Research Paper

Cholesterol enrichment in liver mitochondria impairs oxidative phosphorylation and disrupts the assembly of respiratory supercomplexes

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ABSTRACT

Mitochondrial cholesterol accumulation is a hallmark of alcoholic and non-alcoholic fatty liver diseases and impairs the function of specific solute carriers through changes in membrane physical properties. However, its impact on mitochondrial respiration and organization of respiratory supercomplexes has not been determined so far. Here we fed mice a cholesterol-enriched diet (HC) supplemented with sodium cholate to examine the effect of cholesterol in mitochondrial function. HC feeding increased liver cholesterol content, which downregulated Srebp2 and Hmgcr expression, while sodium cholate administration decreased Cyp7a1 and Cyp8b1 mRNA levels, suggesting the downregulation of bile acid synthesis through the classical pathway. HC-fed mice exhibited increased expression of Stard1 and Mfn64 and enhanced mitochondrial free cholesterol levels (2–3 fold), leading to decreased membrane fluidity. Mitochondria from HC-fed mice displayed increased cholesterol loading in both outer and inner mitochondrial membranes. Cholesterol loading decreased complex I and complex II-driven state 3 respiration and mitochondrial membrane potential. Decreased respiratory and uncoupling control ratio from complex I was also observed after in situ enrichment of mouse liver mitochondria with cholesterol or enantiomer cholesterol, the mirror image of natural cholesterol. Moreover, in vivo cholesterol loading decreased the level of complex II and the assembly of respiratory supercomplexes I1 + III2 + IV and I1 + III2. Moreover, HC feeding caused oxidative stress and mitochondrial GSH (mGSH) depletion, which translated in hepatic steatosis and liver injury, effects that were rescued by replenishing mGSH with GSH ethyl ester. Overall, mitochondrial cholesterol accumulation disrupts mitochondrial functional performance and the organization of respiratory supercomplexes assembly, which can contribute to oxidative stress and liver injury.

1. Introduction

Cholesterol is an integral component of cellular membranes that not only plays an essential role in determining membrane physical properties, but also regulates multiple signaling pathways [1–3]. Due to this key role in modulating membrane structure and function, cholesterol levels in cell membranes are tightly regulated. Cells satisfy their need for cholesterol either through the uptake from nutrients and cholesterol-rich low-density lipoproteins, or by de novo synthesis from acetyl-CoA in the mevalonate pathway controlled by HMG-CoA reductase (HMGCR), the rate-limiting step in cholesterol synthesis. The endoplasmic reticulum (ER)-based transcription factor SREBP-2 is a master regulator of cholesterol synthesis by controlling the expression of HMGCR [3,4].

Abbreviations: ALD, Alcoholic Liver Disease; DHE, Dihydroethidium; FFA, Free Fatty Acids; HC, High-Cholesterol diet; HMGCoA R, HydroxyMethylGlutaryl CoA Reductase; STARD1, Steroidogenic Acute Regulatory protein; TMRM, Tetramethylrhodamine Methyl ester; HPLC, High-Performance Liquid Chromatography; PMH, primary mouse hepatocytes

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Cholesterol is distributed from the plasma membrane and ER to different membrane bilayers, including mitochondria, where it plays key functional and structural roles. Mitochondria are essential organelles with numerous functions in cellular metabolism and homeostasis. They have long been considered as crucial organelles acting as the source of energy in the form of ATP synthesis. Besides this key function, mitochondria are also crucial for other cellular processes, including autophagy, apoptosis, fatty acid synthesis, Ca\(^{2+}\) homeostasis and cell signaling [5]. In addition, mitochondrial function extends beyond the boundaries of the cell and influence the physiology of the organism by regulating communication between cells and tissues [6]. Given this plethora of functions, it is not surprising that mitochondrial dysfunction has emerged as a key factor in a myriad of diseases [7-10]. Furthermore, mitochondria are the main consumers of molecular oxygen in the cell in the respiratory chain, which can result in the collateral generation of reactive oxygen species (ROS) [11-13].

Mitochondria are cholesterol-poor organelles compared to plasma membranes. The levels of cholesterol in mitochondrial membranes are under tight control, particularly in specialized organs, such as steroidogenic tissues or liver, where mitochondrial cholesterol is metabolized into steroid hormones or bile acids, respectively. The rate-limiting step in this metabolic process is determined by the cholesterol availability in mitochondria and therefore its mitochondrial trafficking regulates mitochondrial cholesterol homeostasis under physiological conditions. Mitochondrial cholesterol trafficking is controlled largely by STAR family members, including STAR1D and MLN64 (also known as StARD3) [14-16], and the unbalance between cholesterol trafficking and its metabolism can result in mitochondrial cholesterol accumulation, which can impact mitochondrial membrane physical properties and fluidity [17]. Indeed, mitochondrial cholesterol loading has been shown to perturb the function of specific membrane carriers, such as SLC25A11, which results in mitochondrial GSH (mGSH) depletion through impaired transport of cytosolic GSH into mitochondria, leading to increased oxidative stress [18-20].

Mitochondrial cholesterol accumulation is a hallmark of chronic diseases, including cardiovascular disorders, cancer and Alzheimer's disease [21-25]. In addition, previous studies have shown an association between increased mitochondrial cholesterol levels and liver injury in alcoholic and nonalcoholic liver disease [29-34]. However, the specific role of mitochondrial cholesterol accumulation in mitochondrial respiration and organization of functional respiratory complexes has not been previously examined. Therefore, our aim was to use in vivo and in vitro models of mitochondrial cholesterol enrichment to examine the impact of this event on oxygen consumption rates and the assembly of mitochondrial respiratory supercomplexes.

2. Material and methods

2.1. Animals and treatments

Wild type C57BL/6J male mice were obtained from Charles River Laboratories. All procedures involving animals and their care were approved by the Ethics Committee of the University of Barcelona and were conducted in accordance with institutional guidelines in compliance with national and international laws and policies. Mice were randomly separated in two groups that were fed with a regular chow diet (CTRL) or a high cholesterol diet (HC, 2% cholesterol and 0.5% sodium cholate, custom made Research Diets, Brogaarden Denmark, C18021901) for up to two days, as described previously [19]. In some cases, mice were fed with a diet enriched in 0.5% sodium cholate (SC) (Research Diet) alone. To test the role of mGSH replenishment, mice were fed HC diet and treated with 1.25 mmol/kg of GSH ethyl ester (GSHee), as described previously [35].

2.2. Mitochondria isolation and ROS determination

Livers were minced in 10 vol of cold Buffer A (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 10 mM HEPES and 1 mg/ml fatty acid-free BSA, pH 7.4) supplemented with protease and phosphatase inhibitors (Roche). Tissue was then disrupted with a drill-driven Teflon dounce homogenizer (4 strokes at 1500 rpm). Mitochondria were isolated by differential centrifugation, 15 min at 700 x g followed by 15 min at 10000 x g at 4°C. Pellets containing mitochondria were re-suspended in Buffer B (395 mM sucrose, 0.1 mM EGTA and 10 mM HEPES, pH 7.4).

ROS generation was determined in isolated mitochondria using MitoSox and Amplex Red Hydrogen peroxide/Peroxidase Assay Kit under basal conditions as described previously [36] and detailed in Supplemental Materials.

2.3. Mitoplasts preparation

Mitoplasts were prepared by permeabilization of intact mitochondria by digitonin. Briefly, to a mitochondrial protein suspension of 8 μg/μl in Buffer B (395 mM sucrose, 0.1 mM EGTA and 10 mM HEPES, pH 7.4) an equal volume of digitonin (7.68μg/μl) was added (960μg digitonin/mg mitochondria). After incubation for 30 min at 4°C, the sample was centrifuged for 10 min at 10000 x g at 4°C, and the pellet was resuspended in Buffer B and centrifuged again for washing. Mitoplasts purity was assessed by the presence of Porin or COX IV.

2.4. In vitro cholesterol increase in mouse liver mitochondria

In vitro cholesterol enrichment was performed by incubation of mitochondria with a cholesterol-BSA Complex (CBSAC) or enantiomer cholesterol (Ent-CBSAC) as described previously [32]. Enantiomer cholesterol was described before and generously provided by Scott Rychnowsky (Department of Chemistry, University of California Irvine) [37]. CBSAC and Ent-CBSAC were prepared by dissolving 50 mg of cholesterol or enantiomer cholesterol in 5 ml of absolute ethanol and then diluted with 5 ml of double distilled water. The milk-like solution was then centrifuged at 2000 × g for 10min. The supernatant was discarded, and the pellet was resuspended in 5 ml of Buffer CSH (0.25 M sucrose, 1 mM EDTA, pH 7.3). The white solution was stirred gently and 2 g of Free-Fatty Acid BSA were slowly added at room temperature. Once BSA was completely dissolved, the pH of the solution was adjusted to 7.3, and then centrifuged in the cold at 12000 × g for 10min. The supernatant was collected and used for cholesterol incorporation into mitochondria. CBSAC or Ent-CBSAC was added to mouse liver mitochondria (0.2 mg/mg mitochondria) at 4°C for 1min. Mitochondria were subsequently diluted about 20 times with cold Buffer CSH, and spun down immediately at 12000 × g for 10min to eliminate the unbound cholesterol. Parallel control experiments were performed using only BSA.

2.5. Electron microscopy

Mice were perfused through the portal vein to wash the liver with cold saline and then fixed by perfusion with 2.5% glutaraldehyde in phosphate buffer. Liver tissue fragments were extracted and fixed with glutaraldehyde 2.5% and paraformaldehyde 2% in buffer phosphate (0.1 M, pH 7.4), post-fixed in 1% osmium tetroxide and 0.8% potassium ferrocyanide, dehydrated with acetone, and embedded in epoxy resin. Sections were cut and stained with methylene blue for light microscopy. Ultrathin sections for transmission electron microscopy were cut and stained with 2% uranyl acetate for 10min and with a lead-staining solution for 2min. Images from stained ultrathin sections were acquired by moving randomly across the EM grid using a transmission electron microscope JEOL JEM-1010 fitted with a Gatan Orius SC1000 (model 832) digital camera. ImageJ software was used to quantify the number
and length of mitochondria.

2.6. Measurement of fluorescence anisotropy

Fluidity of mitochondrial membranes was evaluated by fluorescence anisotropy of mitochondria-bound dye DPH. DPH (20 mM in tetrahydrofuran) was first diluted 100 times with 10 mM Tris-HCl, 150 mM KCl, 1 mM EDTA, pH 7.4. Subsequently, DPH was injected into stirred mitochondrial suspensions (0.5 mg/ml) and the mixture was incubated for 30 min at 37 °C. Fluorescence polarization was measured in a Hitachi spectrofluorometer at wavelengths of 425 nm for emission. The results are expressed as anisotropy units (r), Hitachi spectrofluorometer at wavelengths of 366 nm for excitation and 425 nm for emission. 30 min at 37 °C. Fluorescence polarization was measured in a Hitachi spectrofluorometer at wavelengths of 425 nm for emission.

2.7. Blue-Native PAGE

Blue-Native PAGE was performed as described previously [38,39]. Briefly, 200 μg of mitochondria were isolated and solubilized using solubilization buffer A (50 mM Sodium Chloride, 50 mM Imidazole, 2 mM 6-aminohexanoic acid, EDTA 1 mM, pH 7) with digitonin (6 mg digitonin/mg mitochondria). Mitochondrial complexes were subsequently separated using a Criterion™ TGX™ Precast 4–12% gel (Biorad) and Running Buffer 10x Tris/Gly (Biorad), and transferred to a PVDF membrane using the Trans Blot Turbo (Biorad). To detect OXPHOS (super)complexes, the membrane was incubated overnight with the following primary antibodies: COXIV (Cell Signaling, #4844), UQCRC2 (G-10) (Santa Cruz, sc-390378) and MitoProfile™ Total OXPHOS Rodent WB Antibody Cocktail (Abcam, ab110413). Membranes were thoroughly washed with TBS-Tween and incubated for 45 min with HRP-conjugated secondary antibody. Pierce ECL Western Blotting Substrate (ThermoScientific) and converted to cDNA using the High-Capacity Reverse Transcription Kit (ThermoScientific) and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit from Thermofisher. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was subsequently performed using the SensiFAST™ SYBR® No-ROX Kit (Bioline) following the manufacturer’s instructions. Each reaction was run in triplicate to determine the threshold (CT) for each mRNA, and the amount of each cDNA relative to the β2-Microglobulin (B2M) endogenous control was determined using the 2−ΔΔCt method. The primer sequences (Invitrogen) for the expression of Srebp2, Hmgcr, Stard1, Mfn64, Cyp27a1, Cyp7a1, Cyp7b1, Cyp8b1 and B2M are described in Supplementary Table 1.

2.9. Extracellular flux analyses

0.2 μg protein/μl mitochondria stocks were prepared in cold MAS buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1.0 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2) with pyruvate and malate (10 mM) or succinate (10 mM) and rotenone (2 μM) as substrates for respiration. 50 μl of mitochondrial suspension (10 μg) were delivered per well of XFe24 Seahorse plate and plates were spun 15 min at 2000 x g at 4 °C. After centrifugation, 450 μl of warm (37 °C) MAS buffer with substrates were added. Plates were incubated 8–10 min at 37 °C in an incubator without CO2 to allow the plate to warm and then loaded into the XFe24 Analyzer for mitochondrial respiration analysis. Mitochondria began in a coupled state with substrate present; pyruvate and malate (10 mM) or succinate (10 mM) and rotenone (2 μM) (state 2). State 3 was initiated with ADP (4 mM) addition, state 4 was induced with the injection of oligomycin (2.5 μg/ml) (state 4o), and FCCP (4 μM) induced maximal uncoupler-stimulated respiration (state 3u). Non-mitochondrial respiration was assessed by OCR measurement in the presence of antimycin (4 μM). All states were sequentially measured, allowing calculation of respiratory control ratios (OCR: state 3/state 4o, or UCR: state 3u/state 4o). 3–5 mitochondrial preparations per experimental group were analyzed always in triplicates in each plate. Measurement protocol for standard liver mitochondria: 2 cycles of 1 min mix and 3 min measure per state. Measurement protocol for liver mitochondria after incubation with CBSAC: 2 cycles of 1 min mix and 4 min measure (State 2); 1 cycle of 1 min mix and 4 min measure (other States).

2.10. Immunoﬂuorescence and laser confocal imaging

Cultured mouse hepatocytes were fixed for 15 min with 4% paraformaldehyde and permeabilized and blocked for 15 min with 0.2% saponin dissolved in 1% BSA-fatty acid free (FAB) in PBS commercial buffer. Tom20 (Santa Cruz) primary antibody was incubated overnight in BSA 1% followed by a secondary antibody for 1 h at room temperature in BSA 0.1%. Filipin (Sigma) was added at 0.33 mg/ml during the secondary antibody incubation and the following steps were performed in the dark. Stained samples were embedded in fluoromount (Sigma) and digital images were taken in a Leica DM2500 confocal microscope.

2.11. Protein carbonylation

Protein carbonylation was assessed by measuring the levels of carbonyl groups using the OxyBlot Protein Oxidation Detection Kit (Millipore), following manufacturer’s instruction. The DNPH derivatization was carried out on 10 μg of protein for 15 min. One-dimensional electrophoresis was carried out after DNPH derivatization using 4–12% SDS-polyacrylamide gels (SDS-PAGE) (Bio-Rad, XT-Criterion). Proteins were transferred to nitrocellulose membranes (Bio Rad) which were then blocked with 1% BSA in TBS-Tween. Membranes were then incubated in the primary antibody solution (anti-DNP 1:150) followed by incubation with secondary antibody solution (1:300), both incubations during 1 h at room temperature. After every step, membranes were thoroughly washed with TBS-Tween. Immunoreactive bands were visualized by Pierce ECL Western Blotting Substrate (Thermo Scientific) and band signal intensity was quantified by ImageJ software.

2.12. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc). Unpaired Student’s t-test (two tailed) was performed between two groups and one or two-way ANOVA followed by Tukey’s Multiple Comparison test were used for statistical comparisons between three or more groups. The corresponding number of experiments is indicated in the figure legends. Data in graphs are shown as mean ± s.e.m.

3. Results

3.1. HC diet alters hepatic cholesterol homeostasis while sodium cholate feeding represses bile acid synthesis

Although feeding a HC diet supplemented with sodium cholate has been previously used as a nutritional approach to steadily increase liver cholesterol content [19,34], the contribution of sodium cholate in the regulation of cholesterol homeostasis in relationship with bile acid synthesis has not been previously examined. Thus, we first
characterized the regulation of hepatic cholesterol homeostasis and whether dietary cholesterol feeding in the presence of sodium cholate was channeled for bile acids generation. Short-term HC feeding increased cholesterol levels in serum (Fig. 1A) and liver, as determined by HPLC analyses (Fig. 1B), or upon staining of liver sections with filipin to detect free cholesterol levels (Fig. 1C). However, feeding sodium cholate (0.5%) for two days did not significantly increase serum or liver cholesterol levels (Fig. 1A–C). Moreover, the increase of liver cholesterol levels following HC feeding translated in decreased expression of key steps involved in de novo cholesterol biosynthesis, such as Srebp2 (Fig. 1D), a transcription factor that regulates cholesterol neosynthesis, and its target gene Hmgcr (Fig. 1E), which catalyzes the rate-limiting step in the mevalonate pathway. Moreover, as cholesterol is a precursor for bile acids synthesis, we next examined the expression of enzymes
involved in classical (Cyp7a1, Cyp8b1) and the alternative pathway of bile acid synthesis in mitochondria (Cyp27a1, Cyp7b1) (Fig. 1F). While HC feeding markedly decreased the expression of cyp7a1 as well as cyp8b1, this effect was primarily due to the presence of sodium cholate in the diet (Fig. 1G). HC or sodium cholate feeding, however, exerted a modest effect in the expression of Cyp27a1 and Cyp7b1, indicating a minor impact in the alternate pathway of mitochondrial bile acid synthesis. Consistent with these findings, HC feeding did not stimulate the net increase in the total bile acid pool in the liver, likely reflecting the repression by sodium cholate of the enzymes involved in bile acid synthesis (Fig. 1H). Thus, these findings indicate that the presence of sodium cholate in the HC diet contributes to the maintenance of free cholesterol levels, in part, by suppressing its metabolism into bile acid.

3.2. HC-induced mitochondrial cholesterol loading distributes in both mitochondrial membranes and disrupts mitochondrial morphology

Since HC feeding has been shown to increase mitochondrial cholesterol content [19,34], we next examined the expression of putative mitochondrial cholesterol carriers and the distribution of cholesterol between the outer and inner mitochondrial membranes. Confirming previous findings in rat liver mitochondria [19], isolated mitochondria from HC-fed mice exhibited a significant increase in free cholesterol levels (>2-fold) determined by HPLC analyses compared to mitochondria from control mice (Fig. 2A). Feeding a 0.5% sodium cholate did not stimulate cholesterol levels. The increase in mitochondrial cholesterol was also observed by confocal imaging of primary mouse hepatocytes (PMH) isolated from HC-fed mice upon staining with filipin (free-cholesterol marker) and Tom20 (mitochondrial marker). As seen, HC feeding resulted in increased filipin staining, which co-localized with Tom20 indicating increased trafficking of free cholesterol into mitochondria (Fig. 2B). Since STARD1 and MLN64 are known to regulate mitochondrial cholesterol trafficking [14–16,40], we examined their expression in mice fed the HC diet. As shown, Stard1 and Mln64 expression increased after HC feeding (Fig. 2C). We next assessed the relative distribution of cholesterol enrichment within mitochondrial membranes. Mitoplasts were isolated from intact mitochondria after permeabilization of the outer membrane upon digitonin treatment. Mitoplasts purity was confirmed by the de-enrichment of porin and the increase in COX IV, compared to intact mitochondria, which was enriched in both markers (Fig. 2D). While cholesterol levels in mitoplasts were lower than in intact mitochondria, the cholesterol content of mitoplasts from cholesterol-enriched mitochondria was higher than in mitoplasts from control mitochondria, indicating the enrichment of cholesterol in both outer and inner mitochondrial membranes (Fig. 2E). The increase of cholesterol in mitochondrial membranes is known to change membrane physical properties [3,19,20]. Thus, we next confirmed whether the presence of cholesterol in mitochondrial membrane inserted into the lipid bilayers and altered membrane fluidity. Fluorescence anisotropy analysis of mitochondria labeled with the fluorescent probe DPH revealed an increase in membrane order of mitochondria from HC-fed animals compared to control mice (Fig. 2F). Moreover, we next addressed whether the loss of mitochondrial fluidity by cholesterol enrichment altered mitochondrial morphology. Electron microscopy analyses revealed that mitochondria from HC-fed mice appeared rounded and with abnormal cristae compared to mitochondria from control animals (Fig. 2G). Although mitochondrial number was not altered by cholesterol accumulation, a significant decrease in mitochondrial length was observed (Fig. 2H). These findings indicate that nutritional cholesterol feeding traffics to mitochondria and alters mitochondrial membrane fluidity and morphology.

3.3. Mitochondrial cholesterol enrichment impairs complex I and complex II-driven state 3 respiration

In view of the preceding findings indicating increased cholesterol in mitochondrial membranes, we next examined the impact of this event in mitochondrial respiration, determining real-time oxygen consumption rates (OCR) by a flux analyzer. Using pyruvate and malate as substrates of complex I, mitochondria from HC-fed mice showed a significant reduction in mitochondrial performance and respiratory states compared to mitochondria from mice-fed control diet (Fig. 3A). In particular, HC feeding impaired ADP-stimulated state 3 respiration as well as the FCCP-induced maximal uncoupler-stimulated respiration (state 3u) without significant change in state 4o respiration (Fig. 3B). These alterations were reflected in the respiratory control ratio (RCR) (state 3/state 4o) and uncoupling control ratio (UCR) (state 3u/state 4o), decreasing both in mitochondria from HC-fed mice (Fig. 3C). Similar analysis was performed using succinate plus rotenone to examine respiration through complex II. HC feeding impaired OCR from succinate plus rotenone with decreased state 3 and state 3u (Fig. 4A and B). In addition, HC feeding decreased succinate plus rotenone-driven state 4o respiration (Fig. 4B), which translated in unchanged RCR and UCR ratios (Fig. 4C). Thus, these findings indicate that in vitro mitochondrial cholesterol loading significantly impairs mitochondrial complex I and complex II-driven state 3 respiration. Since the protonotive force generated from coupled respiration determines the mitochondrial membrane potential, we next assessed the impact of mitochondrial cholesterol loading in membrane potential. Incubation of PMH from HC-fed mice stained with TMRE revealed a decrease in mitochondrial membrane potential (Fig. 3D). Overall, these findings demonstrate the deleterious effects of cholesterol enrichment on mitochondrial respiration.

3.4. Cholesterol-lipid interactions contribute to decreased complex I-driven state 3 respiration

To determine whether the impairment of mitochondrial respiration by HC-induced mitochondrial cholesterol loading is a direct consequence of cholesterol enrichment in mitochondrial membranes, we used an in situ approach in which cholesterol complexed with BSA (CBSAC) results in the enrichment of cholesterol content in both membranes [32]. Compared to incubation with BSA alone, CBSAC resulted in the increase in cholesterol levels with a 4-fold increase. Real-time OCR driven by pyruvate plus malate revealed that cholesterol did not affect state 3 or state 3u respiration, although it uncoupled mitochondrial respiration as revealed by the increased state 4o respiration (Fig. 5A), which translated in decreased RCR and UCR (Fig. 5B). However, when succinate plus rotenone were used as substrates the enrichment in cholesterol did not affect succinate-driven OCR and hence RCR and UCR remained unchanged (Fig. 5C and D). Since the interaction of cholesterol with membrane proteins is enantioselective but the interaction with membrane lipids is not [41–43], we next enriched mitochondria with enantiomer cholesterol (Ent-CBSAC), the mirror image of natural cholesterol, as an approach to assess the contribution of the interactions between cholesterol with lipids and/or proteins in the disrupting effect of cholesterol in pyruvate and malate-driven respiration. Mitochondria enriched in Ent-CBSAC exhibited an increase in cholesterol levels similar to those found with natural CBSAC (2.5-fold compared to BSA). As CBSAC failed to alter complex II-driven respiration, we tested the role of Ent-CBSAC on complex I-induced OCR. Interestingly, Ent-CBSAC decreased pyruvate and malate-driven state 3 and state 3u respiration, which resulted in lower RCR and UCR (Fig. 5A and B). These in vitro findings suggest that cholesterol-lipid interactions could mediate the decrease in complex I-driven state 3 and state 3u respiration.
A. Liver Mitochondria

B. Filipin, Tom20, Merge
 Filipin-Tom20 Colocalization

C. Stard1, Mfn64

D. CTRL, HC
 Porin, COX IV

E. CTRL, HC

F. Mitochondrial Membrane Order

G. CTRL, HC

H. Mitochondria Number, Mitochondrial Length

(caption on next page)
3.5. Mitochondrial cholesterol enrichment disrupts the assembly of respiratory supercomplexes

In view of the preceding findings, we hypothesized that mitochondrial cholesterol loading in vivo by HC feeding may impact negatively the expression of respiratory complexes. Therefore, we first performed SDS electrophoresis to determine the expression of specific subunits of respiratory complexes. As seen, the expression of specific subunits from complex I (C-I-20), complex II (C-II-30), complex III (C-III-Core 2), complex IV (C-IV-I) and complex V (C-V-α) were unaffected by mitochondrial cholesterol loading by HC feeding (Fig. 6A and B). Since the structural organization of the mitochondrial respiratory complexes as independent entities connected by mobile carriers such as CoQ and cytochrome c has been challenged [39], we next hypothesized that cholesterol-mediated changes in membrane physical properties may impact the organization and assembly of supercomplexes structures, responsible for carrying out cellular respiration. Blue native electrophoresis of mitochondria after digitonin solubilization was carried out with subsequent antibody incubation against specific subunits of each respiratory complex sequentially. Mitochondrial cholesterol accumulation did not change the levels of respiratory complexes CI, CIV and CV but caused a significant downregulation of respiratory complex III2 (Fig. 6C and D). This outcome mirrored the downregulation of supercomplexes I1+III2+IV and I1+III2 in cholesterol-enriched mitochondria (Fig. 6C and D). These data indicating a negative impact of cholesterol on the assembly of respiratory supercomplexes can account for the observed alterations in mitochondrial respiration and emerge as a potential molecular mechanism by which cholesterol accumulation in liver alters mitochondrial function.
3.6. GSH ethyl ester protects against HC-induced oxidative stress and liver injury

As mitochondria are the main consumers of oxygen and a major source of ROS [11–13], we next determined whether HC-induced mitochondrial dysfunction results in increased oxidative stress. As seen, staining of liver sections from HC-fed mice with DHE indicated increased generation of ROS compared to control mice (Fig. 7A and B). This outcome paralleled the depletion of cellular GSH levels (Fig. 7C) as well as the mGSH pool (Fig. 7D), which is primarily due to the defective transport of GSH into mitochondrial matrix [20,29,30]. These findings were accompanied by the stimulation of superoxide anion and especially hydrogen peroxide in isolated mitochondria from HC-fed mice (Supplementary Fig. 1), with the latter effect reflecting the depletion of mGSH induced by mitochondrial cholesterol loading. Consistent with these findings, HC feeding resulted in the increased oxidative stress as shown by enhanced carbonylated proteins (Fig. 7E). HC diet induced a macroscopic change in liver color when compared to control diet. HC livers displayed a steatotic pale appearance compared to control livers (Fig. 7F) that paralleled oil-red staining (Supplementary Fig. 2). In addition, H&E staining revealed a significant dilation of liver perisinusoidal spaces after 2 days of HC feeding (Fig. 7G). These changes were accompanied by the release of AST and ALT in serum indicating that HC-diet induces liver injury (Fig. 7H). We next explored the potential impact of total GSH and mGSH restoration by GSHee in HC-fed mice. GSHee treatment of HC-fed mice restored both total and mGSH pools (Fig. 7C and D). Moreover, GSHee treatment decreased HC-induced DHE increase (Fig. 7A and B), reduced protein carbonylation (Fig. 7E), restored the HC-mediated dilation of liver perisinusoidal spaces (Fig. 7G), and decreased ALT/AST release, indicating that GSHee protected HC-fed mice from liver injury when compared to HC non-treated mice (Fig. 7H). To assess whether the outcome of GSHee administration was accompanied by improved mitochondrial function, we examined the impact of in vivo GSHee administration in mitochondrial performance from pyruvate plus malate-induced OCR. Consistent with previous findings, HC feeding decreased OCR and RCR from complex I that was improved upon GSHee treatment (Fig. 7 I, J). Moreover, GSHee restored the levels of respiratory complex III2, although it did not affect the assembly of respiratory supercomplexes I+III2+IV and I + III2 (Fig. 7K). Thus, these findings indicate that GSHee administration protects mice from HC-induced liver injury and oxidative stress in part by improving mitochondrial respiration.

4. Discussion

Cholesterol accumulation and, particularly its trafficking to mitochondria, has emerged as a key player in different chronic diseases, including fatty liver disease and hepatocellular carcinoma [12,19,24]. Given the critical structural role of cholesterol in membrane bilayers, the contribution of mitochondrial cholesterol enrichment in disease pathogenesis is exerted in part through its effects in the perturbation of membrane physical properties. However, the specific impact of cholesterol in mitochondrial function and routine performance has not been previously addressed. In order to rapidly increase liver cholesterol levels, we used an in vivo approach in which mice were fed a cholesterol-enriched diet supplemented with sodium cholate, which has been shown to sustain liver cholesterol levels [19,34]. Importantly, the presence of sodium cholate in the diet downregulated the expression of
key enzymes involved in the synthesis of bile acids such as Cyp7a1 and Cyp8b1, without effect in the alternate pathway of bile acid synthesis in mitochondria. This finding indicates that sodium cholate is an important component of the HC diet, which contributes to the maintenance of hepatic free cholesterol levels by preventing the metabolism of dietary cholesterol into bile acids.

The in vivo loading of hepatic cholesterol levels by HC feeding attenuated the expression of Srebpg and Hmgr, which reflects the alterations in endogenous cholesterol homeostasis, an effect that was accompanied by its accumulation in mitochondria. This outcome paralleled the increased expression of Star1 and Mito64, which play critical roles in the regulation of mitochondrial trafficking [14–16]. Importantly, mitochondrial fractionation into mitoplasts revealed that cholesterol accumulation occurs in both the outer and inner mitochondrial membranes, consistent with a concerted action between MLN64 and STAR1, which move cholesterol from extramitochondrial sources to the outer mitochondrial membrane and from here to the inner mitochondrial membrane, respectively [14–16]. Of relevance, we show that in vivo cholesterol accumulation impairs mitochondrial oxidative phosphorylation, reflected in decreased ADP-stimulated OCR from complex I, which translated in decreased RCR and UCR. This outcome is accompanied by the defective assembly of respiratory supercomplexes I1 + III2+ IV and I1 + III2. These findings are consistent with the current concept that mitochondrial supercomplexes structures are responsible for carrying out cellular respiration [39,44], as opposed to the long-held view of mitochondrial respiratory complexes as independent entities connected by mobile carriers CoQ and cytochrome c. The vast majority of the subunits of the mitochondrial respiratory complexes are encoded by nuclear DNA, which then traffic to mitochondria by the presence of intramitochondrial sorting signals to undergo a highly-regulated mechanism of import and insertion into mitochondrial membranes [45]. However, hepatic cholesterol loading does not affect the expression of specific subunits of complex I-V, suggesting that the effect of mitochondrial cholesterol in the assembly of the mitochondrial supercomplexes may reflect defects at the mitochondrial membrane level likely due to changes in membrane physical properties. This outcome is consistent with recent findings pointing that membrane lipid composition determines cellular respiration through changes in membrane fluidity and physical properties [46,47]. Indeed, reconstitution of complex I and complex III in proteoliposomes with different lipid composition revealed that the ratio of phospholipid to protein determines the assembly of supercomplex I1 - III2 [46]. Moreover, studies in bacteria engineered to express increased levels of unsaturated fatty acids unraveled that membrane viscosity...
determined by lipid composition controlled the rate of respiration coupled to ADP phosphorylation [47]. Whether the deleterious effect of cholesterol enrichment in mitochondrial function is reversible upon cholesterol extraction or membrane fluidization remains to be further investigated. In line with this possibility, Yu et al. showed that the defective ATPase function from brain mitochondria from NPC knockout mice, which exhibit an increased mitochondrial cholesterol loading, was restored by cholesterol extraction with cyclodextrin [48]. Thus, while mitochondrial cholesterol loading in vivo by feeding the HC diet may reflect the combined effect of cholesterol on established and newly assembled respiratory complexes by membrane mediated changes, we addressed the direct impact of cholesterol enrichment in the existing respiratory complexes in isolated mitochondria and whether this event involves interactions of cholesterol with bilayer’s proteins and/or lipids using enantiomer cholesterol, the mirror image of natural cholesterol.

Enrichment of liver mitochondria in enantiomer cholesterol, which specifically interact with lipids but not proteins due to enantioselective restriction [37,41–43], resulted in decreased complex I-driven state 3/3u respiration, suggesting that cholesterol-lipid interactions in mitochondrial membranes accounts for the impaired ADP-stimulated OCR driven by complex I. Intriguingly, although in vitro cholesterol loading increased complex I-driven state 4o respiration, this outcome was not observed with the in vivo mitochondrial cholesterol loading by HC feeding. Whether cholesterol-lipid interactions is a predominant effect over the interaction of cholesterol with proteins to account for the decreased complex I-driven state 3/3u respiration remains to be further investigated. Interestingly, in situ cholesterol enrichment in mitochondria did not affect complex II-driven OCR although in vivo mitochondrial cholesterol loading did impair state 3 respiration. This differential effect suggests that the impact of cholesterol in impairing complex II-mediated OCR reflects the impact of cholesterol in the assembly of respiratory supercomplexes rather than a direct effect of the changes in membrane physical properties.

A caveat from the in vivo enrichment of mitochondria in cholesterol on mitochondrial respiration by feeding a HC enriched diet is whether the effects truly reflect the actions of cholesterol per se or are determined by cholesterol-derived metabolites. As cholesterol is the precursor of bile acids, it is conceivable that part of the observed effects of cholesterol enrichment in mitochondrial performance could be mediated by the action of bile acids generated from dietary cholesterol. This potential contribution, however, seems unlikely in light of the fact that the presence of sodium cholate in the HC-enriched diet impaired the classical pathway of bile acid synthesis due to decreased the expression of cyp7a1 and cyp8b1, consistent with the lack of increased bile acid pool from HC-fed mice.

As one of the reported consequences of the disruption of the association between complex I and complex III and the impaired assembly of subsequent supercomplexes I1+III2+IV and I1+III2 is the enhanced generation of superoxide anion [46], we analyzed the impact of mitochondrial cholesterol enrichment in oxidative stress and liver injury. While HC feeding caused increased ROS formation, mitochondrial ROS generation and mGSH depletion that was accompanied by steatosis and liver injury, these events were recovered by GSHee administration. Interestingly, the effect of mGSH restoration by GSHee translated in...
replenishing mitochondrial respiration and RCR, in line with previous findings in cerebellar mitochondrial function in NPC disease [35]. Restoration of mGSH levels by GSHee rescued the content of complex III₂, although it failed to normalize the assembly of supercomplexes I + III₂ + IV and I + III₂, which likely reflects the effect of remaining cholesterol-mediated effect on membrane dynamics that are not reversed by mGSH restoration. However, quite intriguingly, the restoration of complex III₂ by GSHee seems to compensate the function of mitochondrial respiration. Further work will be required to determine the mechanisms whereby mGSH depletion by mitochondrial cholesterol...
loading impact the redox-dependent assembly of supercomplexes as assembly and whether the reversible loss of complex II by cholesterol is redox dependent in view of its restoration by mGSH with GSHee.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101214.

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