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Thiol Reduction and Cardiolipin Improve Complex I Activity and Free Radical Production in Liver Mitochondria of Streptozotocin-Induced Diabetic Rats

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Abstract

Mitochondrial reactive oxygen species (ROS) are involved in diabetic liver disease development. Diabetes impairs complex I activity and increases ROS production in liver mitochondria. The complex I produces ROS in forward electron transfer (FET) or in reverse electron transfer (RET) modes depending on the site of electron transfer blocking and the availability of respiratory substrates. Complex I activity depends on the phospholipid cardiolipin and the redox state of reactive thiols in the enzyme. Neither the underlying factors leading to complex I dysfunction nor the mode of ROS production have been elucidated in liver mitochondria in diabetes. We tested in liver mitochondria from streptozotocin (STZ) -induced diabetic rats if the addition of cardiolipin or β-mercaptoethanol, a thiol reducing agent, recovers complex I activity and decreases ROS production with substrates inducing ROS production in FET or RET modes. Decreased complex I activity and enhanced ROS generation in FET mode was detected in mitochondria from diabetic rats. Complex I activity was fully restored with the combined treatment with cardiolipin plus β-mercaptoethanol, which also abated ROS generation in FET mode. This suggest that therapies restoring cardiolipin and reducing mitochondrial thiols might be useful to counteract impaired complex I activity and excessive ROS production in liver mitochondria in diabetes.

Keywords: diabetes, liver disease, electron transport chain, free radicals, NADH dehydrogenase

1. Introduction

Hyperglycemia causes liver injury in diabetic rats by inducing apoptosis via ERK1/2, p38, and NF-κB pathways [1]. These proteins can be activated by ROS [2].

The sources of ROS in the diabetic liver includes activated NADPH oxidases [3] and the mitochondrial electron transport chain (ETC) [4]. Mitochondrial ROS are essential for the development diabetic liver injury, as evidenced by the therapeutic effect of mitochondria-targeted antioxidants on non-alcoholic fatty liver disease (NAFLD) [5], which is the manifestation of liver disease in the metabolic syndrome [6].

Diabetes impairs complex I activity in the ETC of liver mitochondria, leading to electron leak and increased ROS production [7–9]. The complex I is a large, L-shaped, multimeric protein that oxidizes NADH via a flavin mononucleotide (FMN) located at the soluble arm of the complex. Electrons are transferred then through a series of Fe-S clusters to a ubiquinone molecule placed in a binding site at the interface of the soluble and membrane arms of the complex I [10]. Complex I activity depends on interactions with the anionic phospholipid cardiolipin, enabling the access of ubiquinone molecules to its binding site and stabilizing the formation of channels for protonation pathways in the ubiquinone-binding site [11]. Lipid peroxidation, a key feature in the diabetic liver [12], modifies the spatial orientation of cardiolipin in the inner mitochondrial membrane, altering specific interactions between protein domains and cardiolipin [13]. Therefore, lipid peroxidation may disrupt electron transfer in the complex I by impairing cardiolipin function. Another factor decreasing complex I activity is the reversible oxidation of reactive thiols, which occurs by a drop in the ratio of reduced-to-oxidized glutathione (GSH/GSSG) in mitochondria. Complex I activity can be recovered by increasing the levels of GSH with a thiol reducing agent [14].

Decreased complex I activity lead to increased mitochondrial ROS production. ROS are produced in FET mode when NADH is oxidized by FMN but electron transfer is blocked at the ubiquinone-binding site in the complex I [15]. ROS may be also produced in RET mode when high succinate concentrations reduces the pool of ubiquinone and forces reverse electron flow from the ubiquinone-binding site to the FMN [16].

Previously, we have observed that diabetes exacerbates lipid peroxidation and decreases GSH/GSSG ratio in liver mitochondria [9]. As these events causes oxidative damage in cardiolipin and the oxidation of thiol groups in the complex I, respectively, the hypothesis of this study is that impaired complex I activity in liver mitochondria of diabetic rats may be restored by the in vitro treatment with cardiolipin and a thiol reducing agent. To address this hypothesis, we have treated isolated liver mitochondria from type 1, STZ-induced diabetic rats with β-mercaptoethanol, a thiol reducing agent [17], or with exogenous cardiolipin, which restitutes normal cardiolipin levels in mitochondria [18]. The results show that β-mercaptoethanol or cardiolipin partially restored complex I activity, while the combination of these agents fully recovers complex I activity. Moreover, diabetes increased ROS production in the complex I only in FET mode, which was inhibited by the combined treatment with β-mercaptoethanol plus cardiolipin. These results are discussed in the context of known structure–activity relationships for complex I and the importance of impaired liver metabolism in the development of insulin resistance.

2. Materials and methods

2.1 Animals and experimental groups

Male Long-Evans rats weighing 300–350 g were housed in a bioterioum with controlled temperature and 12 h/12 h dark/light cycles. The rats were fed ad libitum with a standard rodent chow (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) plus free access to water. Before diabetes induction, the animals were fasted overnight. Type 1 diabetes was induced by an intraperitoneal injection of

45 mg/Kg STZ. Five days later, blood glucose was measured and the animals with glucose levels ≥150 mg/dL were selected for the study. Blood glucose levels were assessed with an Accu-Chek glucometer (Roche DC México S.A de C.V.). All the procedures with animals were carried out in accordance with Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999) issued by the Mexican Ministry of Agriculture.

2.2 Mitochondria isolation

After 90-days diabetes, rats were fasted by 12 h before the sacrifice. Rats were decapitated, the liver was extracted, weighted, and placed in a medium with 220 mM mannitol, 70 mM sucrose, 2 mM MOPS and 1 mM EGTA (pH 7.4). The liver was cut in small fragments, washed, and homogenized with in a Potter-Elvehjem homogenizer. The liver homogenate was centrifuged at 314 x g for 10 min. The supernatant was recovered and centrifuged at 4410 x g for 10 min. From this centrifugation, the supernatant was discarded, and the pellet was washed and resuspended with a medium containing 220 mM mannitol, 70 mM sucrose and 2 mM MOPS (pH 7.4), and centrifuged at 6350 x g for 10 min. Finally, supernatant from the later centrifugation was discarded and the pellet was resuspended in 500 µL of the latter medium. All the centrifugations were carried out at 4°C. The concentration of mitochondrial protein was assessed by the Biuret method.

2.3 Addition of cardiolipin to isolated mitochondria

Cardiolipin liposomes were fused with liver mitochondria to increase the content of this phospholipid in mitochondrial membranes as reported elsewhere [18]. Briefly, 1.7 mg cardiolipin (Sigma-Aldrich, St. Louis, MO, USA) was added to 1 mL 25 mM KH_2PO_4 (pH 6.7). To obtain liposomes, this mixture was subjected to six cycles of sonication at 40 W for 2.5 min each with a Branson 450 sonifier (Branson Ultrasonics, Danbury, CT USA) under a N_2 stream. Then, 1 mL of freshly prepared liposomes were mixed with 1 mg mitochondrial protein in 25 mM KH_2PO_4 buffer with constant stirring for 40 min. Mitochondria were centrifuged at 13684 x g for 20 min to eliminate cardiolipin excess. Mitochondria were resuspended in 25 mM KH_2PO_4 buffer, and centrifuged again for 10 min at 13684 x g. The pellet was recovered and resuspended in a medium with 250 mM sucrose and 10 mM Tris (pH 7.4).

2.4 Measurement of complex I activity and treatments with β-mercaptoethanol

Mitochondria were solubilized with Triton X-100 as reported previously [9]. Detergent-solubilized mitochondrial protein (0.1 mg/mL) was placed in a quartz cuvette with 1 mL 50 mM KH_2PO_4 buffer (pH 7.6) and incubated with 1 mM KCN and 1 µg antimycin A. After 5 min, 5 mM potassium ferricyanide was added as electron acceptor and the absorbance was registered at 340 nm for 1 min in a Shimadzu UV2550 spectrophotometer (Kyoto, Japan). Next, 1 mM NADH was added and the changes in the absorbance were further registered for 4 min. Complex I activity was calculated from the slopes of the time-traces of NADH oxidation. Mitochondria fused with cardiolipin liposomes were used to determine the effect of exogenously added cardiolipin on complex I activity. For complex I determinations in the presence of a thiol reductant, mitochondria were incubated 15 min before complex I assay in 50 mM KH_2PO_4 buffer (pH 7.6) with 25 or 50 µM β-mercaptoethanol [19]. To evaluate the effect of cardiolipin plus β-mercaptoethanol on complex I activity, mitochondria were first fused with cardiolipin liposomes and then treated with 50 µM β-mercaptoethanol by the procedures described above.

2.5 Determination of ROS production

ROS generation was assessed in isolated mitochondria by measuring the oxidation of the fluorescent probe 2′,7′-dichlorodihydrofluorescein (H$_2$DCF) as previously described [9], except that some modifications in the experimental conditions were made to identify the mode by which ROS were produced. To assess ROS production in complex I by FET mode, 10 mM glutamate-malate was added as substrate and the fluorescence of H$_2$DCF was followed for 20 min. To determine ROS production in the complex I by RET mode, 10 mM succinate was added as substrate and the fluorescence was followed for 20 min. ROS production upstream complex I (i.e. in the complex II - complex IV segment of the ETC) was assessed by inhibiting RET with rotenone, a complex I inhibitor, and using succinate as substrate [16], after which the changes in fluorescence were evaluated for 20 min. The rate of ROS generation was expressed like the change in arbitrary units of fluorescence (ΔF) per min^{-1} per mg mitochondrial protein^{-1}.

2.6 Statistical analysis

The number of independent experiments using different samples is indicated in the legend to each figure. The results are expressed as the mean ± standard error. Statistical differences between means were analyzed with Student's t-test ($P < 0.05$), using the Sigma Plot (v11.0) software (Systat Software, Inc., San Jose, CA, USA).

3. Results

3.1 Blood glucose and relative liver weight

After 90 days of diabetes induction, blood glucose levels in control and diabetic rats were 98.2 and 347.1 mg/dL, respectively, (**Figure 1A**). Moreover, the liver weight in diabetic rats was 1.3- fold higher than in control rats (**Figure 1B**). This effect was more accentuated when liver-to-body weight ratios were compared, as the value of this parameter was almost two - fold higher in the diabetic rats than in the control animals (**Figure 1C**). These results confirm the presence of hyperglycemia and a pathologic phenotype in the livers of STZ-induced diabetic rats.

3.2 Effects of β-mercaptoethanol and cardiolipin on complex I activity

The effect of diabetes on complex I activity of liver mitochondria is shown in the **Figure 2**. Complex I activity was 1.8 – fold lower in the mitochondria of diabetic rats than in mitochondria from the control group. On the other hand, isolated mitochondria from diabetic rats were incubated with 25 or 50 μM β-mercaptoethanol to evaluate at what extent thiol reduction recovers the activity of the complex I (**Figure 2A**). Both β-mercaptoethanol concentrations partially recovered the enzymatic activity, being this effect statistically significant only with 50 μM of β-mercaptoethanol. Thus, 50 μM of β-mercaptoethanol was chosen for the following experiments. Of note, β-mercaptoethanol concentrations higher than 50 μM impaired complex I activity (data not shown).

Liver mitochondria from diabetic rats were incubated with cardiolipin to determine at what extent this phospholipid recovers complex I activity in diabetic rats (**Figure 2B**). It can be observed that cardiolipin addition restored complex I activity
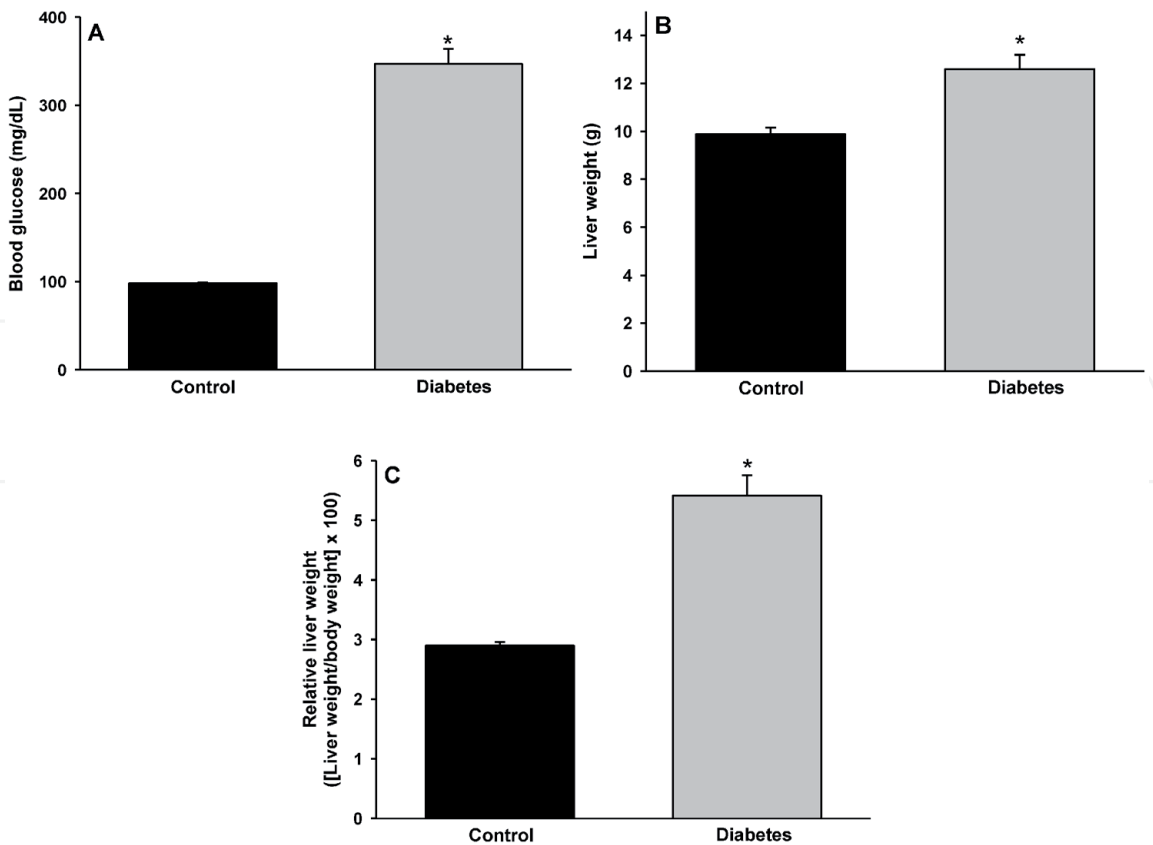
Figure 1.
*Effects of diabetes on blood glucose levels (A), liver weight (B) and relative liver weight (C). Data are presented as the mean ± standard error of n ≥ 4. *P < 0.05 vs. control group (Student's t-test).*
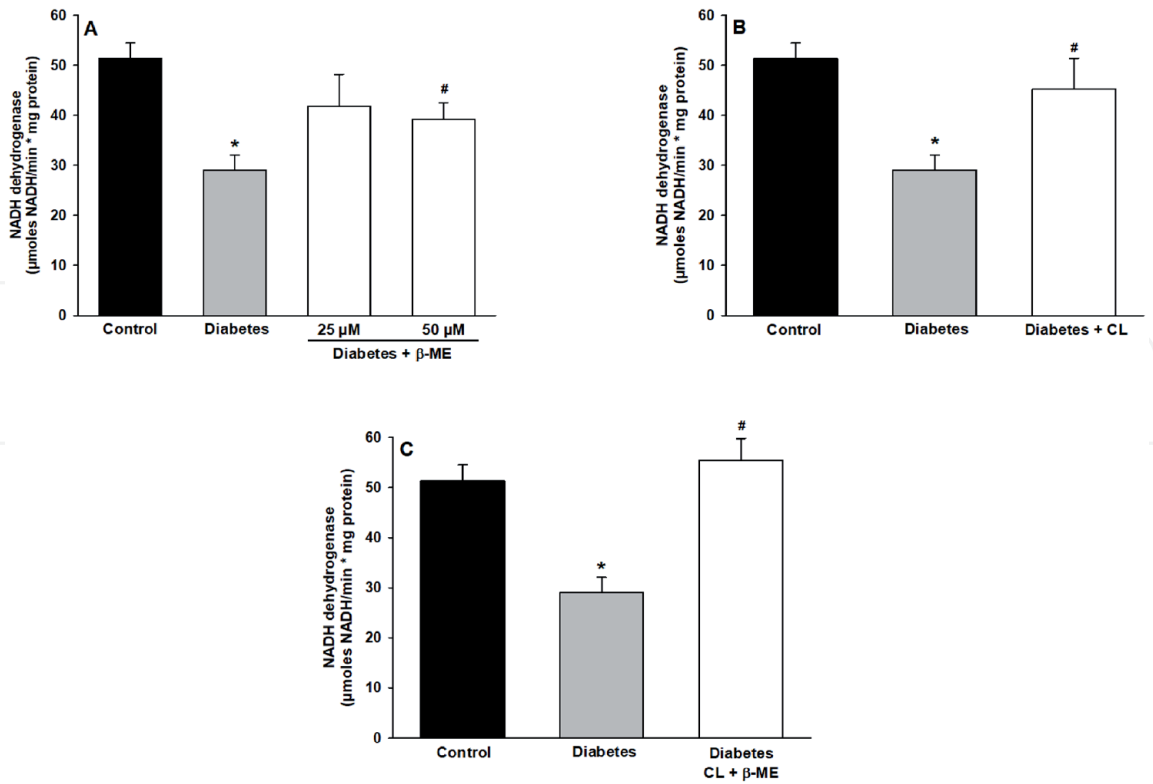

Figure 2.
*Recovery of complex I activity in liver mitochondria from diabetic rats by the incubation with β-mercaptoethanol (β-ME) (A), cardiolipin (CL) (B), or cardiolipin plus 50 μM β-mercaptoethanol (CL+ β-ME) (C). Data are presented as the mean ± standard error of n ≥ 6. *P < 0.05 vs. control; #P < 0.05 vs. Diabetes (Student's t-test).*

without reaching the activity of the control group. In contrast to the individual treatment with β-mercaptoethanol or cardiolipin, the combined treatment with these agents fully recovered complex I activity (**Figure 2C**). These results suggest that impaired complex I activity in mitochondria from diabetic rats may be fully counteracted by supplying cardiolipin and restoring the redox state of mitochondrial thiols.
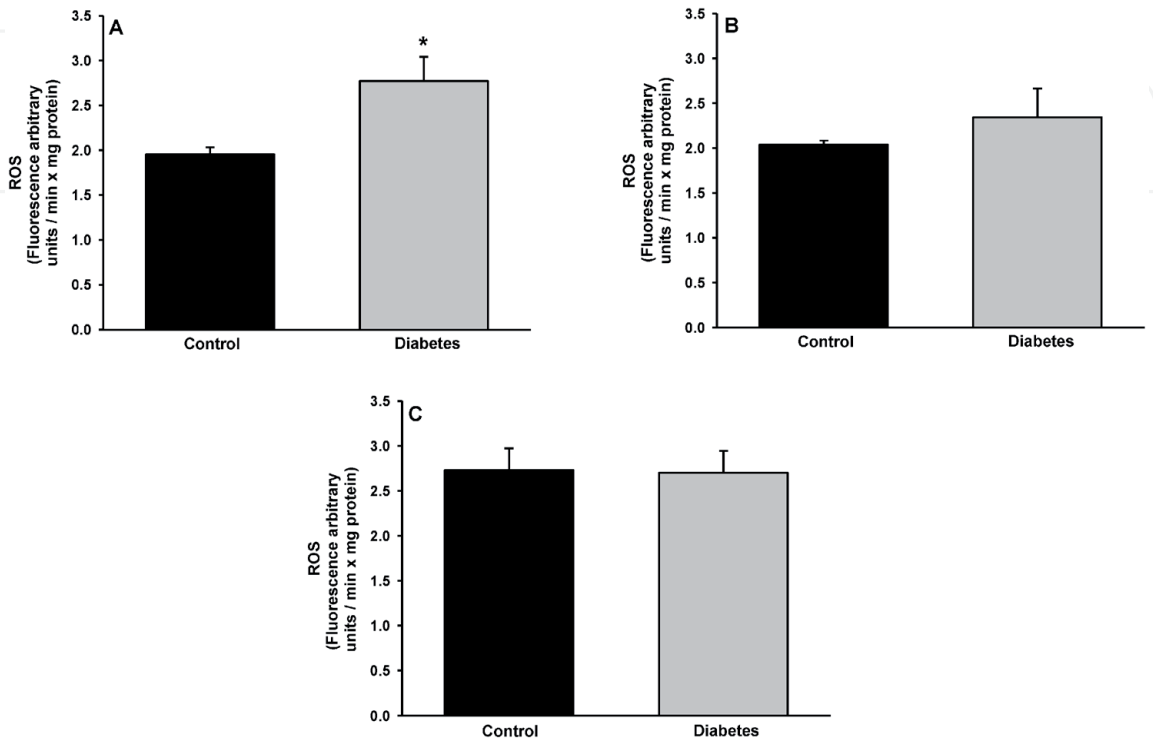
Figure 3.
*ROS production in liver mitochondria in FET mode with glutamate/malate as substrate (A), in RET mode with succinate as substrate (B), and in RET mode blocked with rotenone (C). Data are presented as the mean ± standard error of n = 6. *P < 0.05 vs. control group (Student's t-test).*
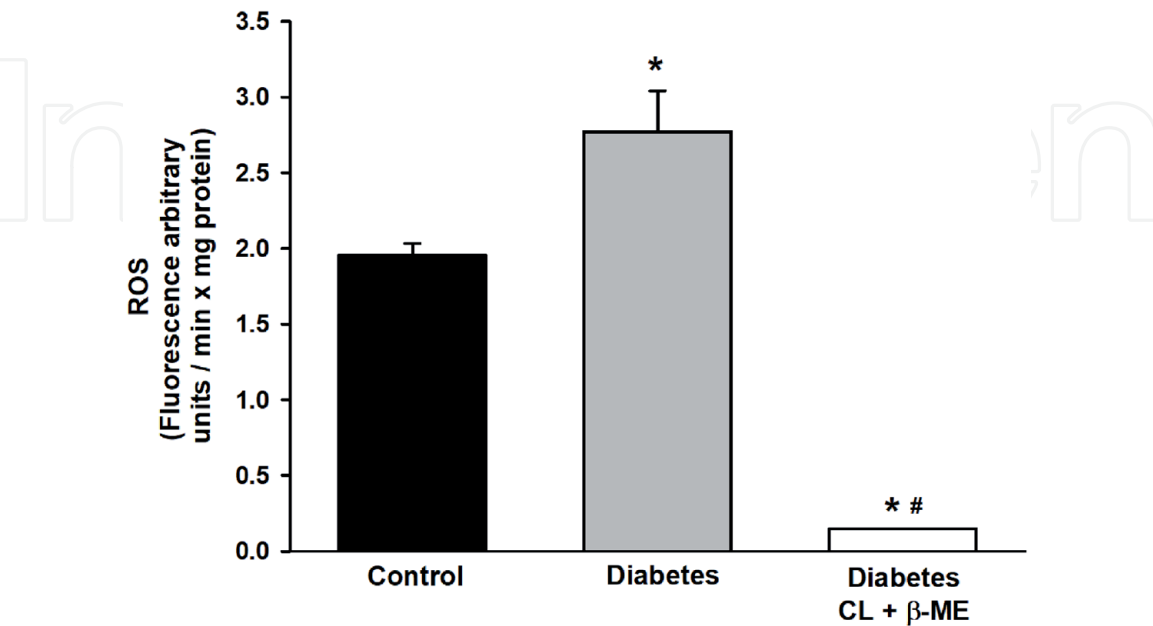
Figure 4.
*Effect of cardiolipin (CL) plus 50 μM β-mercaptoethanol (β-ME) on ROS production in FET mode in liver mitochondria from diabetic rats. Data are presented as the mean ± standard error of n = 6. *P < 0.05 vs. Control; #P < 0.05 vs. Diabetes (Student's t-test).*

3.3 Effect of respiratory substrates on ROS production by complex I

ROS production was determined in isolated liver mitochondria in FET mode using glutamate/malate as substrate for complex I (**Figure 3A**), in RET mode using succinate as complex II substrate (**Figure 3B**), and with succinate plus rotenone (an inhibitor of the ubiquinone-binding site in the complex I) for blocking ROS production in the complex I by RET (**Figure 3C**). In FET mode, ROS production increased 1.4 - fold in mitochondria from diabetic rats in comparison to mitochondria from control rats (**Figure 3A**). No statistically significant changes in ROS production were observed neither in RET mode (**Figure 3B**), nor when RET was blocked with rotenone (**Figure 3C**).

A 19 – fold decrease in ROS production in FET mode was observed in mitochondria from diabetic rats treated with cardiolipin plus β-mercaptoethanol when compared with mitochondria from diabetic rats without any addition (**Figure 4**). These results indicate that diabetes increases ROS production in FET mode, which may be prevented by reducing mitochondrial thiols and supplying mitochondria with cardiolipin.

4. Discussion

The results show that, under our experimental conditions, STZ-induced diabetic rats developed hyperglycemia and abnormal liver weight (**Figure 1**). These events were accompanied by decreased complex I activity (**Figure 2**) and increased ROS generation in FET mode (**Figure 3**), which was reverted by the in vitro treatment with cardiolipin plus β-mercaptoethanol (**Figure 4**). Increased liver weight has been identified as an alteration characterizing liver disease [20]. Moreover, increased blood markers of liver injury have been observed in STZ-induced diabetic rats [1]. All these data indicate that diabetic rats in this study developed liver disease.

There is a close relationship between the development of liver disease and the presence of mitochondrial dysfunction [21]. This agrees with enhanced liver weight (**Figure 1B** and **C**), decreased complex I activity (**Figure 2A**), and higher ROS production (**Figure 3A**) observed in liver mitochondria from diabetic rats. There are two main mechanism modulating complex I activity: the first one is the reversible oxidation of reactive thiols in cysteine residues due to decreased mitochondrial GSH/GSSG ratio. The inhibition of the complex I by thiol oxidation can be reversed in vitro by increasing the concentration of GSH via the addition of thiol reducing agents like dithiothreitol [14]. β-mercaptoethanol is another agent that reduces GSSG to GSH [17], besides, it restores the activity of the ETC complexes after an oxidative insult [19]. Therefore, the partial recovery of complex I activity with 50 μM β-mercaptoethanol (**Figure 2A**) suggests that thiol oxidation in the complex I is part of the mechanism explaining complex I inhibition by diabetes. The second mechanism inhibiting complex I activity is the loss of cardiolipin due to peroxidative damage. An approach to recover impaired complex I activity after cardiolipin loss is the exogenous addition of this phospholipid to isolated mitochondria [18]. This strategy partially recovered complex I activity in liver mitochondria of diabetic rats (**Figure 2B**).

The partial recovery of complex I activity with either β-mercaptoethanol or cardiolipin led us to think that the combined treatment with these two agents may drive to full recovery of complex I activity in liver mitochondria from diabetic rats, which was confirmed in the experiment of the **Figure 2C**. These data suggest that diabetes impaired complex I activity by promoting both the oxidation of reactive thiols in the complex I and the peroxidation of cardiolipin. This suggestion is supported by previous data showing decreased GSH/GSSG ratios and increased

lipid peroxidation in liver mitochondria from STZ-induced diabetic rats [9]. As mentioned before, decreased GSH levels lead to the oxidation of reactive thiols in the complex I [14], while lipid peroxidation affects negatively both the structure and function of cardiolipin in the inner mitochondrial membrane [13].

We acknowledge that one of the main limitations of this study is that we did not measure neither cardiolipin levels nor the redox status of thiols in the complex I. Nevertheless, we believe that our results give a clue about the role of cardiolipin and thiol oxidation in the mechanism of complex I inhibition by diabetes, since the experimental approaches used in this study to replenish cardiolipin in mitochondria and to reduce thiols in complex I have been previously validated by direct determinations of both cardiolipin and the redox state of thiols in complex I [14, 18]. On the other hand, there are conflicting studies showing increased complex I activity [22], enhanced content of cardiolipin [23], and decreased superoxide production by complex I [24] in liver mitochondria from STZ-induced diabetic rats. However, it must be pointed out that diabetes was induced in these studies by a shorter, 8 to 9 -weeks period, in comparison with the 12-weeks period used in this study. Thus, it can be hypothesized that the mechanisms responsible for such adaptive responses seen in shorter periods of diabetes might be impaired at longer time periods like in this study.

The complex I produces ROS in FET mode due to electron leak during electron transfer from NADH to ubiquinone. ROS are also produced in the complex I by RET mode when electrons are forced to flow from ubiquinol to NAD^+ [25]. ROS are produced at least in two different sites of the complex I, one situated at the ubiquinone-binding site (I_Q), and the other one located at the flavin mononucleotide (FMN)-binding site (I_F) [16]. The significant increase in ROS levels in mitochondria from diabetic rats only with a substrate for complex I (**Figure 3A**), suggests that diabetes causes an alteration in the complex I that stimulates ROS production in FET mode. This mode of ROS production occurs when electron transfer to ubiquinone is blocked at the I_Q site [16]. In this regard, the access of ubiquinone to the I_Q site is thought to occur via a cardiolipin-dependent mechanism [11]. The recovery of complex I activity (**Figure 2**) and the decrease in ROS production (**Figure 4**) observed with cardiolipin, suggest that diabetes disrupt electron transfer at the I_Q site by a mechanism involving lipid peroxidation, as suggested by the increased levels of lipid peroxidation found in a previous work in liver mitochondria from STZ-induced diabetic rats [9]. Therefore, impaired electron flow in the I_Q site would drive to a more reduced state of the redox centers upstream the I_Q site (i.e. the iron–sulfur clusters and the FMN in the complex I), leading to electron leak and ROS generation, while the replenishment of mitochondria with cardiolipin might improve both the architecture of the I_Q site and electron transfer to ubiquinone, decreasing in this way the electron leak and ROS production.

The inhibition of the complex I during diabetes is not trivial as this enzyme is the main mechanism to maintain high $NAD^+/NADH$ ratios [26]. High NAD^+ levels activates sirtuins proteins, whose deacetylase activity enhances lipid and glucose catabolism and the antioxidant defenses in the liver [27]. On the contrary, low NAD^+ levels impair hepatic catabolism and increases oxidative stress, leading to the development of liver disease and insulin resistance [28]. This reflect the importance of preserving optimal rates of NADH oxidation by the complex I in the diabetic liver, which, according to our results, might be achieved by strategies simultaneously augmenting the levels of cardiolipin and GSH. In this regard, it has been demonstrated that linoleic acid supplementation increases cardiolipin levels in cultured fibroblasts depleted of cardiolipin [29] and increases complex I activity in HeLa cells [30]. On the other hand, phytochemicals like betaine and β-sitosterol have counteracted the negative impact of liver toxicants like ethanol or carbon tetrachloride on the levels of mitochondrial GSH [31, 32]. Therefore, the concomitant targeting of cardiolipin

and mitochondrial GSH and its impact on both the complex I function and hepatic lipid metabolism deserves further investigation, given the central role of defective hepatic lipid metabolism in peripheral insulin resistance in diabetes [33].

5. Conclusions

Diabetes induced anormal liver weight in STZ-induced rats, accompanied of mitochondrial alterations in the ETC such as decreased complex I activity and increased ROS production in FET mode. These mitochondrial abnormalities were corrected by the in vitro treatment with cardiolipin plus β-mercaptoethanol, which suggest that alterations in the content of cardiolipin and thiol oxidation may be underlying causes of these mitochondrial alterations. On this basis, is proposed that therapies counteracting mitochondrial alterations in cardiolipin and thiol oxidation might be useful to ameliorate hepatic disturbances elicited by diabetes.

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Conflict of interest

The authors declare no conflict of interest.

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