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Autophagy Regulation after Diet and Exercise in Non-alcoholic Fatty Liver Disease

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Autophagy Regulation after Diet and Exercise in Non-alcoholic Fatty Liver Disease

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Abstract

Along with the rise in obesity, rates of non-alcoholic fatty liver disease (NAFLD) have also increased. NAFLD may begin with fat accumulation in the liver, but can progress to non-alcoholic steatohepatitis (NASH), fibrosis, and eventual cirrhosis. With no pharmacological treatment for NASH, lifestyle interventions appear vital to maintaining liver health. Previous work has shown aberrant mitochondrial content/quality and autophagy in models of NAFLD. Exercise is known to improve mitochondrial health and possibly autophagy, thus autophagy may be a key regulatory factor for treatment of obesity induced-NAFLD. **PURPOSE:** The purpose of the study was to examine how weight loss from diet or diet combined with physical activity impacts hepatic mitochondrial content, autophagy and mitochondrial autophagy (mitophagy) in NAFLD. **METHODS:** 48 Male C57BL/6J mice were divided into 1 of 4 groups: low fat diet (LFD, 10% fat, 18 wks), high fat diet (HFD, 60% fat diet, 18 wks.), weight loss by diet (D, 60% fat diet for 10 wks then 10% fat diet for 8 wks) or weight loss by diet and physical activity (D/PA, 60% fat diet for 10 wks, then 10% fat diet plus a running wheel for 8 wks). After interventions, livers were collected and analyzed via Western blot for markers of mitochondrial content and autophagy. Results were analyzed by one-way ANOVA with α set at 0.05. **RESULTS:** COX-IV and PGC-1α protein contents were approximately 50% less in HFD compared to LFD, and were restored and increased with D/PA, respectively. BNIP3 content was 45% lower in HFD compared to LFD; D/PA had 50% more BNIP3 compared to LFD controls. PINK1 content was 40% higher in D and D/PA animals compared to LFD. P-PARKIN/PARKIN levels were 40% lower in HFD, D, and D/PA compared to LFD. Whereas p-Ub^{Ser65} was 3-fold higher in HFD animals. LC3II/I ratio was 50% greater in HFD and D/PA animals, yet p62 protein content was 2.5 fold higher in HFD animals compared to LFD, D, and D/PA, with no

further differences observed. **CONCLUSION:** High-fat diet causes disruptions in mitochondrial content, mitophagy and macroautophagy. Diet combined with physical activity are able to ameliorate these derangements.

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Dedication

I would like to dedicate this thesis to my fiancé, Mr. Aaron Caldwell, who is my forever partner in life and science. Here's to one more degree complete, and one more to go.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the most prominent preventable liver ailment in Western society. Rates of NAFLD have closely mirrored the obesity epidemic, with an estimated 19-30% of adults in Western society diagnosed with NAFLD (10, 60). NAFLD can begin with relatively benign fat accumulation within the liver, but with continual lipid overload, can progress to steatosis, inflammation, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and potentially carcinoma (59). Currently, there are no effective pharmacological interventions for liver disease once progressed to NASH (19) and lifestyle interventions to treat symptoms are the most common treatment option. As such, it is imperative to understand the physiological mechanisms of lifestyle interventions to halt the progression of NAFLD and subsequent NASH. It is currently understood that lifestyle interventions such as diet and exercise mitigate many of the symptoms associated with NAFLD. Diet or physical activity can decrease fat accumulation (90), but exercise appears to offer additional benefits such as increased oxidative metabolism (2, 90) and decreased *de novo* lipogenesis (90) in mice. These findings have been confirmed in human research studies (8).

Mitochondria, the major supplier of ATP to the cell, play a major role in the progression of NAFLD. Previous work has demonstrated derangements in mitochondrial quality control mechanisms in various models of NAFLD (21, 23, 58, 106). Furthermore, work by Rector et al. (89) has also demonstrated mitochondrial dysfunction preceding hepatic steatosis in a genetic model of NAFLD. In addition to being important in oxidative metabolism, mitochondrial quality also plays a major role in regulation of autophagy.

Autophagy is a cellular degradation process serving to remove damaged proteins and organelles (63). Damaged cellular components are engulfed in a cellular autophagosome, fuse

with a lysosome, and are degraded through a lysosomal reaction (63). Disruptions in autophagy can result in the accumulation of damaged cellular by-products (24) or cellular apoptosis (83). Autophagy disruptions have been implicated in multiple diseases such as cancer cachexia and neurological disorders (33). Previous work has demonstrated decreases in autophagy flux (7, 24) in murine models of NAFLD. In muscle, it has been shown that exercise increases markers of autophagy and is necessary for exercise-training adaptations (67).

Mitochondria play an important role in the regulation of autophagy, specifically, shuttling lipid from the mitochondrial membrane for the formation of the autophagosome (92). Additionally, mitochondria also undergo a specific type of autophagy, hereafter referred to as mitophagy. Mitochondria undergo multiple mitophagy pathways, with the two predominant pathways including: BNIP3 mediated and PINK1/PARKIN mediated mitophagy. BNIP3 mitophagy predominantly occurs under hypoxic conditions (30, 76), with dysfunctional mitochondria being tagged by BNIP3 and shuttled to an LC3 tagged autophagosome (30, 76). Knockout of BNIP3, a protein necessary for hypoxia-induced mitophagy, resulted in steatosis in mice fed a normal chow diet (23). PINK1/PARKIN mediated mitophagy was first researched in genetically inherited Parkinson's disease (17, 40). Although a relatively new cellular mechanism, PINK1/PARKIN mitophagy occurs in instances of mitochondrial depolarization (41, 45, 55), with accumulation of PINK1 causing subsequent phosphorylation of PARKIN and ubiquitin, tagging the mitochondria for degradation (41, 45, 55). To our knowledge no studies to date 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 (unpublished findings). Yet it is unclear whether increased autophagy from exercise can fully rescue

maladaptations from prolonged high fat diet in NAFLD. 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 is to investigate autophagy and mitophagy regulation after diet or diet combined with physical activity in a murine model of NAFLD.

Review of Literature

Obesity has reached pandemic levels in the Western world (77). Pathological manifestations as a result of obesity have also dramatically increased, specifically the development of fatty liver (4). Nonalcoholic fatty liver disease (NAFLD) has recently become one of the most common liver ailments (4, 99), and the most common cause of serious liver disease and transplants (93). Lipid overload in the diet along with a sedentary lifestyle result in fat accumulation in the liver (71). Fatty liver can progress to impaired liver function (NAFLD), hepatic inflammation, steatosis, non-alcoholic steatohepatitis (NASH) and potentially fibrosis and cirrhosis. Currently, there are no pharmacological agents for fatty liver once escalated to NASH, as such, it is imperative to prevent liver disease before steatosis has developed. With no effective pharmacological interventions, lifestyle alterations have become the common recommendation for those afflicted with NAFLD or NASH (14). While it is overall understood that lifestyle interventions positively affect the prognosis of those with fatty liver, the physiological mechanisms contributing to the initial disease development and lifestyle based alleviations are not completely understood. As lifestyle interventions are currently the most effective known therapies in NAFLD, they can be utilized to determine potential mechanistic targets in the treatment of such conditions. One particular cellular mechanism of interest is the cell's ability to degrade macromolecules, known as autophagy.

Autophagy serves as the garbage disposal system of the cell, degrading and removing damaged proteins and organelles (63). Through a highly conserved signaling process, damaged organelles are encased in an autophagosome and subsequently degraded via lysosomal reactions. In various pathological conditions it is well established that the autophagy process is negatively affected. For example, in some cancers overactive autophagy may contribute to cancer-cachexia

(83). Ischemia-reperfusion injury causes major increases in hepatic autophagy, leading to increased cytotoxicity (28) and further exacerbation of liver damage. With regards to NAFLD, the significance of autophagy on disease progression and alleviation with respect to lifestyle interventions are not yet completely understood. As such, the purpose of this review is to examine hepatic autophagy models and mechanisms in NAFLD and the potential efficacy of lifestyle interventions in correcting derangements in hepatic autophagy.

Autophagy Regulation

Autophagy is a tightly regulated process mediated through the action and interaction of many autophagy related genes (Atgs). Autophagy related gene-6, Atg6 (also known as and hereafter referred to as Beclin), forms a complex with other Atgs to begin formation of the phagophore and ultimately the autophagosome that encompasses proteins or organelles to be destroyed (80). Atg7 as well as other Atgs facilitates autophagosome formation and starvation-induced degradation of proteins (54, 92). Additionally, microtubule-associated protein (MAP) light chain 3 (LC3), a subunit of MAP1, also assists with autophagosome formation. LC3 is utilized as a signaling protein to bring proteins to the autophagosome (72). First examined in neurons (69), LC3 has two primary conformations, mainly LC3-I and LC3-II. During the autophagy process, LC3-I, located in the cytosol of the cell, undergoes a conformational change via reactions with E1- and E2-ligases enzymes to transform into LC3-II (56). LC3-II is bonded to a phosphatidylethanolamine and attaches to the outer autophagosomal membrane (56). Therefore, the ratio of LC3II/I content can be measured to demonstrate the rate of autophagosome formation, a marker of autophagy flux. Finally, p62 (also known as SQSTM1 protein) is a cargo protein that serves to shuttle proteins/organelles to the autophagosome and is

itself destroyed in the autophagy process and can therefore be utilized to measure the completion of the autophagy process (11). p62 is phosphorylated by ULK1 at Ser407 and Ser403, increasing its affinity for ubiquitinated proteins (43). p62-tagged proteins bind with LC3II-tagged autophagosomes and the proteins are degraded along with p62 (11). As such, the amount of p62 can be measured to determine the completion of autophagy flux, where greater p62 content suggests reduced flux, and less p62 suggests greater flux. This method to interpret autophagy flux has been commonly utilized and validated in literature (53).

Autophagy and NAFLD

Methionine and choline deficient MCD diets as well as high fat diets have been used to demonstrate alterations in hepatic autophagy with most studies finding a decrease in hepatic autophagy (16, 24, 36, 81, 107), with a few showing increases (68, 101). Other dietary models have also been employed to induce NAFLD, such as high sugar diets (7, 18, 44, 62), but only one has examined hepatic autophagy, demonstrating aberrant autophagy in rats fed fructose (7). Current research suggests that some markers of autophagy machinery, such as Beclin, are not affected by either high fat diet nor MCD diet (24). Human studies of NAFLD and autophagy have demonstrated an increase in the number of autophagosome vesicles in patients with NAFLD (20). Paradoxically in that same study, p62 accumulation correlated with serum alanine aminotransferase values and inflammation (20, 24), possibly suggesting that hepatocytes can initiate the autophagy process via increased autophagosome formation, but are unable to complete the degradation progression.

In addition to autophagy machinery and flux, other proteins may have dramatic effects on the cell's capacity for autophagy. For example, PPARα has become a topic of interest in

autophagy regulation in liver (36, 38, 61). PPARα is known to facilitate fatty acid oxidation, and in many models of NAFLD, PPARα content is altered, although some studies have found increases (7) and others decreases (42, 88). Interventions increasing PPARα have been shown to rescue maladaptations in autophagy in NAFLD. Ursolic Acid, a PPARα activator has been found to alleviate NAFLD in high fat fed mice, at least partially due to an increase in hepatic autophagy (36). Other studies have noted increased hepatic autophagy via activation of PPARα, which was sufficient to improve liver damage in an acute liver failure model (38). Yet, PPARα activation does not appear to be the sole activator of autophagy, as long term depletion of PPARα (knockout models) has been shown to attenuate autophagy, but did not completely abolish autophagy (61). Taken together, it appears that either fatty acid oxidation and/or mediators of fatty acid oxidation play a major role in the cell's ability to perform autophagy.

Furthermore, calcium channel derangements have also been associated with autophagy detriments. *In vitro* research has found diet-induced obesity causes increased cytosolic calcium levels (82). The increased calcium level disrupts the binding of the autophagosome to the lysosome, thus causing accumulation of ubiquitinated proteins (82). Treatment of cells or animals with verapamil, a calcium channel blocker, restored autophagy flux and ameliorated symptoms of NAFLD such as lipid accumulation and steatohepatitis (82). Taken together, these findings suggest autophagy is a multi-faceted process that can be affected by multiple cellular components and stimuli, possibly suggesting that promotion of autophagy through various means may alleviate NAFLD.

Autophagy and Lifestyle Interventions

Lifestyle alterations such as exercise are common recommendations for those with liver disease or those with predisposing factors for NAFLD (e.g. obesity). Exercise is known to alleviate some of the symptoms of NAFLD with or without weight loss (2, 31, 44, 91). Yet, the mechanism for how exercise or diet directly affects hepatic autophagy is still not completely understood. Previous work has shown that increases in basal autophagy are necessary for aerobic training adaptations in the muscle (67). Interestingly, my preliminary data demonstrates elevated basal hepatic autophagy following exercise in mice. These training adaptations occurred in spite of lipid overload in Western Diet fed animals, suggesting that exercise may be sufficient to promote autophagic clearance of damaged cellular components despite lipid overload.

Previous work has examined exercise and autophagy in the livers of healthy exercised rats, finding no difference between groups in autophagy flux or machinery (9). Another recent study noted no increases in hepatic autophagy with exercise training (2), although the exercise training protocol was relatively short timeline with only three weeks of exercise training. Both studies utilized treadmill training for exercise. As treadmill training has been demonstrated to be stressful for mice (3, 6, 15), the stress of the training modality may have masked some potential exercise training adaptations. Furthermore, voluntary wheel running has been seen to more closely mimic human exercise training adaptations (25) including increased time to fatigue during graded exercise and muscle mitochondrial content (85) without the additional stress of treadmill running (3, 6). Regardless, the role of hepatic autophagy and exercise has not been thoroughly explored in healthy or NAFLD models.

Various supplements have been examined in relation to NAFLD and autophagy. Curcumin, bergamot polyphenol fraction, capsaicin and caffeine have shown promising results in

cellular and murine models (42, 64, 81, 97), but thus far have not been extended in humans. Pharmacological treatments to induce autophagy have also been investigated with pharmacological increase in autophagy sufficient to mitigate liver steatosis (65). Chang and collegues (12) recently found ezetimibe treatment in rats and cells with lipid overload decreased phenotypic and genotypic symptoms of NAFLD. Furthermore, this was accompanied with increased mRNA content of genes associated with autophagy machinery and increased autophagy flux (12).To our knowledge, little research has directly investigated autophagy in NAFLD after diet interventions. Overall, lifestyle interventions to increase hepatic autophagy and NAFLD appear to have beneficial effects, and these effects appear to carry over to pharmacological treatments, but more research is necessary to substantiate these findings.

Autophagy and Mitochondrial Quality

Currently, it is generally accepted that poor mitochondrial quality, defined as the functionality and health of the mitochondrial network (26), is associated with reduced overall health (5, 47). Mitochondrial quality control encompasses the processes of biogenesis, dynamics (fusing healthy mitochondria together [fusion] and cleaving off unhealthy mitochondria[fission]), and mitochondrial specific autophagy (mitophagy). Prior research in liver has demonstrated dysregulation in these processes during pathological conditions, and is correlated with reduced mitochondrial health (21, 23, 58). It is generally accepted that mitochondrial dysfunction precedes significant changes in fat accumulation and insulin resistance in liver (89, 108).

Furthermore, detriments in hepatic autophagy can affect the mitochondria's capacity for oxidative phosphorylation. In addition to a measure of macroautophagy flux, p62 is a major regulator of transcription of oxidative enzymes and other proteins related to mitochondrial

biogenesis (37). ULK1 can phosphorylate p62, which upon binding with the ubiquitinated organelle, is further phosphorylated by mTOR (37). This phosphorylation allows NRF2 to translocate to the nucleus and promote transcription of oxidative proteins (37). Therefore, dysregulation in the signaling between p62 and NRF2 may result in reduced capacity for oxidative phosphorylation and possible increased oxidative stress.

Reductions in mitochondrial quality have also been associated with concurrent alterations in autophagy regulation (102). Preliminary work suggests other aspects of mitochondrial quality control (biogenesis, content, fusion and fission) are affected by Western Diet before macroautophagy flux detriments occur. Evidence also suggests that some of the lipid utilized for the formation of the autophagosomal membrane are shuttled from the mitochondria via action of Atg9 (92), and deletion of Atg7 in mice has been found to result in misshaped mitochondria (54). Overall, these studies demonstrate that hepatic mitochondrial quality and autophagy are closely tied processes, suggesting that alterations in one process will affect the other.

Mitophagy in NAFLD

Mitophagy is of particular importance for liver health (23, 66, 102).Yet, mitophagy is relatively unexplored in NAFLD with only a few studies to our knowledge investigating mitophagy and NAFLD (23, 66, 102). All of these studies demonstrated major mitophagy disruptions in NAFLD. One major pathway for mitophagy is the BNIP3/Nix pathway. BNIP3 and Nix respond to hypoxic cellular conditions to facilitate mitochondrial binding with LC3-tagged autophagosome (30, 76). One prior study noted hepatic steatosis and fibrosis in normal chow fed mice lacking BNIP3 (23), suggesting BNIP3-mediated mitophagy is necessary for liver function. Though research investigating Nix in relation to NAFLD is not currently available, as

BNIP3-mediated mitophagy has been shown to be important for hepatic health, Nix in relation to NAFLD may provide useful information regarding mitophagy during lipid overload. Our laboratory's preliminary data demonstrates that overall autophagic flux is not altered from 8 weeks of Western Diet. Yet mitochondrial specific autophagy, measured through BNIP3 protein content, did exhibit a marked decrease compared to normal chow fed animals, suggesting reduced capacity for BNIP3-mediated mitophagy. Furthermore, it has recently been demonstrated that loss of ULK1, a protein responsible for autophagy regulation via mTOR and AMPK-mediated pathways, attenuates mitophagy (98). This same study also tied reactive oxygen species (ROS) accumulation to decreased mitophagy and the necessity of mitophagy for adequate oxidative phosphorylation (98). Taken together, these studies suggest mitochondrial quality, autophagy, and hepatic health are all tightly related processes.

In addition to BNIP3-mediated mitophagy, another predominant mitophagy pathway is PTEN-induced putative kinase 1 (PINK1)/PARKIN-mediated mitophagy. The PINK1/PARKIN pathway was first described in neurological diseases, specifically genetically inherited Parkinson's disease (17, 40). PINK1 is a signaling protein that binds with an outer mitochondrial membrane receptor TOM (40). Once bound, PINK1 enters the inner mitochondrial matrix to undergo a series of cleavages to go from a 64 kDa protein to a 52 kDa protein, after which the 52 kDa protein is ejected into the cytosol and degraded (40). When the mitochondria becomes depolarized, PINK1 does not enter the inner mitochondrial matrix, and instead accumulates on the outer mitochondrial membrane (39). The accumulation of PINK1 phosphorylates ubiquitin and PARKIN (41, 45, 55) recruiting PARKIN to the outer mitochondrial membrane (74). PARKIN induces ubiquitination of proteins on outer mitochondrial membrane proteins (17). The ubiquitination causes other autophagy proteins to translocate to the mitochondria such as p62

which then goes to the LC3II-tagged autophagosome (72) and completes the general autophagy process. Mitophagy via PINK1 and PARKIN causes an increase in mitochondrial fission and a decrease in fusion via ubiquitination of MFN1 and 2 and their subsequent degradation (17, 22, 40, 50). Figure 1 pictorially demonstrates the two primary mitophagy processes. As increased mitochondrial fission and fusion have been noted in murine models of NAFLD (21, 106), it is possible that the noted disrupted mitochondrial dynamics is attributable to dysfunctional mitophagy.

However, to our knowledge, no studies to date have investigated the PINK1/PARKIN pathway in NAFLD, despite the apparent ties between the two. Although the PINK1/PARKIN pathway has been investigated in other liver diseases (50, 51). Hepatitis B causes increases in PINK1, PARKIN, and LC3II gene expression, while silencing PARKIN induced apoptosis (50). In hepatitis C studies, a similar pattern was seen with increases in PINK1 and PARKIN, while silencing PINK1 and PARKIN inhibited progression of the disease (51). These few studies in liver of the PINK1/PARKIN pathway suggest that the pathway may be of critical importance for overall liver health. In the brain, recent studies have demonstrated exercise's potential to increase the tissue's capacity for mitophagy as evidenced by greater PINK1 content and LC3II/I ratios, but without complete resolution of autophagy as evidenced by no changes in PARKIN or p62 (70). A recent study in mice brains by Wei et. al (105) suggested that mitophagy alterations may be attributable to reactive oxygen species (ROS) causing decreased mitochondrial membrane polarization and increased mitophagy via PINK1/PARKIN pathway. In the brains of mice fed a 60% high fat diet, decreases in PARKIN were noted that were also correlated with a reduction in PGC1-α (48), the major regulator of mitochondrial biogenesis. This may suggest that alterations in mitophagy may cause decreases in mitochondrial biogenesis. In human

exercise studies in muscle acute ultra-endurance exercise elicited no acute changes in mitophagy as measured in both the PINK1/PARKIN-mediated pathway as well as BNIP3 pathway, yet did illicit alterations in overall autophagy (35). This may be suggestive that alterations in mitophagy are long-term effects and not seen acutely. Taken together these studies suggest that mitophagy may play a major role in overall cellular health, however the extent that mitophagy affects, or is affected by, stimuli such as hepatic lipid overload (NAFLD) or exercise remains elusive.

Summary

Autophagy is a cellular degradation process necessary for liver health. NAFLD causes disturbances in hepatic autophagy via the inhibition or activation of various cellular signaling mechanisms. Lifestyle interventions can ameliorate symptoms of NAFLD, specifically with regard to autophagy, although the specific mechanisms are not completely understood. It is currently not well known how mitophagy is related to NAFLD or if activation of mitophagy by lifestyle interventions is sufficient to rescue maladaptations associated with NAFLD. Therefore future research should seek to investigate the mechanism of autophagy and mitophagy derangements in NAFLD and how these mechanisms are altered with diet or exercise.

Purpose

The purpose of this study is to examine autophagy and mitophagy regulation in high fat diet-induced NAFLD and how lifestyle interventions of diet or exercise affect autophagy in NAFLD.

Methods

Animal Interventions

All animal work was performed at and approved by the Southern Illinois University at Edwardsville Institutional Animal Care and Use Committee. 48 C57BL/6J (Jackson Laboratories, Bar Harbor, ME) mice were evenly divided into two groups at 6 wks of age, one group of 12 animals consumed low fat diet (LFD, 10% of kcal from fat, Research Diets, New Brunwick, NJ) and 36 animals consumed high fat diet to induce obesity (60% of kcal from fat, Research Diets). Animals consumed chow for 10 wks, after which, high fat diet-induced obese animals were further divided into three groups, one (HFD) continued to consume the 60% high fat diet, one group was placed back on the 10% fat diet to induce weight loss (D) and the final group was placed on the 10% fat diet and given a freely movable running wheel to provide physical activity to induce weight loss (D/PA). Wheel running activity was monitored via computer running wheel software. LFD animals continued consuming 10% fat chow. Animals continued interventions for an additional 8 wks. Final groups included: LFD, HFD, D, D/PA with n=12 in each group (Figure 2). After interventions, animals were humanly euthanized, blood and livers collected and snap frozen in liquid nitrogen for later analysis. 6 hours before tissue harvest animals' food was removed; 24 hours before tissue harvest running wheels were removed from cages of D/PA animals. Liver lipid content, blood analysis of triglycerides and cholesterol were assessed as previously described (86).

Isolation of Protein and Immunoblotting

Isolation of protein and immunoblotting were performed as previously described (26). Briefly, 40 mg of liver was homogenized in glass dounce type homogenizers in 0.30 ml of complete protein loading buffer (50 mM Tris·HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 10%

glycerol, 20 mM dithiothreitol, 127 mM 2-mercaptoethanol, and 0.01% bromophenol blue supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO) according to Alliance for Cellular Signaling protocols and as described elsewhere (26). After homogenization, samples were transferred to sterile 1.5mL microcentrifuge tubes, heated for 5 minutes at 95°C to denature protein and then centrifuged for 5 minutes at 13,000 rpm. Protein concentrations were determined using a commercially available RC/DC assay kit following manufacturer instructions (Bio-Rad, Hercules, CA). 40 µg of liver protein were loaded into 6-15% SDS-polyacrylamide gels, depending on size of protein of interest, and transferred onto PVDF membranes (Thermo Scientific, Rockford, IL, USA). Membranes were blocked for an hour in 5% milk solution in 0.5% TBST, and then incubated in primary antibody blocking solution at 4°C. After 12-48 hour primary incubation, membranes were washed in 0.5% TBST and incubated with appropriate secondary antibody solutions for 1 hour (Li-Cor Biosciences, Lincoln, NE). Membranes were imaged using a FlourChem M (Protein Simple, San Jose, CA) and protein content normalized to *Ponceau S*. Primary antibodies included: COX-IV, p62/SQSTM1, LC3A/B, BNIP3, PINK1, PARKIN, P-PARKIN-S65, Ubiquitin, and P-Ubiquitin-S65.

Statistical Analysis

Independent variables were intervention (LFD v. HFD v. D v. D/PA). Results were analyzed by one-way analysis of variance (ANOVA), with α set at 0.05. Duncan's multiple range test was used to distinguish differences among means. All data were analyzed using the Statistical Analysis System (SAS, version 9.3, Cary, NC) and expressed as mean ± SEM.

Results

HFD resulted in NAFLD phenotype, weight loss interventions ameliorated many phenotypic symptoms of NAFLD

HFD animals were approximately 50% heavier compared to LFD or D animals (Figure 3A, $p<0.05$). D restored body weights to LFD levels (Figure 3A, $p>0.05$). D/PA animals were approximately 4 grams lighter compared to LFD or D animals (Figure 3A, $p<0.05$). Liver weights followed a similar pattern, with HFD liver weights about 2-fold greater than LFD, D, or D/PA (Figure 3B, $p<0.05$).HFD and D/PA animals consumed approximately 30% more calories compared to LFD and D during the last 8 wks of protocol (Figure 3C, $p<0.05$). By weight, D/PA animals consumed approximately 25% more food compared to the other three groups during the last 8 wks of protocol (Figure 3D, $p<0.05$). D/PA animals ran approximately 8.8 ± 0.1 km per day. Liver lipid content was slightly more than 2-fold greater in HFD compared to LFD animals, and more than 5-fold greater compared to D and D/PA (Figure 3E, $p<0.05$). HFD resulted in over 2-fold increase in hepatic triglyceride levels compared to LFD (Figure 3F, $p<0.05$). D as well as D/PA caused lower levels of triglycerides compared to LFD, with D/PA having slightly reduced levels compared to D (Figure 3F, $p<0.05$). HFD animals had approximately 30% greater hepatic cholesterol levels compared to LFD (Figure 3G, $p<0.05$), while D was not different from either HFD or LFD (Figure 3G, $p>0.05$). D/PA resulted in 60% lower cholesterol levels compared to D, but was not different from LFD (Figure 3G, $p<0.05$).

Mitochondrial content and biogenesis markers were negatively affected by HFD, weight loss interventions rescued some of these maladaptations

COX-IV content, a common surrogate marker of mitochondrial content (26, 27), was approximately 50% lower in HFD compared to LFD animals (Figure 4A, $p<0.05$). COX-IV content in D animals was not different from either LFD or HFD (Figure 4A, $p>0.05$). D/PA restored COX-IV content, with no difference between D/PA and LFD (Figure 4A, $p>0.05$). Protein content of PGC1-α was 50% lower in HFD animals compared to LFD animals (Figure 4B, $p<0.05$). D restored PGC1-α levels with no difference between LFD and D (Figure 3B, $p>0.05$). D/PA animal had approximately 50% more PGC1-α compared to LFD animals (Figure 4B, $p<0.05$).

BNIP3 mediated autophagy was impaired by HFD and enhanced by D/PA

BNIP3 protein content was 45% lower in HFD animals compared to LFD animals (Figure 5A, $p<0.05$). D was not different from LFD or HFD ($p>0.05$), whereas D/PA had about 50% greater content compared to LFD (Figure 5A, $p<0.05$).

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

PINK1 protein content was unaffected by HFD ($p>0.05$), whereas, D and D/PA each resulted in approximately 40% greater PINK1 compared to LFD (Figure 6A, $p<0.05$). PARKIN content was 4-fold greater in D animals compared to LFD (Figure 6B, $p<0.05$). Whereas, PARKIN content in HFD and D/PA did not differ from LFD (Figure 6B, $p>0.05$). With regards to p-PARKIN^{Ser65} no interventions were statistically different from LFD, although, D was

approximately 50% lower compared to HFD and D/PA (Figure 6C, $p<0.05$). Comparing p-PARKIN^{Ser65}/PARKIN ratios, all three experimental groups (HFD, D, and D/PA) were roughly 40% lower compared to LFD (Figure 6D, $p<0.05$). Total Ubiquitin protein content was unaffected by HFD compared to LFD (Figure 6E, $p>0.05$). D and D/PA had about 50% greater ubiquitin content compared to HFD ($p<0.05$), but only D was statistically different from LFD (Figure 5E, $p<0.05$). p-Ubiquitin^{Ser65} was 2.5 times greater in HFD compared to LFD, D, and D/PA (Figure 6F, $p<0.05$), with no other differences noted between groups. p-Ubiquitin^{Ser65}/Ubiquitin was 3-fold greater in HFD animals compared to the three other experimental groups (Figure 6G, $p<0.05$), with no other differences between groups.

Macroautophagy flux was decreased in HFD animals and rescued with weight loss interventions

As LC3I is lipidated into LC3II to become a part of the autophagosome membrane, the ratio of LC3II/I can be utilized to determine the rate of autophagosome formation (53). This methodology is commonly utilized to analyze autophagy membrane formation and has been validated by Klionsky et al. in murine models (52), LC3II/I ratio was approximately 2-fold greater in HFD and D/PA animals compared to LFD (Figure 6A, $p<0.05$). D was not different from LFD (Figure 6A, $p>0.05$). LC3II content was approximately 100% greater in D/PA compared to LFD and D animals (Figure 7B, $p<0.05$), although HFD was not statically different from any of the three other groups (Figure 7B, $p>0.05$). Total LC3, found by adding the density of both the LC3I and LC3II bands, was 2-fold greater in D/PA animals compared to LFD, HFD, and D (Figure 7C, $p<0.05$), with no further differences noted in the other three experimental groups. Since p62 is taken into the autophagosome along with the organelle or protein to be degraded, the amount of p62 protein content can by utilized as a measure of autophagy

resolution, with greater p62 content indicative of reduced autophagy flux and reduced p62 indicative of greater autophagy flux (52). p62 content was 2.5-fold greater in HFD compared to LFD, D, or D/PA (Figure 7D, $p<0.05$), there were no other differences between groups.

AMPK activity was unaffected by interventions

AMPK acts as a major regulator of autophagy initiation by activating ULK1 (49) , therefore we sought to determine if changes in AMPK activity could explain noted differences between groups. Total AMPK content was unaffected by HFD compared to LFD, D was approximately 50% greater compared to HFD ($p<0.05$), but no other differences were noted between interventions (Figure 8A, $p>0.05$). Neither p-AMPK^{Thr172} or p-AMPK/AMPK content was different between groups (Figure 8B & 8C, $p>0.05$).

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 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 on autophagy and mitophagy, whereas diet does not appear to have as prominent effects with regard to mitophagy. Our results suggest that in NAFLD hepatocytes may forgo mitophagy in efforts to maintain mitochondria albeit likely dysfunctional, which may increase liver susceptibility to progression of fatty liver disease into more serious conditions.

First, our high fat diet was sufficient to induce NAFLD, similar to previous research (13, 84, 89, 90), demonstrating increased body weight, liver weight, circulating triglycerides, and cholesterol levels. D and D/PA rescued most of these phenotypic maladaptations. Our data follows our previous findings with reductions in PGC1-α content (unpublished findings). Although diet was sufficient to rescue PGC1-α content, it was not sufficient to restore COX-IV content. Contrastingly, D/PA increased PGC1-α content above baseline and completely restored mitochondrial content as measured by COX-IV. While PGC1-α content does not specifically measure mitochondrial biogenesis, as the major regulator of mitochondrial biogenesis (87), PGC1-α content provides valuable insight into promotion of mitochondrial biogenesis in this model. Additionally, previous studies have corroborated our findings of reduced mitochondrial biogenesis and content in NAFLD utilizing multiple methods (1, 58, 84). Taken together, these data demonstrate diet combined with physical activity are more effective for treating disrupted

hepatic mitochondrial biogenesis and content compared to diet alone in murine models of NAFLD.

HFD severely attenuated the hepatocyte's capacity for BNIP3-mediated mitophagy, as measured by reduced BNIP3 content. This follows our previous observations in pre-NAFLD animals (unpublished findings). 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-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 above basal levels (LFD). Our findings align with previous research in murine skeletal muscle demonstrating increased mitophagy markers (suggestive of increase mitochondrial turnover) in exercised animals (35, 67). Recent reports have also suggested that increased mitophagy is necessary for increased mitochondrial biogenesis (34, 96), therefore it is unsurprising that BNIP3 content mirrored PGC1-α content in exercised animals. Taken together, our findings suggest that D/PA is more effective in restoring BNIP3-mediated mitophagy compared to D alone.

Interestingly, while BNIP3 is suppressed in HFD and 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 increased PINK1 content, the entire PINK1/PARKIN mitophagy process may be attenuated in HFD, D, and D/PA, as evidenced by decreased p-PARKIN/PARKIN content. Yet, p-Ub^{Ser65} appears to suggest the opposite in HFD animals, with p-Ub^{Ser65}/Ub ratios greatly increased compared to all other groups. The complicated interplay between p-Ub^{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 Serine 65 (32). In addition to phosphorylation by PINK1, PARKIN is further activated by p-Ub^{Ser65} (79, 104). Specifically, p-Ub^{Ser65} binds to p-PARKIN^{Ser65} leading to a conformational shift (46, 95, 103), allowing PARKIN to mediate ubiquitination of the outer mitochondrial membrane proteins, and tagging the mitochondria for degradation (73, 94). Currently, PINK1 is the only known protein to phosphorylate Ubiquitin at Ser65, suggesting that p-Ub^{Ser65} is specific to PINK1 activity; although it is possible that another protein may perform the same function (32, 100). We noted increased p-Ub^{Ser65} with a concurrent reduction in P-PARKIN/PARKIN ratio. p-Ub^{Ser65} is currently known to be predominantly utilized for mitophagy (41) and p-Ub^{Ser65} binds to PARKIN to trigger E3 ligase activity (46, 57, 78). This may suggest that in NAFLD, hepatocytes may attempt to increase mitophagy through accumulation of p-Ub^{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 resolution of PINK1/PARKIN-mediated mitophagy in an effort to maintain some of the mitochondrial network, regardless of the mitochondria's functionality. Or there may be some unknown dysfunction in the signaling between p-Ub^{Ser65} and p-PARKIN^{Ser65}, resulting in an accumulation of p-Ub^{Ser65} without the synchronized increase in p-PARKIN^{Ser65} activity. Correspondingly, mitochondria in D or D/PA animals, although having 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 hours prior to harvest and food removed 6 hours before to remove 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 some of our mitophagy specific markers, our markers of macroautophagy, suggested a slightly greater capacity for autophagosome formation in D/PA animals, as evidenced by increased total LC3 content (52). Interestingly, HFD animals had 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, suggesting impaired resolution of autophagy via accumulation of p62. Taken together, these markers suggest an attempted increase in macroautophagy flux, but 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 accumulation of reactive oxygen species (ROS). Unrestrained ROS production has been associated with decreased liver health (75) and possible progression of fatty liver disease (29, 75). Therefore, it is possible that decreased mitophagy through high fat diet-induced NAFLD, may be a major regulatory point for progression of NAFLD. As such, therapeutics to promote mitophagy in NAFLD, may be possible treatment options.

Taken, together our research demonstrates fluctuating mitophagy responses to HFD-induced NAFLD as well as weight loss therapeutic interventions. Whereas BNIP3-mediated mitophagy appears to be blunted in NAFLD, 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 this

claim. With no current FDA approved pharmacological methods to specifically target NAFLD, it appears lifestyle interventions are the best available option to increase hepatic autophagy and thereby improve symptoms of NAFLD, with an emphasis on physical activity in addition to diet.

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Figures

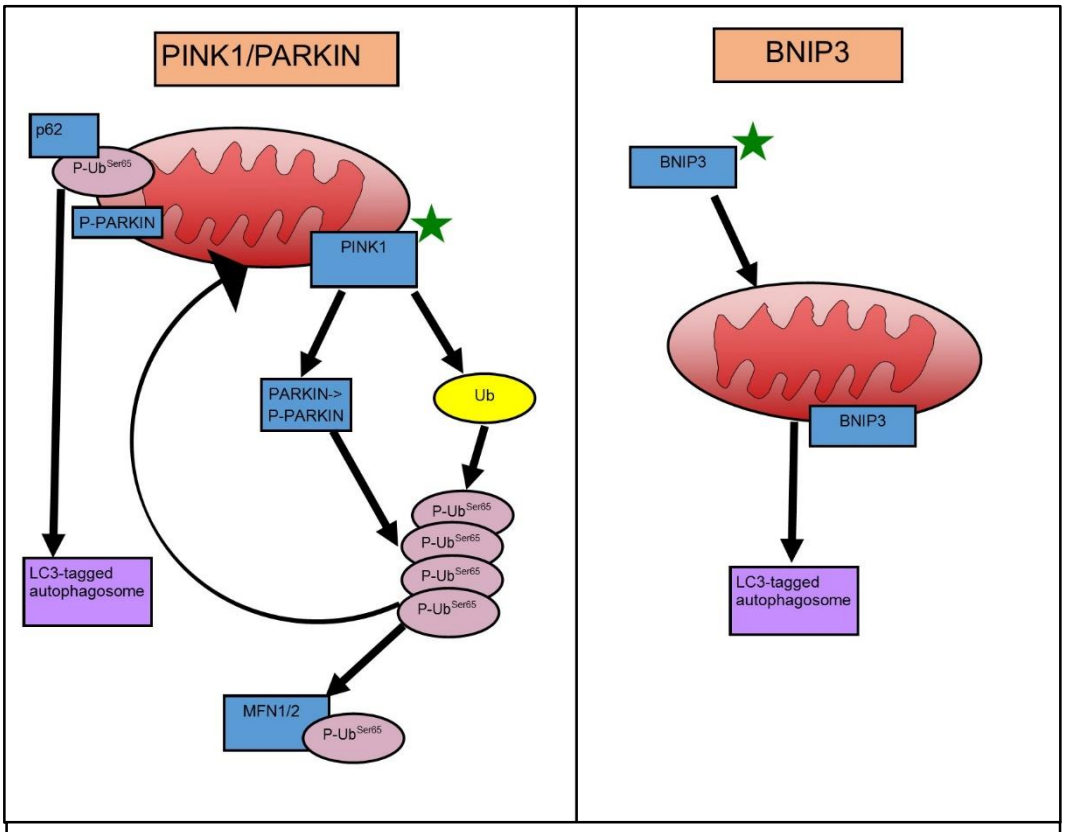

Figure 1: Flow chart of PINK1/PARKIN and BNIP3 mediated pathways, green star indicates starting point

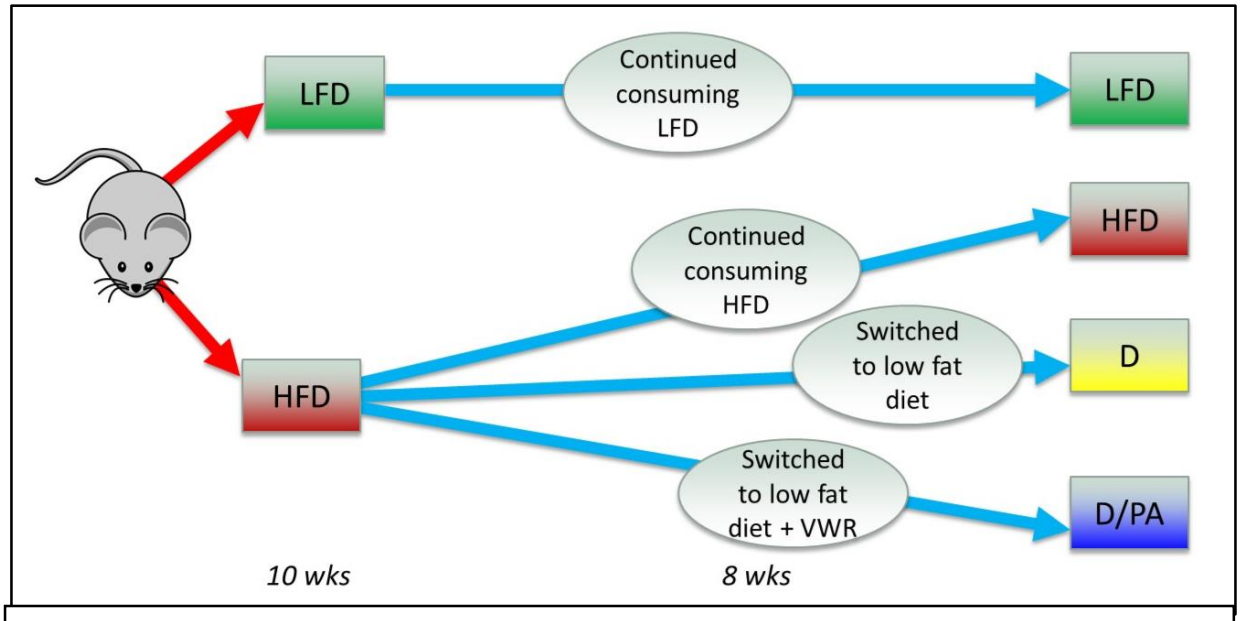

Figure 2: Flow chart of experimental protocol.

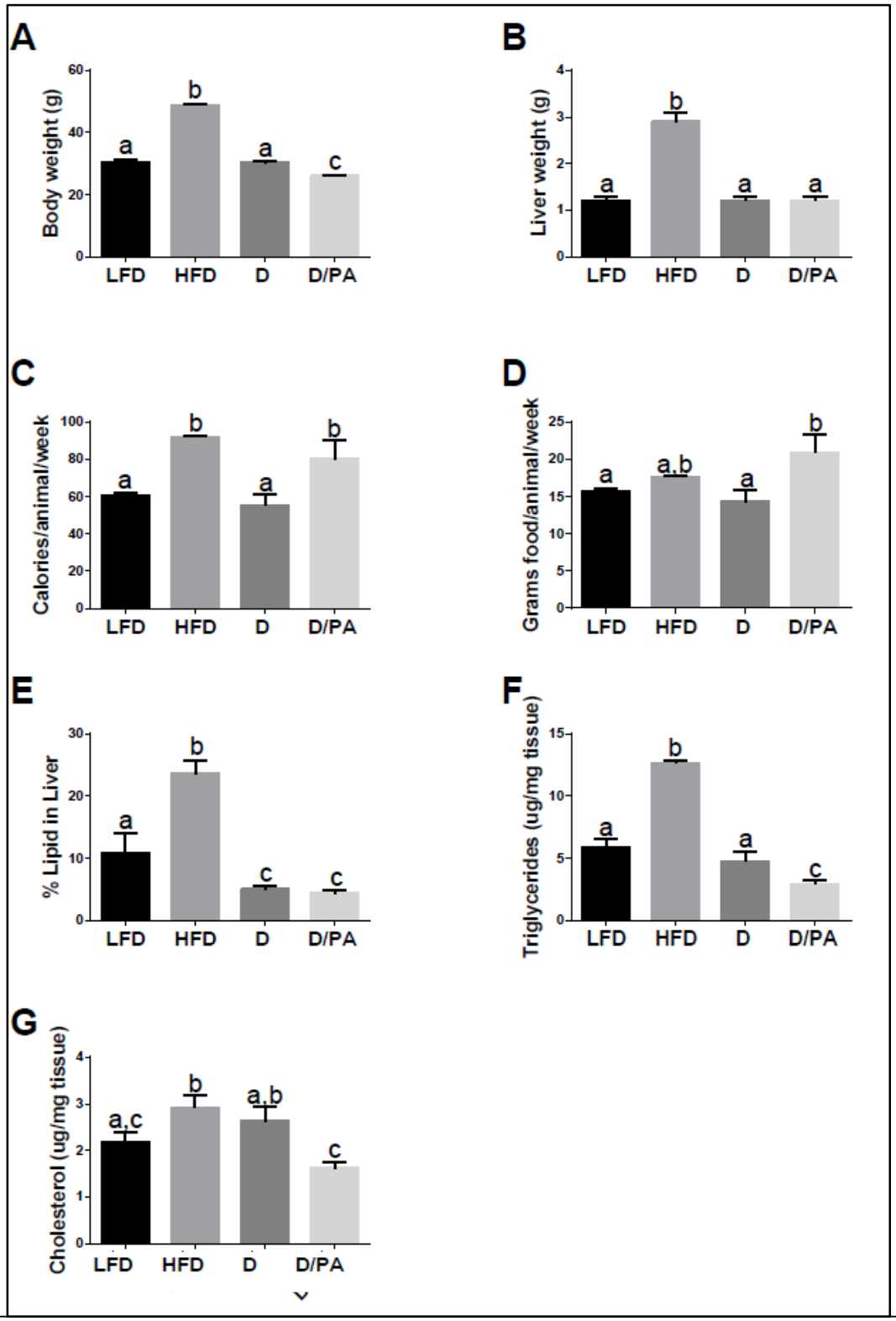

Figure 3: Phenotypic data from current study. **A:** Body weights of animals. **B:** Liver weights of animals. **C:** Calories consumed per animal per week in the last 8 weeks of experiment. **D:** Food weight consumed per animal per week in the last 8 weeks of experiment. **E**: % lipid content in the livers of animals. **F:** Triglyceride levels in animals. **G:** Cholesterol levels in animals.

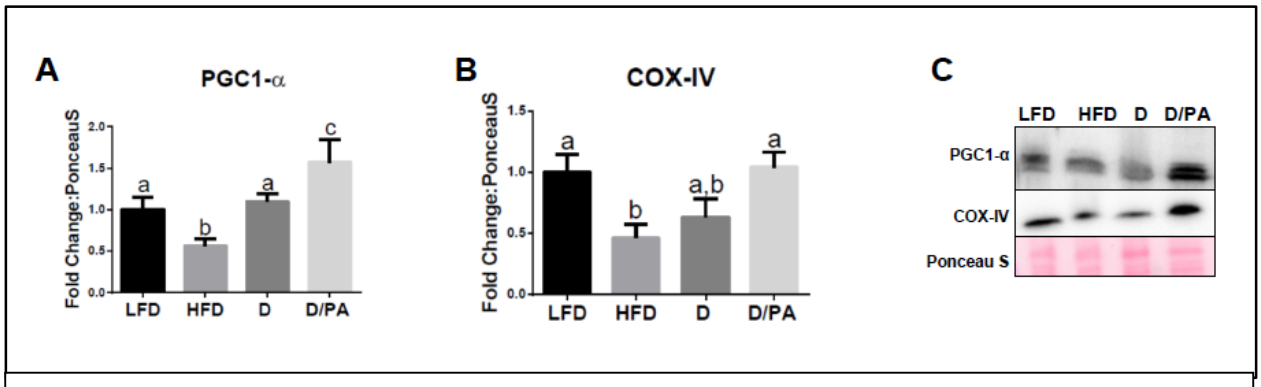

Figure 4: Markers of mitochondrial biogenesis and content. A: PGC1-α protein content. B: COX-IV protein content. C: Representative blots for PGC1-α and COX-IV.

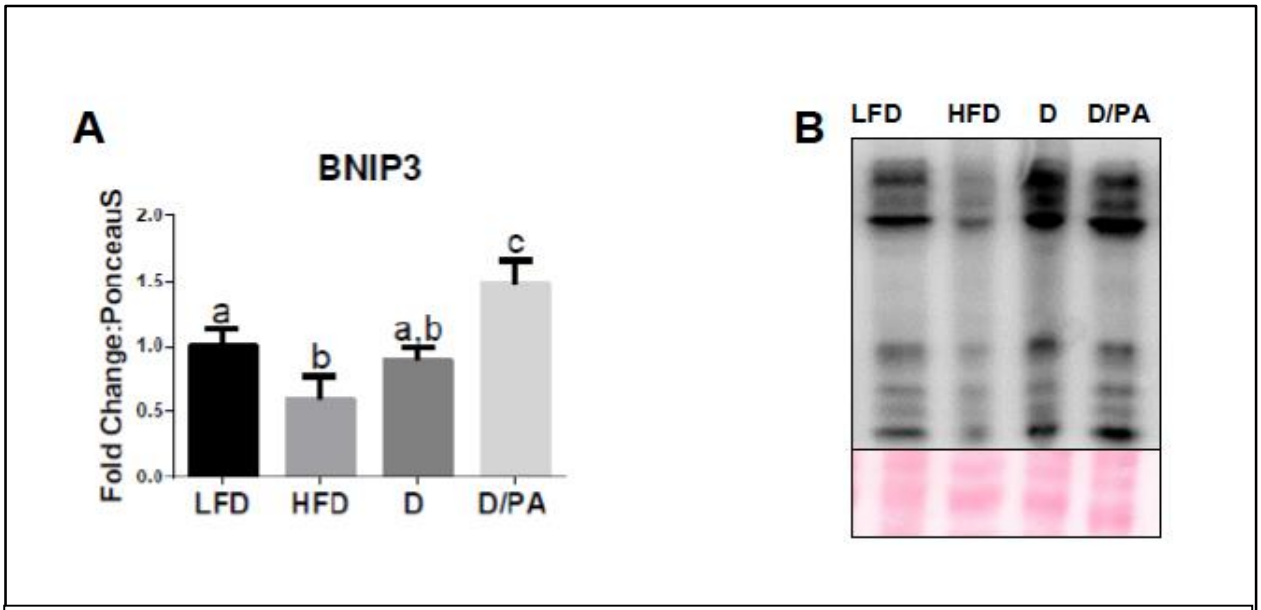

Figure 5: BNIP3 mediated mitophagy. A: BNIP3 protein content. B: Representative blot of BNIP3.

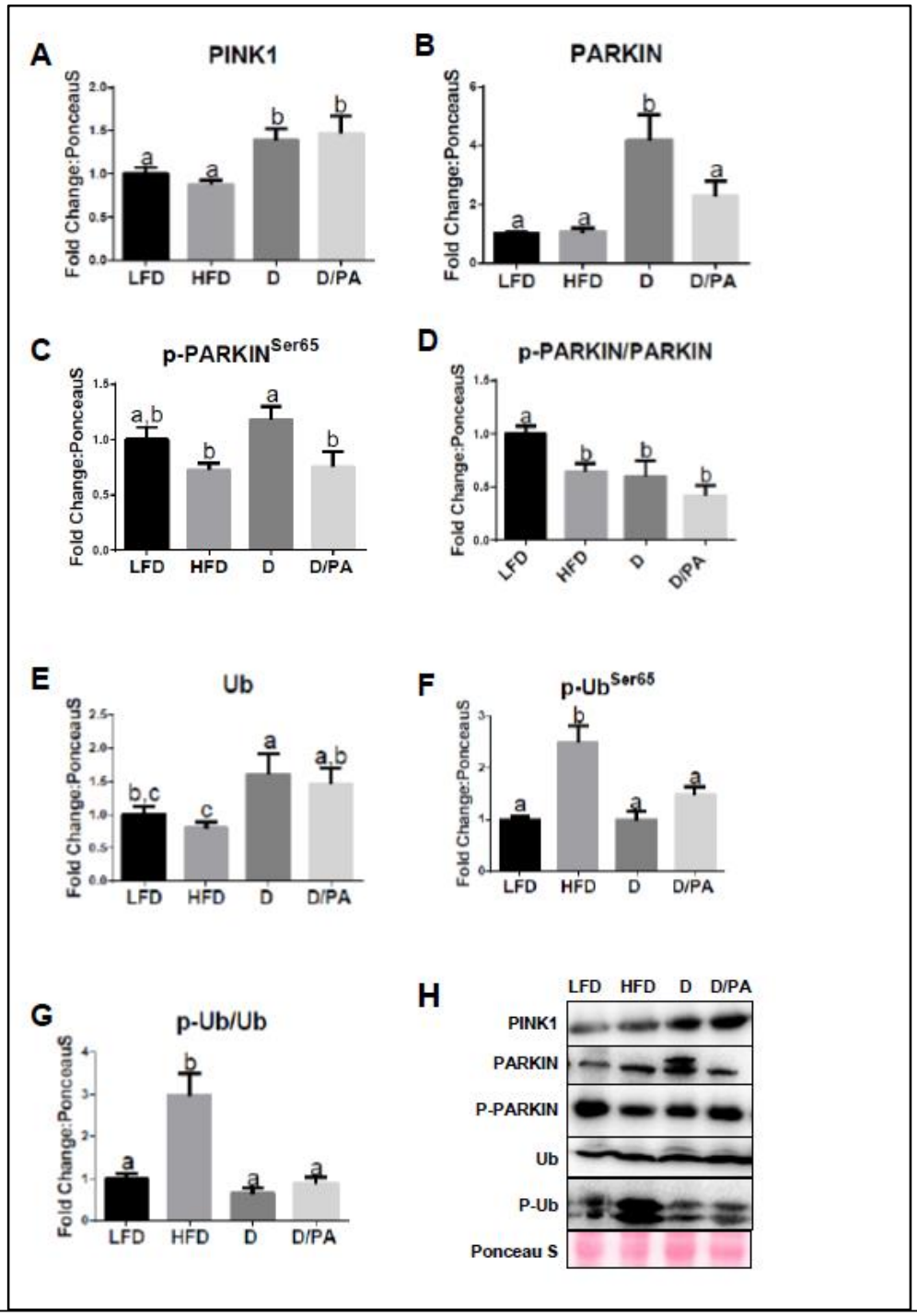

Figure 6: PINK1/PARKIN mediated mitophagy data. A: PINK1 protein content. B: PARKIN protein content. C: p-PARKIN^{Ser56} protein content. D: p-PARKIN/PARKIN protein content. E: Ubiquitin (Ub) protein contend. F: p-Ub^{Ser65} protein content. G: Ratio of: p-Ub/Ub protein content. H: Representative blots for PINK1, PARKIN, P-PARKIN, Ub, and p-Ub.

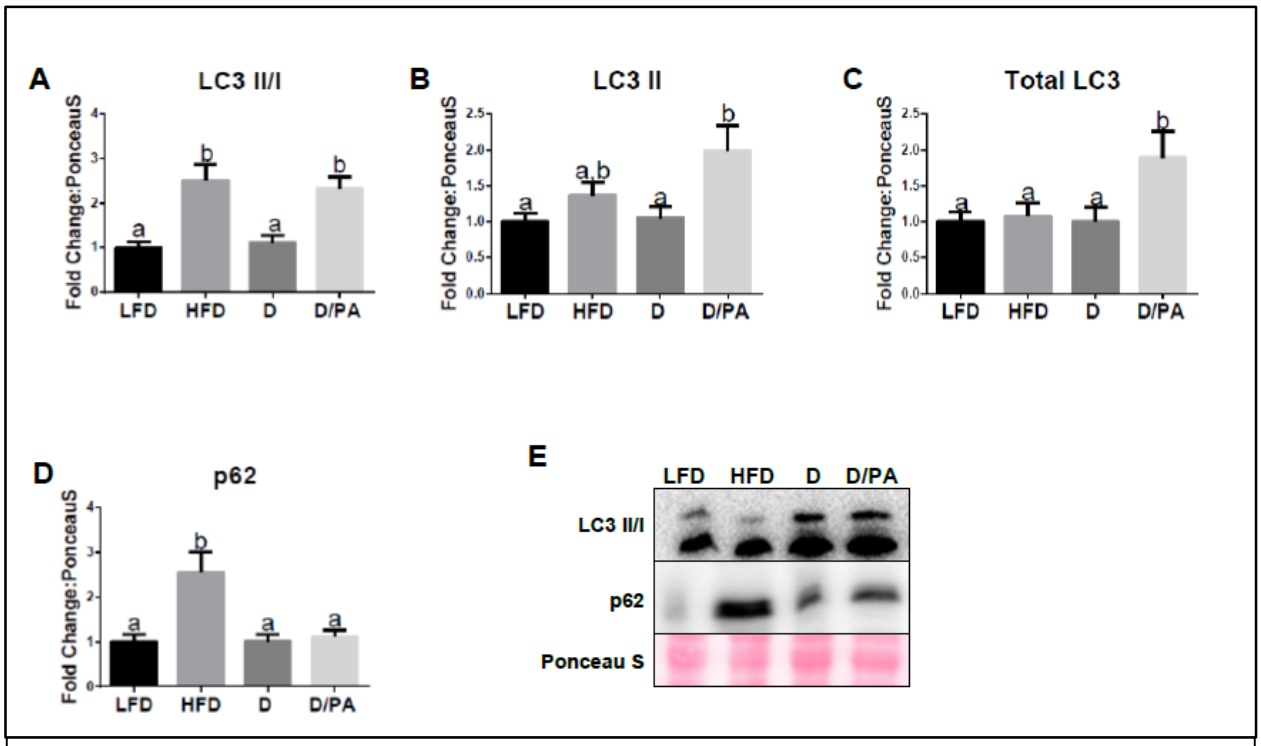

Figure 7: Markers of macroautophagy. A: LC3II/I ratio. B: LC3II content. C: Total LC3 content. D: p62 protein content. E: Representative blots for LC3 and p62.

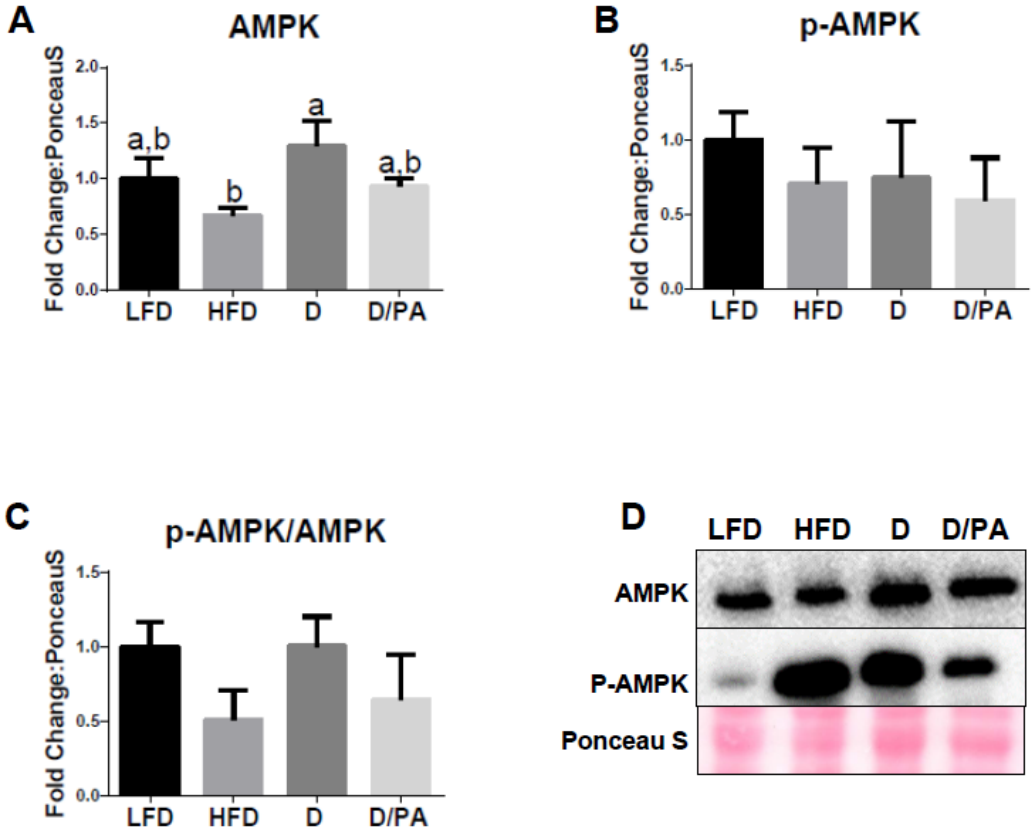

Figure 8: AMPK activity. A: Total AMPK protein content. B: p-AMPK^{Thr172} protein content. C: p-AMPK/AMPK ratio. D: Representative images for AMPK and p-AMPK^{Thr172}.

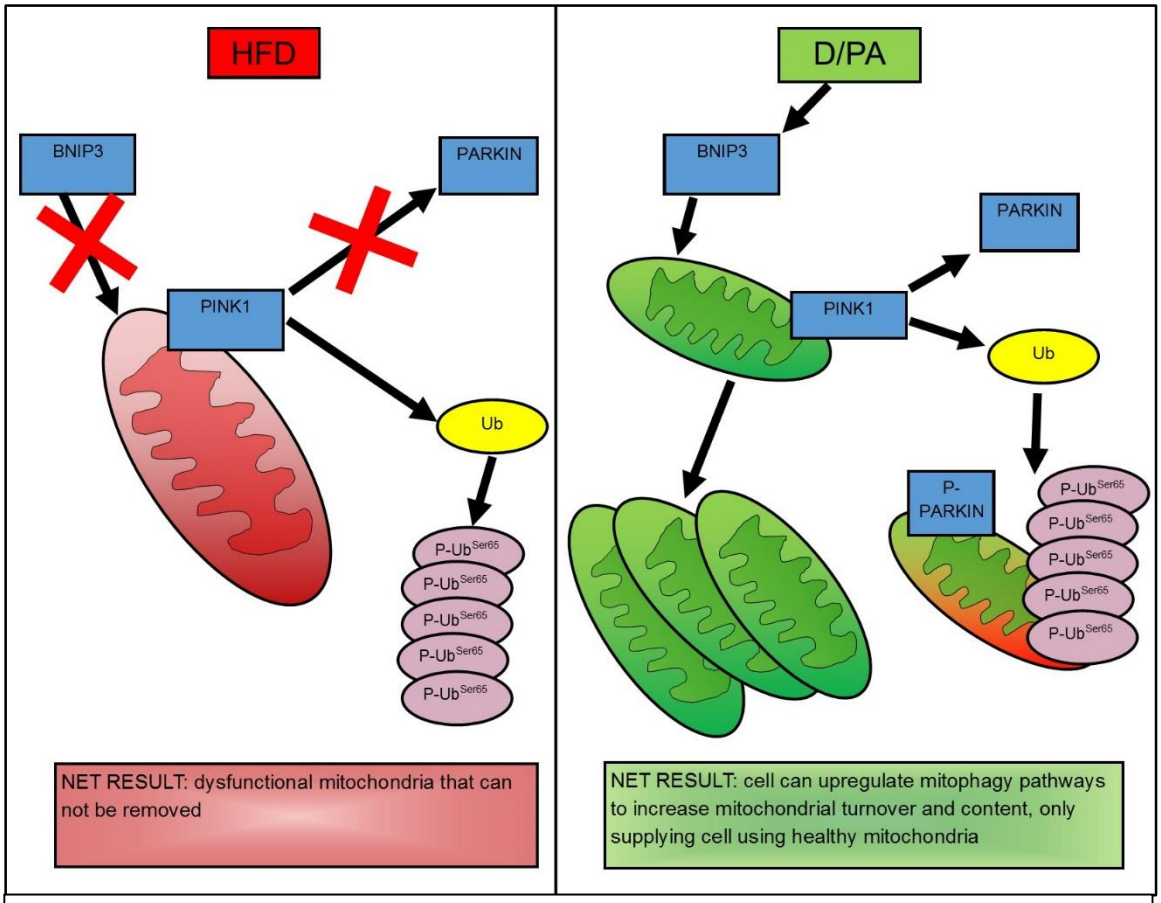

Figure 9: Conclusions from this study. HFD does not appear to affect overall PINK1 content or PARKIN activation, yet does appear to ramp up PINK1 activation via the phosphorylation of Ub^{Ser65}, yet the total mitophagy process does not resolve itself, suggesting that HFD may decrease capacity for completing the mitophagy process. Further, HFD decreases cellular capacity for BNIP3 mediate mitophagy. Taken together, it appears HFD mitigates multiple pathways for mitophagy in the liver. D/PA had overall greater effects than D itself, D/PA appears to predominantly affect the BNIP3 mitophagy process. Compared to HFD animals, D/PA animals are capable of adequately completing PINK1/PARKIN mediated mitophagy, though in this group PINK1/PARKIN does not appear to be as active compared to BNIP3. Taken together, this study suggests D/PA can restore aspects of mitophagy that become dysfunctional with prolonged HFD.

Appendix

A. Institutional Animal Care and Use Committee Approval Letter

SOUTHERN ILLINOIS UNIVERSITY
EDWARDSVILLE
OFFICE OF RESEARCH AND PROJECTS

Office of Research & Projects
Campus Box 1046,
Phone: 618-650-2958
Fax: 618-650-3523
e-mail: lskelto@siue.edu

SIUE IACUC
Animal Welfare Assurance
No: A3486-01

MAY 16, 2014

[redacted]
Department of Kinesiology and Health Education
Campus Box 1126
Southern Illinois University Edwardsville
Edwardsville, IL 62026

Your proposal to conduct research involving animals entitled: "The Cardioprotective Role of Physical Activity During Weight Cycling." was received by the Institutional Animal Care and Use Committee (IACUC), reviewed, and approved on May 9, 2014 according to the federal regulations on the care and use of animals in research. The following Protocol I.D.# has been assigned:

I.D. # 042314-JW2

No further action is required unless you change your methods as they are stated in your protocol. If you plan to make such changes you must notify the IACUC by contacting Linda Skelton in the Office of Research and Project (ORP) at lskelto@siue.edu to update your protocol and to determine whether further IACUC review is warranted.

The IACUC requires that you submit a "Continuing Review Report" each year during this approval period (9/15/2012-9/15/2015) of your project and/or at the completion of your project. At that time, please complete the form at: http://www.siue.edu/orp/researchpolicies/animal.shtml and send to ORP at campus box 1046.

Thank you for cooperating with the Institutional Animal Care and Use Committee. If you have any questions about your research with animals, please contact the IACUC. Complete contact information appears at the top of this memo.

Sincerely,

[redacted]
IACUC Chair

CC: [redacted]

PROTOCOL FOR USE OF ANIMALS IN RESEARCH, INSTRUCTION AND OTHER ACTIVITIES
Southern Illinois University Edwardsville

For Graduate School Use
PROPOSAL NUMBER 042314-JW2
Approval Date: may 9, 2014
Expiration Date: 5/14/2017

A. ADMINISTRATIVE DATA

Principal Investigator: [redacted]

Department: Kinesiology and Health Education

Mailing Address: Campus Box 1126, Edwardsville, IL 62026-1126

Telephone: [redacted] Fax: [redacted] Email: [redacted]

Project Title: The Cardioprotective Role of Physical Activity During Weight Cycling

Check One ☒ Initial Submission ☐ Renewal ☐ Modification

Duration of the Project (not to exceed 3 years): Begin Date: 5/15/2014 End Date: 5/14/2017
Continuing Review Reports must be submitted to Graduate Studies and Research at intervals of not less than once per year.

List the names of all individuals authorized to conduct procedures involving animals under this proposal and identify key personnel (e.g., co-investigator(s), providing their department, telephone, fax, and email:

Key Personnel's Name [redacted]
Department: Kinesiology and Health Education, School of Education
Mailing Address: Campus Box 1126, Edwardsville, IL 62026-1126
Telephone: [redacted] Fax: [redacted] Email: [redacted]

Key Personnel's Name
Department:
Mailing Address:
Telephone: Fax: Email:
Funding Source:

B ANIMAL REQUIREMENTS

Genus: *Mus* *[e.g., Mus]* Species: *musculus* *[e.g., musculus]*
Strain, subspecies, or breed: *C57BL/6* *[e.g., C57BL]* Common name: *C57 black laboratory mouse* *[e.g., black laboratory mouse]*
Approximate age, weight or size: 6 weeks, 20-22g

Source(s): *Jackson Laboratories*
[e.g., name of vendor or breeder, bred in-house]
Primary housing location(s): *[If animals will be housed in lab or anywhere else outside central facility for more than 12 hours, provide building and room number.]*
Room 2305F Vadalabene Center
Location(s) where manipulation will be conducted: Room 2305D Vadalabene Center

L. PRINCIPAL INVESTIGATOR CERTIFICATIONS

1. **I certify that I have attended the institutionally required investigator training course.**

Year of Course Attendance: 2012 **Location:** SIUE

2. I certify that I have determined that the research proposed herein is not unnecessarily duplicative of previously reported research.
3. I certify that all individuals working on this proposal who are at risk are participating in the Institution's Occupational Health and Safety Program.
4. I certify that the individuals listed in Section A. are authorized to conduct procedures involving animals under this proposal, have attended the institutionally required investigator training course, and received training in: the biology, handling, and care of this species; aseptic surgical methods and techniques (if necessary); the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics, analgesics, and tranquilizers (if necessary); and procedures for reporting animal welfare concerns.
5. For all USDA Classification D and E proposals (see section G.1.): I certify that I have reviewed the pertinent scientific literature and the sources and/or databases as noted in Section G.2. and have found no valid alternative to any procedures described herein which may cause more than momentary pain or distress, whether it is relieved or not.
6. I certify that I will obtain approval from the IACUC before initiating any significant changes in this study.
7. I certify that I will notify the IACUC regarding any unexpected study results that impact the animals. Any unanticipated pain or distress, morbidity or mortality will be reported to the attending veterinarian and the IACUC.
8. I certify that I am familiar with and will comply with all pertinent institutional, state, and federal rules and policies.

M. REQUIRED SIGNATURES

Principal Investigator:
Name: [redacted] Signature: [redacted] Date: 4/22/2014

Department Chair:
Name: [redacted] Signature: [redacted] Date: _____

Safety Office/Committee Certification of Review and Concurrence:
(Required of all studies utilizing hazardous agents.)

Name: _____ Signature: _____ Date: _____

Facility Manager
(Facility manager must certify below that facility has the resource capability to support the study)

Name: [redacted] Signature: [redacted] Date: 5/9/14

Attending Veterinarian certification of review and consultation on proper use of anesthetics and pain relieving medications for any painful procedures:

Name: [redacted] Signature: [redacted] Date: 5/9/14

N. FINAL APPROVAL:
Certification of review and approval by the Institutional Animal Care and Use Committee:

IACUC Chair [redacted] Signature: [redacted] Date: 5/9/14