HHS Public Access

Author manuscript

Obesity (Silver Spring). Author manuscript; available in PMC 2023 May 01.

Published in final edited form as:

Obesity (Silver Spring). 2022 May ; 30(5): 1066–1078. doi:10.1002/oby.23414.

Hepatocyte-specific eNOS deletion impairs exercise-induced adaptations in hepatic mitochondrial function and autophagy

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Abstract

Objective: Endothelial nitric oxide synthase (eNOS) is a potential mediator of exercise-induced hepatic mitochondrial adaptations.

Methods: Here, male and female hepatocyte-specific eNOS knockout ($eNOS^{hep-/-}$) and intact hepatic eNOS ($eNOS^{fl/fl}$) mice performed voluntary wheel running exercise (EX) or remained in sedentary cage conditions for 10 weeks.

Results: EX resolved the exacerbated hepatic steatosis in $eNOS^{hep-/-}$ male mice. Elevated H_2O_2 emission (~50% higher in $eNOS^{hep-/-}$ vs $eNOS^{fl/fl}$ mice) was completely ablated with EX. Interestingly, EX increased [1-^{14}C] palmitate oxidation in male $eNOS^{fl/fl}$, but this was blunted in the $eNOS^{hep-/-}$ male mice. $eNOS^{hep-/-}$ mice had lower markers of the energy sensors pAMPK/AMPK and mTOR and p-mTOR, and the autophagy initiators ULK1 and pULK1 compared to $eNOS^{fl/fl}$ mice. Females showed elevated ETC protein content and markers of mitochondrial biogenesis (TFAM, *pgc1α*).

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Author Contributions

R.P.C. and R.S.R. were involved in the study concept and design. T.T. provided the eNOS floxed mouse model. R.P.C., M.P.M., R.J.D., G.M.M., V.J., and R.S.R. helped with acquisition of data. R.P.C., M.P.M., R.J.D., G.M.M., and R.S.R analyzed and interpreted results. R.P.C., and R.S.R. provided statistical analysis of the data. R.P.C. drafted the manuscript, while R.P.C., M.P.M., G.M.M., R.J.D., V.J.V., J.A.K, F.W.B., and R.S.R. revised the manuscript and provided important intellectual content. R.P.C., M.P.M., R.J.D., G.M.M., V.J., T.T., V.J.V., J.A.K, F.W.B., and R.S.R. approved final version of manuscript. R.P.C., R.J.D., and R.S.R. obtained funding.

Disclosures

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

Conclusions: Collectively, we demonstrate for the first time the requirement of eNOS in hepatocytes in the EX-induced increases in hepatic fatty acid oxidation in male mice. Deletion of eNOS in hepatocytes also appears to impair the energy sensing ability of the cell and inhibit the activation of the autophagy initiating factor ULK1. These data uncover the important and novel role of hepatocyte eNOS in exercise-induced hepatic mitochondrial adaptations.

Keywords

NAFLD; eNOS; mitochondria; exercise; autophagy

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a progressive liver disease, with diet and exercise the cornerstone of treatment. Studies from our lab have demonstrated that exercise reduces hepatic steatosis and NAFLD, with corresponding improvements in hepatic mitochondrial fatty acid oxidation and respiration (1, 2, 3, 4). However, the precise mechanisms causing mitochondrial dysfunction as well as exercise-induced improvements in mitochondrial function in NAFLD remain unresolved.

Given its well established role in regulating mitochondrial biogenesis (5, 6, 7, 8), endothelial nitric oxide synthase (eNOS) may play a significant role in hepatic mitochondrial adaptations to exercise. Whole body eNOS null mice display blunted exercised-induced improvements in mitochondrial biogenesis and content in both adipose and cardiac tissue (9, 10). We have also demonstrated via multiple approaches that loss of eNOS exacerbates hepatic mitochondrial dysfunction and NAFLD development (11, 12, 13). Recently, we have generated a hepatocyte-specific eNOS knockout mouse that exhibits exacerbated NAFLD development and reduced hepatic mitochondrial function (14). While the link between eNOS and hepatic mitochondrial dysfunction is clear, whether hepatocyte eNOS is required for hepatic mitochondrial adaptations to exercise is yet to be determined.

Exercise is also a potent inducer of autophagy – the ability to clear dysfunctional proteins and mitochondria (mitophagy) (15, 16). Exercise-induced autophagy is mediated primarily through AMP-activated protein kinase (AMPK) activation of Unc-51 Like Autophagy Activating Kinase 1 (ULK1) (17), while this is also regulated by the inhibitory effects of mammalian target of rapamycin (mTOR) (18). Indeed, fully functioning autophagy/mitophagy is required for beneficial exercise-induced adaptations in skeletal muscle (19, 20, 21), although its role in exercise adaptations in the liver is relatively unknown. While the role of eNOS in mitochondrial biogenesis with exercise is clear, its role in exercise-induced autophagy/mitophagy is relatively unknown.

Our group has also demonstrated that female rodents are not only protected from hepatic steatosis compared to male mice, but also display elevated markers of hepatic mitochondrial biogenesis and mitophagy (22, 23). Additionally, female mice possess elevated hepatic mitochondrial content, respiratory capacity, and lower ROS emission compared to male mice, regardless of physical activity status (24, 25, 26). The mechanisms conferring benefit to hepatic mitochondria in female rodents has yet to be resolved.

Based on the phenotype from our previously characterized hepatocyte-specific eNOS knockout mice ($eNOS^{hep-/-}$) (14), we hypothesize that these mice will display blunted hepatic mitochondrial adaptations to exercise. To assess this, we tested whether voluntary wheel running exercise (EX) could rescue the exacerbated NAFLD phenotype and reduce hepatic mitochondrial function observed in $eNOS^{hep-/-}$ mice (14). Further, we evaluated the molecular signals involved in hepatic mitochondrial improvements with exercise in both male and female mice, allowing us to determine if hepatocyte specific eNOS is required for the exercise-induced improvements in hepatic mitochondrial function.

RESEARCH DESIGN AND METHODS

Animal protocol.

The hepatocyte-specific eNOS knockout (KO) ($eNOS^{hep-/-}$) mouse model has been generated and characterized by our group previously (14). Briefly, homozygous eNOS floxed ($eNOS^{fl/fl}$) mice on a C57BL/6J background (27) were crossed with albumin-Cre recombinase transgenic mice (Jackson Labs no. 002684; Bar Harbor, ME). At 10 weeks of age, male and female $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice were randomized into either sedentary (SED) or voluntary wheel running exercise (EX) conditions. SED mice were group housed and separated by sex with no access to running wheels, while EX mice were individually housed and provided a running wheel. It is worth noting that comparing group versus single housed mice could be a confounding variable, as single housed mice often present with lower body weights (28). Importantly, our housing conditions were consistent across sex and genotype. This resulted in a total of 8 groups (n = 10–14/group): male $eNOS^{fl/fl}$ SED, male $eNOS^{fl/fl}$ EX, male $eNOS^{hep-/-}$ SED, male $eNOS^{hep-/-}$ EX, female $eNOS^{fl/fl}$ SED, female $eNOS^{fl/fl}$ EX, female $eNOS^{hep-/-}$ SED, female $eNOS^{hep-/-}$ EX. All mice received a semi purified control diet (CON; no. D12110704; Research Diets, New Brunswick, NJ) containing 10% kcal fat, 70% kcal carbohydrate (3.5% kcal sucrose), and 20% kcal protein, and kept in these conditions for 10 weeks. Total distance ran and time spent on the running wheel was measured by daily monitoring of the number of running wheel revolutions continuously throughout the intervention using a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA). The voluntary running wheel approach represents a less stressful model of increasing physical activity versus forced treadmill exercise training.

For all animal experiments, room temperature was kept constant at 21–22°C with a 12:12 light/dark cycle. Food intake and body weight of the animals was recorded weekly, and body composition (4in1–1100 Analyzer; EchoMRI, Houston, TX) measured monthly. Running wheels were locked 48 hr prior to terminal procedure to avoid the confounding effects of acute exercise on hepatic physiology. On the day of euthanasia, mice were fasted overnight for 12 hr (2000–0800), before being anesthetized with pentobarbital sodium (80–100 mg/kg). Blood was collected via cardiac puncture, and the animals were euthanized via removal of the heart. Livers were quickly excised from anesthetized mice and prepared for mitochondrial isolation, nuclear extraction, homogenization for palmitate oxidation, and fixed in 10% formalin or snap-frozen in liquid nitrogen for later processing as described in

detail in the following sections. All animal protocols were approved by the University of Missouri and the Harry S Truman Animal Care and Use Committees.

Mitochondrial isolation and respiration

Hepatic mitochondria were isolated as previously described (3, 4, 13, 29). Hepatic mitochondrial respiration and H_2O_2 production was assessed in isolated hepatic mitochondria using high-resolution respirometry (Oroboros Oxygraph-2k; Oroboros Instruments; Innsbruck, Austria), via oxygen consumption and Amplex™ UltraRed reagent, respectively (#A36006, Thermo Fisher Scientific), with substrates as described previously (1, 3, 4, 13, 14). For H_2O_2 emission, male and female mice were combined due to a lower sample size for this measure. Mitochondrial coupling efficiency was calculated as 1 – (leak respiration/ADP stimulated respiration of complex I+II), with a higher number indicative of increased mitochondrial coupling.

Hepatic fatty acid oxidation

Using isolated hepatic mitochondria and whole liver homogenate, hepatic fatty acid oxidation capacity was determined by measuring [1-^{14}C] palmitate oxidation to $^{14}CO_2$ (complete oxidation) and [1-^{14}C] containing acid-soluble metabolites (complete oxidation), as previously described (3, 4, 14, 29, 30).

Gene and protein quantification

Western blot analyses were completed in whole liver homogenate and isolated hepatic mitochondria. A list of primary antibodies used are included in the supplemental data provided. Total protein was assessed with Amido black (0.1%, Sigma) to control for differences in protein loading and transfer as previously described (3, 22). Initially, RNA was extracted from frozen liver tissue with a commercially available kit (no. 74104, Qiagen), and quantitative real-time PCR (qPCR) was conducted using Sybr Green reagents (172–5121, BioRad) and primer pairs (Sigma) listed in supplemental table 1. PCR product melt curves were used to assess primer specificity. Data are represented relative to *18S* using the $2^{-\Delta\Delta CT}$ method.

Liver histology

To histologically determine hepatic steatosis development, the fresh liver placed in 10% formalin for 24 hr was then imbedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) by IDEXX RADIL (Columbia, MO). NAFLD activity score (NAS) – the aggregate of hepatic steatosis, ballooning, and inflammation histology scores of liver sections (31), were conducted by a trained and blinded observer.

Enzymatic assays

Further examination of mitochondrial content was assessed in whole liver homogenate with enzymatic assays. Citrate synthase activity, a well-established surrogate marker of mitochondrial content, was measured as previously described in whole liver homogenate (3).

Statistical analysis

Statistical analyses were completed in SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY) with an alpha level of P<0.05 used to determine statistical significance of all comparisons. Comparison across all three variables (sex, EX, and genotype) were analyzed via three-way (3×2) ANOVA. When comparing just two variables, i.e. removing sex as a variable for males and females combined due to insufficient sample size for some outcomes, or EX when comparing within EX groups only (e.g. daily running distance), a two-way (2×2) ANOVA was used. A Fisher's least significant difference post hoc test was used when a three-way or two-way ANOVA detected a significant interaction term (P<0.05). Figures were made using GraphPad Prism 8.3. All data are presented as means ± standard error of the mean (SEM).

RESULTS

Animal Characteristics

We have previously characterized this model (14), confirming ablation of eNOS from hepatocytes and a slight but non-significant reduction of nitrate and nitrite concentration. Importantly, we also show no compensatory upregulation of iNOS or nNOS in the liver or decreased eNOS in other tissues. In the current study, male mice weighed significantly more than female mice (~20%) and had higher fat mass, and significantly higher food intake than female mice regardless of genotype (main effect of sex, $P < 0.05$, table 1). EX did not reduce body weight, likely due to the increase in the average weekly food intake with EX (main effect of exercise, $P < 0.05$, table 1). As expected, EX increased heart to body weight ratio, as well as decrease final body fat, delta body fat change, and retroperitoneal (RP) fat mass (main effect of exercise, $P < 0.05$, table 1). Unlike whole body eNOS null mice who have limited exercise capacity (32), voluntary running distance did not differ between $eNOS^{hep-/-}$ and $eNOS^{fl/fl}$, with no effect of sex (table 1). This suggests that the limited exercise capacity in whole body eNOS null mice is being driven by lack of eNOS in the vasculature and other tissues.

Liver histology

Confirming findings in our recent report (14), $eNOS^{hep-/-}$ mice displayed elevated histological hepatic inflammation and NAS score compared to $eNOS^{fl/fl}$ mice, regardless of sex or EX (main effect of genotype, $P < 0.05$, fig 1A-B). There was a trend for a sex by exercise interaction for inflammation ($P = 0.062$), although given that the inflammation score was remarkably low for all females regardless of condition, this may not be of physiological relevance. Similar to previous publications (22, 23), male mice had elevated hepatic steatosis compared to female mice regardless of other conditions (main effect of sex, $P < 0.05$, fig. 1A-B). Histological ballooning score was zero for all groups, likely due to the low-fat diet and relatively young age of the animals (fig. 1B). EX reduced hepatic steatosis and NAS in all groups (main effect of EX, $P < 0.05$, fig. 1A-B). The EX-induced reduction in NAS was driven by the males (fig. 1B), with low NAS in all female groups.

Mitochondrial function

Our group has previously shown EX increases whole liver fatty acid oxidation (3, 4). Here, EX significantly increased complete, incomplete, and total palmitate oxidation in $eNOS^{fl/fl}$ males (main effect of EX, $P < 0.05$, fig. 2A-C). This EX-induced increase in fatty acid oxidation was completely absent in $eNOS^{hep-/-}$ males, who had significantly reduced complete, incomplete, and total palmitate oxidation compared to $eNOS^{fl/fl}$ regardless of EX ($P < 0.05$, fig. 2A-C). EX increased only complete palmitate oxidation in females (fig. 2A-C). Further, females had lower complete and total palmitate oxidation compared to males, regardless of EX or genotype (main effect of sex, $P < 0.05$, fig. 2A, C).

In isolated hepatic mitochondria, $eNOS^{hep-/-}$ mice had lower state 3 – complex I+II respiration compared to $eNOS^{fl/fl}$ mice (main effect of genotype, $P < 0.05$, fig. 3D). Notably, $eNOS^{hep-/-}$ mice had a ~50% increase in H_2O_2 emission compared to $eNOS^{fl/fl}$ mice, and this was completely ablated with EX (main effect of genotype, $P < 0.05$, fig. 3G). This cannot be explained by differences in antioxidant defense, as gene expression of *arg1*, *cat*, *gpx1*, *nqo1*, *nfe212, sod1, sod2, and nrf1* were not affected by genotype (table 2). Females displayed elevated expression of *nqo1, nfe212, cat, arg1, gpx1, sod2, and nrf1* (table 2). Interestingly, EX slightly reduced basal, state 3 – complex I, and maximal uncoupled respiration, regardless of genotype or sex (main effect of EX, $P < 0.05$, fig. 3A, C, E). Mitochondrial coupling efficiency was calculated and revealed that female $eNOS^{hep-/-}$ mice did not exhibit lower mitochondrial coupling efficiency compared with $eNOS^{fl/fl}$ mice as was seen in male counterparts (fig. 3F).

Mitochondrial content

In concurrence with our previous report (14), there was no effect of genotype on protein expression of electron transport chain (ETC) complexes (fig. 4A-F). EX increased protein content of CI, while decreasing CIV (main effect of EX, $P < 0.05$, fig, 4 A, D). Females displayed elevated protein content of all complexes apart from CIV compared to males, regardless of EX or genotype (main effect of sex, $P < 0.05$, fig. 4A-F). There was no effect of EX, sex, or genotype on whole liver citrate synthase activity, a classical marker of mitochondrial content/mass (fig. 4G).

Markers of mitochondrial biogenesis

Similar to what we have reported previously (14), there was no effect of genotype on protein expression of PGC1α or TFAM (fig. 5B-C; table 2). There was a sex by genotype interaction for PGC1α protein, such that the modest reduction in PGC1α in $eNOS^{hep-/-}$ vs $eNOS^{fl/fl}$ males was not observed in $eNOS^{hep-/-}$ females (sex by genotype interaction, $P < 0.05$, fig. 5B). EX increased *pgc1α* and *tfam* mRNA expression in all groups, but not protein content (main effect for EX, $P < 0.05$, fig. 5B, C; table 2). Interestingly, $eNOS^{hep-/-}$ mice showed reduced activation of AMPK (pAMPK to total AMPK ratio), regardless of EX or sex (main effect of genotype, $P < 0.05$, fig. 5A), suggesting hepatocytes from these mice have an impaired energy sensing ability compared to $eNOS^{fl/fl}$ mice. Similar to our previous work (22, 23), females exhibited marked elevation in TFAM protein compared to males, (main effect of sex, $P < 0.05$, fig. 5C), as well as increased mRNA expression of

mitochondrial biogenesis markers (*pgc1α, tfam,* and *sirt1*) (main effect of sex, $P < 0.05$, table 2).

Markers of mitochondrial turnover

Both total and phosphorylated mTOR were reduced in eNOS$^{hep-/-}$ mice compared to eNOS$^{fl/fl}$ mice, regardless of EX or sex (main effect of genotype, $P < 0.05$, fig. 6A-B). Additionally, both total and phosphorylated ULK1 were significantly reduced in eNOS$^{hep-/-}$ mice (main effect of genotype, $P < 0.05$, fig. 6C-D) and the robust EX-induced elevation in these markers as seen in male eNOS$^{fl/fl}$ mice ($P < 0.05$) was completely absent in eNOS$^{hep-/-}$ mice (fig. 6C-D). Surprisingly, this reduction in the autophagy initiator ULK1 did not affect the selected downstream markers of autophagy/mitophagy – whole liver Parkin, BNIP3, and mitochondrial LC3-II were not affected by genotype (fig. 6E-G). Similar to what we have previously reported (23), female mice exhibit elevated markers of autophagy/mitophagy compared to males (elevated Parkin and BNIP3 protein expression, main effect of sex, $P < 0.05$, fig. 6E-F). Although females also showed a higher pULK/ULK1 ratio compared to males (data not shown), female mice had markedly lower phosphorylated and total ULK1 compared to males (main effect of sex, $P < 0.05$, fig. 6C-D), indicating a lower total abundance of ULK1 in female livers. The lower pULK1 content may be explained by the increase in *mtor* gene expression in females (main effect of sex, $P < 0.05$, table 2), a potent inhibitor of ULK1 (17, 18). However, there was no effect of sex on mTOR and p-mTOR protein content or p-mTOR/mTOR (fig. 6A-B), suggesting sex differences in ULK1 may not be regulated by mTOR activity.

DISCUSSION

eNOS plays a known role in the regulation of mitochondrial biogenesis and in exercise-induced adaptations in heart, skeletal muscle, and adipose tissue (5, 6, 7, 8, 9, 10). Here, we demonstrate for the first time that while wheel running exercise attenuates hepatic steatosis and hepatic ROS regardless of genotype, exercise-induced increases in hepatic fatty acid oxidation required intact eNOS in hepatocytes. This was coupled with reduced AMPK activation in eNOS$^{hep-/-}$ mice, resulting in a blunted induction of the autophagy initiator ULK1 with exercise. Female mice displayed elevated markers of mitochondrial biogenesis and altered autophagy markers compared to male mice, with little added response to exercise. These novel data collectively suggest a requirement of eNOS in hepatocytes for the full beneficial effects of exercise on hepatic mitochondrial function and turnover.

Whole-body eNOS knockout mice have increased hepatic steatosis and inflammation compared to their wild-type counterparts (33), and we have shown mice lacking eNOS at the whole-body level are more susceptible to western diet-induced NASH (13). Additionally, we recently reported that hepatocyte eNOS deficient eNOS$^{hep-/-}$ mice displayed exacerbated histological NAFLD compared to eNOS$^{fl/fl}$ mice with compromised hepatic BNIP3 and mitophagic flux (14). Our current findings are in support of this, as eNOS$^{hep-/-}$ male mice had greater histological inflammation and overall NAS compared to eNOS$^{fl/fl}$ mice (fig. 1) with minimal histological findings in female mice fed a low-fat diet. This is consistent with literature showing female rodents are protected against hepatic steatosis and NAFLD

development versus their male counterparts (22, 23, 24, 25, 26). Despite these mice being on a low-fat diet, male sedentary eNOS$^{hep-/-}$ mice still developed steatosis and inflammation, highlighting the importance of hepatocyte eNOS in preventing the initial onset of NAFLD. We also have recently reported that when challenged with a NASH inducing western diet, eNOS$^{hep-/-}$ mice are more susceptible to NASH and fibrosis (14). Importantly, EX resolved inflammation and NAS score in eNOS$^{hep-/-}$ mice, suggesting hepatocellular eNOS is dispensable for EX-induced histological improvements in the liver, at least on a low-fat diet.

We have shown previously that exercise robustly increases hepatic mitochondrial fatty acid oxidation (1, 2, 3, 4). Similarly, here EX increased fatty acid oxidation in eNOS$^{fl/fl}$ mice, but this increase was completely ablated in male eNOS$^{hep-/-}$ mice (fig. 2). This is in accordance with whole-body eNOS null mice having a blunted response to swim training-induced elevations in mitochondrial biogenesis and content in cardiac and adipose tissue (9, 10). Surprisingly, females had lower complete and total fatty acid oxidation compared to males and did not respond to EX for these outcomes. This may be due to increased mitochondrial coupling control in females, as shown previously (24, 25), where females utilize fatty acids more efficiently, requiring less oxidation. In fact, female eNOS$^{hep-/-}$ mice did not exhibit lower mitochondrial coupling efficiency compared with eNOS$^{fl/fl}$ mice as was seen in male counterpart eNOS$^{hep-/-}$ mice. The lack of response to EX in females is supported by previous work, unlike males, who required EX to achieve the elevated mitochondrial function observed in females (24). These novel data indicate the requirement of eNOS in hepatocytes for exercise-mediated increases in fatty acid oxidation.

A hallmark of NAFLD/NASH progression is the increase in ROS emission and oxidative stress. Previous reports have shown that exercise attenuates NAFLD/NASH-induced elevated oxidative stress in rodents (24, 25). As seen here and previously by our group (14), hepatic mitochondrial H_2O_2 emission in eNOS$^{hep-/-}$ mice was ~50% higher compared to eNOS$^{fl/fl}$ mice (fig. 3). This effect was completely abrogated with EX, accompanied by induction of a number of genes involved in mitigating H_2O_2 production (*arg1, cat, gpx1*), indicating eNOS in hepatocytes does not appear to be required for the EX-induced reduction of ROS emission or antioxidant defense in the liver. Previous studies found females to possess lower H_2O_2 emission than males, and lends support to the notion of higher quality mitochondria in females (24, 25, 26). Unfortunately, our samples size was insufficient for statistical power to examine sex differences in H_2O_2 emission; however, genes associated with attenuating H_2O_2 (*arg1, cat, gpx1*) were significantly elevated in females compared to males (table 2).

Fully functional autophagy/mitophagy is required for exercise training-induced improvements in skeletal muscle mitochondrial adaptations and physical performance (19, 21). This is regulated primarily by the interplay between the activating and inhibitory effects of AMPK and mTOR on the autophagy initiator ULK1, respectively (17, 18). Here, eNOS$^{hep-/-}$ mice presented with dysregulated markers of energy sensing proteins – with reduced AMPK and mTOR regardless of sex or EX (fig. 5, 6). Decreased AMPK activity may explain the marked attenuation of exercise-induced increases in both total and Ser555 phosphorylated ULK1 in male mice, given that it is directly phosphorylated by AMPK during exercise (17). Despite dysregulated markers of energy sensing ability in

eNOS$^{hep-/-}$ mice, this did not affect mitochondrial LC3-II content (fig. 6), a marker of end stage autophagy/mitophagy. Thus, AMPK activation alone may not be sufficient to regulate autophagy/mitophagy, a concept supported by previous work (34). Collectively, these novel data indicate that hepatocellular eNOS may regulate exercise-induced autophagy/mitophagy in the liver, at least in males, although more robust and dynamic measurements of autophagic/mitophagic flux are required to confirm this.

Our group and others have shown that females confer some protection for hepatic steatosis development due, in part, to increased mitochondrial function (22, 23, 24, 25, 26). Here, females showed elevated markers of mitochondrial content (ETC) and biogenesis (TFAM protein, *pgc1α* mRNA) compared to males. This is likely due to increased hepatic *esr1* mRNA expression (table 2), the gene encoding estrogen receptor α – known to induce mitochondrial biogenesis and content (35, 36). This may also explain how the reduction in PGC1α and BNIP3 in eNOS$^{hep-/-}$ males was not witnessed in eNOS$^{hep-/-}$ females (fig. 5). Notably, females had markedly lower total and phosphorylated ULK1, indicating decreased autophagic initiation compared to males despite having elevated downstream autophagy/mitophagy markers (Parkin, BNIP3) (fig. 6). This is consistent with previous literature, with females presenting with reduced mitophagy flux despite elevated Parkin and BNIP3 (24). The authors suggested that females have increased mitochondrial coupling efficiency, and therefore less need for mitophagy. As indicated above, while we did not see increased mitochondrial coupling efficiency with EX, female eNOS$^{hep-/-}$ mice maintained mitochondrial coupling efficiency compared with eNOS$^{fl/fl}$ mice, a phenomenon which was not observed in male eNOS$^{hep-/-}$ mice. Although females are likely displaying similar mitophagic capacity here, more thorough methods of mitophagic flux are required to confirm this.

It is unclear whether the blunted responses to exercise are mediated by lack of eNOS-derived nitric oxide (NO). We recently reported that there was no compensatory upregulation of hepatic iNOS or nNOS expression in eNOS$^{hep-/-}$ mice, nor was eNOS expression altered in other tissues we assessed in eNOS$^{hep-/-}$ mice (14). However, we did report hepatocytes from eNOS$^{hep-/-}$ mice tended to have a slight reduction in nitrate/nitrite levels, a surrogate measure of NO (14). In addition, treatment of eNOS$^{hep-/-}$ mice with the liver-specific NO donor (PYRRO/NO) increased palmitate oxidation and decrease histological steatosis (14). These data suggest that the addition of NO in eNOS$^{hep-/-}$ exercised mice may restore the ability of physical activity to increase hepatic mitochondrial function in these mice, and further studies in this area are warranted.

Collectively, this study demonstrates for the first time the requirement of eNOS specifically in hepatocytes for the full hepatic mitochondrial adaptations to exercise. While exercise improved liver histology and reduced mitochondrial H_2O_2 emission in both groups of mice, hepatocellular eNOS deficient male mice failed to exhibit exercise-induced increases in hepatic fatty acid oxidation. Further, we uncover a role for hepatocyte eNOS in EX-induced autophagy/mitophagy where eNOS is required for normal hepatic AMPK activation and exercise-induced increases in activation in the autophagy initiator ULK1. These novel data help to further uncover the important role of eNOS in hepatocytes in NAFLD development and the molecular mechanisms behind exercise-induced hepatic mitochondrial adaptations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding.

Dr. Rector is supported by a VA-Merit Grant I01BX003271 and an NIH R01 DK113701–01. Dr. Cunningham was partially supported by ACSM Foundation Doctoral Student Research Grant #18–00754. Dr. Dashek is partially supported by NIH T32 OD011126. This work was supported with resources and the use of facilities at the Harry S Truman Memorial Veterans Hospital in Columbia, MO.

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KEY POINTS

- What is already known about this subject? Exercise increases hepatic mitochondrial function, although the precise mechanisms are unresolved.
- What does this study add? Using a hepatocyte specific eNOS null mouse model, we showed that exercise was unable to increase hepatic fatty acid oxidation in these mice compared to those with intact eNOS.
- These data demonstrate that hepatocyte eNOS is required for the full exercise-induced improvements in hepatic mitochondrial function.

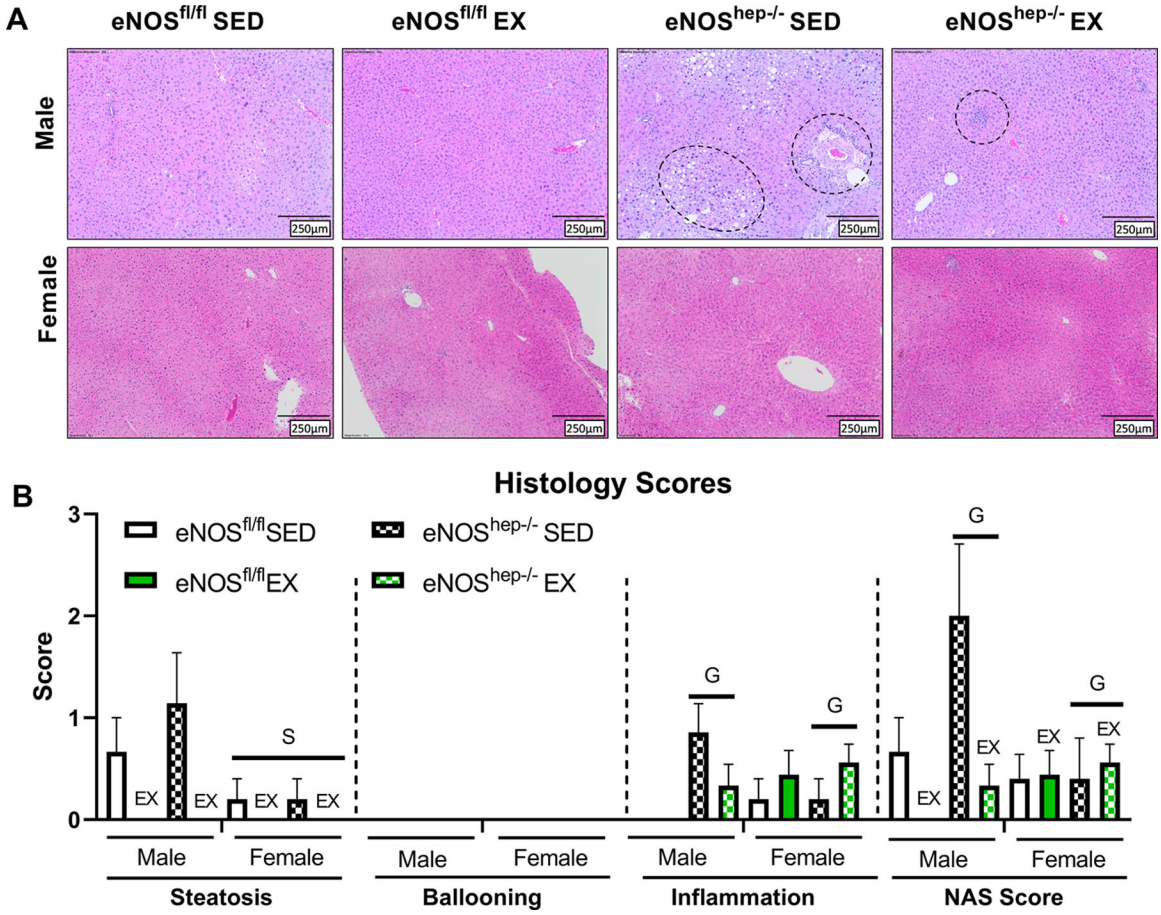

Figure 1:

Effects of hepatocellular eNOS deficiency, sex, and EX on liver histology and inflammation. A) Representative liver H&E slides from the indicated mice at 20–24 weeks of age. B) Histological steatosis scoring, inflammation scoring, and total NAS for all groups (n = 10–14/group). Ballooning scores were included in total NAS but no represented as the score was zero for all groups. Data are presented as mean ± SEM. S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$); G, main effect of genotype. EX, voluntary wheel running exercise; H&E, haemotoxylin and eosin; NAS, NAFLD activity score.

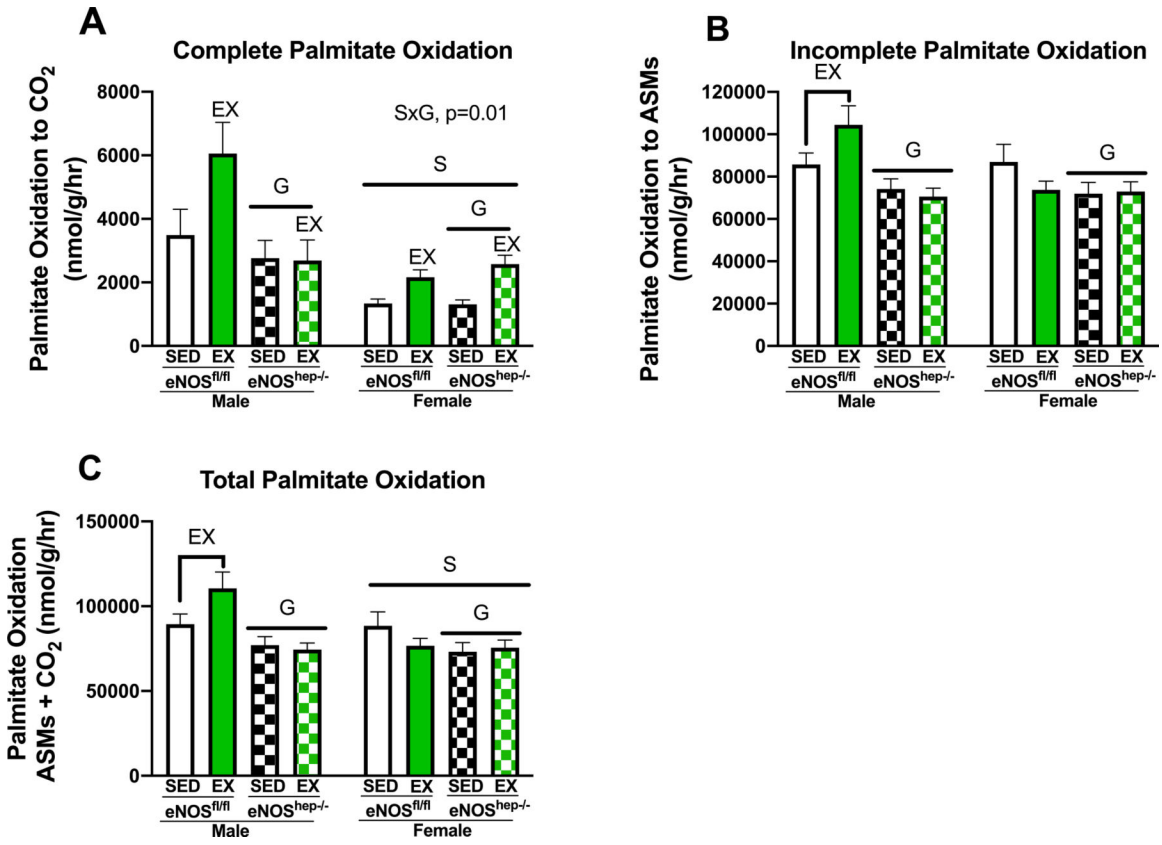

Figure 2:

Effects of hepatocellular eNOS deficiency, sex, and EX on whole liver homogenate fatty acid oxidation. A) Whole liver complete, B) incomplete, and C) total [1-^{14}C] palmitate oxidation to CO_2 (n = 10–14/group). Data are presented as mean ± SEM. S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$); G, main effect of genotype ($P < 0.05$). EX, voluntary wheel running exercise; ASMs, acid soluble metabolites.

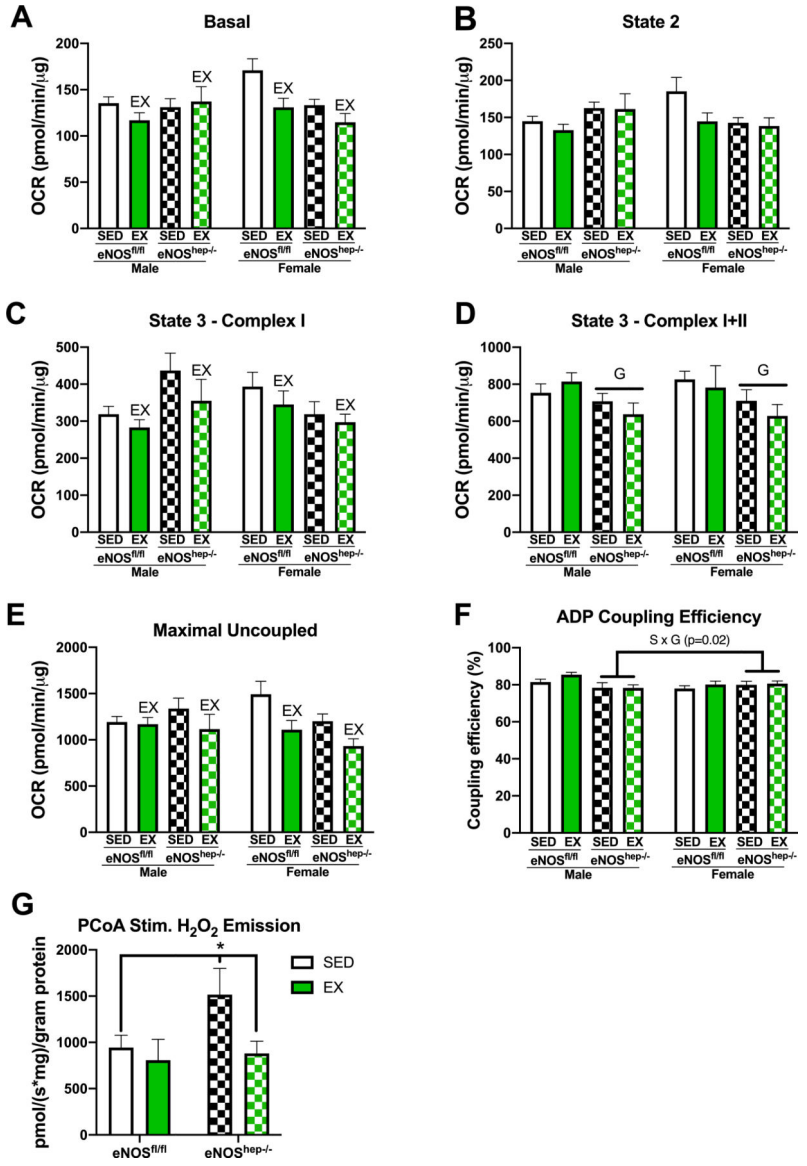

Figure 3:

Effects of hepatocellular eNOS deficiency, sex, and EX on isolated hepatic mitochondrial respiration. A) Basal oxygen consumption rate (OCR), B) state 2 OCR, C) state 3 complex I OCR D) state 3 complex I+II OCR, E) maximal uncoupled OCR. F) ADP coupling efficiency. E) PCoA stimulated H_2O_2 emission in isolated liver mitochondria from male and female combined (n = 7–9/group). Data are presented as mean ± SEM. S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$); G, main effect of genotype ($P < 0.05$). EX, voluntary wheel running exercise; OCR, oxygen consumption rate; PCoA, palmitoyl-CoA.

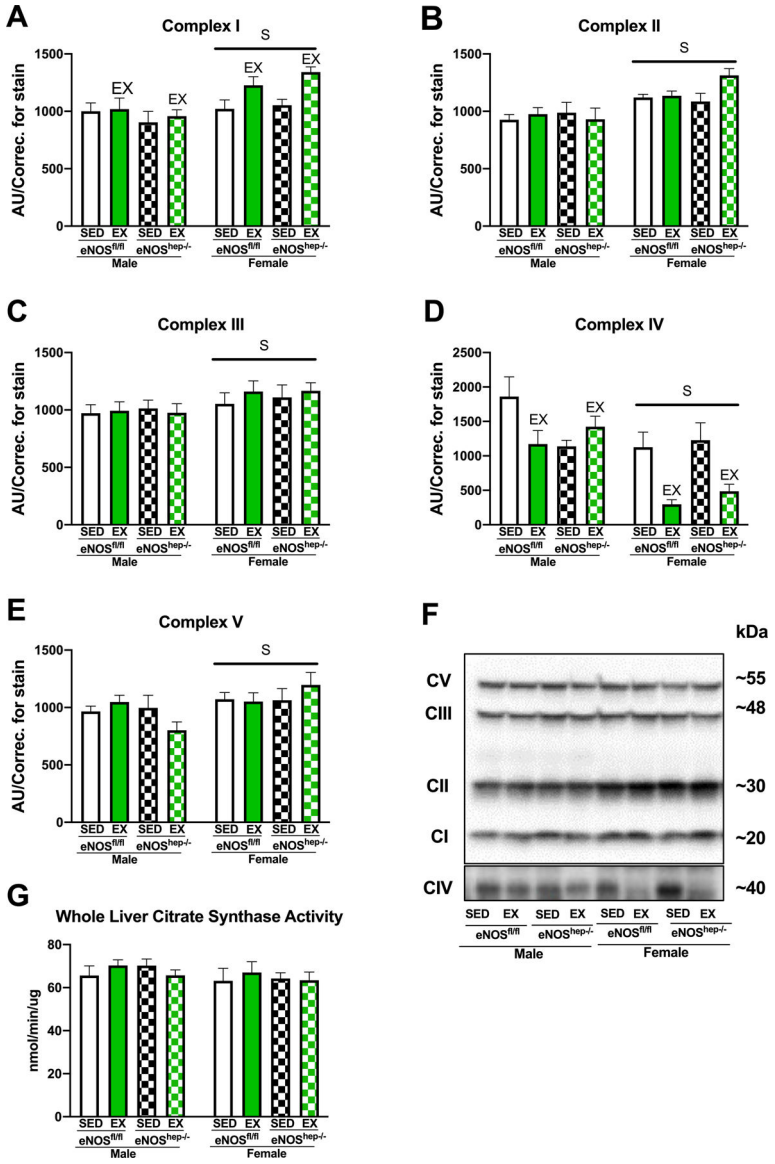

Figure 4:

Effects of hepatocellular eNOS deficiency, sex, and EX on markers of hepatic mitochondrial content in whole liver homogenate. A-E) Protein abundance of the electron transport chain complexes I-V (n = 10–14/group), and F) their representative Western blot images. G) Citrate synthase activity (n = 9–10/group). Data are presented as mean ± SEM. S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$); EX, voluntary wheel running exercise; C, complex.

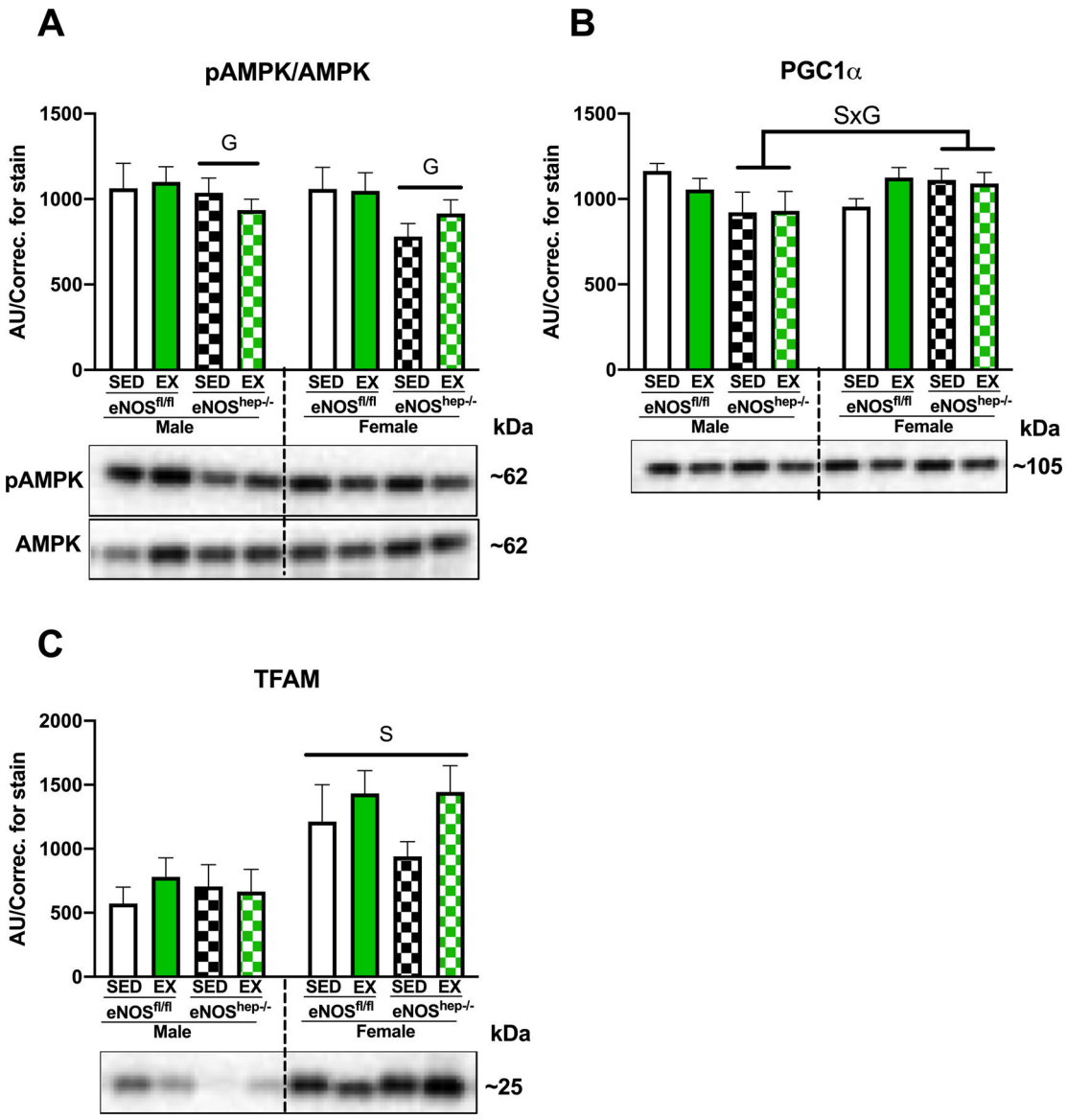

Figure 5:

Effects of hepatocellular eNOS deficiency, sex, and EX on markers of hepatic mitochondrial biogenesis in whole liver homogenate. Protein abundance and their representative Western blot images of; A) the ratio of phosphorylated AMPK to total AMPK, B) PGC1α, C) TFAM (n = 10–14/group). Data are presented as mean ± SEM. S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$); G, main effect of genotype ($P < 0.05$); SxG, sex and genotype interaction ($P < 0.05$). EX, voluntary wheel running exercise.

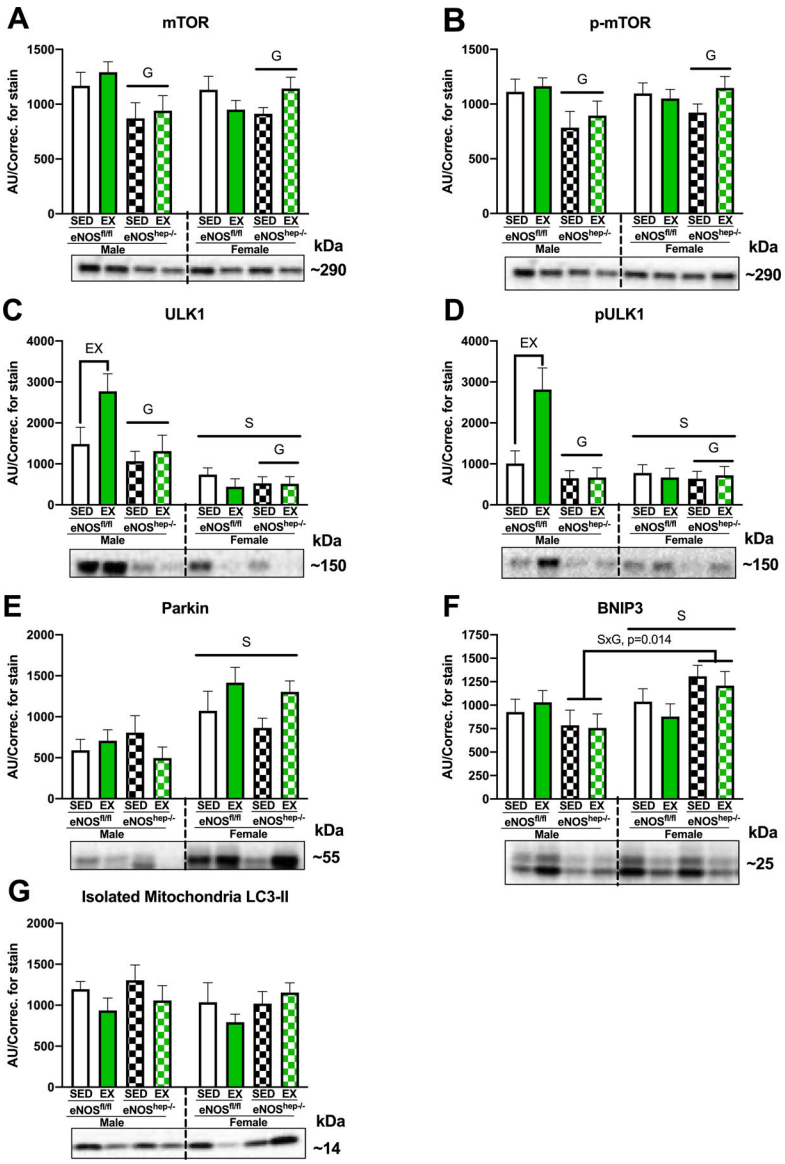

Figure 6:

Effects of hepatocellular eNOS deficiency, sex, and EX on markers of hepatic mitochondrial turnover. Protein abundance of markers of mitochondrial turnover and their representative Western blot images in whole liver homogenate; A) mTOR, B) p-mTOR, C) ULK1, D) pULK1, E) Parkin F) BNIP3, and G) isolated hepatic mitochondria LC3-II (n = 10–14/group). Data are presented as mean ± SEM. S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$); G, main effect of genotype ($P < 0.05$); SxG, sex and genotype interaction ($P < 0.05$). EX, voluntary wheel running exercise.

Table 1:

Animal Characteristics for all groups following 10 weeks sedentary (SED) or voluntary wheel running exercise (EX)

	Male eNOS$^{fl/fl}$ SED	Male eNOS$^{fl/fl}$ EX	Male eNOS$^{hep-/-}$ SED	Male eNOS$^{hep-/-}$ EX	Female eNOS$^{fl/fl}$ SED	Female eNOS$^{fl/fl}$ EX	Female eNOS$^{hep-/-}$ SED	Female eNOS$^{hep-/-}$ EX
Final Body weight (g)	27.93 ± 0.46	26.23 ± 0.89	27.31 ± 0.81	26.68 ± 1.03	21.67 ± 0.61^{S}	21.86 ± 0.44^{S}	22.02 ± 0.7^{S}	23.23 ± 0.6^{S}
Body fat (%)	15 ± 0.014	11 ± 0.014^{EX}	17 ± 0.011	11 ± 0.015^{EX}	19 ± 0.01^{S}	13 ± 0.012^{S,EX}	20 ± 0.025^{S}	14 ± 0.01^{S,EX}
Delta change body fat (%)	3 ± 0.009	−1 ± 0.015^{EX}	5 ± 0.016	−2 ± 0.019^{EX}	5 ± 0.01	−3 ± 0.015^{EX}	5 ± 0.02	−2 ± 0.011^{EX}
Liver (mg)	898.84 ± 38.2	863.37 ± 24.8	877.5 ± 51.8	868.24 ± 54.2	905.67 ± 52.9	851.22 ± 20.6	856.86 ± 27	817.07 ± 51.5
RP fat (mg)	729.83 ± 63.6	579.16 ± 96.3^{EX}	753.9 ± 79.7	608.29 ± 102.8^{EX}	480.4 ± 58.5^{S}	316.8 ± 47.8^{S,EX}	434.2 ± 48^{S}	405.7 ± 51.6^{S,EX}
Heart:body weight (mg/g)	4.5 ± 0.16	5.15 ± 0.15^{EX}	3.95 ± 0.37	5.14 ± 0.19^{EX}	4.54 ± 0.15	4.97 ± 0.13^{EX}	4.3 ± 0.08	5.15 ± 0.15^{EX}
Average weekly food intake (g)	18.6 ± 0.12	24.67 ± 0.49^{EX}	18.44 ± 0.76	24.62 ± 0.56^{EX}	15.52 ± 0.4S	22.99 ± 0.5^{S,EX}	14.95 ± 0.51^{S}	22.78 ± 0.5^{S,EX}
Average Daily Running Distance (km/day)	-	6.38 ± 0.46	-	6.31 ± 0.54	-	6.4 ± 0.42	-	6.09 ± 0.34

Animal characteristic following 10 weeks of SED or EX on a control diet. Data are presented as mean ± SEM (n = 10–14/group). S, main effect of sex (P<0.05); EX, main effect of exercise (P<0.05). EX, voluntary wheel running exercise; RP, retroperitoneal.

Table 2:

Gene expression of markers of mitochondrial biogenesis, turnover, and oxidative stress.

	Male eNOS$^{fl/fl}$ SED	Male eNOS$^{fl/fl}$ EX	Male eNOS$^{hep-/-}$ SED	Male eNOS$^{hep-/-}$ EX	Female eNOS$^{fl/fl}$ SED	Female eNOS$^{fl/fl}$ EX	Female eNOS$^{hep-/-}$ SED	Female eNOS$^{hep-/-}$ EX
nqo1	1.08 ± 0.16	1.19 ± 0.13^{EX}	1.02 ± 0.21	1.51 ± 0.22^{EX}	3.36 ± 0.66^{S}	5.31 ± 0.55^{S,EX}	4.32 ± 0.85^{S}	5.35 ± 0.58^{S,EX}
nfe212	1.01 ± 0.19	1.47 ± 0.2^{EX}	1.22 ± 0.31	1.76 ± 0.25^{EX}	2.56 ± 0.57^{S}	3.44 ± 0.35^{S,EX}	2.64 ± 0.6^{S}	3.4 ± 0.4^{S,EX}
esr1	1.03 ± 0.19	1.05 ± 0.15^{EX}	0.77 ± 0.15	1.2 ± 0.22^{EX}	1.74 ± 0.52^{S}	2.03 ± 0.16^{S,EX}	1.48 ± 0.4^{S}	2.6 ± 0.39^{S,EX}
pgc1α	1.05 ± 0.12	1.29 ± 0.22^{EX}	1.03 ± 0.2	1.34 ± 0.23^{EX}	1.43 ± 0.27^{S}	2.04 ± 0.28^{S,EX}	1.57 ± 0.29^{S}	2.8 ± 0.38^{S,EX}
tfam	1.08 ± 0.16	1.24 ± 0.11^{EX}	0.92 ± 0.18	1.19 ± 0.1^{EX}	1.82 ± 0.37^{S}	2.4 ± 0.16^{S,EX}	1.65 ± 0.31^{S}	2.22 ± 0.18^{S,EX}
sirt1	1.1 ± 0.16	1.13 ± 0.1^{EX}	0.82 ± 0.14	1.2 ± 0.15^{EX}	1.75 ± 0.28^{S}	2.02 ± 0.15^{S,EX}	1.5 ± 0.23^{S}	2.14 ± 0.2S^{S,EX}
mtor	1.09 ± 0.15	0.97 ± 0.12	0.95 ± 0.16	1.24 ± 0.1	1.71 ± 0.29^{S}	1.8 ± 0.09^{S}	1.59 ± 0.27^{S}	1.63 ± 0.18^{S}
arg1	1.07 ± 0.14	1.13 ± 0.17	0.83 ± 0.19	1.05 ± 0.15	1.48 ± 0.28^{S}	1.74 ± 0.18^{S}	1.2 ± 0.23^{S}	1.89 ± 0.23^{S}
cat	1.33 ± 0.34	1.98 ± 0.34^{EX}	0.88 ± 0.22	1.57 ± 0.28^{EX}	1.65 ± 0.42^{S}	2.77 ± 0.23^{S,EX}	1.76 ± 0.46^{S}	2.76 ± 0.23^{S,EX}
gpx1	1.04 ± 0.1	1.45 ± 0.17^{EX}	1.06 ± 0.22	1.51 ± 0.19^{EX}	1.28 ± 0.2^{S}	2.3 ± 0.21^{S,EX}	1.72 ± 0.3^{S}	2.35 ± 0.18^{S,EX}

Gene expression was determined using RT-PCR. Relative expression of all hepatic mitochondrial biogenesis, turnover, and oxidative stress genes were normalized to *18S,* and presented as mean ± SEM (n = 8–10/group). S, main effect of sex ($P < 0.05$); EX, main effect of exercise ($P < 0.05$). EX, voluntary wheel running exercise.