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Hepatic steatosis impairs mitochondrial electron transfer in female rats

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Introduction

Fatty liver develops when hepatic lipid input and synthesis exceed oxidation and export. Accumulation of triacylglycerols (TAGs), cholesterol and other lipids may progress from simple steatosis to inflammatory and fibrotic injury. Mitochondria are central to this transition because they integrate fatty-acid oxidation, the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS). Their inner membrane contains electron transfer-pathway complexes that conserve redox energy as protonmotive force for ATP synthesis. Experimental models can therefore help determine whether hepatic lipid accumulation is merely a marker of stress or a direct cause of bioenergetic failure [1].

Ethionine provides a rapid model of hepatic steatosis. This non-proteinogenic methionine analogue disrupts methyl-group metabolism and phosphatidylcholine-dependent very-low-density lipoprotein export, promoting retention of neutral lipid in hepatocytes [2]. Diet-induced models develop more gradually and model chronic lipid exposure. Comparing acute ethionine-induced and longer diet-induced steatosis can reveal shared mitochondrial consequences despite different disease triggers.

Clinical and experimental studies link steatotic liver injury to impaired mitochondrial-complex activities and reduced ATP-generating capacity [3,4]. Complex I is a plausible early target because it accepts electrons from NADH, transfers them through flavin and iron-sulfur centers to ubiquinone, and contributes strongly to the protonmotive force. We therefore tested whether hepatic lipid accumulation alters mitochondrial electron transfer in female Sprague-Dawley rats. Native-complex separation, in-gel activity staining, spectrophotometric assays and immunoblotting were combined with biochemical and lipidomic assessment. We hypothesized that both models would impair OXPHOS, with complex I as a major site of dysfunction.

Methods

Thirteen female Sprague-Dawley rats were allocated to control chow (n = 5), diet-induced fatty liver (D-FL; n = 5), or ethionine-induced fatty liver (E-FL; n = 3). Animals were housed under controlled temperature, humidity and 12 h light-dark cycles. The D-FL group received standard chow soaked in extra-virgin olive oil for 12 weeks after

weaning. The E-FL group received four intraperitoneal injections of 3 mL D,L-ethionine (17 mg mL^{-1} in isotonic saline) at 3 h intervals, beginning 24 h before euthanasia. The Scientific Research Ethics Committee of Birzeit University approved the study, which followed NIH animal-care guidance.

Livers were inspected macroscopically and used for lipid extraction and mitochondrial isolation. Lipids were separated by thin-layer chromatography using hexane-diethyl ether-formic acid (80:20:2, by volume); TAG species were assessed by tandem mass spectrometry and cholesterol by the Liebermann-Burchard reaction.

Mitochondria were isolated by differential centrifugation in mannitol-sucrose-HEPES buffer and standardized to 2 mg mL^{-1} protein. Isolation of mitochondria through differential centrifugation in mannitol-sucrose-HEPES buffer to a final concentration of 2 mg mL^{-1} protein was done. Intact OXPHOS complexes were fractionated by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) followed by activity staining of complexes I, II and IV, and ATP synthase [5]. Quantification of band intensity was performed using ImageJ. Assays for citrate synthase, complex I, II and IV, complex I + III linked to NADH and complex II + III linked to succinate were performed by spectrophotometry using defined substrate and inhibitor combination [6]. All experiments were at 37 °C and performed in technical triplicates with three biological replicates if available. Immunoblotting was used to assess NDUFS1 and NDUFA9 proteins with VDAC1 as mitochondrial loading control. Results are presented as means ± standard deviation. In the absence of full statistical analysis information from the source data, conclusions based on change direction and magnitude only are provided.

Results and Discussion

In both cases (D-FL and E-FL), significant hepatic accumulation of lipids was observed. Livers of D-FL and E-FL rats were paler and possessed more evident lipid deposition than those from controls. Lipid profiling by thin layer chromatography revealed enhanced TAG and cholesteryl-ester peaks, and several TAG classes were detected by targeted analysis. The results also showed an increase in the content of cholesterol in both models, which was stronger in D-FL animals. An increase in the overall content of sphingolipids is reported.

Mitochondrial dysfunction was associated with the development of steatosis but lipid accumulation differentially affected mitochondrial complexes (Table 1). Activity of citrate synthase was decreased in both models. Most affected was the complex I: it showed decreased spectrophotometric activity in both cases and close to complete absence of spectrophotometric activity in D-FL mice. Independent confirmation of reduced activity of native complex I was provided by BN-PAGE. The amount of NDUFS1 protein was decreased in both models, while that of NDUFA9 and VDAC1 proteins remained high.

The activity of complex II was found to be reduced in D-FL mitochondria but remained normal or was even elevated in E-FL mitochondria. The activity of succinate-dehydrogenase-linked electron transport in complexes II and III showed no significant

changes, but the activity of NADH-dehydrogenase-linked electron transport in complexes I and III was significantly reduced in both models. There were no changes in complex IV activity in the general data set.

Table 1. Direction of change relative to control liver mitochondria.

Endpoint	D-FL	E-FL
Hepatic TAG and cholesterol	Higher	Higher
Citrate synthase activity	Lower	Lower
Complex I activity / NDUFS1	Strongly lower	Lower
Complex II activity	Lower	Preserved/slightly higher
NADH-linked I+III transfer	Lower	Lower
Succinate-linked II+III and complex IV	No clear change	No clear change

D-FL, diet-induced fatty liver; E-FL, ethionine-induced fatty liver; TAG, triacylglycerol.

These data concur with and extend previous descriptions of impaired complex I function in fatty livers induced by an experimental diet and decreased ability of OXPHOS in steatotic liver [3,4]. In the diet-induced model, the pronounced defect is more likely due to prolonged lipid exposure while the ethionine model demonstrates that changes in mitochondrial physiology can appear rapidly with the impairment of lipid export. Remodeling of lipids can affect the conditions for proper enzyme assembly by changing the environment of the inner mitochondrial membrane but not directly proved in this study.

It is interesting that decreased level of NDUFS1 was observed because this subunit is involved in the iron-sulfur-containing peripheral subcomplex of complex I which transfers electrons to ubiquinone. Normal levels of NDUFA9 and VDAC1 argue that the general loss of proteins is not possible. Further studies are necessary to determine if decreased level of complex I protein subunits is connected with its assembly defects.

The model specific effects lend further weight to this interpretation. Ethionine has an acute effect on methylation and lipoprotein export, while the olive oil diet involves exposing the liver to a lipid excess for 12 weeks. Thus, convergence in complex I implies that it is the outcome of lipid loading, rather than ethionine toxicity, per se. In the case of E-FL, the maintenance of complex II indicates that not all OXPHOS proteins are equally compromised by steatosis. The selective impairment of NADH but not succinate transfer supports this idea.

Impairment of complex I might reduce the oxidation of NADH and disturb the redox balance and cause electron leakage. While this potential downstream effect was not addressed experimentally, one can use the results in combination with previous information regarding lipid loading and NADH oxidation to conclude about the specific bioenergetic defect in the liver, which needs to be validated by high-resolution respirometry and analysis of ROS production.

Limitations of this study include uneven sample size and the use of female rats only, as well as incomplete statistics for data analysis. However, the consistent results from kinetic analysis and native gel and western blots indicate the hypothesis that the development of hepatic steatosis negatively impacts mitochondrial respiration,

especially through complex I. Further research is needed to measure oxygen consumption rate, ATP production, reactive oxygen species and intermediates of complex I assembly in balanced groups to see if stabilization of complex I can help prevent liver damage.

Keywords: hepatic steatosis; mitochondria; complex I; BN-PAGE; ethionine

Author contributions: JS designed the study and obtained funding. BMI, RJ, YA and JS performed data collection and evaluation. YAH provided lipidomic analysis. BMI drafted the full manuscript; JS revised it. All authors reviewed and approved this short communication.

Conflicts of interest: All authors declare no conflicts of interest.

Data availability: Laboratory data are available from the corresponding author upon reasonable request.

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