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Cytochrome c release from rat liver mitochondria is compromised by increased saturated cardiolipin species induced by sucrose feeding

Angélica Ruiz-Ramírez,1 Miguel-Angel Barrios-Mayá,1 Ocarol López-Acosta,1 Dora Molina-Ortiz,2 and Mohammed El-Hafidī1

1Departamento de Biomedicina Cardiovascular, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City, Mexico; and 2Laboratorio de Toxicología Genetica, Instituto Nacional de Pediatria, Mexico City, Mexico

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Ruiz-Ramírez A, Barrios-Mayá MA, López-Acosta O, Molina-Ortiz D, El-Hafidī M. Cytochrome c release from rat liver mitochondria is compromised by increased saturated cardiolipin species induced by sucrose feeding. Am J Physiol Endocrinol Metab 309: E777–E786, 2015. First published September 8, 2015; doi:10.1152/ajpendo.00617.2014.—Cytochrome c release from mitochondria has been described to be related to reactive oxygen species (ROS) generation. With ROS generation being increased in fatty liver from sucrose-fed (SF) rats, we hypothesized that cytochrome c release might be positively associated with H2O2 generation from SF mitochondria. Surprisingly, cytochrome c release from mitochondria of SF liver was found to be significantly lower compared with control (C) mitochondria oxidizing pyruvate/malate or succinate. Exposure of mitochondria to exogenous superoxide radical generated by the xanthine/xanthine oxidase system elicits a dose-response cytochrome c release in both control and SF mitochondria, but cytochrome c release remains lower in SF mitochondria compared with C mitochondria. Furthermore, the addition of ebselen, PEG-catalase, or catalase, a H2O2 scavenger, significantly reduces cytochrome c release from C and SF mitochondria. Our results suggest that both intramitochondrial H2O2 are involved in cytochrome c release, but the persisting difference between C and SF levels can be attributed to the differences in cardiolipin compositions. Indeed, the ratio of palmitic acid-rich cardiolipin species was found to be increased in lipid membrane from SF mitochondria compared with C mitochondria, whereas that of linoleic acid-rich cardiolipin species was found decreased. In addition, the content of tafazzin, a protein responsible for cardiolipin remodeling, was decreased in SF mitochondria. Therefore, we conclude that the changes observed in the composition of cardiolipin molecular species in SF mitochondria may be involved in cytochrome c interaction with mitochondrial inner membrane lipid and in its reduced release from SF mitochondria.

Mitochondria are essential for cell life by producing ATP and maintaining redox status, but they also play an essential role in the process of programmed cell death by releasing cytochrome c (cyt c) and other proapoptotic proteins (5). Accumulating data suggest that oxidative stress and reactive oxygen species (ROS) generated by mitochondrial respiratory chain are important factors to determine the release of cyt c from the intermembrane space into the cytosol (33). Cyt c is bound to the inner membrane by anionic phospholipids, specifically cardiolipin (CL). This binding involves electrostatic and hydrophobic attractions that have been shown to be due to two distinct sites named A-site and C-site that are involved in the electrostatic and hydrophobic interactions with negatively charged phosphate groups and with fatty acids of CL, respectively (1, 4). CL is a unique phospholipid with a dimeric structure containing two negative charges and four linoleic acid-rich fatty acids, which are susceptible to peroxidation. It was proposed that CL bound-cyt c acquires peroxidase activity in the presence of H2O2 and oxidizes CL, contributing to the dissociation of cyt c from the mitochondrial inner membrane (23). The dissociation of cyt c from the mitochondrial inner membrane is proposed as a first step for the mechanism by which cyt c is released outside the mitochondria. The second step is the release of cyt c from the intermembrane space to the cytosol across the outer membrane by a mechanism involving Bcl2-associated X protein (BAX) and BAK pore formation and outer membrane permeability transition (31). Thus H2O2 generation and cyt c release from mitochondria are considered the first steps in programmed cell death (15), and they appear to be involved in the development and progression of liver diseases from simple fatty liver to serious nonalcoholic fatty liver diseases (NAFLD) such as steatohepatitis and liver deficiency (6, 7, 12, 17).

Fatty liver is considered to be the hepatic manifestation of metabolic syndrome and the early stage of NAFLD by the accumulation of both triglycerides and free fatty acids (FFAs) (40), which are more toxic than triglycerides because they can induce H2O2 generation in liver mitochondria (22). Many animal models of metabolic syndrome and NAFLD have been developed by dietary regimens simulating human dietary habits. The main diet-induced models in rats are high-fat diet, high-carbohydrate diet (e.g., sucrose or fructose diets), and combined high-fat and high-carbohydrate diet. All these models are suitable to study metabolic syndrome because they all develop hyperlipidemia, obesity, insulin resistance, and fat accumulation in liver, a risk of NAFLD development. However, a model of the combined high-fat/high-carbohydrate diet is the one used most frequently, because it develops hepatic inflammation, tissue damage, and apoptosis (19, 39) and may exhibit a mitochondrial dysfunction and high ROS generation to develop an advanced fatty liver disease (26, 32). Our sucrose-fed (SF) rat model does not show mitochondrial dysfunction maintaining a normal respiratory control because it seems to be protected against oxidative stress by overexpres-
sion of uncoupling protein 2 and antioxidant enzymes as a response to lipotoxicity and increased mitochondrial ROS generation (35). In addition, our SF rats develop high intracellular fat accumulation and increased FFA levels in both mitochondria and liver wall, and it can be considered as a model for an initiation step of fatty liver disease. Thus, we hypothesize that increased H$_2$O$_2$ generation and availability in liver mitochondria and its relation to CL contents and composition in a model of obesity induced by a high-sucrose diet.

**MATERIALS AND METHODS**

Animals. The animal experiments were approved by the Laboratory Animal Care Committee of the National Institute of Cardiology Ignacio Chávez and conducted in compliance with the institution’s ethical guidelines for animal research. Male Wistar rats, aged 28 days and weighing $\pm$65 $\pm$ 5 g, were housed with a 12:12-h light-dark cycle and randomly separated into two groups of eight to 10 animals: group 1, control (C) rats given tap water for drinking; and group 2, SF rats given drinking water containing 30% sucrose for 24 wk. All animals were fed ad libitum with commercial rat chow (LabDiet 5008; PMI Nutrition International, Richmond, IN).

Mitochondria isolation. Rat liver mitochondria were isolated in ice-cold medium containing 250 mM sucrose, 10 mM Tris, and 1 mM EGTA, pH 7.4, by differential centrifugation. Liver homogenate was centrifuged for 10 min at 600 $\times g$ and the supernatant was centrifuged for 250 mM sucrose and 10 mM Tris, pH 7.4, supplemented with 0.1% fatty acid free BSA. Finally, the mitochondrial fraction was washed in the same buffer without BSA and stored in ice. Mitochondrial protein concentration was measured by the Lowry method (27), using BSA as standard.

Mitochondria oxygen uptake. Mitochondrial respiratory rates were measured by using a Clark-type O$_2$ electrode and incubating 0.50 mg protein/ml of fresh mitochondria in an air-saturated medium containing 125 mM KCl, 10 mM HEPES (pH adjusted to 7.4 with KOH), 10 mM EGTA, 2 mM K$_2$HPO$_4$, 5 mM MgCl$_2$, and 10 mM succinate oxidative substrate in the presence of rotenone (2 $\mu$M) at 30°C.

Cyt c release. Mitochondria (0.5 mg/ml) were incubated in a medium containing 100 mM sucrose, 75 mM KCl, 5 mM Tris-base, pH 7.4, 10 mM EGTA, and 1 mM KH$_2$PO$_4$ for 15 min at 30°C with continuous stirring. Cyt c release was assessed in forward and reverse electron transport fluxes by energizing mitochondria with pyruvate/malate (P/M: 5:3 mM) or succinate (10 mM). The reverse electron transport rates were measured by reverse electron transport fluxes by energizing mitochondria with pyruvate/malate (P/M: 5:3 mM) or succinate (10 mM), respectively. The reverse electron transport rates were calculated by 10.22 $\pm$ 3.5 on November 7, 2017 http://ajpendo.physiology.org/ Downloaded from
rate of H2O2 generation was lower than when oxidizing succinate, whereas the difference between C and SF remained significant in the respiratory control (RC) that corresponds to the ratio of state III and state IV. *P < 0.05 vs. C.

Western blotting for cyt c, BAX, apoptosis-inducing factor, cardiolipin synthase, and tafazzin (phospholipid-lysophospholipid transacylase). Proteins were separated using 13% SDS-polyacrylamide gel (SDS-PAGE) and electrotransferred to PVDF membrane (Innompilon-P; Millipore). Cyt c, BAX, and apoptosis-inducing factor (AIF) were measured in mitochondria and supernatant. The following amounts of protein were loaded in each lane for the different blots: 100 µg for cyt c, BAX, tafazzin, and cardiolipin synthase (CLS) analysis in mitochondria. In the supernatant, 150–180 µg of protein were loaded in each lane for the analysis of BAX and AIF. All proteins were analyzed in 13% SDS-PAGE and transferred to PVDF membrane. After transfer, membranes were blocked with 5% nonfat dry milk in TBS-T buffer (10 mM Tris-base, 10 mM NaCl, and 0.1% Tween-20) for 2 h at room temperature. The blocked membranes were incubated overnight at 4°C in the same buffer without Tween containing mouse polyclonal antibody to cyt c (1/1,000), to BAX (1/500), to AIF (1/500), to CLS (1/200), and to tafazzin (1/200) (Santa Cruz Biotechnology, Santa Cruz, CA). For control load, goat polyclonal anti-ANT (1/1,000) (Santa Cruz Biotechnology) and rabbit anti actin (1/1,000) were used for the mitochondria and supernatant, respectively. The membranes were rinsed with TBS-T and then incubated with horseradish peroxidase coupled to anti-mouse, anti-goat, or anti-rabbit IgG as a secondary antibody (1/2,000), depending on the primary antibody used. The bands were visualized using enhanced chemiluminescence detection reagent (Millipore) and exposed on Kodak Biomax ML scientific image film for 5–10 min. The band density was analyzed by the photodocumenter GelDoc (UVP).

Data analysis. All values are presented as means ± SE. The differences between groups were determined by one-way ANOVA for selected variables. The number of animals used for each analysis is indicated in the figure and table legends. Group mean differences were considered significant when P < 0.05.

RESULTS

Mitochondrial oxygen uptake. The rate of oxygen uptake by mitochondria-oxidizing succinate in the presence of ADP (state III) and after ADP was exhausted (state IV) is significantly increased by sucrose feeding (Table 1), and no change was observed in the respiratory control (RC) that corresponds to the ratio between the rates of oxygen uptake in states III and IV between SF and C mitochondria. Thus, the no change of RC indicates that mitochondria from liver of SF rats have a normal function with normal synthetize ATP compared with mitochondria from C rat and as it has been found previously [35].

Mitochondria H2O2 generation and cyt c release. Figure 1A shows that mitochondria from SF rat liver generated H2O2 at a higher rate than mitochondria from the C group liver when oxidizing succinate or pyruvate/malate. In the presence of ADP, H2O2 generation was significantly decreased in both C and SF mitochondria. When mitochondria oxidized P/M, the rate of H2O2 generation was lower than when oxidizing succinate, whereas the difference between C and SF remained statistically significant independently of the type of substrate. However, cyt c release experiments, performed in the same conditions as described above for H2O2 generation, showed a significant increase in cyt c release from both group mitochondria-oxidizing succinate compared with its release without succinate (Fig. 1A). Nevertheless, cyt c release was significantly lower in SF than in C mitochondria. In the same way, Fig. 1B shows that cyt c release from both types of mitochondria-oxidizing P/M was also increased compared with its release without P/M and that cyt c release remained lower in mitochondria from SF than from C. Furthermore, no difference was observed in cyt c release from mitochondria oxidizing P/M or succinate despite the higher generation rate of H2O2 by mitochondria oxidizing succinate than when oxidizing P/M. The addition of ADP to mitochondria significantly reduced the release of cyt c in both C and SF mitochondria oxidizing succinate. However, when mitochondria oxidized P/M, ADP significantly decreased cyt c release in C mitochondria (P < 0.01) and did not affect it in SF mitochondria. In regard to total cyt c, the analysis by Western blot using anti-cyt c antibody

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<th>Table 1. Mitochondria oxygen uptake</th>
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<td>State IV</td>
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Values are expressed as means ± SE (n = 8 different animals). C, control; SF, sucrose fed; RC, respiratory control. The rate of O2 consumption was expressed as nmol-min⁻¹·mg protein⁻¹ and it was performed in the presence of 10 mM succinate + rotenone. RC corresponds to the ratio of state III and state IV. *P < 0.05 vs. C.

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showed no significant difference in cyt c level between mitochondria from C and SF rats (Fig. 2).

The addition of ebselen, a synthetic lipid-soluble seleno-organic compound having potent antioxidant capacity and glutathione peroxidase-like activity (36, 38) to mitochondria oxidizing P/M, reduced the generation rate of H$_2$O$_2$ in a dose response manner (Fig. 3A) in both C and SF mitochondria. At low concentrations assayed for H$_2$O$_2$ generation (from 0.5 to 2.5 μM), ebselen reduced cyt c release from C mitochondria in a dose response manner and had no more effect reaching a plateau at 5 and 10 μM in C mitochondria. Nevertheless, it had no effect on cyt c release from SF mitochondria at any of the concentrations assayed (Fig. 3B).

A second H$_2$O$_2$ scavenger, PEG-catalase (catalase conjugated to polyethylene glycol), was assayed, which can cross the mitochondrial outer membrane to catalyze the degradation of H$_2$O$_2$ generated inside mitochondria. Figure 4 shows that PEG significantly reduced cyt c release from C and SF mitochondria while being more effective in C than SF mitochondria.

**Effect of X/XO on cyt c release.** In the xanthine/xanthine oxidase (X/XO) system, the production of superoxide anion or H$_2$O$_2$ depends on the concentration of O$_2$ in the medium (24); therefore, we tested the effect of superoxide dismutase (SOD) and catalase on cyt c release. Figure 5 shows that X/XO induced cyt c release from both SF and C mitochondria in a dose response manner, but this release remained lower in SF compared with C. In the presence of SOD to dismutate the superoxide into H$_2$O$_2$, a moderate decrease in cyt c release was observed in SF mitochondria, but it did not reach statistical significance. The addition of catalase to the incubation medium to scavenge H$_2$O$_2$ produced by SOD and X/XO significantly decreased cyt c release in dose response in both C and SF mitochondria. At 10 and 50 U/ml, catalase reduces cyt c release to the level of mitochondria incubated without substrate. When catalase was added to mitochondria without SOD, a significant inhibition of cyt c release induced by X/XO was observed in both C and SF mitochondria.

**Effect of Ca$^{2+}$ on cyt c release.** Because Ca$^{2+}$ is well known to induce cyt c release outside mitochondria by inducing mitochondrial membrane permeability transition (MPT), which is sensitive to cyclosporine A (CsA) (18), and by affecting the electrostatic interaction between positive charges of cyt c and negative charge of CL (21), and because the sensitivity to CsA of cyt c release from liver mitochondria in response to Ca$^{2+}$ has been described to depend on the buffer used (14), the experiments were conducted in the same buffer described above containing sucrose (100 mM) and KCl (75 mM) compared with a buffer containing 150 mM KCl in the presence of 100 μM Ca$^{2+}$ and using different concentrations of CsA as an inhibitor of MPT. Cyt c release induced by Ca$^{2+}$ has been described to be sensitive to CsA in a buffer containing KCl (150 mM), Tris (5 mM), and KH$_2$PO$_4$ (1 mM). However, in a buffer containing manitol, sucrose, and HEPES, cyt c release induced by the Ca$^{2+}$ sensitivity to CsA is lost (14).

Figure 6 shows that the amount of cyt c released from C and SF mitochondria is twofolds higher in a buffer containing 150 mM KCl (Fig. 6B) compared with our buffer containing sucrose (100 mM) and KCl (75 mM) (Fig. 6A). In addition,
Ca\(^{2+}\) at 100 \(\mu\)M significantly increases cyt \(c\) release in both buffers. This increase in cyt \(c\) release from both C and SF rat mitochondria was inhibited in a dose response by CsA in a buffer containing 150 mM KCl (Fig. 6B), and it was not significantly affected by CsA in a buffer containing sucrose and 75 mM KCl. However, cyt \(c\) release remains lower in SF than C mitochondria in both buffers used.

**BAX and AIF content.** Another mechanism involved in cyt \(c\) release toward cytosol is the insertion of BAX into the outer mitochondrial membrane to form a pore by which cyt \(c\) crosses the mitochondria outer membrane. The Western blot analysis of mitochondria fraction using anti-BAX antibody showed that there was a significant decrease in the density of the band corresponding to BAX in SF rat mitochondria compared with controls (Fig. 7). On the other hand, the content of BAX in the supernatant fraction, obtained after the total liver homogenate to sediment mitochondrial fraction was centrifuged, was increased in SF compared with control rats. Regarding AIF, which also resides in mitochondrial intermembrane space and may be released by the same way as cyt \(c\), Fig. 7 shows that the bands corresponding to AIF were not different in their density between C and SF in either mitochondria or supernatants of liver homogenate after mitochondria sedimentation.

**Mitochondrial CL content.** CL is a complex phospholipid specific to mitochondria. It is an anionic phospholipid involved in cyt \(c\) anchoring to the mitochondrial inner membrane, and it is involved in several other functions such as stability and activity of complex II (37). The HPLC-MS analysis shows no significant difference in the amount of CL between C and SF (12.5 ± 2.5 vs. 10.7 ± 3.2 nmol/mg protein; \(n = 4\) different animals). Figure 8 illustrates the spectra of different CL species in simple negatively charged ion \([M-H]^−\) together with the ion corresponding to the IS [(C14:0)\(_4\)CL; \(m/z\) 1,242.4] in control and SF mitochondria. In all spectra, the ions at \(m/z\) 1,401.5, 1,425.5, 1,450.3, and 1,472.4 correspond to CL species that contain (C68)CL, (C70)CL, (C72)CL, and (C74)CL, respectively. The cluster (C72)CL \((m/z\) 1,450.3) corresponds to different CL species rich in linoleic acid, such as (C18:2)\(_4\)CL \((m/z\) 1,450.3) and (C18:2)(C16:0)CL \((m/z\) 1,452.5). The (C70)CL cluster \((m/z\) 1,425) corresponds to CL species enriched with palmitic \((C16:0)\) or palmitoleic acids \((C17:1)\) at \(m/z\) 1,425.5 or palmitoleic acids \((C17:1)\) at \(m/z\) 1,427.5. The (C68)CL cluster contains double palmitic acids such as (C18:2)(C16:0)CL with the ion at \(m/z\) 1,401 or (C18:2)(C16:0)(C16:1)CL, which corresponds to \(m/z\) 1,403.8. The cluster (C74)CL at \(m/z\) 1,472.7 and 1,474.3 corresponds to different CL species such as (C18:2)(C20:4)CL.

**Fig. 4.** Effect of PEG-catalase on cyt \(c\) release from liver mitochondria. Open bars correspond to C; black bars correspond to SF mitochondria. Data correspond to the means ± SE of 6 experiments from 6 separate animals. *\(P < 0.01\) vs. C without P/M; **\(P < 0.01\) vs. SF without P/M; ***\(P < 0.05\) vs. all C + P/M with and without PEG-catalase; \(\Delta P < 0.01\) vs. C + P/M; \#\(P < 0.05\) vs. SF + P/M.

**Fig. 5.** Effect of xanthine/xanthine oxidase (X/XO) on cyt \(c\) release from SF mitochondria (black bars) and from C mitochondria (open bars). \(+, +, +\), and +++ correspond to 8, 16, and 32 mU/ml of XO, respectively. In superoxide dismutase (SOD) assay, XO was used at 16 mU/ml (+ +), and SOD was used as 10 (+), 50 (+ +), and 100 U/ml (+ + +). In the catalase (Cat) assay, XO and SOD were used at 16 mU/ml and 50 U/ml, respectively. Cat was used at 5, 10, and 50 U/ml. The results are represented as the mean ± SE of 6 preparation from 6 separate animals. *\(P < 0.05\) vs. C + X; **\(P < 0.01\) vs. SF + X; \(\Delta P < 0.01\) vs. C + SOD (+ +); \#\(P < 0.01\) vs. C + X + XO (16 mU/ml); ###\(P < 0.05\) vs. SF + X/XO (16 mU/ml).
and (C18:2)2(C18:1)(C20:4)CL, which are enriched in linoleic (C18:2) and arachidonic (C20:4) acids. Regarding the proportions of the major molecular species of CL detected in all group mitochondria, they are summarized in Table 2. In mitochondria from SF rats, there was a general increase in the proportion of (C68)CL to (C70)CL clusters that were enriched in palmitic acid. From SF rats, there was a general increase in the proportion of CL species from SF mitochondria may be due to the defect in CL remodeling that involves the mitochondrial protein tafazzin, a phospholipid-lysophospholipid transacylase that incorporates linoleic acid into lysocardiolipin to produce tetralinoleil-cardiolipin, a major CL species found in mitochondria. Tafazzin Western blot analysis shows a significant decrease in its content in SF compared with C mitochondria (Fig. 9), whereas no difference in the content of CLS was observed between C and SF mitochondria.

**DISCUSSION**

A large body of experiments suggests that mitochondria play an important role in the development of oxidative stress and cell death in liver disease and other tissues (29, 34). In a previous study, we reported that mitochondria from liver of SF rats generate H2O2 at a higher rate than their corresponding control (35). In the present work, a high rate of H2O2 generation from SF mitochondria was again observed, but surprisingly, it was accompanied by a weaker release of cyt c from SF mitochondria than from control animals (despite the large amount of H2O2 generated by SF mitochondria oxidizing P/M or succinate as substrates of complex I and II, respectively). In addition, the lower cyt c release from SF mitochondria cannot be attributed to lower cyt c content because no difference was indeed observed between C and SF mitochondria. The mechanism underlying ROS-mediated cyt c release from mitochondria is not well established. However, it is proposed that cyt c release from mitochondria proceeds through a loss of interaction between cyt c and CL at the outer leaflet of the mitochondrial inner membrane due to cardiolipin peroxidation, followed by cyt c release through the outer mitochondrial membrane (33). CL peroxidation in the presence of H2O2 modifies the interaction of cyt c with CL, resulting in its dissociation from the inner membrane (21). Thus, it was suspected that the high availability of endogenous H2O2 due to its high generation rate in SF rat mitochondria may result in increased cyt c release from SF mitochondria. However, our results suggest that the availability of H2O2 does not affect cyt c release from SF, as it does from C mitochondria. This suggestion is supported by the experiments performed in SF mitochondria in the presence of H2O2 scavengers such as ebselen or PEG-catalase, which have no effect on mitochondrial functions such as oxygen uptake or membrane potential at the low concentration used (data not shown). Thus, the decreased cyt c release from C mitochondria is related to the decreased H2O2 generation due to ebselen. Nevertheless, in SF mitochondria, this relationship between cyt c release and H2O2 generation is lost. When mitochondria from both C and SF animals are exposed to extramitochondrial H2O2 generated by the X/XO system, cyt c release is exacerbated but remains lower in SF mitochondria compared with control. This finding is in good agreement with a study describing the increased cyt c release by exposure of mitochondria to the X/XO system, except that superoxide anion was considered to be a species involved in cyt c release by inducing the mitochondrial outer membrane permeability, depending on voltage-dependent anion channel (25). In our study, the effect of catalase abolishing cyt c release from both types of mitochondria in the presence of X/XO indicates that H2O2 is the species involved in cyt c release.
release from both types of mitochondria. Indeed, H$_2$O$_2$ has been described as a major reactive oxygen species produced by the X/XO system (24). Extramitochondrial H$_2$O$_2$ may induce cyt c release from mitochondrial intermembrane space probably by the same mechanism as that which endogenously generated H$_2$O$_2$ because it can diffuse across the mitochondrial outer membrane. It was reported that overexpression of cytosolic catalase protects against antimycin-induced H$_2$O$_2$, and when overexpressed in mitochondria it protects mitochondria from extramitochondrial H$_2$O$_2$ in HepG2 cells, suggesting that mitochondrial or cytosolic catalase might act as attraction points for H$_2$O$_2$ and promote H$_2$O$_2$ movement down to its gradient concentration (3). Even if these results suggest that intra- or extramitochondrial H$_2$O$_2$ is involved in cyt c release in C mitochondria, it does not explain the reduced cyt c release from SF compared with C mitochondria. The dissociation of cyt c from the inner membrane is also a key process in the release of cyt c and may depend on the electrostatic and hydrophobic interactions between cyt c and membrane lipid, specifically with cardiolipin. Indeed, it was reported that cyt c peroxidase activity depends on the fatty acid composition of the CL molecule species (4). In liposomes formed by dioleyl-

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**Fig. 7.** Western blotting analysis of Bcl2-Associated X protein (BAX) and apoptosis-inducing factor (AIF) levels in mitochondria and supernatant. ANT and β-actin were used as a control load for mitochondria fraction and supernatant, respectively. Open bars correspond to C mitochondria; black bars correspond to SF mitochondria. Data correspond to means ± SE of 4 different mitochondria and supernatant preparations from 4 separate animals. *P < 0.001 vs. SF; **P < 0.001 vs. C.

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**Fig. 8.** Mass spectra of cardiolipin molecule species of mitochondria from C (A) and from SF (B). A scan between m/z 1,000 and 1,500 was performed to identify the different cardiolipin molecule species. The cluster of ions at m/z 1,242.5 corresponds to the internal standard tetrameristoylcardiolipin (C14)4CL. In both spectra, the clusters of ions at m/z 1,401.5, 1,425.5, 1,450.3, and 1,472.4 match CL species that contain (C68), (C70), (C72), and (C74), respectively, and represent the total number of carbon related to 4 fatty acids of cardiolipin. The cluster (C72)CL (m/z 1,450.3) corresponds to different cardiolipin species containing 4 linoleic acids [(C18:2)4CL]. The cluster (C70)CL (m/z 1,425.5) corresponds to (C18:2)3(C16:0)CL, where linoleic acid is substituted by 1 palmitic acid. The cluster 1,472.4 corresponds to the replacement of 1 linoleic acid by arachidonic acid (18:2)3(C20:4)CL.

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**E783 CYTOCHROME c RELEASE AND OXIDATIVE STRESS**

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H2O2, because tetralinoleyl CL, as a polyunsaturated CL, may affect cyt portion and palmitic acid-enriched CL species in SF mitochondria. Lowered tetralinoleyl-CL proportion of CL molecule species enriched in palmitic acid may be involved in the mechanism by which cyt c release from mitochondria by competing with the positive binding sites of cyt c, as described elsewhere (21). Indeed, Ca2+ increases cyt c release in both types of mitochondria. However, cyt c release from SF mitochondria remains lower than from C mitochondria. The sensitivity to CsA of cyt c release in response to Ca2+ depends on the buffer used, as has been described elsewhere (14). In a buffer containing KCl (150 mM), cyt c release induced by Ca2+ is sensitive to CsA. However, when the buffer contains sucrose and the reduced concentration of KCl (75 mM), the release of cyt c induced by Ca2+ is not sensitive to CsA, suggesting a mechanism of cyt c release that involves Bax insertion to mitochondrial outer membrane, as proposed in the literature (14). The insertion of BAX into mitochondrial outer membrane and the induction of a specific pore formed by BAX constitute the second step by which cyt c and other apoptotic proteins such as AIF cross the outer membrane outside the mitochondria. The decreased BAX amount in SF rat liver mitochondria may contribute to the reduced cyt c release outside mitochondria. However, the lack of difference found between C and SF in the amount of AIF in both mitochondria and supernatant of liver homogenate suggests that the reduced BAX level in outer membrane is not the only mechanism involved in the release of cyt c from SF mitochondria. The decreased BAX insertion in the membrane may also be due to changes observed in CL composition, as suggested by the study using liposomes and showing that CL appears to be important for the recruitment and oligomerization of BAX in lipid membrane (28). However, another study using yeast mutants that lack either CL synthase (crd1Δ) or tafazzin

Table 2. Cardiolipin molecular species in mitochondria

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<th>m/z</th>
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<th>SF</th>
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<tr>
<td>(C68)CL</td>
<td>1,401.5</td>
<td>0.3 ± 0.15</td>
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<tr>
<td>(C70)CL</td>
<td>1,403.8</td>
<td>0.6 ± 0.4</td>
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<tr>
<td>(C72)CL</td>
<td>1,425.5</td>
<td>4.9 ± 1.1</td>
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<tr>
<td>(C74)CL</td>
<td>1,427.5</td>
<td>3.3 ± 1.7</td>
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<tr>
<td>(C76)CL</td>
<td>1,428.3</td>
<td>2.2 ± 1.5</td>
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<tr>
<td>(C78)CL</td>
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<td>3.5 ± 1.5</td>
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<tr>
<td>(C84)CL</td>
<td>1,474.6</td>
<td>5.2 ± 2.4</td>
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<tr>
<td>1,477.3</td>
<td>4.2 ± 1.7</td>
<td>5.4 ± 1.6</td>
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Values correspond to the percentage ± SE from 4 different experiments. The percentage of each species was calculated from the abundance counts of each species in relation to the total abundance of all species identified. (C68)CL contains one C16:0 carbon chain and three C18 carbon chains; (C70)CL contains one C16:0 carbon chain and three C18 carbon chains; (C72)CL contains four C18 carbon chains; (C74)CL contains one C20 carbon chain and three C18 carbon chains. C16 could be either palmitic or palmitoleic acid; C18 corresponds to oleic or linoleic acid, and C20 is consistent with arachidonic acid.

*P < 0.05, SF vs. control.

phosphatidycholine and several CL species at different proportions, the cyt c-CL complex acquires a high peroxidase activity when CL is enriched with unsaturated fatty acids, and this ability decreases according to the type of CL-composing fatty acid in the following order: polyunsaturated tetralinoleoyl-CL [(C18:2)4CL] ≥ monounsaturated tetraoleoyl-CL [(C18:1)4CL] ≥ saturated tetramyristoyl-CL [(C14:0)4CL] (4). Therefore, the changes in CL molecule species found in SF mitochondria may involve in the mechanism by which cyt c dissociation from the mitochondrial inner lipid membrane is decreased in SF mitochondria. Lowered tetralinoleoyl-CL proportion and palmitic acid-enriched CL species in SF mitochondria may affect cyt c release despite the high availability of H2O2, because tetralinoleoyl CL, as a polyunsaturated CL, may be more sensitive to the oxidation by H2O2 in the presence of cyt c than saturated CL. These results suggest that SF mitochondria CL species enrichment with palmitic acid may be responsible for the resistance of CL to oxidative stress and for its dissociation from the inner mitochondrial membrane. Moreover, cyt c-binding affinity for CL-containing liposomes was recently described to be higher when liposomes were formed from dioleoylphosphatidylcholine-tetrastearoyl CL than when they contained tetraoleoyl or tetralinoleoyl CL (1). These observations lead us to suggest that a specific hydrophobic interaction arising from the saturated fatty acid chain in CL is important for strongly stabilizing the cyt c-CL complex. The increased proportion of CL molecule species enriched in palmitic acid may be due to decreased remodeling mechanisms via the deacylation-reacylation cycle mediated by tafazzin, a phospholipid-lysophospholipid transacylase. Indeed, Western blot analysis of tafazzin showed a significant decrease in its content in SF mitochondria, whereas no difference was observed in the content of CL synthase. The high availability of palmitic acid in SF rat liver (35) and the increased palmitic acid-rich CL did not affect the content of total CL in SF mitochondria. Palmitic acid has been described to induce apoptosis in rat neonatal cardiomyocytes by increasing cyt c release and diminishing total CL because there was an accumulation of both saturated phosphatidic acid and saturated phosphatidylglycerol, poor substrates for cardiolipin synthase (30). The decrease in CL level may affect cyt c binding to the inner membrane, increasing the soluble cyt c level in the mitochondrial intermembrane space. In our SF mitochondria, total CL, CL synthase, and total cyt c contents are unchanged, and consequently, cyt c may be well associated with inner membrane by both hydrophobic and electrostatic interaction. The possible mechanism involving the electrostatic interaction between cyt c and lipid membrane has been explored using high concentrations of Ca2+ to enhance cyt c release from mitochondria by competing with the positive binding sites of cyt c, as described elsewhere (21). Indeed, Ca2+ increases cyt c release in both types of mitochondria. However, cyt c release from SF mitochondria remains lower than from C mitochondria.
taz1Δ) shows that cardiolipin is not required for BAX-mediated cyt c release from yeast mitochondria (20). Moreover, the presence of CL in the outer membrane has been reported (13). However, the role of CL on BAX insertion is not definitively established, and more studies are needed to clarify the role of CL and its different molecule species on BAX insertion and cytochrome release across the mitochondria outer membrane.

In summary, the decreased cyt c release from SF mitochondria can be due to the increased availability of saturated fatty acids, which may induce changes in the composition of CL, affecting the interaction of cyt c with lipid membrane. The interaction of cyt c with CL and the insertion of BAX into the mitochondrial outer membrane appear to be the key processes in the mechanism of cyt c release from mitochondria to the cytosol in SF. On the other hand, the decreased cyt c release from SF mitochondria can be considered an adaptive mechanism of mitochondria to avoid the apoptosis process that is associated with the progression of liver diseases from simple fatty liver to serious NAFLDs such as steatohepatitis. However, additional investigations are required to understand at the molecular level how changes in CL distribution are the cause of or the effect of cyt c release in SF mitochondria.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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