Exercise improves lipid metabolism disorders induced by high-fat diet in a SESN2/JNK- independent manner

Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2021-0241.R2
Manuscript Type:	Article
Date Submitted by the Author:	19-May-2021
Complete List of Authors:	Wang, Tianyi; Tianjin Medical University, Physiology and Pathophysiology Hu, Wenqing; Tianjin Medical University Niu, Yanmei; Tianjin Medical University Liu, Sujuan; Tianjin Medical University Fu, Li; Tianjin Medical University School of Medicine, Department of Physiology and Pathophysiology
Novelty bullets: points that summarize the key findings in the work:	☐ Exercise improved lipid disorder induced by HFD feeding and SESN2 knockout., ☐ SESN2 and JNK play a vital role in lipid biosynthesis in vivo and in vitro., ☐ SESN2 suppressed JNK to improve lipid metabolism in skeletal muscle cells
Keyword:	SESN2, JNK, Lipid biosynthesis, Lipid disorder, High-fat diet, exercise < exercise
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

Exercise improves lipid metabolism disorders induced by high-fat diet in a SESN2/JNK-independent manner

Tianyi Wang¹, Wenqing Hu², Yanmei Niu², Sujuan Liu^{3*}, Li Fu^{1, 2*}

1. Department of Physiology and Pathophysiology, School of Basic Medical Science, Tianjin Medical University, Tianjin 300070, China.

2. Department of Rehabilitation, School of Medical Technology, Tianjin Medical University, Tianjin 300070, China

3. Department of Anatomy and Histology, School of Basic Medical Science, Tianjin Medical University, Tianjin 300070, China

***Corresponding authors Address:** Department of Physiology and Pathophysiology, School of Medicine; Tianjin Medical University, 22 Qixiangtai Road, Tianjin 300070, China. Tel: 86-22-83336831

E-mail address: lifu@tmu.edu.cn (L. Fu)

Or Department of Anatomy and Histology, School of Basic Medical Science, Tianjin Medical University, 22 Qixiangtai Road, Tianjin 300070, China. Tel: 86-22-83336819

E-mail address: liusujuan@tmu.edu.cn (SJ. Liu)

Abstract

SESN2 and JNK are emerging powerful stress-inducible proteins in regulating lipid metabolism. The aim of this study was to determine the underlying mechanism of SESN2/JNK signaling in exercise improving lipid disorder induced by high-fat diet (HFD). Our data showed that HFD and SESN2 knockout resulted in abnormalities including elevated body weight, increased fat mass, serum total cholesterol (TC), lipid biosynthesis related proteins, and a concomitant increase of pJNK-Thr183/Tyr185. The above changes were reversed by exercise training. SESN2 silencing or JNK inhibition in palmitate-treated C2C12 further confirmed that SESN2 and JNK play a vital role in lipid biosynthesis. Rescue experiment further demonstrated that SESN2 reduced lipid biosynthesis through inhibition of JNK. SESN2/JNK signaling axis regulates lipid biosynthesis in both animal and cell models with abnormalities of lipid metabolism induced by HFD or palmitate treatment. This study provided evidence that exercise ameliorated lipid metabolic disorder induced by HFD feeding or by SESN2 knockout. SESN2 may improve lipid metabolism through inhibition JNK expression in skeletal muscle cells, providing a molecular mechanism that may represent an attractive target for the treatment of lipid disorder.

Keywords: SESN2, JNK, Lipid biosynthesis, Lipid disorder, Exercise, High-fat diet

Novelty

- Exercise improved lipid disorder induced by HFD feeding and SESN2 knockout.
- SESN2 and JNK play a vital role in lipid biosynthesis in vivo and in vitro.
- SESN2 suppressed JNK to improve lipid metabolism in skeletal muscle cells.

Introduction

Diet-induced obesity is an increasingly prevalent major global health and economic burden. Body fat accumulation increases the risk of developing metabolic syndrome, type 2 diabetes mellitus (T2DM), and cardiovascular disease (Harada

2018). Exercise reduces fat mass by improving lipid metabolism and has been used as an important intervention for the clinical prevention and treatment of metabolic diseases (Lehnig and Stanford 2018). However, the underlying mechanisms of exercise training improve lipid disorder induced by HFD is still elusive.

Sestrins represent a small family of proteins that involved in stress response and metabolic regulation (Lee et al. 2012). In mammals, Sestrins are encoded by SESN1, SESN2 and SESN3 genes (Ho et al. 2016). The expression of SESN2 is upregulated in cell exposed to environmental stresses such as DNA damage, oxidative stress, and hypoxia (Park et al. 2014). SESN2 is necessary for maintaining metabolic homeostasis and preventing obesity-associated pathologies (Lee et al. 2012; Park et al. 2014; Li et al. 2017). SESN2 expression elevated in the liver under conditions of hypernutrition, obesity and hepatosteatosis; and ablation of SESN2 promotes hepatic lipid deposition (Lee et al. 2012). Moreover, loss of Sestrin results in age-associated pathologies, including triglyceride accumulation, mitochondrial dysfunction, muscle degeneration, and cardiac malfunction in *Drosophila* (Lee et al. 2010). These findings indicate that SESN2 has an important homeostatic function in the control of mammalian lipid metabolism. Although relatively few exercise training studies have been conducted on SESN2, our previous data revealed that physical exercise enhances SESN2 protein expression in the skeletal muscle of mice not only in improvement of abnormal glucose-lipid metabolism but also in sarcopenia and mitochondrial dysfunction (Liu et al. 2015; Wang et al. 2018; Liu et al. 2021). Interestingly, although its underlying mechanism is still elusive, Sestrins mimic the physiological effects of exercise, suggesting that it could be a major effector of exercise on human metabolism (Kim and Sujkowski 2020). Similar to exercise, Sestrins also are involved in mechanistic target of rapamycin complex (mTOR), AMP-activated protein kinase (AMPK), FoxO and PRXs pathways that are associated with age-related pathologies and lipid metabolism (Liu et al. 2015; Liu et al. 2021; Lee et al. 2010; Sun et al. 2020). SESN2 may serve a key role in maintaining lipid metabolism during/after exercise training. Therefore, in this study, we focused on investigating whether exercise can

affect lipid metabolism through SESN2, as well as the potential underlying mechanisms.

c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK), comprises a subclass of the mitogen-activated protein kinase (MAPK) signaling pathway in mammalian cells. JNK plays an important role in many physiological and pathological processes, such as the cell cycle, reproduction, apoptosis, and cell stress. The JNK subgroup consists of three members: JNK1 and JNK2, which are widely expressed throughout the body, and JNK3, the expression of which is restricted to the brain, heart, and testes. Studies have reported that JNK may be involved in the regulation of lipid metabolism (Lawan and Bennett 2017). JNK has been shown to be activated in liver of mice fed by HFD (Vernia et al. 2014) and in liver biopsies of human (Koliaki et al. 2015) presenting with obesity and nonalcoholic steatohepatitis (NASH). Moreover, inhibition of JNK phosphorylation can significantly reduce parenteral nutrition- induced liver lipid deposition in rat (Xiao et al. 2017). Recent evidence indicates that SESN2 and JNK may represent two antagonistic forces governing lipid metabolism. In liver injury of mice, SESN2 inhibits Toll like receptor-induced proinflammatory signaling and protects cells by inhibiting JNK- or p38-mediated c-Jun phosphorylation (Yang et al. 2015). Moreover, in mouse RAW264.7 macrophage cell line, the oxidized low-density lipoprotein-induced of SESN2 expression is mediated by the activation of the JNK/c-Jun signaling pathway (Hu et al. 2015), suggesting SESN2 might be an effective pharmacological target for the treatment of lipid-related cardiovascular diseases. However, direct evidence of the molecular link between SESN2 and JNK, especially in state of impairments in lipid metabolism, and their roles in the regulating process of aerobic exercise ameliorating lipid metabolism still need to be determined.

In the present study, we used the lipid disorder model induced by long-term HFD-feeding in C57BL/6 or SESN2^{-/-} mice, and palmitate treatment in C2C12 cell, to observe the responses of regular exercise and to explore the interaction of SESN2 and JNK. In addition, si-SESN2 or JNK inhibitor was used in palmitate-incubated

C2C12 to confirm their vital roles in lipid metabolism. Rescued experiment was performed via JNK activation plus SESN2 overexpression administration to further explore the molecular link between SESN2 and JNK in palmitate-treated cell. In this study, we postulated that both SESN2 and JNK may be harnessed as an important treatment strategy for lipid disorder. We aimed to better understand the relationship and the possible mechanism between SESN2 and JNK in exercise-related metabolic benefits in lipid metabolism.

Materials and methods

Animals

All animal protocols were approved by the Tianjin Medical University Animal Care and Use Committee (approval number: SYXK-2019-0004) under the guidelines of the animal welfare and experimental protocol of the Chinese Academy of Sciences. SESN2^{-/-} mice were provided by Model Animal Research Centre of Nanjing University, China (GemPharmatech Co., Ltd). SESN2^{-/-} mice were generated on the C57BL/6 background from ESCs. The ESCs were created by CRISPR/Cas9 approach with a strong splice acceptor site that disrupts the SESN2 Open Reading Frame (ORF). Male C57BL/6 mice (wild type, WT) were purchased from SPF (Beijing) Biotechnology Co., Ltd. All mice were housed in a controlled environment with a reversed 12/12 h light-dark cycle. Four-week-old male WT mice and SESN2^{-/-} mice were randomly divided into normal chow control groups (WT Ctrl and SESN2^{-/-} Ctrl) and HFD groups (WT HFD and SESN2^{-/-} HFD). Mice from HFD groups were fed high-fat diet (45% calories from fat, #D12451, Research Diets) for 8 weeks. After 8-week HFD, those mice were randomly subdivided into sedentary groups (WT NC, SESN2^{-/-} NC, WT HC and SESN2^{-/-} HC) and exercise groups (WT NE, SESN2^{-/-} NE, WT HE and SESN2^{-/-} HE). Mice from exercise groups underwent a 6-week of exercise training on a motor-driven rodent treadmill. The running intensity and time were 75% VO₂max (12 m/min) (Fernando et al. 1993) for 5 days per week (60 min/day). During the 6-week of exercise training period, HFD-fed mice continued to

eat a high-fat diet. Body weight of mice was monitored weekly throughout the dietary intervention. After exercise training, the body composition including fat mass (FM) and fat-free mass (FFM) were determined with an ImpediVET analyzer (ImpediMed). Mice were then anesthetized intraperitoneally with a Ketamine (110 ml/kg)–Xylazine (10 ml/kg) mixture, and blood, quadriceps muscle, and liver were harvested for analysis.

Serum lipid profiles analyses

Serum was obtained by centrifugation at 3,000 rpm for 30 min at 4°C. The serum contents of total cholesterol (TC), triglycerides (TG), and free fatty acids (FFA) were enzymatically determined using individual commercial kits (Jiangsu Meimian Industrial Co., Ltd, Yancheng, Jiangsu, China). All experiments were run in triplicate.

Cell culture and treatments

C2C12 mouse myoblasts cell line (ATCC CRL-1772) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a humidified atmosphere with 5% CO₂ at 37°C until passage 6. C2C12 myoblasts were differentiated into myotubes in DMEM with 2% horse serum for 6 days. C2C12 myotubes were incubated with 0.8 mM palmitate in ethanol vehicle for 18 hours in DMEM with 1% fatty acid-free BSA (Liu et al. 2012). For siRNA silencing, C2C12 myoblasts were transfected by siRNA against SESN2 with 50 pmol using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and harvested at 48 hours after administration. For adenovirus experiments, C2C12 myotubes were infected by SESN2 adenoviruses (Ad-SESN2) using 100 multiplicity of infection (MOI) and harvested at 48 hours after treatment. For JNK inhibition and activation experiments, myotubes were incubated with 30 µM SP600125 (a JNK inhibitor, Selleck) for 24 hours (Rousseau et al. 2016) or 10 µM Anisomycin (a JNK agonist, Selleck) for 6 hours in DMEM supplemented with 1% fatty acid-free BSA (Hilder et al. 2005). All experiments were performed in triplicate.

Cell Counting Kit-8 (CCK-8) assay

C2C12 cells were seeded at a density of 6000 cells per well in five replicate

wells of 96-well plates. Palmitate-incubated C2C12 cells were treated by Anisomycin with or without Ad-SESN2. C2C12 myotubes were incubated with 0.8 mM palmitate in ethanol vehicle for 18 hours. For JNK activation experiments, myotubes were incubated with 10 μ M Anisomycin for 6 hours. For adenovirus experiments, C2C12 myotubes were infected by Ad-SESN2 (100 MOI) for 48 hours. The CCK-8 (Proteintech, PF00004) reagent (10 μ l) and DMEM (90 μ l) was added per well. Cell viability at 6-hour post-transfection was determined, and the absorbance values at 450 nm were measured by using the Quant Microplate Spectrophotometer (BioTek, Winooski, VT).

Oil Red O (ORO) staining

Oil Red O staining was used to detect lipid droplets accumulation. The livers of mice were dissected into the different lobes and immediately immersed in optimum cutting temperature compound. Afterwards, tissues were cut into 8 μ m section using a cryostat microtome, placed on glass slides, air dried, and stored at -80°C. Myotubes were washed with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, and then rinsed with PBS. The liver frozen-sections and the myotubes were then stained with ORO (in isopropanol) for 5 min, rinsed in 60% isopropanol, and then washed with PBS. ORO images were taken by using light microscope (Olympus, Tokyo, Japan). Number of lipid droplets was quantified by Image J.

Western blot analysis

Total protein was extracted from C2C12 cells and skeletal muscle using NP-40 lysis buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris [pH 8.0], protease inhibitor cocktail, and phosphatase inhibitor cocktail). The extract was diluted in SDS loading buffer and heated at 75°C for 10 min. Equal amounts of protein were subjected to 10% SDS/PAGE at 110 V and transferred onto 0.45 μ m polyvinylidene difluoride (PVDF) membranes at 300 mA for 2 hours. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies against SESN2 (Abcam, ab178518), SESN1 (Proteintech, 21668-1-AP), SESN3 (Proteintech, 11431-2-AP), SREBP-1c (Abcam, ab28481),

CPT-1 (Proteintech, 221701-AP), FASN (Proteintech, 10624-2-AP), pACC-Ser79 (Cell Signaling Technology, #3661s), ACC (Cell Signaling Technology, #3662s), pJNK-Thr183 Tyr185 (Cell Signaling Technology, #4668S), or β -tubulin (Cell Signaling Technology, #2146). Protein immunoreactivity was detected with a chemiluminescent horseradish peroxidase (HRP) substrate (Proteintech, SA00001-2), analyzed with a scanner, and digitalized using Quantity One image analysis software (Hercules, CA, USA). All results are representative of three independent experiments.

Statistical analysis

Data were presented as means $\pm$ SEM. We performed the unpaired Student's 2-tailed *t* test for comparison of 2 experimental groups and one-way or two-way ANOVA in more than 2 groups followed by Tukey's post hoc test. Data were considered significantly different when $P < 0.05$. Statistical analyses were performed using SPSS 22.1.

Results

Exercise training attenuates lipid profiles disorder induced by HFD and SESN2 knockout

Our results showed that 8-week HFD feeding significantly increased the body weight both in WT and SESN2^{-/-} mice (Fig. 1A). The mice were then performed a 6-week aerobic treadmill exercise. The body weight curve showed that SESN2 knockout significantly decreased the body weight of mice with or without exercise training (Fig. 1B). While aerobic exercise had no significant effect on the body weight both in WT and SESN2^{-/-} mice fed by normal chow diet (Fig. 1B). In addition, the body weight of SESN2 KO mice was lower on a chow diet (Fig. 1A and 1B), and was higher on a HFD (red dotted line, Fig. 1A), indicating that SESN2 KO mice were more sensitive to HFD. We further detected the body weight response to exercise after HFD both in WT and SESN2^{-/-} mice. Compared to WT HC, the body weight of mice from WT HE group significantly reduced after 6-week treadmill exercise (Fig. 1C). The similar trend was observed after comparison with SESN2^{-/-} HC and SESN2^{-/-} HE groups (Fig. 1C), indicating that regular exercise can reverse HFD-induced weight

increase. In addition, the body weight of SESN2^{-/-} HC significantly increased compared to WT HC, suggesting again that SESN2^{-/-} mice displayed a greater weight gain after HFD (Fig. 1C). Serum lipid profiles (TC, TG and FFA) and body composition (Fat Mass and Lean Mass) were further examined in WT and SESN2^{-/-} mice fed normal chow. Although no significant difference in TG and FM has been observed between WT and SESN2^{-/-} mice, SESN2 knockout did lead to TC and FFA increase, and LM decrease (Fig. 1D). The above data demonstrated that SESN2 knockout induces lipid metabolic disorder. Therefore, we focused on the role of SESN2 and further detected serum lipid profiles, body composition and lipid deposition of liver in SESN2^{-/-} mice after HFD and aerobic exercise. Our data suggested that exercise training largely abolished the HFD-induced increase in TC, TG, FFA, FM, LM and liver lipid deposition in SESN2^{-/-} mice (Fig. 1E-J).

Exercise training reduced lipid synthesis induced by HFD and SESN2 knockout

Given the important role of SESN2 in lipid metabolism, we further examined some proteins related to fatty acid synthesis and oxidation in quadriceps muscle of WT and SESN2^{-/-} mice. The results confirmed that SESN2 protein was completely deleted in SESN2^{-/-} mice (Fig. 2A and 2B). Moreover, consistent with our previous study (Wang et al. 2018), 6-week of aerobic exercise led to SESN2 protein increase in quadriceps muscle of WT mice (Fig. 2A and 2B). Interestingly, HFD also induced SESN2 protein increase in this study (Fig. 2A and 2B). This result is agreement with the report of Lee et al (Lee et al. 2012), and this may be a self-protection mechanism of organism. Acetyl-CoA carboxylase (ACC) catalyses the formation of malonyl-CoA, an essential substrate for fatty acid synthesis in lipogenic tissues, and its activity is regulated by substrate supply, allosteric ligands, the phosphorylation of multiple serine residues and interactions with other proteins (Brownsey et al. 2006). We have previously demonstrated that AMPK suppressed ACC-dependent de novo lipogenesis through the down regulation ACC activity by increasing ACC phosphorylation on serine 79 in C2C12 myotubes (Liu et al. 2012). Our data showed that the rate of pACC-Ser79 and ACC was significantly decreased after HFD when compared to NC

group mice; and it was significantly increased after aerobic exercise not only in wild-type mice but also in SESN2^{-/-} mice (Fig. 2A and 2C). The result suggested that exercise training can reverse HFD-induced fatty acid synthesis in quadriceps muscle. In addition, after normal chow feeding, exercise significantly increased pACC-Ser79/ACC in quadriceps muscle only in WT but not in SESN2^{-/-} mice, indicating that exercise reduces fatty acid synthesis in quadriceps muscle depending on SESN2 (Fig. 2A and 2C). Carnitine palmitoyltransferase 1 (CPT-1) is the enzyme in the outer mitochondrial membrane that converts long-chain acyl-CoA species to their corresponding long-chain acyl-carnitines for transport into the mitochondria. We found that HFD and aerobic exercise had no effect on CPT-1 protein expression in WT mice (Fig. 2A and 2D). In SESN2^{-/-} mice, aerobic exercise significantly increased CPT-1 protein expression in quadriceps muscle (Fig. 2A and 2D). Unexpectedly, HFD also increased CPT-1 expression in SESN2^{-/-} mice (Fig. 2A and 2D), suggesting that the regulatory mechanisms of SESN2 in fatty acid beta-oxidation is more complex and may involve in multiple pathways. We measured other markers related to lipid synthesis such as fatty acid synthase (FASN) and sterol regulatory element-binding protein 1 (SREBP-1) in skeletal muscle. The variation trends were similar. Compared with WT mice, FASN and SREBP-1c protein were significantly increased after SESN2 knockout (Fig. 2A, 2E and 2F), suggesting that SESN2 plays an important role on inhibition of fatty acid synthase. In addition, HFD significantly increased FASN and SREBP-1c expressions both in WT and SESN2^{-/-} mice, and those changes were reversed by regular exercise (Fig. 2A, 2E and 2F). Consistent with previous studies (Yang et al. 2015; Jegal et al. 2017), SESN2 knockout significantly increased pJNK-Thr183/Tyr185 content in quadriceps muscle (Fig. 2A and 2G) after HFD and exercise training, suggesting SESN2 inhibited JNK activity after several challenges. Aerobic exercise significantly reduced the increase of pJNK-Thr183/Tyr185 expression induced by HFD not only in wild-type mice but also in SESN2^{-/-} mice (Fig. 2A and 2G). Given three family members of Sestrins, we further detected the SESN1 and SESN3 proteins in SESN2^{-/-} mice. SESN1 protein

significantly increased after SESN2 knockout (Fig. 2A and 2H), while there was no significant effect on SESN3 protein (Fig. 2A and 2I). This result suggested that SESN1 may play a compensatory role after SESN2 knockout.

SESN2 inhibits JNK activity in palmitate-treated C2C12 myotubes

To determine the regulatory relationship of SESN2 and JNK, si-SESN2 was transfected into C2C12 myotubes incubated with or without palmitate. As shown in Fig. 3A-B, oil red O staining confirmed that increased lipid droplet was observed in Palmitate and si-SESN2 treated groups compared with Con group, and with particularly increase in Palmitate + si-SESN2 treated group. These findings indicated that SESN2 silencing or palmitate treatment can induce lipid metabolic disorder in C2C12 myotubes. Indeed, SESN2-siRNA administration resulted in a decrease of SESN2 expression (Fig. 3C and 3D). Consistent with HFD-induced increase of SESN2 in Fig. 2A and 2B, palmitate treatment significantly increased SESN2 protein expression in vitro (Fig. 3C and 3D). Similar effect of HFD on JNK activity in vivo, pJNK-Thr183/Tyr185 expression was significantly elevated in palmitate- incubated C2C12 myotubes with or without SESN2-siRNA transfection (Fig. 3C and 3E). In addition, silencing of SESN2 led to a significant increase of pJNK-Thr183/Tyr185 in C2C12 myotubes with or without palmitate treatment, indicating that SESN2 inhibits JNK activity (Fig. 3C and 3E).

Inhibition of JNK decreases lipid biosynthesis in palmitate-treated C2C12 myotubes

Studies have reported that JNK may be involved in the regulation of lipid metabolism (Lawan and Bennett 2017; Vernia et al. 2014). To explore the effect of JNK on lipid metabolism, SP600125, a JNK inhibitor, was added to palmitate-treated C2C12 myotubes, and lipid metabolism-related proteins were assessed in cell lysates (Fig. 4A). The inhibitory effect of SP600125 on JNK was confirmed in Fig. 4B. Our data demonstrated that ACC expression was significantly reduced after SP600125 administration (Fig. 4C), suggesting that lipid synthesis was suppressed in C2C12 myotubes after JNK inhibition. Interestingly, CPT-1 protein was also reduced after

JNK inhibition (Fig. 4D), suggesting that fatty acid beta-oxidation was also decreased after JNK inhibition. It seems discrepant observation that JNK inhibition led to a decrease of lipid synthesis and also availability. We further detected the changes of FASN and SREBP-1c in palmitate -treated cell. Those changes were similar to ACC showed in Fig. 4E and F. We found that FASN and SREBP-1c proteins were significantly reduced after SP600125 administration (Fig. 4E and 4F). Those findings demonstrated that inhibition of JNK may suppress lipid biosynthesis not lipid availability in palmitate-cultured C2C12.

SESN2 overexpression decreases lipid biosynthesis induced by JNK activator in palmitate-treated C2C12 myotubes

We further designed a rescue experiment to explore the role of SESN2 and JNK in lipid metabolism. Anisomycin, an agonist of JNK, was used in palmitate-cultured C2C12 and then Ad-SESN2 was infected in palmitate-treated myotubes. pJNK-Thr183/Tyr185 protein expression was significantly elevated after Anisomycin administration, suggesting that JNK was effectively activated by Anisomycin (Fig.5A and 5B). As predicted, activation JNK resulted in an increase of lipid biosynthesis related proteins including ACC, FASN and SREBP-1c in palmitate-treated C2C12 myotubes, while concomitant an increase of CPT-1 content (Fig.5C-5F). Lipid biosynthesis induced by JNK activator was partly reversed by Ad-SESN2 treatment (Fig.5C, 5E and 5F), demonstrating that SESN2 overexpression can improve lipid metabolic disorder induced by activation of JNK. Herein, CPT-1, lipid availability related protein, increased after JNK activation and reduced after SESN2 overexpression in palmitate-treated C2C12 myotubes (Fig.5D). Although it was consistent with the data showed in Fig.2D and Fig.4D in this study, the result of CPT-1 seems discrepant and intricate. One possible explanation is that SESN2/JNK may mainly mediate lipid biosynthesis in the process of lipid metabolism; while regulation of lipid availability may include other proteins or signaling pathways. In addition, the cell viability significantly reduced after JNK activation in palmitate-treated C2C12 myotubes, while it was significantly increased after SESN2

overexpression (Fig.5G). This result showed that SESN2 overexpression can rescue C2C12 myotubes and largely reverse the decrease of cell viability induced by JNK activation.

Discussion

Systemic knockout of SESN2 in mice (Lee et al. 2012) or dSesn in *Drosophila* (Lee et al. 2010) leads to obesity-related pathological changes, including TG accumulation, increased generation of reactive oxygen species, and mitochondrial dysfunction (Liu et al. 2021). In this study, we found that 8-week HFD feeding significantly increased the body weight both in WT and SESN2^{-/-} mice. Moreover, compared with WT mice, SESN2^{-/-} mice displayed a greater weight gain after HFD. Furthermore, increase of weight gain was accompanied with increased serum TC and FFA, decreased LM and increased liver lipid deposition in SESN2^{-/-} mice after HFD feeding. Those findings suggested that mice are vulnerable to HFD induced lipid disorder after SESN2 knockout. We further examined the effect of regular exercise in WT and SESN2^{-/-} mice after HFD feeding. Although aerobic exercise had no significant effect on the body weight in both WT and SESN2^{-/-} mice after normal chow feeding, exercise training significantly reduced the body weight after HFD. In addition, our data showed that exercise training largely ameliorated the HFD-induced increase in TC, TG, FFA, FM, LM, and liver lipids accumulation in SESN2^{-/-} mice. Those findings demonstrated that exercise training attenuates lipid metabolism disorder induced by HFD and SESN2 knockout.

Beside the above indicators including serum lipid profiles, body composition and liver lipid deposition, we further detected some target proteins related to fatty acid synthesis and oxidation in quadriceps muscle. As our previous studies, Sestrins, an emerging family of stress-inducible proteins, is modulated by exercise training and HFD (Li et al. 2017; Liu et al. 2015; Wang et al. 2018; Liu et al. 2021). In this study, we also found that long-term HFD-feeding and regular exercise increased SESN2 expression in skeletal muscle. Knockout of dSesn led to a significant increase in the expression of the dSrebp, dFas, and dAcc genes in *Drosophila* (Lee et al. 2010). ACC

is mainly responsible for fatty acid synthesis and its phosphorylation regulates fatty acid beta-oxidation. We found that exercise training can reverse HFD-induced decreased of pACC-Ser79/ACC in quadriceps muscle both in WT and SESN2^{-/-} mice. In addition, under the condition of normal chow feeding, exercise significantly increased pACC-Ser79/ACC in quadriceps muscle only in WT but not in SESN2^{-/-} mice, indicating that SESN2 plays an important role in exercise reducing fatty acid synthesis in quadriceps muscle. CPT-1 plays a major regulatory role in β -oxidation, and muscle-specific CPT-1 knockout mice exhibit reduced fatty acid oxidation and increased lipid accumulation (Wicks et al. 2015). Our results showed in WT mice, HFD and aerobic exercise had no effect on CPT-1 protein expression; while in SESN2^{-/-} mice, aerobic exercise significantly increased CPT-1 protein expression in quadriceps muscle. Unexpected, HFD also enhanced CPT-1 expression in SESN2^{-/-} mice, suggesting that the regulatory mechanism of SESN2 in lipid availability is involved in other pathways and more complex. As important markers related to lipid synthesis, the changes of FASN and SREBP-1 were consistent with recent reports (Wicks et al. 2015; Ok et al. 2018) that HFD significantly increased FASN and SREBP-1 expressions both in WT and SESN2^{-/-} mice, and those changes were reversed by regular exercise. In addition, FASN and SREBP-1c protein were significantly increased after SESN2 knockout, suggesting that SESN2 plays an important role on inhibition of fatty acid biosynthesis. These results indicated that other compensatory mechanisms may be involved in the absence of SESN2 after aerobic exercise. We further detected SESN1 and SESN3 in WT and SESN2^{-/-} mice following exercise training or HFD. Our results demonstrated that SESN1 may play a compensatory role after SESN2 knockout.

JNK is a key regulator of inflammatory signal transduction and knockout of JNK prevents HFD-induced obesity in genetically predisposed mice (Hirosumi et al. 2002). High-fat diet can promote JNK activity in multiple tissues. JNK activation rose markedly in response to HFD feeding, in parallel with mitochondrial dysfunction and oxidative stress in liver of male rat (Galmés-Pascual et al. 2020) and in brain of mice

(Khan et al. 2019). Similarly, our data showed that pJNK-Thr183/Tyr185 expression significantly increased in HFD feeding mice or palmitate-incubated C2C12 myotubes. In addition, the expression of ACC, FASN and SREBP-1c in palmitate-treated C2C12 myotubes was significantly reduced after SP600125 administration, revealing that inhibition of JNK may suppress lipid biosynthesis and JNK plays a vital role in lipid metabolism.

Being an important mediator of stress response, the molecule link between SESN2 and JNK remains disputed. Several studies have shown that JNK/c-Jun pathway can regulate SESN2 expression in the nasopharyngeal carcinoma cell lines (Zhang et al. 2013), in human umbilical vein endothelial cells (Yi et al. 2014), in the mouse RAW264.7 macrophage cell line (Hu et al. 2015) and in *Drosophila* nerve cells (Singh and Chowdhuri 2018). However, some studies have reported that SESN2 can affect JNK activity. Overexpression of SESN2 in HepG2 cells reduce JNK phosphorylation and block the cytotoxic effects resulting from excessive endoplasmic reticulum stress (Jegal et al. 2017). In RAW264.7 cells stably expressing SESN2, LPS-induced increase in JNK phosphorylation were suppressed, suggesting that SESN2 can inhibit JNK activity (Yang et al. 2015). Overexpression of SESN2 can significantly reduce p-aminoethylphenol-induced JNK phosphorylation in the mouse liver (Kim et al. 2017). In this study, we found that SESN2 knockout significantly increased pJNK-Thr183/Tyr185 content in quadriceps muscle. Consistent with this *in vivo* result, silencing of SESN2 led to a significant increase of pJNK-Thr183/Tyr185 in C2C12 myotubes with or without palmitate treatment. Moreover, the rescue experiment was performed via JNK activation plus SESN2 overexpression. The result of cell viability showed that SESN2 overexpression can largely reverse the decrease of cell viability induced by JNK activation. JNK plays an important role in the regulation of apoptosis and lipid metabolism (Vernia et al. 2014; Lawan and Bennett 2017). We and others have verified that inhibition of JNK decreases lipid biosynthesis in skeletal muscle and liver tissues (Xiao et al. 2017). Therefore, the reasons of cell viability reduced after JNK activation may be the lipotoxicity-induced by increase of

lipid biosynthesis and the apoptosis-induced by cell stress. SESN2, a newly discovered stress-inducible protein, is involved in stress response and metabolic regulation (Lee et al. 2012). Some evidences indicated that SESN2 has an important homeostatic function in the improvement of lipid metabolism (Lee et al. 2010; Li et al. 2017; Wang et al. 2018). Therefore, the reasons of cell viability increased after SESN2 overexpression may be involved in improvement of lipid metabolism and anti-stress response.

In conclusion, our study provided evidence that exercise improved lipid disorder induced by HFD feeding and SESN2 knockout. SESN2 may improve lipid metabolism through inhibition JNK expression in skeletal muscle cells, which providing a molecular mechanism that may represent an attractive target for the treatment of lipid disorder.

Conflict of interest statement

The authors declare that there is no duality of interest associated with this manuscript.

Acknowledgements

This study was supported by grants from the Natural Science Foundation of China (NSFC) 31871206 (to LF), 31671237 (to YMN), and Tianjin Medical University Graduate student innovation fund YJSCX201709 (to TYW).

Reference

- Brownsey, R. W., Boone, A. N., Elliott, J. E., Kulpa, J. E., and Lee, W. M. 2006. Regulation of acetyl-CoA carboxylase. *Biochem. Soc. Trans.* **34** (Pt 2): 223-227. doi: 10.1042/BST20060223. PMID: 16545081.
- Fernando, P., Bonen, A., and Hoffman-Goetz, L. 1993. Predicting submaximal oxygen consumption during treadmill running in mice. *Can. J. Physiol. Pharmacol.* **71** (10-11): 854-857. doi: 10.1139/y93-128. PMID: 8143245.
- Galmés-Pascual, B. M., Martínez-Cignoni, M. R., Morán-Costoya, A.,

- Bauza-Thorbrügge, M., Sbert-Roig, M., Valle, A., et al. 2020. 17 β -estradiol ameliorates lipotoxicity-induced hepatic mitochondrial oxidative stress and insulin resistance. *Free Radic. Biol. Med.* **150**: 148-160. doi: 10.1016/j.freeradbiomed.2020.02.016. PMID: 32105829.
- Harada, N. 2018. Role of androgens in energy metabolism affecting on body composition, metabolic syndrome, type 2 diabetes, cardiovascular disease, and longevity: lessons from a meta-analysis and rodent studies. *Biosci. Biotechnol. Biochem.* **82** (10): 1667-1682. doi: 10.1080/09168451.2018.1490172. PMID: 29957125.
- Hilder, T. L., Baer, L. A., Fuller, P. M., Fuller, C. A., Grindeland, R. E., Wade, C. E., and Graves, L. M. 2005. Insulin-independent pathways mediating glucose uptake in hindlimb-suspended skeletal muscle. *J. Appl. Physiol.* (1985). **99** (6): 2181-2188. doi: 10.1152/japplphysiol.00743.2005. PMID: 16099889.
- Hirosumi, J., Tuncman, G., Chang, L., Görgün, C. Z., Uysal, K. T., Maeda, K., et al. 2002. A central role for JNK in obesity and insulin resistance. *Nature*, **420** (6913): 333-336. doi: 10.1038/nature01137. PMID: 12447443.
- Ho, A., Cho, C. S., Namkoong, S., Cho, U. S., and Lee, J. H. 2016. Biochemical Basis of Sestrin Physiological Activities. *Trends Biochem. Sci.* **41** (7): 621-632. doi: 10.1016/j.tibs.2016.04.005. PMID: 27174209.
- Hu, H. J., Shi, Z. Y., Lin, X. L., Chen, S. M., Wang, Q. Y., and Tang, S. Y. 2015. Upregulation of Sestrin2 expression protects against macrophage apoptosis induced by oxidized low-density lipoprotein. *DNA Cell Biol.* **34** (4): 296-302. doi: 10.1089/dna.2014.2627. PMID: 25692450.
- Jegal, K. H., Park, S. M., Cho, S. S., Byun, S. H., Ku, S. K., Kim, S. C., et al. 2017. Activating transcription factor 6-dependent sestrin 2 induction ameliorates ER stress-mediated liver injury. *Biochim. Biophys. Acta. Mol. Cell Res.* **1864** (7): 1295-1307. doi: 10.1016/j.bbamcr.2017.04.010. PMID: 28433684.
- Khan, M., Rutten, B. P. F., and Kim, M. O. 2019. MST1 Regulates Neuronal Cell Death via JNK/Casp3 Signaling Pathway in HFD Mouse Brain and HT22

- Cells. *Int. J. Mol. Sci.* **20**(10): 2504. doi: 10.3390/ijms20102504. PMID: 31117242.
- Kim, M., and Sujkowski, A. 2020. Sestrins are evolutionarily conserved mediators of exercise benefits. *Nat. Commun.* **11** (1): 190. doi: 10.1038/s41467-019-13442-5. PMID: 31929512.
- Kim, S. J., Kim, K. M., Yang, J. H., Cho, S. S., Kim, J. Y., Park, S. J., et al. 2017. Sestrin2 protects against acetaminophen-induced liver injury. *Chem. Biol. Interact.* **269**:50-58. doi: 10.1016/j.cbi.2017.02.002. PMID: 28209544.
- Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., et al. 2015. Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell metab.* **21** (5): 739-746. doi: 10.1016/j.cmet.2015.04.004. PMID: 25955209.
- Lawan, A., and Bennett, A. M. 2017. Mitogen-Activated Protein Kinase Regulation in Hepatic Metabolism. *Trends Endocrinol. Metab.* **28** (12): 868-878. doi: 10.1016/j.tem.2017.10.007. PMID: 29128158.
- Lee, J. H., Budanov, A. V., Park, E. J., Birse, R., Kim, T. E., Perkins, G. A., et al. 2010. Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science*, **327**(5970):1223-1228. doi:10.1126/science.1182228. PMID: 20203043
- Lee, J. H., Budanov, A. V., Talukdar, S., Park, E. J., Park, H. L., Park, H. W., et al. 2012. Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. *Cell metab.* **16** (3): 311-321. doi: 10.1016/j.cmet.2012.08.004. PMID: 22958918.
- Lehnig, A. C., and Stanford, K. I. 2018. Exercise-induced adaptations to white and brown adipose tissue. *J. Exp. Biol.* **221** (Pt Suppl 1): jeb161570. doi: 10.1242/jeb.161570. PMID: 29514893.
- Li, H., Liu, S., Yuan, H., Niu, Y., and Fu, L. 2017. Sestrin 2 induces autophagy and attenuates insulin resistance by regulating AMPK signaling in C2C12 myotubes. *Exp. Cell Res.* **354** (1):18-24. doi: 10.1016/j.yexcr.2017.03.023. PMID: 28300563.

- Liu, S., Yu, C., Xie, L., Niu, Y., and Fu, L. 2021. Aerobic exercise improves mitochondrial function in sarcopenia mice through Sestrin2 in an AMPK α 2-dependent manner. *J. Gerontol. A Biol. Sci. Med. Sci.* **glab029**. doi: 10.1093/gerona/ghab029. PMID: 33512470.
- Liu, X., Niu, Y., Yuan, H., Huang, J., and Fu, L. 2015. AMPK binds to Sestrins and mediates the effect of exercise to increase insulin-sensitivity through autophagy. *Metabolism*. **64** (6): 658-665. doi: 10.1016/j.metabol.2015.01.015. PMID: 25672217
- Liu, X., Yuan, H., Niu, Y., Niu, W., and Fu, L. 2012. The role of AMPK/mTOR/S6K1 signaling axis in mediating the physiological process of exercise-induced insulin sensitization in skeletal muscle of C57BL/6 mice. *Biochim. Biophys. Acta.Mol.Basis. Dis.* **1822** (11): 1716-1726. doi: 10.1016/j.bbdis.2012.07.008. PMID: 22846606.
- Ok, D. P., Ko, K., and Bae, J. Y. 2018. Exercise without dietary changes alleviates nonalcoholic fatty liver disease without weight loss benefits. *Lipids Health Dis.* **17** (1): 207. doi: 10.1186/s12944-018-0852-z. PMID: 30172252.
- Park, H. W., Park, H., Ro, S. H., Jang, I., Semple, I. A., Kim, D. N., et al. 2014. Hepatoprotective role of Sestrin2 against chronic ER stress. *Nat. Commun.* **5**: 4233. doi: 10.1038/ncomms5233. PMID: 24947615.
- Rousseau, A. S., Sibille, B., Murdaca, J., Mothe-Satney, I., Grimaldi, P. A., and Neels, J. G. 2016. α -Lipoic acid up-regulates expression of peroxisome proliferator-activated receptor β in skeletal muscle: involvement of the JNK signaling pathway. *FASEB J.* **30** (3): 1287-1299. doi: 10.1096/fj.15-280453. PMID: 26655383.
- Singh, P., and Chowdhuri, D. K. 2018. Modulation of sestrin confers protection to Cr(VI) induced neuronal cell death in *Drosophila melanogaster*. *Chemosphere*. **191**: 302-314. doi: 10.1016/j.chemosphere.2017.10.037. PMID: 29045932.
- Sun, X., Han, F., Lu, Q., Li, X., Ren, D., Zhang, J., et al. 2020. Empagliflozin Ameliorates Obesity-Related Cardiac Dysfunction by Regulating

- Sestrin2-Mediated AMPK-mTOR Signaling and Redox Homeostasis in High-Fat Diet-Induced Obese Mice. *Diabetes*. **69** (6): 1292-1305. doi: 10.2337/db19-0991. PMID: 32234722.
- Vernia, S., Cavanagh-Kyros, J., Garcia-Haro, L., Sabio, G., Barrett, T., Jung, D. Y., et al. 2014. The PPAR α -FGF21 hormone axis contributes to metabolic regulation by the hepatic JNK signaling pathway. *Cell metab.* **20** (3): 512-525. doi: 10.1016/j.cmet.2014.06.010. PMID: 2504381.
- Wang, T., Niu, Y., Liu, S., Yuan, H., Liu, X., and Fu, L. 2018. Exercise improves glucose uptake in murine myotubes through the AMPK α 2-mediated induction of Sestrins. *Biochim. Biophys. Acta. Mol. Basis. Dis.* **1864** (10): 3368-3377. doi: 10.1016/j.bbadis.2018.07.023. PMID: 30048751.
- Wicks, S. E., Vandanmagsar, B., Haynie, K. R., Fuller, S. E., Warfel, J. D., Stephens, J. M., et al. 2015. Impaired mitochondrial fat oxidation induces adaptive remodeling of muscle metabolism. *Proc. Natl. Acad. Sci. U S A.* **112** (25): E3300-3309. doi: 10.1073/pnas.1418560112. PMID: 26056297.
- Xiao, Y., Wang, J., Yan, W., Zhou, K., Cao, Y., and Cai, W. 2017. p38 α MAPK antagonizing JNK to control the hepatic fat accumulation in pediatric patients onset intestinal failure. *Cell Death Dis.* **8** (10): e3110. doi: 10.1038/cddis.2017.523. PMID: 29022907.
- Yang, J. H., Kim, K. M., Kim, M. G., Seo, K. H., Han, J. Y., Ka, S. O., et al. 2015. Role of sestrin2 in the regulation of proinflammatory signaling in macrophages. *Free Radic. Biol. Med.* **78**: 156-167. doi:10.1016/j.freeradbiomed.2014.11.002. PMID: 25463278.
- Yi, L., Li, F., Yong, Y., Jianting, D., Liting, Z., Xuansheng, H., et al. 2014. Upregulation of sestrin-2 expression protects against endothelial toxicity of angiotensin II. *Cell Biol. Toxicol.* **30** (3): 147-156. doi: 10.1007/s10565-014-9276-3. PMID: 24838122.
- Zhang, X. Y., Wu, X. Q., Deng, R., Sun, T., Feng, G. K., and Zhu, X. F. 2013. Upregulation of sestrin 2 expression via JNK pathway activation contributes to

autophagy induction in cancer cells. *Cell. Signal.* **25** (1): 150-158. doi: 10.1016/j.cellsig.2012.09.004. PMID: 22982090.

Draft

Figure legends

Fig.1. Exercise training attenuates lipid disorder induced by HFD and SESN2 knockout.

(A) Body weight was examined after 8-week HFD in WT and SESN2^{-/-} mice. Values are means $\pm$ SEM ($n = 6$). * $P < 0.05$ WT HFD vs. WT Ctrl; # $P < 0.05$ SESN2^{-/-} HFD vs. SESN2^{-/-} Ctrl. (B) Body weight was examined after 6-week treadmill exercise in WT and SESN2^{-/-} mice. Results were expressed as means $\pm$ SEM ($n = 6$). * $P < 0.05$ SESN2^{-/-} NC vs. WT NC; # $P < 0.05$ SESN2^{-/-} NE vs. WT NE. (C) Body weight was examined after 8-week HFD plus 6-week treadmill exercise in WT and SESN2^{-/-} mice. Values are means $\pm$ SEM ($n = 6$). * $P < 0.05$ SESN2^{-/-} HC vs. WT HC; ** $P < 0.05$ WT HE vs. WT HC; # $P < 0.05$ SESN2^{-/-} HE vs. SESN2^{-/-} HC. (D) Fold change of serum lipid profiles (TC, TG and FFA) and body composition (Fat Mass and Lean Mass) were examined in WT and SESN2^{-/-} mice. Values are means $\pm$ SEM ($n = 6$). * $P < 0.05$; # $P < 0.01$. (E-I) TC (E), TG (F), FFA (G), Fat Mass (H) and Lean Mass (I) were examined in SESN2^{-/-} mice after HFD and treadmill exercise. Values are means $\pm$ SEM ($n = 6$). * $P < 0.05$; # $P < 0.01$. (J) Representative images stained with oil red O of the liver in SESN2^{-/-} mice ($n = 3$). Scale bars = 100 μ m. P value by two-way ANOVA in A-B; P value by the unpaired Student's 2-tailed t test in D; P value by one-way ANOVA in E-I. HFD, high-fat diet; WT, wild type; TC, total cholesterol; TG, triglycerides; FFA, free fatty acids; FM, fat mass; LM, lean mass.

Fig.2. Exercise training reduced lipid synthesis induced by HFD and SESN2 knockout.

(A) Blotting and (B-G) quantitative analysis of proteins expression of SESN2, SESN1, SESN3, pACC-Ser79, ACC, CPT-1, FASN, SREBP-1c, and pJNK-Thr183/Tyr185 in quadriceps muscle of WT and SESN2^{-/-} mice after 8-week HFD and then 6-week treadmill exercise. β -tubulin was used as an internal control for loading. Data are means $\pm$ SEM ($n = 6$); * $P < 0.05$; # $P < 0.01$. P value by two-way ANOVA. HFD, high-fat diet; WT, wild type.

Fig.3. SESN2 inhibits JNK activity in palmitate-treated C2C12 myotubes. (A-B)

Representative images and quantitative analysis of lipid droplets number of C2C12

myotubes stained with oil red O ($n = 3$). C2C12 myotubes were incubated in medium with or without 0.8 mM palmitate for 18 hours, plus with or without si-SESN2 treatment for 48 hours. Scale bars = 100 μ m. (C-E) Blotting and quantitative analysis of protein expression of SESN2 and pJNK-Thr183/Tyr185 in lysates of C2C12 cells. β -tubulin was used as the internal housekeeping protein control. Data are means $\pm$ SEM ($n = 6$); # $P < 0.01$. P value by two-way ANOVA.

Fig.4. Inhibition of JNK decreases lipid biosynthesis in palmitate-treated C2C12 myotubes. (A) Blotting and (B-F) quantitative analysis of proteins expression of pJNK-Thr183/Tyr185, ACC, CPT-1, FASN and SREBP-1c in palmitate treated C2C12. C2C12 myotubes were incubated with 0.8 mM palmitate for 18 hours, plus with or without 30 μ M SP600125 treatment for 24 hours. β -tubulin was used as the internal control. * $P < 0.05$; # $P < 0.01$; P value by the unpaired Student's 2-tailed t test. All data are means $\pm$ SEM ($n = 6$).

Fig.5. SESN2 overexpression decreases lipid biosynthesis induced by JNK activator in palmitate-treated C2C12 myotubes. (A) Blotting and (B-F) quantitative analysis of proteins expression of pJNK-Thr183/Tyr185, ACC, CPT-1, FASN and SREBP-1c in palmitate-incubated C2C12 myotubes. C2C12 myotubes were infected with or without Ad-SESN2 for 48 hours, plus with or without 10 μ M Anisomycin treatment for 6 hours. β -tubulin was used as the internal control. * $P < 0.05$; # $P < 0.01$; Results are means $\pm$ SEM ($n = 6$). (G) The cell viability was detected by CCK-8 assay in five replicate wells in a 96-well plate. * $P < 0.05$; # $P < 0.01$; Results are means $\pm$ SEM ($n = 5$). P value by one-way ANOVA.

Figure 1

252x169mm (300 x 300 DPI)

Figure 2

427x428mm (300 x 300 DPI)

Figure 3

Figure 4

Figure 5