Exercise during weight loss improves hepatic mitophagy

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) has recently become a public health concern concurrent with the obesity crisis. Previous work has shown aberrant mitochondrial content/quality and autophagy in models of NAFLD, whereas exercise is known to improve these derangements. The purpose of this study was to examine the effect of different weight-loss modalities on hepatic mitochondrial content, autophagy and mitophagy in NAFLD. Forty-eight male C57BL/6J mice were divided into 1 of 4 groups: low fat diet (LFD, 10% fat, 18 weeks), high fat diet (HFD, 60% fat diet, 18 weeks), weight-loss by diet (D, 60% fat diet for 10 weeks then 10% fat diet for 8 weeks) or weight-loss by diet and physical activity (D/PA, 60% fat diet for 10 weeks, then 10% fat diet plus a running wheel for 8 weeks). Immunoblot data were analyzed by one-way analysis of variance (ANOVA) with significance denoted at \( p < 0.05 \). COX-IV protein contents were approximately 50% less in HFD compared to LFD. D/PA had 50% more Bnip3 compared to HFD. PINK1 content was 40% higher in D and D/PA compared to LFD. P-Parkin/Parkin levels were 40% lower in HFD, D, and D/PA compared to LFD. Whereas p-UbSer65 was 3-fold higher in HFD. LC3II/I ratio was 50% greater in HFD and D/PA, yet p62 protein content was 2.5 fold higher in HFD. High-fat diet causes disruptions in markers of mitochondrial quality control. Physical activity combined with diet were able to ameliorate these derangements and seemingly improve hepatic mitochondrial quality above control values.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the leading preventable liver ailment in Western society.1,2 Rates of NAFLD have closely mirrored the obesity epidemic, with an estimated 19%–30% of adults in Western society diagnosed with NAFLD.1,2 NAFLD can begin with relatively benign fat accumulation within the liver, but with continued lipid overload, can progress to steatosis, inflammation, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and potentially failure.3 Currently, there are no effective pharmacological interventions for the liver disease once progressed to NASH.4 As such, it is imperative to halt the progression of NAFLD and subsequent NASH. It is currently understood that lifestyle interventions such as weight-loss, diet and exercise mitigate many of the symptoms associated with NAFLD5,6 and are therefore a favorable therapeutic modality for halting the progression hepatic pathologies before NAFLD develops.

Mitochondria, the major supplier of ATP (adenosine triphosphate) to the cell, play a major role in the progression of NAFLD.7 Previous work has demonstrated derangements in mitochondrial quality control mechanisms in various models of hepatic lipid overload.8–11 Work by Rector et al.12 has also demonstrated mitochondrial dysfunction preceding hepatic steatosis in a genetic model of NAFLD. To maintain quality control, dysfunctional mitochondria must be removed by mitochondrial-specific autophagy, hereafter referred to as mitophagy. Mitochondria undergo multiple mitophagy pathways,13 with the two predominant pathways including Bnip3 mediated and PINK1/Parkin mediated mitophagy. Bnip3 mitophagy predominantly occurs under pathological conditions,14,15 with the accumulation of PINK1 causing subsequent phosphorylation of PARKIN and ubiquitin at serine-65, tagging the mitochondria for degradation.16–18 To our

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knowledge, few studies have investigated hepatic PINK1/PARKIN mitophagy during lipid-overload or exercised conditions. Previous work from our laboratory has suggested increased hepatic autophagy with exercise may provide protective effects from NAFLD despite lipid overload,19,20 and that Western diet can decrease BNIP3 mediated mitophagy.19 Yet, it is unclear how physical activity combined with dietary alterations (reduced caloric intake) may differentially affect hepatic mitophagy compared to dietary alterations alone. Furthermore, it is unclear what, if any impact PINK1/PARKIN mitophagy has on hepatic health in NAFLD and with weight-loss interventions to improve NAFLD. Therefore, the purpose of the study was to investigate autophagy and mitophagy regulation after weight-loss by diet or diet combined with physical activity in a murine model of NAFLD.

Materials and methods

Animal interventions

All animal work was performed at and approved by the Southern Illinois University at Edwardsville Institutional Animal Care and Use Committee as previously described.21 Further details of both the animal protocol and other phenotypic outcomes can be found in other works using these same animals.21 Briefly, male C57BL/6J (n = 48; Jackson Laboratories, Bar Harbor, ME) mice were evenly divided into two groups at 8 weeks of age, one group of 12 animals consumed a low fat diet (LFD, 10% of kcal from fat, Research Diets #D12450J, New Brunswick, NJ) and 36 animals consumed high fat diet to induce obesity (60% of kcal from fat, Research Diets #D12492). Animals consumed diets for 10 weeks, after which, high fat diet-induced obese animals were further divided into three groups, one (HFD, n = 12) continued to consume the 60% high fat diet, one group was placed back on the 10% fat diet to induce weight-loss (D, n = 12) and the final group was placed on the 10% fat diet and given a freely movable running wheel to induce physical activity to improve weight-loss (D/PA, n = 12). Wheel running activity was monitored daily with the Vital View Data Acquisition System and daily distance travelled was recorded for data analyses (Mini-Mitter, Bend, OR). LFD animals continued consuming a 10% fat diet. Animals continued interventions for an additional 8 weeks. The final groups included: LFD, HFD, D, and D/PA. After interventions, animals were euthanized by an overdose of isoflurane and confirmed by cardiac puncture. Following perfusion with saline (0.9% w/v NaCl), livers were collected and snap-frozen in liquid nitrogen for later analysis. Six hours before tissue

Fig. 1. Mitochondrial biogenesis Western blot data. A.) Western blot analysis for PGC1α content. B.) Western blot analysis for COX-IV content. C.) Western blot analysis for BNIP3 content. D.) Representative Western blot images. n = 8–12/group Different letters represent differences between groups at p < 0.05. PGC1α: Peroxisome proliferator-activated receptor-gamma coactivator, COX-IV: cytochrome c oxidase subunit IV, BNIP3: BCL2 and adenovirus E1B 19-kDa-interacting protein 3.
harvest, animals' food was removed and 24 h before tissue harvest running wheels were removed from cages of D/PA animals.

As acknowledged in our previous publication, it is possible animals exhibited excessive energy expenditure due to housing temperatures between 22.5 °C and 23.5 °C. Due to males being more physiologically susceptible to lipid-induced hepatic alterations, and epidemiological data implying males have slightly greater rates of NAFLD compared to females, only males were used in this investigation.

Isolation of protein and immunoblotting

Isolation of protein and immunoblotting were performed as we have previously described. For analysis at least one sample from every group was run on each membrane. Membranes were imaged using a FlourChem M (Protein Simple, San Jose, CA) and protein content normalized to Ponceau S. Primary antibodies included: PGC1α (Santa Cruz, CAT# sc-13067), COX-IV (Cell Signaling, CAT# 4844S), BNIP3...
(Cell Signaling, CAT# 3769), LC3 (Cell Signaling, CAT# 4108), p62/SQSTM1 (Cell Signaling, CAT# 5114s), PARKIN (Santa Cruz, CAT# sc-33796), PARKIN Ser65 (Abcam, CAT# ab154995), Ubiquitin (Cell Signaling, CAT# 3933), and P-UbiquitinSer65 (R & D Systems, CAT# A-110). All antibodies were diluted with 1:1000 or 1:500 per manufacturer recommendations.

**Statistical analysis**

Independent variables were the interventions (LFD v. HFD v. D v. D/PA). Results were analyzed by one-way analysis of variance (ANOVA), with a set at 0.05. If the global F test was significant, Tukey’s post-hoc-adjustment was used to determine differences between groups. All data were analyzed using the Statistical Analysis System (SAS, version 9.3, Cary, NC) and expressed as mean ± standard error of the mean (SEM). All statistical code and data are available upon request to the corresponding author.

**Results**

**HFD lowered mitochondrial content, D/PA restored multiple components of mitochondrial turnover**

Other phenotypic data on these animals has previously been reported.21 In brief, HFD animals had significantly larger livers compared to LFD, D, or D/PA (~3 g v. ~1 g). Correspondingly, HFD also had significantly greater hepatic lipid mass and triglyceride concentrations compared to LFD, D, or D/PA (~24% total lipid mass compared to 5%–10% lipid mass in other groups and ~25 μg/mg tissue compared with 5–11 μg/mg in other groups).21 Finally, as reported in the prior study,21 D/PA animals ran ~8 km/day during the 8-week running wheel phase of the study, corresponding to an additional estimated energy expenditure of ~25kCal/week in D/PA animals compared to D.21 In this study, HFD did not result in lower PGC1α content (p = 0.383); however, D/PA had ~1.5-fold greater PGC1α content compared to HFD (p < 0.001), which also was ~1-fold greater than LFD animals (p = 0.010) and ~1-fold greater than D animals (p = 0.019, Fig. 1A and D). COX-IV content, a common surrogate marker of mitochondrial content,25,26 was approximately 50% lower in HFD compared to LFD animals (p = 0.049, Fig. 1B and D). COX-IV content in D animals was not different from either HFD (p = 0.811), LFD (p = 0.237) or D/PA (p = 0.163, Fig. 1B and D). D/PA appeared to restore COX-IV content, with no difference between D/PA and LFD (p = 0.997) and ~50% greater COX-IV content compared to HFD (Fig. 2B and D, p = 0.031). Similar to PGC1α content, BNIP3 content was not statistically different between LFD and HFD (p = 0.348, Fig. 1C and D). However, D/PA had ~84% greater BNIP3 content compared to HFD (p = 0.001). D was not significantly different compared to all groups (p = 0.147–0.994, Fig. 1C and D).

**PINK1/PARKIN-mediated mitophagy was altered at different regulatory points in all three experimental conditions**

PINK1 protein content was unaffected by HFD (p = 0.865, Fig. 2A and H), whereas, D and D/PA each resulted in approximately 50% greater PINK1 compared to HFD (p = 0.011 & p = 0.048, Fig. 2A and H). PARKIN content was not different between LFD and HFD animals (p = 0.997, Fig. 2B and H). However, PARKIN content was ~3–4 fold greater in D animals compared to LFD (p < 0.001), HFD (p < 0.001), and D/PA (p = 0.043, Fig. 2B and H). With regards to p-PARKINSer65, no interventions were statistically different from LFD (p = 0.312–0.643). However, D had ~45% greater p-PARKINSer65 compared to both HFD (p = 0.029) and D/PA (p = 0.047, Fig. 2C and H). Comparing p-PARKINSer65/Parkin ratios, both intervention groups (D and D/PA) were roughly 45% lower compared to LFD (p = 0.050 & p = 0.002, Fig. 2C and H).
Total Ubiquitin (Ub) protein content was unaffected by HFD compared to LFD \( (p = 0.893, \text{Fig. 2D and H}) \). D had \( \sim 80\% \) greater Ub compared to HFD \( (p = 0.038, \text{Fig. 2D and H}) \). p-Ub\text{Ser65} was \( \sim 100\% - 150\% \) greater in HFD compared to LFD, D, and D/PA \( (p < 0.001, p < 0.001) \), and \( p = 0.006, \text{Fig. 2F and H}) \), with no other differences noted between groups. Additionally, p-Ub\text{Ser65}/Ubiquitin was \( \sim 1.5\)-fold greater in HFD animals compared to LFD, D, and D/PA \( (p < 0.001, p < 0.001, \& p < 0.001 \text{ respectively, Fig. 2G and H}) \), with no other differences between groups.

### Macroautophagy resolution was altered in HFD animals and rescued with weight-loss interventions

LC3II/I ratio was approximately 1.5-fold greater in HFD and D/PA animals compared to LFD \( (p < 0.001 \& p < 0.001, \text{Fig. 3A and D}) \), with no other differences noted. Total LC3, found by adding the density of both the LC3I and LC3II bands, was \( \sim 80\% \) greater in D/PA animals compared to LFD and D \( (p = 0.056 \& p = 0.043) \); additionally the \( \sim 80\% \) differences between D/PA and HFD approached statistical significance \( (p = 0.085, \text{Fig. 3B and D}) \). p62 content was \( \sim 1.5\)-fold greater in HFD compared to LFD, D, or D/PA \( (p = 0.001, p = 0.002, \& p = 0.004, \text{Fig. 3C and D}) \), there were no other differences between groups.

### Discussion

To our knowledge, our group is the first to report on PINK1/PARKIN-mediated mitophagy in high-fat diet-induced NAFLD in addition to utilizing weight-loss interventions to lessen NAFLD symptoms. Our results demonstrate concurrent diet and physical activity during weight-loss provides greater benefits on aspects of mitochondrial biogenesis and content compared to diet alone as a treatment for NAFLD. Furthermore, D/PA appears to provide benefits to autophagy and mitophagy, whereas diet does not appear to have as prominent effects. Our results suggest that in NAFLD hepatocytes attempt to initiate mitophagy without resolution of the process, allowing dysfunctional mitochondria to remain and potentially exacerbating the disease.

First, our high fat diet was sufficient to induce hepatic lipid overload,\(^1\) a known precursor to the development of NAFLD and similar to previous research.\(^6,12,27,28\) Our data follows our previous findings with reductions in mitochondrial content.\(^15\) Overall, D had a limited effect on all measures of both mitochondrial content and biogenesis. Contrastingly, D/PA increased PGC1\(\alpha\) content above baseline and completely restored mitochondrial content as measured by COX-IV. While PGC1\(\alpha\) content does not specifically measure mitochondrial biogenesis, as the major regulator of mitochondrial biogenesis,\(^9\) PGC1\(\alpha\) content provides valuable insight into the promotion of mitochondrial biogenesis in this model. Additionally, previous studies have corroborated our findings of reduced mitochondrial content in NAFLD utilizing multiple methods.\(^10,28,32\) Taken together, these data demonstrate diet combined with physical activity is more effective for treating disrupted hepatic mitochondrial content compared to diet alone in murine models of NAFLD.

HFD appeared to attenuate the hepatocyte's capacity for BNIP3-mediated mitophagy, as measured by reduced BNIP3 content. As directly measuring the mitophagy process was not a viable option for this study, we interpret alterations in BNIP3 protein content as reflective of the hepatocyte's capacity for mitophagy. While HFD reduced BNIP3-mediated mitophagy, D was not different from either HFD or LFD animals, thus the total impacts of D on BNIP3-mediated mitophagy are inconclusive. Yet, D/PA increased BNIP3-mediated mitophagy capacity compared to HFD. Our findings align with previous research in murine skeletal muscle demonstrating increased mitophagy markers (suggestive of increased mitochondrial turnover) in exercised animals.\(^31,32\) Recent reports have also suggested that increased mitophagy is necessary for increased mitochondrial biogenesis,\(^33,34\) therefore it is unsurprising that BNIP3 content mirrored PGC1\(\alpha\) content in exercised animals. Taken together, our findings suggest that D/PA is more effective in restoring and improving BNIP3-mediated mitophagy.

Interestingly, while BNIP3 increased in D/PA animals, the PINK1/PARKIN pathway is not as easily interpreted. While D or D/PA appear to increase capacity for mitophagy through this mechanism as evidenced by greater PINK1 content, the entire PINK1/PARKIN mitophagy process may be attenuated in HFD, D, and D/PA, as evidenced by lower p-PARKIN/PARKIN content. Yet, p-Ub\text{Ser65} appears to suggest the opposite in HFD animals, with p-Ub\text{Ser65} ratios greatly increased compared to all other groups. The complicated interplay between p-Ub\text{Ser65}, PARKIN and PINK1 is not yet entirely understood, but current literature suggests that for optimal activation of PARKIN, PINK1 needs to phosphorylate both ubiquitin and PARKIN at the corresponding Serine 65 of each target.\(^11\) In addition to phosphorylation by PINK1, PARKIN is further activated by p-Ub\text{Ser65}.\(^36,37\) Specifically, p-Ub\text{Ser65} binds to p-PARKIN\text{Ser65} leading to a conformational shift,\(^38-40\) allowing PARKIN to mediate ubiquilination of the outer mitochondrial membrane proteins, and tagging the mitochondria for degradation.\(^41,42\) Currently, PINK1 is the only known protein to phosphorylate Ubiquitin at Ser65, suggesting that p-Ub\text{Ser65} is specific to PINK1 activity; although it is possible that another protein may perform the same function.\(^43,44\) We noted increased p-Ub\text{Ser65} with a concurrent reduction in P-PARKIN/PARKIN ratio. p-Ub\text{Ser65} is currently known to be predominantly utilized for mitophagy,\(^45\) and p-Ub\text{Ser65} binds to PARKIN to trigger E3 ligase activity.\(^35,44,45\) This may suggest that in NAFLD, hepatocytes may attempt to increase mitophagy through the accumulation of p-Ub\text{Ser65} in order to dispose of damaged mitochondria and/or replace damaged mitochondria with new mitochondria. Yet, due to decreased mitochondrial biogenesis, the cell may forgo the resolution of PINK1/PARKIN-mediated mitophagy in an effort to maintain some of the mitochondrial networks, regardless of the mitochondria's functionality. Or there may be some unknown dysfunction in the signaling between p-Ub\text{Ser65} and p-PARKIN\text{Ser65}, resulting in an accumulation of p-Ub\text{Ser65} without the synchronized increase in p-PARKIN\text{Ser65} activity. Consequently, mitochondria in D or D/PA animals, although having a greater capacity for PINK1/PARKIN mitophagy, may forgo PINK1/PARKIN mitophagy in favor of BNIP3-mediated mitophagy. Although future research investigating total mitochondrial density and function is required to substantiate these claims. We should note that our data are collected in a relatively basal state as running wheels were removed 24 h prior to harvest and food removed 6 h before to remove the effects of acute PA and feeding. Considering this we believe, our examination is of basal phosphorylation and content of these targets which may impact interpretations of the effects of these stimuli.

Corroborating mitophagy specific markers, markers of macro-autophagy suggested a slightly greater capacity for autophagosome formation in D/PA animals, as evidenced by increased total LC3 content.\(^46\) Interestingly, HFD animals had a greater LC3II/I ratio compared to LFD and D, potentially suggesting enhanced autophagy initiation through increased autophagosome formation. Yet HFD had significantly greater p62 levels, compared to all other groups, implying an impaired resolution of autophagy via accumulation of p62. Taken together, these markers suggest an attempted increase in macroautophagy flux but a decreased resolution in high fat diet-induced NAFLD.

Maintaining dysfunctional hepatic mitochondria via decreased mitophagy at the BNIP3 and PINK1/PARKIN levels, may lead to the accumulation of reactive oxygen species (ROS). Unrestrained ROS production has been associated with decreased liver health\(^47\) and the progressive progression of fatty liver disease.\(^48\) Therefore, it is possible that decreased mitophagy through high fat diet-induced NAFLD may be a major regulatory point for the progression of NAFLD. As such, therapeutics to promote mitophagy in NAFLD may be possible treatment options.

We should acknowledge some limitations to the present study. As noted in the previous study using these animals,\(^21\) although the high fat diet interventions were sufficient to induce greater hepatic lipid and triglyceride content, histological confirmation of NAFLD or NASH was
not possible in this study. Therefore, the present work should be interpreted as lipid-induced alterations that would precede the development of diagnosable NAFLD or NASH. Moreover, we were not able to delineate the potential interactions between HFD consumption when performed in conjunction with physical activity. However, given our previous work in animals fed a 45% Western Diet and given access to a running wheel,19 we anticipate the relatively greater volumes of physical activity in the present study (~500 m/day v. ~8 800 m/day) would be sufficient to confer similar hepatic protections noted in the previous study.

Finally, we also were only able to conduct this study on male rodents. Recent works have begun to elucidate the nuances of hepatic health in relation to biological sex.24,49,50 For example, epidemiologically, females tend to have a lower incidence of NAFLD.24 Mechanistically, given the same HFD stimulus, females have lower hepatic H2O2 emission compared to males,49,50 and almost no difference in H2O2 measurements compared to low fat fed females.50 Additionally, given access to a running wheel, females have a greater response in mitochondrial maximal respiratory capacity compared to males.50 Therefore, given the results of prior research, it is likely females would have a slightly different (likely greater) mitochondrial response to physical activity and diet interventions. However, this hypothesis would require further validation along with data collection collected in a more thermoneutral (i.e. 30 °C) environments.22

Taken together, our research demonstrates fluctuating mitophagy responses to HFD-induced NAFLD as well as weight-loss therapeutic interventions. PINK1/PARKIN mitophagy appears to be induced but not fully resolved in NAFLD. D and D/PA both appear to restore some aspects of mito/macroautophagy, with an overall greater effect of D/PA. Increasing autophagy may partially ameliorate symptoms of NAFLD, though more research is necessary to substantiate these findings. These conclusions are summarized in Fig. 4. With no current FDA approved pharmacological methods to specifically target NAFLD, it appears lifestyle interventions incorporating both diet and physical activity are the best available option to increase hepatic autophagy and thereby improve symptoms of NAFLD, with an emphasis on physical activity in addition to the diet.

Submission statement

All authors have read and agree with manuscript content. While this manuscript is being reviewed for this journal, the manuscript will not be submitted elsewhere for review and publication.

Ethical approval statement

All animals were housed at 22.5 °C and 23.5 °C temperatures and a 12 h dark and 12 h light cycle. Food and water were provided ad libitum during experimental period. All procedures were performed in accordance with the guidelines established by Animal Care and Committee Guidelines of Southern Illinois University at Edwardsville.

Authors’ contributions

Assistance with animal experiments—KEP, AS, MPH, JSW, Collection and Analysis of data—MER, KEP, AS, MPH, JSW. Data Analysis and Interpretation—MER, NPG, JSW. Editing/Revising of Manuscript—MER, KEP, AS, MPH, NPG, JSW.

Conflict of interest

The authors declare no financial or other conflicts of interest that could influence the interpretations of this work.

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