids and/or by improving ETC functionality and decreasing ROS generation. Streptozocin-induced diabetes elicited a noticeable increase in the content of C22:6, leading to augmented mitochondrial peroxidizability index and higher levels of lipid peroxidation. Mitochondrial respiration and complex I activity were impaired in diabetic rats with a concomitant increase in ROS generation using a complex I substrate. This was associated to a more oxidized state of glutathione, All these alterations were prevented by avocado oil except by the changes in mitochondrial fatty acid composition. Avocado oil did not prevented hyperglycemia and polyphagia although did normalized hyperlipidemia. Neither diabetes nor avocado oil induced steatosis. These results suggest that avocado oil improves mitochondrial ETC function by attenuating the deleterious effects of oxidative stress in the liver of diabetic rats independently of a hypoglycemic effect or by modifying the fatty acid composition of mitochondrial membranes. These findings might have also significant implications in the progression of NAFLD in experimental models of steatosis.

Keywords Diabetes · *Persea americana* · Respiratory chain · Liver disease · Fatty acids · Oxidative damage

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Introduction

Diabetes is characterized by a generalized state of ROS overproduction, leading to oxidative damage in biomolecules and triggering signaling pathways that ultimately lead to the development of diabetic complications in target organs (Giacco and Brownlee 2010). In the liver of diabetic models, ROS overproduction is associated, along with other factors, to impaired function of the mitochondrial electron transport chain (ETC) (Lukivskaya et al. 2007; Satav and Katyare 2004;

Bouderba et al. 2012) and increased lipid peroxidation (Kristal et al. 1997). Moreover, diabetes is a risk factor for the development of nonalcoholic fatty liver disease (NAFLD), which includes the appearance of steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Musso et al. 2011). Diabetes and NAFLD shares features of mitochondrial dysfunction and oxidative stress, being lipid peroxidation highlighted as a key factor that activates signaling pathways leading to inflammation and fibrosis (Dey and Swaminathan 2010). On this basis, it has been hypothesized that protection of liver mitochondrial function from the deleterious effects of oxidative damage may be beneficial to slow the progression of NAFLD. Hence, the improvement of mitochondrial function and oxidative stress may also be valuable against hepatic injury in diabetes (Grattagliano et al. 2012).

Some efforts have been done to improve liver mitochondrial function and oxidative stress during diabetes through the use of compounds with antioxidant properties. For example, ursodeoxycholic acid, a secondary bile acid used for the treatment of cholestatic liver diseases, prevents the decrease in the mitochondrial content of reduced glutathione and the generation of intermediate products of lipid peroxidation in alloxan-induced diabetic rats with an improvement of mitochondrial respiration (Lukivskaya et al. 2007). Moreover, the administration of boldine, an alkaloid extracted from *Peumus boldus*, restores the activity of glutathione peroxidase and decreases lipid peroxidation and ROS generation in liver mitochondria from STZ-induced diabetic rats, although these effects might be related with the partial hypoglycemic effect of that alkaloid (Jang et al. 2000). These two studies exemplify the potential of natural products against the deleterious effects of diabetes on liver mitochondrial bioenergetics and oxidative stress, supporting its usefulness as a complimentary strategy to conventional pharmacological approaches to prevent and/or delay liver damage during diabetes.

Our group has recently reported the properties of avocado oil against the harmful effects of diabetes on mitochondrial oxidative stress and ETC function in kidney from STZ-induced diabetic rats (Ortiz-Avila et al. 2013). It was observed that diabetes inhibited the activity of the complex III due to impaired electron transfer at cytochrome $c+c_1$, causing increased ROS generation without changes in the levels of lipid peroxidation. 90-days of dietary avocado oil intake prevented these effects and augmented the resistance of the complex III to the inhibition by in vitro-induced oxidative stress. This was attributed to the wide variety of lipophilic antioxidants present in the oil (Ashton et al. 2006). Oleic acid (C18:1) is the main fatty acid in avocado oil, constituting 50–70 % of its total fatty acids (Ozdemir and Topuz 2004). Besides the favorable effects of monounsaturated fatty acids (MUFA) from avocado on serum lipid profile and cardiovascular risk (Alvizouri-Muñoz et al. 1992; Carranza et al. 1995), MUFA sources like olive oil decreases oxidative damage in some components of the ETC from liver mitochondria by increasing MUFA content of mitochondrial membranes and enhancing its resistance to peroxidative damage (Quiles et al. 2006).

In view of the role of mitochondrial ROS generation and lipid peroxidation in liver damage during diabetes, the high content of MUFA and antioxidants in avocado oil and its protective effect against kidney mitochondrial dysfunction in diabetic rats, the main goal of this study was to explore whether avocado oil consumption decreases mitochondrial oxidative stress in liver mitochondria of diabetic rats in association with improved function of the ETC, lower ROS generation and remodeling of the fatty acid composition of liver mitochondrial membranes. In addition, we explored whether avocado oil promotes hepatic steatosis in control and diabetic rats.

Materials and methods

Animals and experimental design

Male Wistar rats weighing 300–350 g were used and kept in a room under controlled temperature and cycles of 12 h light/dark. Animals were feed with a standard rodent diet and water ad libitum. For rat care, we followed the recommendations of the Mexican Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico). This research was also approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo. Diabetes was induced by intraperitoneal administration of STZ (45 mg/kg) to rats subjected to 12-h fasting. Five days after STZ injection, blood glucose levels were determined and the rats exhibiting glucose levels higher than 300 mg/dL were considered diabetic. Control rats were treated in the same way except that it were administered only with vehicle.

After the determination of glucose levels, the animals were randomly assigned to 4 groups of 8 rats each: Group 1, consisting of normoglycemic rats feed only with rodent diet; group 2, consisting of diabetic rats feed only with rodent diet; group 3, consisting of normoglycemic rats feed with rodent diet plus avocado oil; group 4, consisting of diabetic rats feed with rodent diet plus avocado oil. Avocado oil was orally administered daily at a dose of 1 mL/250 g weight during 90 days, using a bottled, commercial presentation of avocado oil (Ahuacatlan, DIRI COM, S.A. de C.V., México), purchased from a local grocery. The fatty acid composition of this oil was assessed by gas chromatography and consisted of 5.92±0.1 % C16:0, 0.2±0.02 % C16:1, 1.86±0.02 % C18:0, 55.9 %±0.2 C18:1, 24.9±0.1 % C18:2, 10.1±0.1 % C18:3 and 0.92±0.1 % of a non-identified fatty acid. Food and water intake, as well as the weight of the

animals, were recorded before the beginning and at the end of the treatment with avocado oil.

Determination of serum glucose and lipids

The animals were fasted 12 h before the sacrifice, after which blood was recollected and serum was obtained for the enzymatic determination of glucose and total cholesterol and triglycerides with kits from BioSystems (Barcelona, Spain), according to the manufacturer's instructions.

Histological evaluation of liver

Immediately after the sacrifice of the animals, liver sections were fixed in 10 % formalin, embedded in paraffin blocks and sectioned for hematoxylin-eosin staining in glass slides. Preparations were blindly examined by an independent researcher by light microscopy for evaluation of steatosis, lobular inflammation and hepatocyte ballooning, following the criteria of the nonalcoholic fatty liver disease activity score (NAS) described by Kleiner et al. (2005).

Isolation of mitochondria

Mitochondria were isolated by differential centrifugation of liver homogenates as described elsewhere (Saavedra-Molina and Devlin 1997). The mitochondrial pellets were stored at −80 °C until used, except for assays of mitochondrial respiration, where mitochondria were used immediately after isolation. Protein concentration was assessed before each assay by the Biuret method.

Determination of mitochondrial fatty acid composition

Lipids from mitochondrial samples were obtained by the method of Bligh and Dyer (1959) and the resultant fatty acids were derivatized for its analysis according to the method of Morrison and Smith (1964). Subsequently, the methyl-esters of fatty acids were analyzed by gas chromatography under the conditions previously described by Ortiz-Avila et al. (2013).

Evaluation of lipid peroxidation levels

This determination was carried out in 0.1 mg/mL of mitochondrial protein by measuring the levels of thiobarbituric acid reactive substances (TBARS), according to the protocol of Buege and Aust (1978). To avoid false positive results due to the interaction of thiobarbituric acid with the carbohydrates present in mitochondria isolation buffers, mitochondrial pellets were washed twice and resuspended with 50 mM KH_2PO_4 buffer (pH 7.6) immediately before TBARS assays. In experiments where the sensitivity to in vitro lipid peroxidation was tested, mitochondria were incubated before the assay during 30 min with Fe^{2+} μM in 50 mM KH_2PO_4 buffer at the concentrations indicated in the Fig. 5b.

Evaluation of oxidative stress

This parameter was estimated by measuring the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). First, to measure the concentration of total glutathione (GSH+GSSG), mitochondrial samples (0.1 mg/mL) were deproteinized through the addition of 5 % sulfosalicilic acid and centrifuged at 7840 g during 10 min. Then, the pellet was discarded and 90 μL of the supernatant were added to a reaction mixture containing 0.1 M phosphate buffered solution, 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 M EDTA and 0.115 U/mL glutathione reductase. After 5 min of incubation at room temperature, 2 mM NADPH was added and DTNB reduction was followed spectrophotometrically at 412 nm in a Shimadzu UV2550 spectrophotometer, being the rate of DTNB reduction directly proportional to the concentration of GSH (Akerboom and Sies 1981). GSSG was determined by the procedure described above, except that GSH was derivatized with 3 % 4-vinylpyridine during 60 min. Finally, the amount of reduced glutathione was obtained by subtracting the concentration of GSH+GSSG minus the concentration of GSSG.

Measurement of ROS production

ROS generation was determined by evaluating the oxidation of the ROS probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) into the fluorescent compound 2′,7′-dichlorofluorescein (DCF) after its intramitochondrial hydrolysis into dichlorodihydrofluorescein (H_2DCF). Briefly, 0.5 mg/mL freeze-thawed mitochondrial protein and 500 mM H_2DCFDA were added to a buffer containing 100 mM KCl, 10 mM HEPES, 3 mM KH_2PO_4 and 3 mM $MgCl_2$ (pH 7.4) and incubated during 20 min with shaking at 4 °C. This mixture was placed in a quartz cuvette and basal fluorescence was recorded in a Shimadzu RF5301PC spectrofluorometer (λ_{ex} 491 nm; λ_{em} 518 nm). After 1 min, 10 mM glutamate-malate (Complex I-linked ROS) or 10 mM succinate plus rotenone (Complex II-linked ROS) were added and the changes in H_2DCF fluorescence were further followed by 20 min. Alternatively, to catalyze the reaction of H_2DCF with the oxidants produced by the activity of the ETC, mitochondria were incubated during 30 min with 25 μM of Fe^{2+}. All the determinations were done at room temperature. The results were expressed as the difference of the fluorescence (ΔF) in arbitrary units, calculated by subtracting the maximum fluorescence reached 20 min after substrate addition minus the basal fluorescence (i.e., the fluorescence before substrate addition).

Determination of the activities of the ETC complexes and mitochondrial respiration

To evaluate the activities of ETC complexes, mitochondria were solubilized with Triton X-100 (Hallberg et al. 1993) before each assay in order to enhance the accessibility of substrates and inhibitors to the redox sites of the ETC complexes. Complex I activity was assayed by a modification of the technique reported by Chomova et al. (2012). Briefly, 0.1 mg/mL solubilized mitochondria were resuspended in 1 mL 50 mM KH_2PO_4 buffer and incubated with 1 μg antimycin A plus 1 mM KCN. After 5 min, 5 mM $K_3Fe(CN)_6$ was added and absorbance was followed during 1 min at 340 nm in a Shimadzu UV2550 spectrophotometer. Then, NADH was added and its oxidation was measured during 4 min. The rate of NADH oxidation was calculated using a molar extinction coefficient of 16.3 mM^{-1} cm^{-1} for NADH. No changes in absorbance were detected in the absence of NADH. The activities of succinate-DCIP oxidoreductase (complex II) and antimycin A-sensitive succinate-cytochrome *c* oxidoreductase (complex III), were determined as described previously (Ortiz-Avila et al. 2013), while cytochrome *c* oxidase (complex IV) activity was evaluated by the protocol described by Cortés-Rojo et al. (2009).

Mitochondrial respiration was assayed polarographically in intact, freshly isolated mitochondria with a Clark-type electrode connected to a YSI5300 biological oxygen monitor and a computer for data acquisition. 0.5 mg/mL mitochondrial protein was placed in a sealed glass chamber with constant stirring containing 2 mL respiration buffer (100 mM KCl, 10 mM HEPES, 3 mM KH_2PO_4, 3 mM $MgCl_2$, pH 7.4). 10 mM glutamate-malate was added as substrate to start oxygen consumption registration in basal, state 2. After 5 min, 300 μM ADP was added to stimulate state 3 respiration and oxygen consumption was further registered during 5 min.

Evaluation of F_1F_0-ATPase activity

This activity was estimated by quantifying the release of phosphorus from ATP hydrolysis following the methodology described by Fiske and SubbaRow (1925). To distinguish the phosphorus released by F_1F_0-ATPase activity from the phosphorus from other sources, this experiment was also carried out in mitochondria pre-treated before ATP addition with the F_1F_0–ATPase inhibitor oligomycin (50 μg) as a negative control. The phosphorous released by the activity of the enzyme was calculated by subtracting the concentration of phosphorous in the presence of oligomycin to that obtained in the absence of the inhibitor.

Statistical analyses

Results are expressed as the mean±standard error. Statistical differences of the data were determined with Student's *t* test using Sigma Plot software v11.0. Statistically significant differences were defined as $P<0.05$.

Results

Effects of diabetes and avocado oil on physiological parameters and liver histology

At the end of the treatments, glucose levels of STZ-induced diabetic rats were 7.8-fold higher than in control rats. Avocado oil supplementation did not have any effect on this parameter, although normoglycemic rats treated in the same way exhibited a decrease of 23.8 % in glucose levels with respect to control animals (Fig. 1a). Diabetic rats also developed hyperlipidemia as serum levels of cholesterol and triglycerides were 1.3- and 4.4-fold higher, respectively, than in control rats, with avocado oil normalizing these alterations (Fig. 1b and c). Moreover, avocado oil did not modify the levels of these lipids in control rats.

Signs of polyphagia and polydipsia were detected in diabetic rats at the beginning of the treatment since food and water intake were almost two and three-fold higher, respectively, in comparison to control animals (Table 1) and these differences remained constant at the end of the study. No changes in polyphagia and polydipsia were observed when avocado oil was supplemented in diabetic rats. Regarding to the body weight of the groups, diabetic rats exhibited a loss weight of 68 g at the end of the treatment, while the opposite was observed in control rats with a weight gaining of 163.3 g. The treatment with avocado oil induced an apparent attenuation in loss weight in diabetic animals, as loss weight was 34 g lower in diabetic animals treated with avocado oil, although the differences between the final values of weight of the diabetic group and the diabetic group plus avocado oil were not statistically significant. In the same way, avocado oil caused an apparent higher weight gaining in control rats, but the differences in the final weight between the rats from the control group and the control group plus avocado oil were not statistically significant.

Histological analyses were carried out in order to assess whether avocado oil intake induced liver injury during the experimental period tested. No signs of steatosis, hepatocyte ballooning or lobular inflammation were observed due to avocado oil treatment in control or diabetic rats. Moreover, diabetes did not induce histological alterations although the elevated levels of serum lipids and glucose in that group (Fig. 2). No alterations were detected even when microscopic analysis were done at higher magnification (Fig S1).

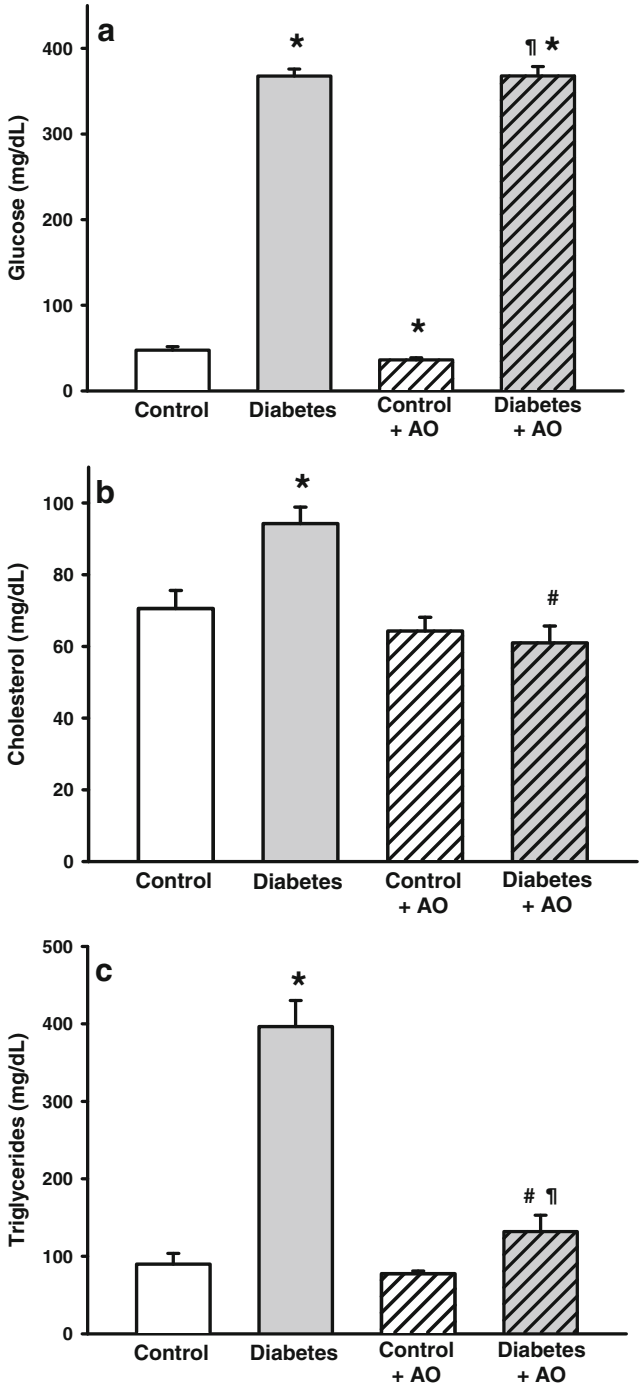


Fig. 1 Serum levels of glucose (**a**), cholesterol (**b**) and triglycerides (**c**) at the end of 90-days of treatment with avocado oil. The results are presented as the mean±S.E. of $n>5$. *$P<0.05$ vs. control; $^{¶}P<0.05$ vs. control + AO; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

Effects of diabetes and avocado oil on mitochondrial fatty acid composition and fatty acid indexes

Profound alterations in fatty acid composition were detected in diabetic rats (Table 2). Changes were detected in the content of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), with decrements of 10, 87.5 and 40.4 % in palmitic (C16:0), palmitoleic (C16:1) and oleic (C18:1) acids, respectively, while the content of stearic acid (C18:0) exhibited an increment of 33.9 %. Regarding to the changes in PUFA, the content of linolenic acid (C18:3) decreased 44.4 %, whereas among all the fatty acid species, docosahexaenoic acid (22:6) underwent the most dramatic change with an increment in 104.4 %. Avocado oil intake failed to prevent these alterations and no significant changes were neither observed in mitochondria from control animals consuming avocado oil.

Concerning to fatty acid indexes, increased C18:0 content and the diminution in both C16:1 and C18:1 led to a slight increase in the total content of SFA (Fig. 3a) and a ~50 % decrease in the total content of MUFA, respectively (Fig. 3b). In contrast, the percentage of total polyunsaturated fatty acid (PUFA) remained without changes despite the prominent increase in the percentage of C22:6 (Fig. 3c). Avocado oil did not have any effect on these parameters. On the other hand, peroxidizability index (PI), a parameter that estimates membrane sensitivity to lipid peroxidation on the basis of its fatty acid composition, was 21 % higher in mitochondria from diabetic rats than in mitochondria from control rats, irrespectively of avocado oil treatment (Fig. 3d).

Influence of avocado oil over the effects of diabetes on liver mitochondrial oxidative stress and lipid peroxidation

Mitochondrial GSH/GSSG ratios were assessed in order to determine the degree of oxidative stress in liver mitochondria in response to diabetes and avocado oil (Fig. 4a). Mitochondria from diabetic rats displayed exacerbated oxidative stress as the GSH/GSSG ratio was 2.2-fold lower than in control mitochondria. Avocado oil fully prevented this effect without having any influence in control mitochondria. Lower GSH/GSSG ratio induced by diabetes may not be attributed to impaired glutathione synthesis since there were no statistically significant differences in the concentration of total glutathione among all the experimental groups, except by the diabetic + avocado oil group, which exhibited a 19.2 % increment with respect to the diabetic group (Fig. 4b). Regarding to the levels of lipid peroxidation (Fig. 5), a similar behavior with respect to oxidative stress levels was observed, with mitochondria from diabetic rats displaying a two-fold increase in TBARS levels and avocado oil fully inhibiting this effect (panel a). Moreover, avocado oil also aided to prevent excessive TBARS production when mitochondria were challenged against an acute oxidative stimulus (Fig. 5b), since mitochondria from animals supplemented with avocado oil were several-fold less sensitive to TBARS generation at concentrations up to 100 μM Fe^{2+}, independently of diabetes.

Table 1 Effect of diabetes on food and water intake and weight before and after 90-days of avocado oil supplementation

		Control	Control + AO	Diabetes	Diabetes + AO
Weight	Before treatment	339.0±13.8	333.5±8.3	314.3±2.5	316.0±8.3
	After treatment	502.3±9.6	547.8±15.3	246.3±16.0*	282.0±19.3*
Food	Before treatment	16.5±0.8	18.3±2.3	32.9±2.4*	33.9±0.9*
	After treatment	17.7±1.7	19.6±1.0	36.0±0.8*	38.6±1.7*
Water	Before treatment	51.3±2.6	48.7±2.1	156.2±12.3*	166.1±9.4*
	After treatment	49.0±1.6	43.6±2.7	171.5±4.4*	166.6±2.0*

These parameters were determined 5 days after STZ injection, just before the start of the treatment with avocado oil and after 90-days of the treatment. Data are presented as the mean±S.E. of n=4. *P<0.05 vs. control (student's t-test)

Effects of avocado oil on ROS generation and ETC functionality

Aimed to test whether increased oxidative stress and lipid peroxidation are related to enhanced ROS production at the ETC, this parameter was measured in the presence of glutamate-malate, a complex I-linked substrate (Fig. 6a) or succinate, a complex II-linked substrate (Fig. 6b). In the first case, liver mitochondria from diabetic animals produced 1.7-fold more ROS than mitochondria from normoglycemic rats. Avocado oil intake inhibited ROS generation to the levels of control mitochondria. In contrast, when succinate was used as substrate (Fig. 6b), ROS generation was marginal in comparison with that observed with glutamate-malate (Fig. 6a) and no differences were observed between all the experimental groups. These experiments were also carried out in the presence of 25 μM Fe^{2+} to catalyze the reaction of the oxidants produced at the ETC with H_2DCF. As observed in the Fig. 6c and d, a similar trend was observed under this condition, although the levels of DCF fluorescence were 10-fold higher than in the absence of exogenously added Fe^{2+}, which reflects the catalyzing effect of Fe^{2+}.

The activity of the complexes from the ETC was determined to further characterizing the site of ROS generation in liver mitochondria of diabetic rats and the effects of avocado oil. As expected from the results of ROS generation with glutamate-malate (Fig. 6a), the activity of the complex I from mitochondria of diabetic animals was almost 50 % lower in comparison to the activity of mitochondria from control animals (Fig. 7a). Moreover, this effect was fully prevented by avocado oil supplementation. In contrast, complex II activity increased 1.6 times in the mitochondria from the diabetic rats and a similar effect was observed in mitochondria from control animals supplemented with avocado oil (Fig. 7b). On the contrary, the activities of the complexes III and IV (Fig. 7c and d, respectively), remained without significant changes.

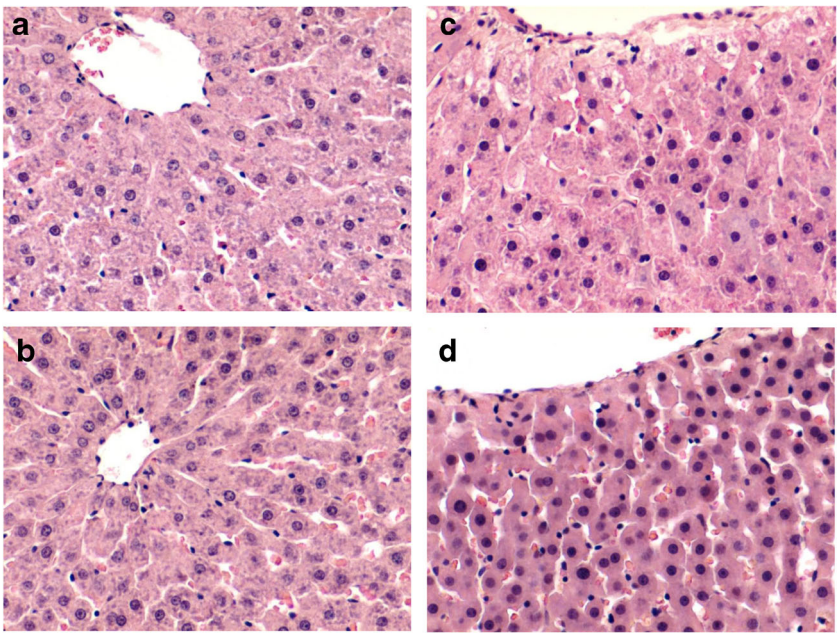


Fig. 2 Histological analyses by hematoxylin-eosin staining of liver sections of rats from control (**a**), Diabetes (**b**), control + AO (**c**) and diabetes + AO (**d**) groups. Pictures were taken at 400× magnification and are representative of n=4

Table 2 Effect of avocado oil on fatty acid composition of liver mitochondria from control and diabetic rats

	C16:0	C16:1	C18:0	C18.1	C18:2	γ-C18:3	C18:3	C20:4	C20:5	C22:1	C22:6	N.I.
Control	21.2±0.5	0.8±0.1	15.3±0.4	9.9±0.3	25.2±0.3	0.4±0.1	0.9±0.0	18.7±0.4	0.4±0.0	0.2±0.1	4.5±0.2	2.3±0.4
Diabetes	19.2±0.6*	0.1±0.1*	20.5±0.8*	5.9±0.2*	22.9±1.8	0.3±0.0	0.5±0.1*	18.0±0.7	0.2±0.1	0.4±0.3	9.2±1.2*	2.8±0.3
Control + AO	19.8±0.5	0.7±0.1	15.4±1.3	11.5±0.9	23.6±1.1	0.3±0.0	0.9±0.1	20.1±1.4	0.3±0.1	0.7±0.5	4.8±0.2	1.9±0.3
Diabetes + AO	19.2±0.2	0.2±0.1¶	20.6±0.8¶	6.3±0.2¶	22.3±1.0	0.3±0.0	0.4±0.0¶	17.5±0.7	0.2±0.1	0.1±0.1	10.3±0.4¶	2.6±0.4

Data are presented as the mean±S.E. of n=5. *P<0.05 vs control; ¶ P<0.05 vs control + AO (student's t-test)

Basal (state 2) and phosphorylating (state 3) states of respiration were evaluated in order to determine whether complex I inhibition have an impact in the overall function of the ETC and its ability to modulate oxygen consumption in response to exogenous ADP addition. Decreased respiration in both state 2 and state 3 was observed in mitochondria from diabetic rats when compared to control (Fig. 8a, gray line vs. black line, respectively, and Fig. 8b). As observed in the gray dashed trace, supplementation with avocado oil improved the decline in respiration observed in diabetic rats in both states, being statistically significant this effect only in state 3 (Fig. 8b). In contrast, avocado oil becomes mitochondria from control rats unresponsive to ADP addition (black dashed trace), although respiration rate in state 2 was similar to control mitochondria (Fig. 8b).

To test whether decreased stimulation of state 3 respiration was the result of impaired F_1F_0-ATP synthase in mitochondria from either diabetic rats or control rats supplemented with avocado oil, we measured the activity of this enzyme in the sense of ATP hydrolysis. As depicted in the Fig. 8c, ATPase activity remained unaltered in mitochondria from control rats treated with avocado oil; however, diabetes induced a ~40 %

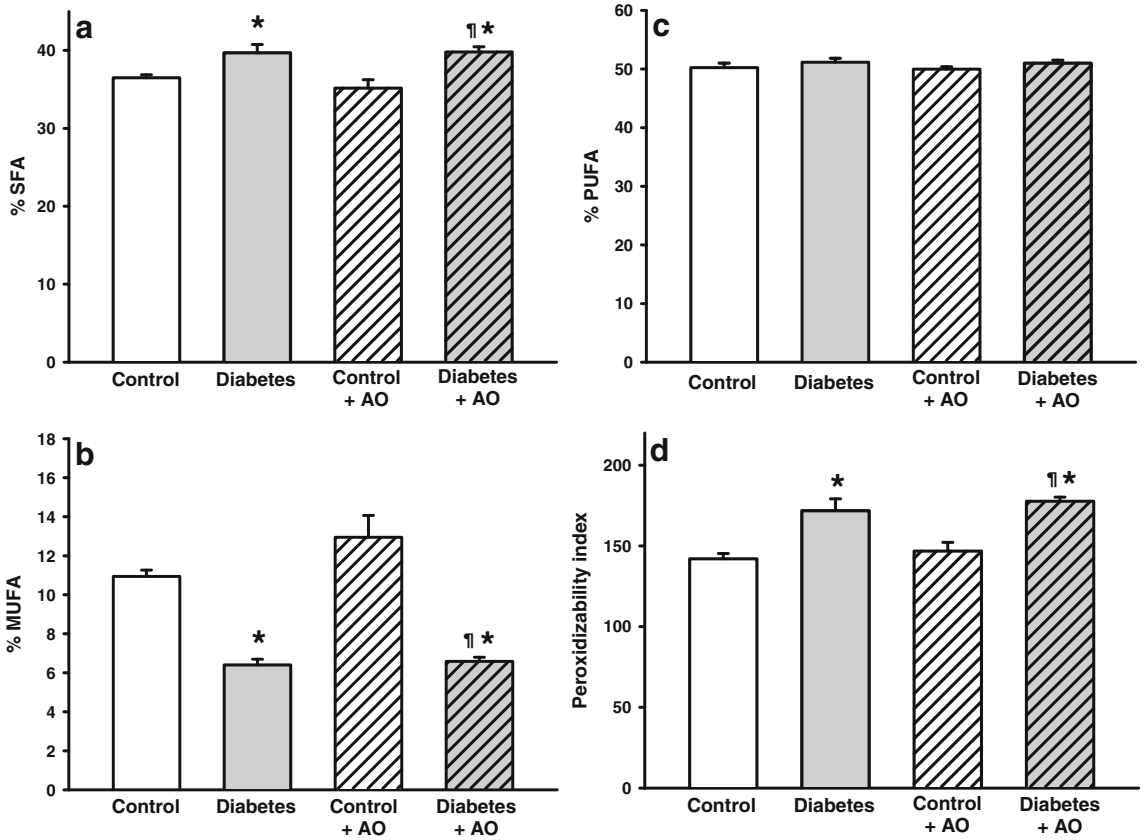


Fig. 3 Mitochondrial fatty indexes: Percentages of saturated fatty acids (SFA) (**a**), monounsaturated fatty acids (MUFA) (**b**), polyunsaturated fatty acids (PUFA) (**c**), and peroxidizability indexes (PI) (**d**). Indexes were calculated using the data from the Table 1. PI were calculated with the following equation reported by Pamplona et al. (1998): PI = [(%Monoenoic × 0.025) + (%Dienoic × 1) + (%Trienoic × 2) + (%Tetraenoic × 4) + (%Pentaenoic × 6) + (%Hexaenoic × 8)]. Data are presented as the mean±S.E. of n=5. *P<0.05 vs. control; ¶P<0.05 vs. control + AO (student's t-test)

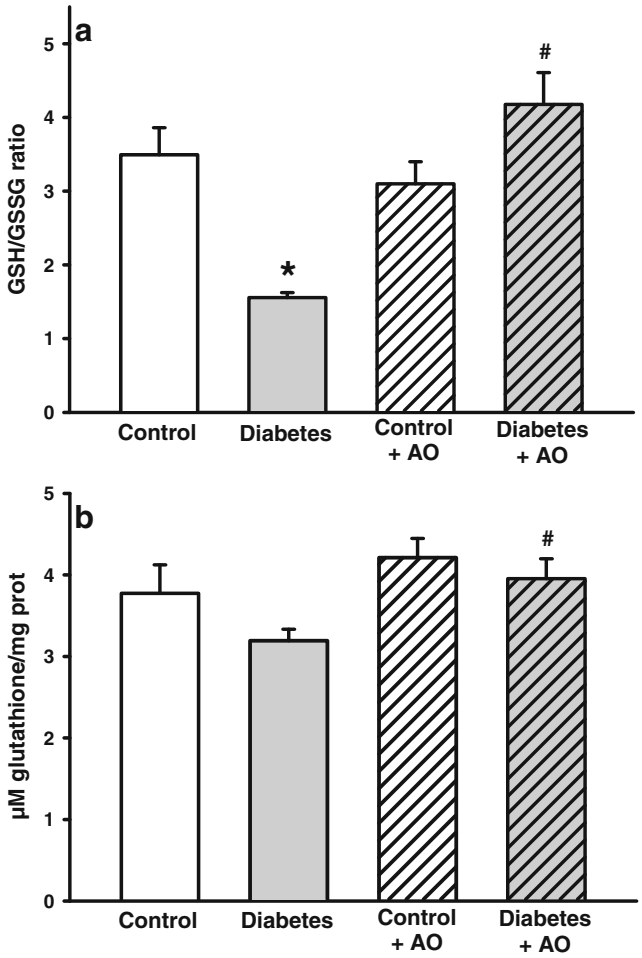


Fig. 4 Influence of avocado oil and diabetes on mitochondrial GSH/GSSG ratios (**a**) and total levels of glutathione (**b**). The results are presented as the mean±S.E. of n=4. *P<0.05 vs. control; $^{\#}P$<0.05 vs. diabetes (student's t-test)

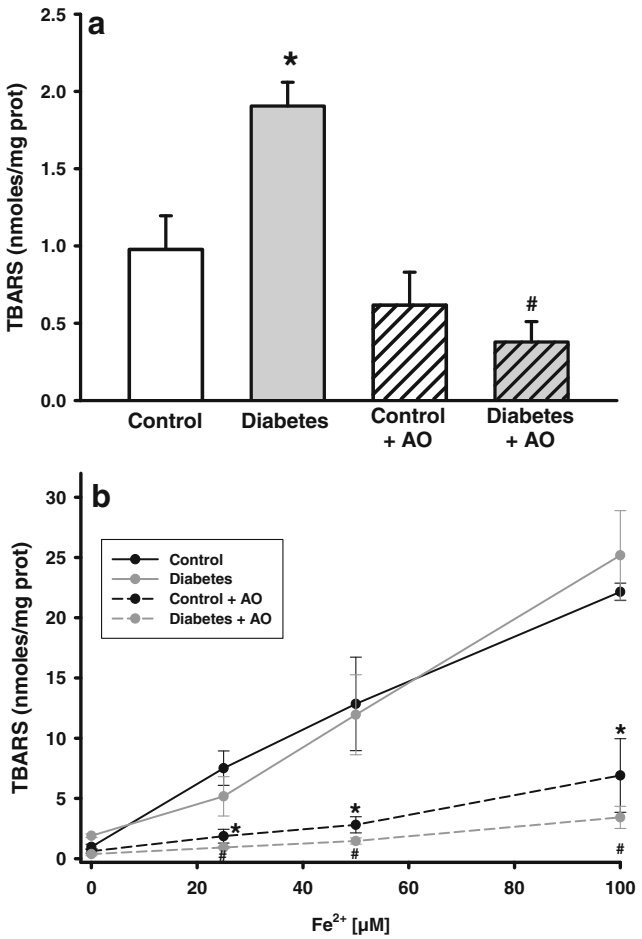


Fig. 5 Influence of avocado oil over the alterations induced by diabetes on mitochondrial lipid peroxidation. **a** Basal levels of lipid peroxidation. **b** Mitochondrial sensitivity to in vitro-induced lipid peroxidation with increasing concentrations of Fe^{2+} during 30 min. Data are represented as thiobarbituric acid reactive substances (TBARS) and presented as the mean±S.E. of n=4. *P<0.05 vs. control; $^{\#}P$<0.05 vs. diabetes (student's t-test)

increase in this activity, which was statistically significant only in mitochondria from the diabetic animals treated with avocado oil.

Discussion

Global prevalence and incidence of diabetes are in sharp growth, which has urged for the implementation of novel strategies for the management of this disease and their devastating complications (Danaei et al. 2011). Glycemic control is fundamental for the management of diabetes; however, due to the complexity of this disease, diabetes care is more complex and requires strategies beyond to only addressing glycemic control (Standards of medical care in diabetes-2014 2014). The results from this study shows that avocado oil intake did not improved hyperglycemia (Fig. 1a) and, consequently, polyphagia and polydipsia remained unaltered (Table 1). However, avocado oil intake decreased liver mitochondrial oxidative stress and improved ETC function in diabetic rats, which might be the result of the antioxidant actions of the components of avocado oil independently of glycemic control. Alternatively, since mitochondrial function and ROS production also depends on other processes like mitophagy or mitochondrial fusion/fission (Lee et al. 2012; Westermann 2012), the possibility remains that the antioxidants constituting avocado oil may modulate signaling pathways favoring these processes, as it has been shown that some components from avocado modify intracellular signaling independently of their antioxidants properties (D'Ambrosio et al. 2011).

Despite the lack of effects on hyperglycemia, avocado oil normalized the rise in serum lipids caused by diabetes (Fig. 1b and c). This is in concordance with the hypocholesterolemic and hypotriglyceridemic effects of diets enriched with avocado in subjects with type-2 diabetes (Lerman-Garber et al. 1994) or hypercholesterolemia (Carranza et al. 1995; López

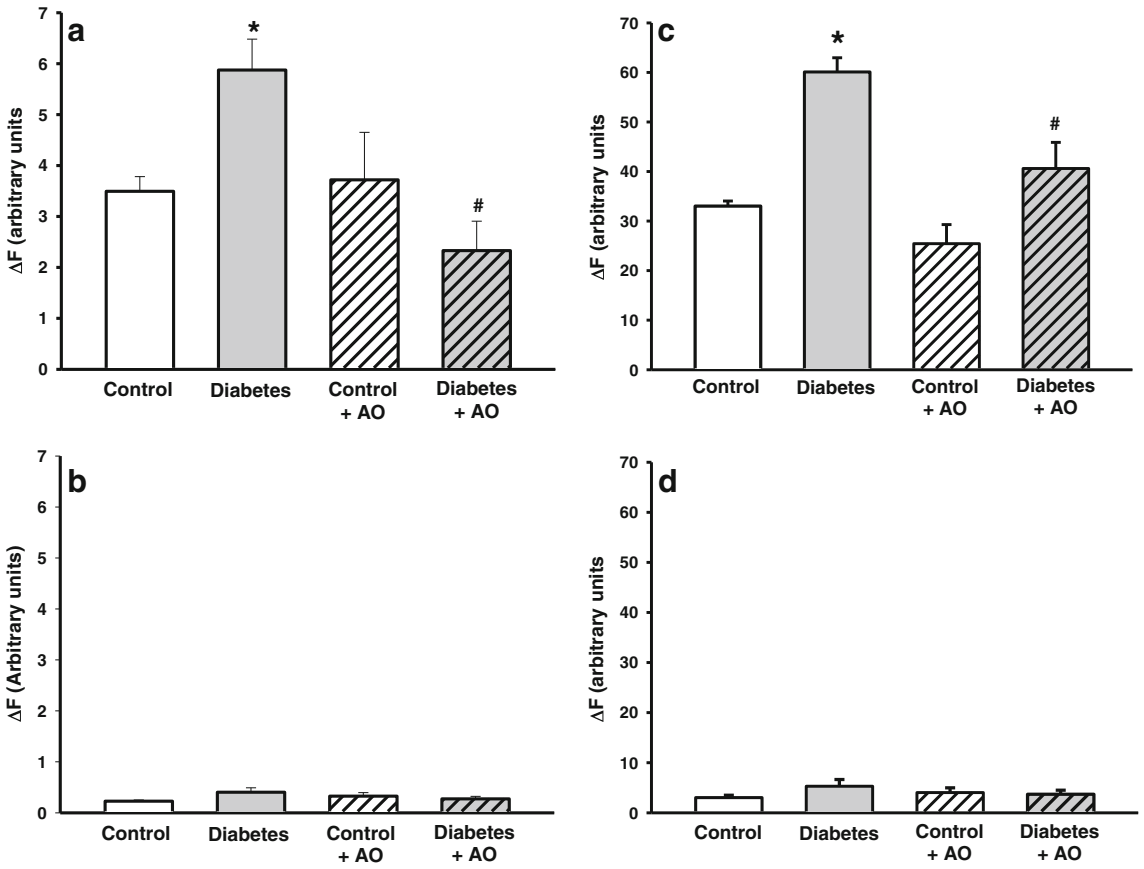


Fig. 6 Influence of avocado oil over the alterations induced by diabetes on mitochondrial ROS generation. H_2DCF oxidation was monitored in the absence (panels **a** and **b**) or the presence of 25 μM Fe^{2+} (panels **c** and **d**) to catalyze H_2DCF oxidation by ETC-generated oxidants. Glutamate-malate (panels **a** and **c**) or succinate (**b** and **d**) were used as substrates. Data are presented as the mean±S.E. of $n>3$. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

Ledesma et al. 1996). It has been demonstrated that C18:1 promotes intracellular cholesterol esterification and increased expression of low-density lipoprotein (LDL) receptors (Rumsey et al. 1995). Moreover, enriched diets with C18:1 decrease serum triglycerides in mice by a mechanism involving lower hepatic triglyceride production and inhibition of hormone-sensitive lipase (Kotake et al. 2004). Thus, as C18:1 constitutes more than 50 % of the fatty acids from avocado oil, increased LDL uptake and decreased hepatic triglyceride synthesis may be underlying causes of normalization of serum cholesterol and triglycerides, respectively, by avocado oil. The molecular mechanism of avocado oil for controlling lipids levels might be related with peroxisomes proliferator activated receptors (PPARs). It has been reported that C18:1 is capable to induce PPAR-δ (Wu et al. 2012), which has been associated with degradation of lipids by enhancing fatty acid catabolism (Wang et al. 2003). Another interesting characteristic of avocado oil is the relative high concentration of linolenic acid (around 10.1 %), an omega-3 fatty acid precursor of long chain fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fatty acids are widely recognized as PPARs activators with several effects on lipids metabolism, leading to increased fatty acid oxidation not only in liver but also in skeletal muscle, regulating in this way lipid concentrations on serum (Grygiel-Górniak 2014).

Another probable mechanism accounting for the effects of avocado oil on elevated lipids levels of diabetic rats might reside in the protective effects of avocado oil over the complex I activity of these animals. In this regard, utilization of triglycerides through mitochondrial β-oxidation requires a constant influx of NAD^+ to sustain the activity of the 3-hydroxyacyl-CoA dehydrogenase. NADH equivalents produced by this enzyme are re-oxidized by complex I and its inhibition lead to diminished β-oxidation flux and accumulation of 3-hydroxyacyl-CoA and carnitine esters (Eaton et al. 1994). Therefore, the rise in triglycerides levels detected in diabetic rats may be related with the inhibition of complex I activity (Fig. 7a), leading to limited β-oxidation and enhanced fatty acid efflux from liver to circulation. Therefore, it is possible to suggest that normalized complex I activity by avocado oil may enhance mitochondrial β-oxidation by favoring NADH reoxidation, leading to lowered levels of triglycerides as observed in the Fig. 1c. This is in agreement with the notion that

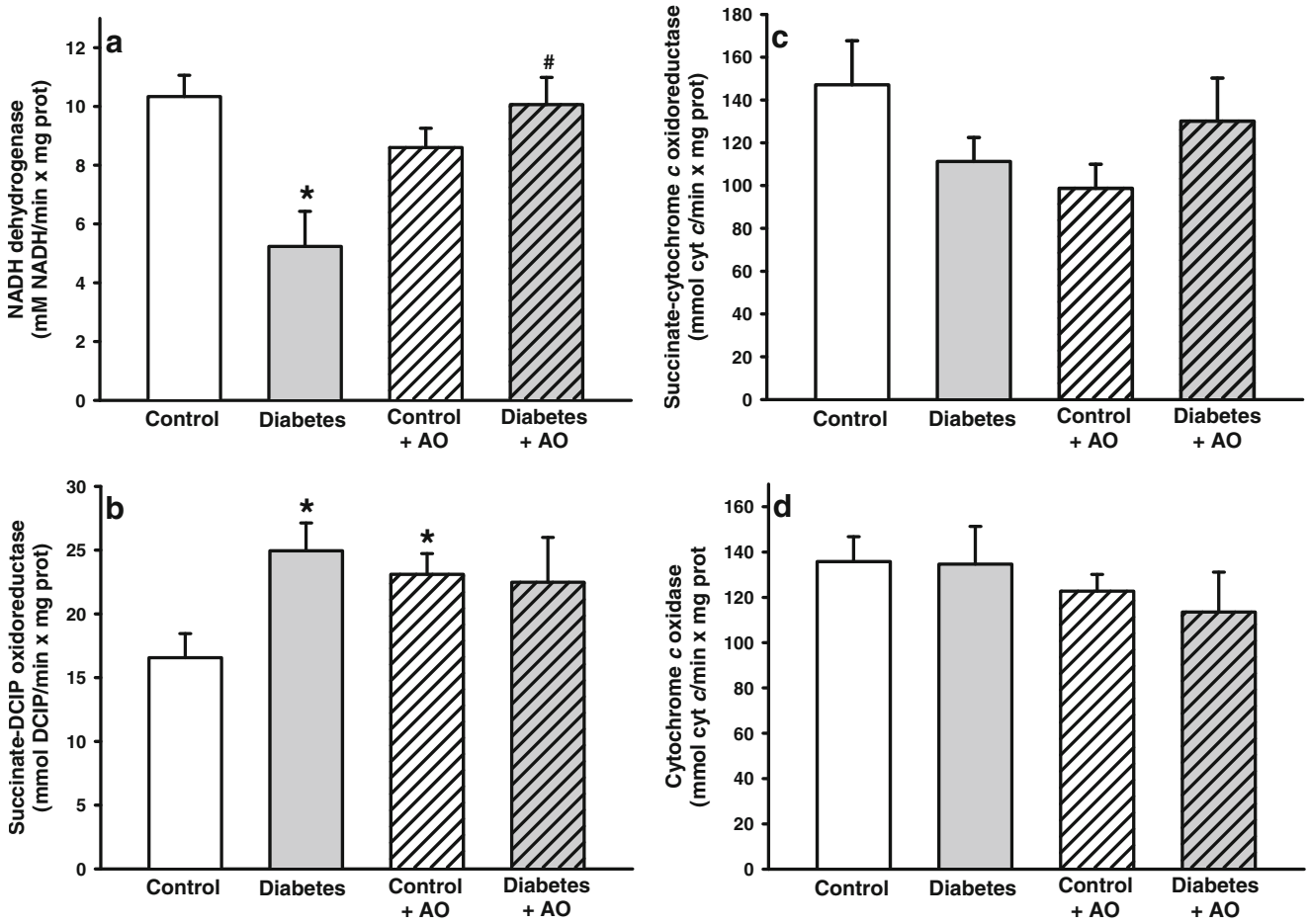
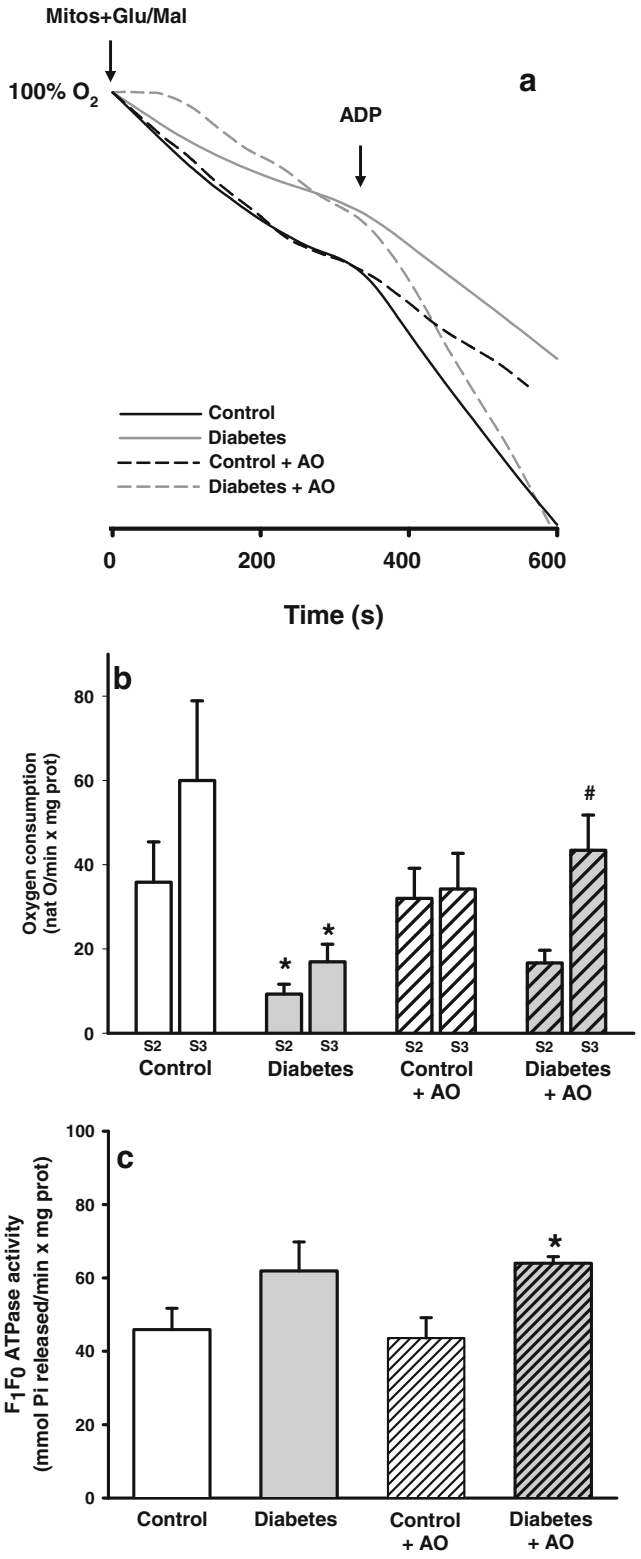


Fig. 7 Effects of diabetes and avocado oil on the activities of the ETC complexes. Complex I (**a**), complex II (**b**), complex III (**c**), complex IV (**d**). Data are presented as the mean±S.E. of $n>4$. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

the augment of the catabolism of lipids causes a diminution in the circulating levels of triglycerides and free fatty acids (Wang et al. 2003), and is also related with activation of PPARs as was discussed above. Thus, the role of avocado oil consumption in PPARs activation, mitochondrial β-oxidation flux and NADH/NAD^+ ratios deserves further investigation.

No evidence for steatosis or inflammation was observed in the diabetic animals, even considering the "extra load" of fat when avocado oil was supplemented (Fig. 2). Although it has been paid more attention to hepatic steatosis in type-2 diabetes than in type-1 diabetes, there is substantial evidence about the occurrence of this condition in the latter case (Regnell and Lernmark 2011). Even though this may be contradictory with our results about the absence of steatosis in diabetic rats (Fig. 2b), a previous study reported that STZ-induced type-I diabetes lead to a very low degree of steatosis in Sprague–Dawley rats, being this attributed to an absence of hyperinsulinemia despite elevated levels of serum free fatty acids and hyperglycemia (Wang et al. 2011). Thus, the probable hypoinsulinemia induced by STZ in diabetic rats due to the destruction of pancreatic β-cells, along with the different rat strain used in this study (i.e., Wistar rat), the lower dose of STZ (45 vs. 55 mg/Kg) and the shorter time of diabetes duration (12.8 vs. 14 weeks), may account together for the absence of steatosis seen in the diabetic animals.

Regarding to lipid peroxidation, diabetic rats exhibited a prominent augment in this parameter (Fig. 5), which is in agreement with a similar effect observed also in STZ-induced (Kristal et al. 1997) or alloxan-induced (Lukivskaya et al. 2007) diabetic rats. In order to elucidate if this effect was due to a remodeling of the fatty acid composition of mitochondrial membranes leading to enhanced sensitivity to peroxidation, we analyzed mitochondrial fatty acid composition and calculated some fatty acid indexes. Accordingly, peroxidizability index (PI) was higher in diabetic rats (Fig. 3d). This can be explained mainly as a consequence of an inferior percentage of MUFA (Fig. 3b) and higher amounts of n-3 PUFA, which in turn was the result of a diminution in C16:1 and C18:1 proportions and a noticeable increment in C22:6, the more unsaturated fatty acid present in the membranes (Table 2). Furthermore, the changes in fatty acid composition induced by STZ are in concordance with the suggestion by Kristal et al. (1997) about the influence of probable



◀ **Fig. 8** Effects of diabetes and avocado oil on mitochondrial respiration (panels **a** and **b**) and F_1F_0-ATPase activity (panel **c**). In the panel **a**, basal (state 2 (S2)) respiration was stimulated in 0.5 mg/mL mitochondrial protein using glutamate-malate as substrate. Where indicated, 300 μM ADP was added to stimulate respiration in phosphorylating state (state 3 (S3)). The quantification of oxygen consumption rates is shown in the panel **b**. Data of oxygen consumption and F_1F_0-ATPase activity are presented as the mean±S.E. of $n \geq 4$. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

changes in fatty acid composition on augmented rates of lipid peroxidation in STZ-induced diabetic rats.

Avocado oil fully abrogated the effect of diabetes on lipid peroxidation (Fig. 5) but this was not attributable to an influence of avocado oil on fatty acid composition of diabetic rats (Table 2) and, hence, on the PI of membranes, as this parameter and the fatty acid indexes remained without changes, even in control, normoglycemic rats. This is contrary to a previous report from our group where avocado oil supplemented under the same conditions, elicited an increase in the percentage of MUFA in kidney mitochondrial membranes of both normoglycemic and STZ-induced diabetic rats (Ortiz-Avila et al. 2013). The latter is in agreement with the notion that fatty acid residues of mitochondrial membranes are modified at some extent according to the type of fat supplemented in the diet, in such way that rats supplemented with a source of MUFA exhibited higher proportions of MUFA in their mitochondrial membranes in comparison with animals supplemented with a source of PUFA (Ochoa et al. 2003). However, as stated by the same authors, mitotic tissues like the liver are more resilient to the influence of exogenous fatty acids on membrane lipid composition than post-mitotic tissues (Quiles et al. 2002). Therefore, this may explain the differences observed in MUFA accumulation between the membranes of liver and kidney mitochondria from rats supplemented with avocado oil. Avocado oil also lowered ostensibly the sensitivity to lipid peroxidation induced by a short-term, exogenous, oxidant challenge, which suggest that its administration enables a more reduced redox state of the mitochondrial milieu that aid to attenuate the deleterious effect of oxidative stress. The results of the later experiment were also important to conciliate the use of the TBA technique with some concerns about the suitability of this assay for measurements of lipid peroxidation levels in biological samples (Janero 1990; Trevisan et al. 2001). Although it is true that TBA exhibits a high unspecificity as react with many aldehyde different from malondialdehyde (i.e., one of the final products of lipid peroxidation), it has been recognized that methods measuring free MDA like the TBA assay are applicable to short-term in vitro preparations of biological materials, in which free MDA accumulates, (Draper et al. 1993), which would be applicable for the experiment of the Fig. 5b. In support for the use of TBA assay in this study, the usefulness of this method has been recognized when lipid peroxidation is estimated in isolated membrane systems (i.e., isolated mitochondria) (Halliwell and Gutteridge 1999). Furthermore, when the extent of lipid peroxidation in biological samples measured by the TBA assay has been compared to more refined assays like

gas chromatography-mass spectroscopy (GC-MS), it has been concluded that both methods yields parallel results, although the TBA assay exhibits a lower sensitivity (Liu et al. 1997). In summary, all these arguments, along with our findings under Fe^{2+} stress, suggests that avocado oil might decrease the in vivo damage to mitochondrial lipids as reflected by the lower levels of TBARS found in mitochondria from diabetic rats consuming avocado oil. Nevertheless, the need to confirm this result by a more sophisticated technique is not denied.

Mitochondria from diabetic rats underwent a condition of severe oxidative stress as reflected by a twofold decrease in GSH/GSSG ratio (Fig. 4a). Thus, increased mitochondrial lipid peroxidation in diabetic rats may also be the result of diminished availability of GSH. In mitochondrial membranes, phospholipid hydroperoxide glutathione peroxidase 4 (GPX4) protects from lipid peroxidation by reducing lipid hydroperoxides at the expense of GSH (Ursini and Bindoli 1987; Arai et al. 1996). Therefore, the mechanism by which avocado oil decreased lipid peroxidation in diabetic rats might also involve enhanced reduction of lipid hydroperoxides by GPX4, as increased availability of GSH (Fig. 4a) may improve the ability of this enzyme to counteract lipid peroxidation. Nonetheless, it cannot be discarded that some lipophilic antioxidants from avocado oil may also protect membrane phospholipids against peroxidative damage by directly scavenging ROS at the lipophilic core of the membranes. In this regard, lutein and zeaxanthine, two xanthophyll carotenoid reported to be present in avocado oil (Ashton et al. 2006), possess notable protective effects against membrane peroxidative injury without altering the mechanical properties of the membranes (Sujak et al. 1999), which in turn may be advantageous for the preservation of the ETC functionality. In summary, the relative contributions of enhanced GPX4 activity or the direct actions of xanthophyll carotenoids against increased lipid peroxidation in the liver of diabetic animals supplemented with avocado oil deserves further research.

Besides GSH/GPX4 system, mitochondrial oxidative stress is also under the control of the thioredoxine (Trx) system (Stanley et al. 2011; Aon et al. 2012). In mitochondria, the Trx system is constituted by peroxiredoxin 3 (Prx3), which scavenges H_2O_2 produced at the ETC and its activity is dependent on the reduced form of thioredoxin 2 (Trx2), which in turn, is converted back to its reduced form by the action of thioredoxin reductase 2 (TrxR2) using NADPH as reducing power (Ribas et al. 2014). When ETC operates in forward mode (i.e., as tested in this work when glutamate-malate was used as complex I substrate), it is believed that Trx2 is the main controller of ROS production by virtue of the low Trx2 concentrations in comparison with GSH concentrations and the prominent increase of H_2O_2 production when Trx2 is selectively inhibited (Stanley et al. 2011). However, these systems exhibits an interdependence between them since when GSH is depleted, Trx2 becomes oxidized and enhanced susceptibility to ROS is observed (Zhang et al. 2007; McCommis et al. 2011). Recently, it was confirmed that both systems act in a concerted and continuous way by relieving each other when the antioxidant capacity of one of these system becomes saturated during mitochondrial H_2O_2 overproduction (Aon et al. 2012). Based on these reports, it can be proposed that a probable mechanism by which avocado oil decreases ROS production in mitochondria from diabetic rats is by enhancing the activity of Trx2 system through augmented availability of reduced GSH, as reflected by the increased GSH/GSSG ratio observed with avocado oil (Fig 4a), although this possibility was not directly demonstrated. Nonetheless, in agreement with this idea, it has been shown that exogenous addition of GSH to cardiac mitochondria from diabetic rats reverts lowered activity of Trx2 system (Tocchetti et al. 2012).

Importantly, in the aforementioned study, it was shown that diabetic rats exhibited impaired bioenergetics function as reflected by altered state 2–state 3 respiratory transition, which is in concordance with our results (Fig. 8a and b). Moreover, it was also found that diabetic mitochondria exhibited a decrease in the reduction of Trx2 stimulated by addition of respiratory substrates, which was related with impaired activity of the ETC (Tocchetti et al. 2012). Reduced status of GSH in liver mitochondria is also dependent on respiration by increasing the levels of NADPH (Garcia et al. 2010). Therefore, the decreased GSH/GSSG ratio in mitochondria from diabetic rats and the prevention of this effect by avocado oil (Fig. 4a) may be also explained by decreased respiratory capacity in states 4 and 3 and the prevention of this effect by avocado oil (Fig. 8a and b), respectively. These speculations reflect the need to evaluate the target(s) of avocado oil in mitochondria by further determining its effect on the redox couples $Trx2(SH_2)$/Trx2SS, $NADH/NAD^+$ and $NADPH/NADP^+$, as well as the identity of the molecule(s) from avocado oil possibly targeting these systems. Exacerbated oxidative stress in mitochondria from diabetic rats seems to be related to a remarkable increment in ROS production when the ETC oxidizes complex I substrates (Fig. 6a). Of note, a marginal production of ROS was observed when succinate, a complex II substrate, was used in combination with rotenone (an inhibitor of complex I) (Fig. 6b), confirming that ROS generation proceeds exclusively at the complex I. This is in full agreement with the twofold inhibition of the complex I activity observed in mitochondria from diabetic rats (Fig. 7a). Moreover, the treatment with avocado oil abolished both the exacerbation in ROS production and the inhibition of the complex I activity observed in diabetic rats, which further supports the hypothesis that complex I was the solely site of ROS production in these animals. Diabetes also decreases NADH dehydrogenase and complex I activity in liver mitochondria from alloxan-treated rats (Lukivskaya et al. 2007) and diabetic, obese, *Psammomys obesus* gerbils (Bouderba et al. 2012), respectively, further

suggesting that complex I is an important target in the liver of diabetic models. However, our data of ETC complex activities in diabetic rats seems to be in conflict with some reports about this topic. For example, Raza et al. (2011) have found that 8 weeks after STZ administration, liver mitochondria displayed declined activities of both complex III and complex IV along with an upregulation of complex II, the latter being in agreement with our results. However, another discrepant result from this report is that complex I activity was also increased. We ignore the reasons explaining those differences with our results, and it can be only conjectured that the different strain of rat (Sprague Dawley vs, Wistar in that paper and our manuscript, respectively) and a shorter time of treatment with STZ may account for these differences. The issue about the duration of treatment with STZ and the effects on ETC function was addressed by other study (Satav and Katyare 2004), where it was found that respiration with complex I and complex II-linked substrates was inhibited at the end of 1-month STZ treatment, but longer times were not tested. Moreover, the diabetes-prone *Psammomys obesus* gerbil, a rodent model of nutritional diabetes, displayed an inhibition in the activity of the complexes I and III and upregulation of complex II activity after 18 weeks of feeding with a hypercaloric diet (Bouderba et al. 2012). From this report and our results, it seems that after several weeks of hyperglycemia (~8–12, according to the report of Raza et al. 2011 and our results), complex II is upregulated probably to increase the flux of electrons towards ETC to compensate an eventual decrease in electron transfer at complex I. Thus, the kinetics of ETC impairment at longer times than 3-months needs to be studied to explore this hypothesis and to verify whether avocado oil has beneficial effects on mitochondrial function throughout the entire lifespan of the rat.

It has been argued that H_2DCFDA may not be the more convenient probe to measure ROS generation due to the inability of the hydrolyzed probe (i.e., H_2DCF) to cross the inner mitochondrial membrane and its dependence on catalyst such as transition metals or cytochrome *c* to detect H_2O_2. Moreover, under certain circumstances, it has been considered that H_2DCF fluorescence may reflects catalyst level as much as ROS production (Karlsson et al. 2010; Dikalov and Harrison 2014; Wardman et al. 2002). To circumvent all these disavantages, we addressed the issue of inner mitochondrial membrane impermeability to H_2DCF by doing all the experimentation in freeze-thawed mitochondria. On the other hand, to discard that higher H_2DCF oxidation in mitochondria from diabetic animals and the prevention by avocado oil simply reflect changes in the pool of either cytochrome c or iron instead estimating oxidants production at the ETC, we carried out additional experiments by incubating mitochondria with 25 μM Fe^{2+}. The rationale for this approach was that an excess of exogenous catalyst might circumvent any constrain in H_2DCF oxidation (and therefore, in ROS detection) imposed by variable amounts of endogenous catalyst. The Fig. 6c and d show that the differences in H_2DCF oxidation seen in the Fig. 6a and b were not due to parallel variations in endogenous catalyst since the same trend in fluorescence changes was observed than in the absence of added iron. Moreover, the parallel, ~10-fold increase in the levels of H_2DCF fluorescence reflect a higher catalyzing effect by exogenous iron.

The substrate-dependent rise in the fluorescent signal of H_2DCF constitute another line of evidence supporting that, under our experimental conditions, this parameter reflects the production of oxidants due to the function of the ETC instead of being a result of the method unspecificity. This is displayed in the representative time-traces of H_2DCF fluorescence in mitochondria from all the samples using glutamate-malate or succinate as substrate (Fig S2). The panel b of the Figure S2 is a "zoom" of the Fig S2a that allows a better appreciation of the effects of substrate addition on fluorescence. In mitochondria from diabetic rats, it can be observed that upon addition of glutamate-malate, there is an instantaneous increase in H_2DCF fluorescence, while in the rest of the samples there is a biphasic behavior, first, with a lag phase of different extent among all the samples and then, a phase of rapid rise after several seconds. In contrast, it was observed that H_2DCF fluorescence begins to increase several minutes after succinate addition in a discrete fashion. Additional control experiments were done to discard that fluorescence changes elicited by substrate were the results of an artifact and not by mitochondrial electron transport (Fig S2, panels c and d). In the panel c it is observed that fluorescence remained unchanged in mitochondria from control rats when substrate addition was omitted. The same behavior was observed for heat-denatured mitochondria or when substrates were added in a reaction mix not containing mitochondria. These results are better observed in the Fig S2d. These data demonstrate that rising in H_2DCF fluorescence is dependent on both the availability of substrates for electron transport and a functional ETC.

On the other hand, in concordance with the notable inhibition of complex I, respiration in both state 2 and state 3 exhibited a notable repression, which was prevented in an important extent by avocado oil (Fig. 8a and b). Therefore, it seems that complex I is the main step limiting the flux of electrons through the ETC in the diabetic animals. Repression of respiration in state 3 might be attributable to decreased F_1F_0-ATP synthase activity; however, this scenario was discarded as this enzyme showed a tendency to increase its activity in mitochondria from diabetic animals independently of avocado oil treatment (Fig. 8c). This reinforces the idea that the overall activity of the ETC in diabetic rats was impaired at the level of complex I and that avocado oil prevented this effect by preserving the activity of this respiratory complex. The tendency of incremented F_1F_0-ATPase activity might be interpreted as a probable compensatory effect to counteract the

deleterious effects of diabetes in bioenergetic status of mitochondria due to ETC dysfunction in order to supply adequate amounts of ATP to sustain the intense anabolic activity of liver. However, it is important to point out that such increment was not statistically significant.

The insensitivity to ADP addition seen in mitochondria from control rats supplemented with avocado oil was neither due to inhibition of F_1F_0-ATP synthase (Fig. 8c). An uncoupling of respiration does not seems the cause of this effect as respiration in state 2 was similar to mitochondria from control animals. Thus, suppressed activity of adenine nucleotide translocase or limited availability of inorganic phosphate may be probable candidates responsible for this effect. However, it is important to note that neither ROS generation, lipid peroxidation nor oxidative stress was higher in mitochondria from control animals supplemented with avocado oil despite its inhibitory effect on state 3 respiration.

Cardiolipin plays a crucial role in both the structure and function of the complex I (Lenaz et al. 2006). Peroxidative damage to this phospholipid has been linked to impaired complex I activity and exacerbated ROS generation during in vitro-induced oxidative stress (Paradies et al. 2002), in hepatic steatosis (Petrosillo et al. 2007) and cardiac isquemia-reperfusion injury (Paradies et al. 2004). Despite these data, it is difficult to envisage that lipid peroxidation inhibition has a primary role on the inhibition of complex I during diabetes neither in the mechanism by which avocado oil prevented this effect and excessive ROS generation. In this regard, it is well known that cardiolipin is essential for the activity of a myriad of proteins embedded in mitochondrial membranes, including all the ETC complexes (Chicco and Sparagna 2007). By this reason, lipid peroxidation has an inhibitory role in all the ETC complexes (Forsmark-Andrée et al. 1997; Cortés-Rojo et al. 2009). Thus, the lack of inhibitory effects by diabetes on the activity of the complexes II and IV, along with the weak, non-statistically decline in complex III activity, argues against the idea about a central participation of lipid peroxidation in the effects of diabetes on the ETC. Instead, selective complex I inhibition and enhanced ROS generation at this site fits better with probable glutathionylation of this enzyme due to a more oxidized state of glutathione, as reflected by decreased GSH/GSSG ratio in diabetic mitochondria (Fig. 4a). Protein glutathioylation occurs in response to decreased GSH/GSSG ratio and consist in the reaction of oxidized glutathione (GSSG) formed during oxidative stress with protein thiols ($Pr\text{-}S^-$) to yield mixed disulfides (Pr-S-SG), inhibiting in this way the activity of a variety of proteins. This reaction occurs in a non-enzymatic fashion when GSH/GSSG approach to a value of 1.0 and ROS generation is high. Alternatively, glutaredoxin enzymes (Grx) can catalyze this process (Ribas et al. 2014). In mitochondria, complex I is the solely enzyme from the ETC that becomes persistently glutathionylated, being its activity inhibited by ~50 % when the GSH/GSSG ratio has a value of 2.0 (Beer et al. 2004). Besides, glutathionylation increases ROS production in complex I and this has been associated with anomalous ROS production during pathologic states (Taylor et al. 2003). These numbers are in good agreement with the obtained in this study, since in diabetic rats, GSH/GSSG ratio decreased to 1.5±0.06 and complex I activity was inhibited in 49.3 % with a concomitant augment in ROS production only with a complex I-linked substrate (i.e., glutamate-malate). Thus, the mechanism underlying the beneficial effects of avocado oil in mitochondria from diabetic rats might consist in the maintenance of a more reduced GSH/GSSG ratio similar to that observed in control mitochondria (4.1±0.4 vs 3.5±0.3, respectively), which would prevent and/or revert complex I inhibition as high GSH/GSSG ratio enables Grx2-mediated deglutathionylation (Ribas et al. 2014). Despite this mechanism is merely speculative because we have not measured complex I gluthationylation, there is increasingly evidence supporting the pathologic importance of complex I inhibition by glutahionylation, as demonstrated in a recent report where complex I inhibition due to homozygotic Grx2 deletion caused cardiac impairment and hypertension and this was prevented by restoration of mitochondrial redox environment (Mailloux et al. 2014).

Although under our experimental conditions diabetic rats did not developed liver steatosis, some consideration may be done in relation with the probable beneficial effects of avocado oil in the context of NAFLD. The two-hits hypothesis of NAFLD pathogenesis states that lipid accumulation in liver constitutes a "first" hit that sensitize this organ to a "second" hit where oxidative stress and inflammation lead to the progression of steatosis to NASH and fibrosis (Day and James 1998). Despite this theory has been subjected to several modifications (Dowman et al. 2010; Takaki et al. 2013), mitochondrial dysfunction and lipid peroxidation remain as central players of this hypothesis, with these processes contributing to the activation of inflammatory pathways (Day 2002; Jaeschke et al. 1996; Baeuerle and Henkel 1994) and the development of fibrosis (Letteron et al. 1996), respectively. As indicated above, diabetic rats did not exhibited the first hit (i.e., steatosis); nevertheless, some components of the second hit such as mitochondrial dysfunction, ROS generation and increased lipid peroxidation does. Thus, it can be hypothesized that avocado oil or some of its fractions might delay the progression of NAFLD in diabetic models where steatosis is observed (e.g., Zucker diabetic rats (Forcheron et al. 2009)), by inhibiting the factors involved in the second hit. Further research is underway in our laboratory to test this hypothesis.

Regarding to the utility of avocado oil to improve liver health during diabetes, the possible implications of our findings is that the antioxidant properties of avocado oil at mitochondrial level might be useful to delay the deterioration of liver function during this disease, mainly the aspects related

with ROS-activated signaling pathways inducing tissue degeneration. However, considering its high fat load, this suggestion must be taken with caution because we still do not know whether avocado oil consumption can be deleterious due to lipotoxicity when steatosis is already established. Thus, one of the next steps in this research is to test the effects of avocado oil on liver function in animals with steatosis or steatohepatitis. Another possibility is that the probable disadvantages of fat from avocado oil may be bypassed by administering a fatty acid-free extract. In these regards, intensive work is being carried out in our group to answer these questions. Finally, other possibility is that regular consumption of avocado oil in the diet may delay the development of liver dysfunction in people with predisposition to suffer metabolic syndrome before the clinical and biochemical manifestations of this disease begin.

In conclusion, avocado oil decreases oxidative stress and lipid peroxidation in mitochondria from STZ-induced diabetic rats in association with enhanced complex I activity and attenuation of ROS production, independently of a hypoglycemic effect or by prevention of the alterations in mitochondrial fatty acid composition elicited by diabetes, but with amelioration of hyperlipidemia.

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