Current Research in Physiology 5 (2022) 63–72

Contents lists available at ScienceDirect

Current Research in Physiology

journal homepage: www.sciencedirect.com/journal/current-research-in-physiology

The effects of voluntary wheel running during weight-loss on biomarkers of hepatic lipid metabolism and inflammation in C57Bl/6J mice

Joshua S. Wooten *, Kaylee E. Poole, Matthew P. Harris, Brianne L. Guilford, Megan L. Schaller, David Umbaugh, Andrew Seija

Department of Applied Health, Southern Illinois University Edwardsville, Edwardsville, IL, USA

ARTICLE INFO

Keywords:
Lipotoxicity
Inflammation
Obesity
Lipid metabolism
Metabolic syndrome
Exercise

ABSTRACT

The aim of this study was to determine the effect of voluntary wheel running (VWR) during weight-loss on hepatic lipid and inflammatory biomarkers using a murine model. To induce obesity, male C57Bl/6 mice were fed a 60% high-fat diet (HF) for 10 weeks. At 10 weeks, weight-loss was promoted by randomizing HF-fed mice to a normal diet (ND) either with (WL + VWR) or without (WL) access to running wheels for 8 weeks. Age-matched dietary control mice were fed either a ND or HF for 18 weeks. Following weight-loss, WL + VWR had a lower body mass compared to all groups despite an average weekly caloric consumption comparable to HF mice. WL + VWR had an increased adiponectin concentration when compared to WL, but no difference between WL and WL + VWR was observed for plasma glucose and lipid biomarkers. When compared to HF, the lower hepatic total lipids in both WL and WL + VWR were associated with increased pAMPK:AMPK and reduced pACC-1:ACC-1 ratios. When compared to WL, WL + VWR resulted in lower hepatic cholesterol and trended to lower hepatic triglyceride. In both WL and WL + VWR, pNF-κB p65:NF-κB p65 ratio was lower than HF and comparable to ND. TGFβ1 and BAMBI protein levels were evaluated as biomarkers for hepatic fibrosis. No differences in TGFβ1 was observed between groups; however, WL and WL + VWR had BAMBI protein levels comparable to ND. Overall, the addition of voluntary exercise resulted in greater weight-loss and improvements in hepatic cholesterol and triglyceride levels; however, limited improvements in hepatic inflammation were observed when compared to weight-loss by diet alone.

1. Introduction

The growing prevalence of obesity worldwide affects more than 40% of people in industrialized countries (Afshin et al., 2017). Obesity is the result of a disruption in energy balance leading to weight gain and increased risk for the development of cardiovascular and metabolic diseases (Koliaki et al., 2019). Obesity paired with insulin resistance plays a pivotal role in the progression of cardiometabolic diseases leading to elevated circulating lipids and proinflammatory cytokines and chemokines (Koliaki et al., 2019). Paralleling the rise in lipid concentrations is an increased ectopic lipid deposition in organs such as the liver, skeletal muscle, and heart (Ferrara et al., 2019; Koliaki et al., 2019).

The accumulation of hepatic triglycerides during obesity is accelerated in patients with insulin resistance and type 2 diabetes mellitus (Donnelly et al., 2005) and is the result of hepatic lipid dysregulation characterized as an imbalance between lipid accumulation and lipid disposal. The sources of excess free fatty acids that make up the hepatic triglyceride lipid pool includes increased uptake of circulating free fatty acids from adipose tissue lipolysis, *de novo* lipogenesis, and dietary lipids (Donnelly et al., 2005; Santoleri and Titchenell, 2019). The majority of hepatic free fatty acids, about 60%, are due to the excess mobilization of free fatty acids from adipose tissue to the liver, whereas *de novo* lipogenesis and dietary lipids account for about 26% and 15%, respectively of the free fatty acids used to synthesize hepatic triglycerides (Donnelly et al., 2005). In contrast, hepatic free fatty acid disposal is accomplished by mitochondrial β-oxidation or very low-density lipoprotein (VLDL) export of triglycerides into the circulation (Kuchay et al., 2020). During obesity, hepatic mitochondrial respiration is increased, as well as increased VLDL-triglyceride secretion (Koliaki et al., 2015; Sparks and Sparks, 2012).

Hepatic lipid dysregulation and the accumulation of hepatic

* Corresponding author. Department of Applied Health Southern Illinois University Edwardsville, Campus Box 1126, Edwardsville, IL, 62026-1126, USA
E-mail address: jwooten@siue.edu (J.S. Wooten).

https://doi.org/10.1016/j.crphys.2022.01.003
Received 26 July 2021; Received in revised form 23 December 2021; Accepted 18 January 2022
Available online 25 January 2022

triglycerides creates an environment prone for the formation of cytotoxic lipid species or lipotoxicity (Alkhouri et al., 2009; Cusi, 2012). These lipotoxic species increase the livers susceptibility to inflammation and fibrogenesis (Grunhut et al., 2018) characterized by the up-regulation of nuclear factor-κB p65 (NF-κB p65) and subsequent transcription and translation of proinflammatory cytokines and chemokines IL-1β, TNFα, and MCP-1 (Liu et al., 2017). Chronic elevation of the proinflammatory cytokines and chemokines have been attributed to the activation of resident macrophage Kupffer cells and hepatic stellate cells associated with hepatocellular remodeling and fibrogenesis (Heymann and Tacke, 2016).

In patients with obesity, weight-loss is the primary treatment strategy for the reduction in adipose tissue mass and reduction in ectopic lipid deposition that is related to improvements in biomarkers associated with metabolic syndrome (Eckel et al., 2014; Goldstein, 1992; Watson et al., 2016). In general, supplementing a weight management program with exercise or physical activity has been tied to greater improvements in cardiometabolic risk when compared to using diet alone (Balk et al., 2015; Oliveira et al., 2016). Several studies have evaluated the benefits of exercise and physical activity on hepatic lipid metabolism, but few studies have examined the concomitant effects on markers of hepatic inflammation (Kwak and Kim, 2018; Romero-Gomez et al., 2017). In these studies examining changes in both hepatic lipid metabolism and inflammation, the reductions in hepatic lipid are not always associated with improvements in hepatic inflammation (Kwak and Kim, 2018). Due to the complexity of the pathogenesis of obesity-related hepatic metabolic diseases, it remains unclear how the therapeutic effects of exercise or physical activity during weight-loss will affect hepatic inflammation. Using previous studies evaluating exercise, weight-loss, and hepatic lipid metabolism and inflammation (Houghton et al., 2017; Wang et al., 2011), we hypothesized that the addition of voluntary wheel running during weight-loss would result in lower hepatic lipids than the weight-loss by diet alone. In addition, the physically active weight-loss mice would have lower measures of hepatic and systemic inflammation. To test our hypotheses, we sought to determine the effect of voluntary wheel running during weight-loss on biomarkers of hepatic lipid metabolism and inflammation in C57Bl/6 mice.

2. Materials and methods

2.1. Experimental animals and protocols

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Southern Illinois University Edwardsville. All animal experimentation was conducted in accordance with accepted standards of humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (2011). Male C57Bl/6J mice were acquired from Jackson Laboratory (Bar Harbor, ME) at 6-weeks of age and maintained on a 12-h light:12-h dark cycle at a room temperature between 22.5 °C and 23.5 °C. Only male mice were used for this study due to sex-specific metabolic differences in response to dietary-induced obesity (Gonzalez-Granillo et al., 2019; Lee et al., 2016). In general, male mice are more susceptible to obesity induced hepatic lipid dysregulation and inflammation than female mice (Gonzalez-Granillo et al., 2019). All cages were provided approximately 4–6 g of nesting (Bed-r'Nest; The Andersons, Maumee, OH) for enrichment and to reduce the metabolic effects of room temperature housing (Gaskill et al., 2013; Maher et al., 2015). Despite the attempts to reduce the effects of housing temperature on whole body metabolism, mice may have displayed greater total energy expenditure to in order to maintain body temperature (Fuller and Thyfault, 2020). Animals were housed (n = 2 per cage) in an individually ventilated cage system and fed *ad libitum* on a normal diet (ND; D12450J) until 8 weeks of age. Research Diets, Inc. (New Brunswick, NJ) formulated all diets for this study.

At 8 weeks of age, mice (n = 48) were randomly placed on one of two diets for 10-weeks: a ND (10% of calories from lard and soybean oil; n = 12) or a high-fat diet (HF; D12492, 60% of calories from lard and soybean oil; n = 36) to induce obesity (n = 2 per cage). The composition and ingredients for each diet are shown in Table 1. The energy content of the ND and HF diets were 3.85 kcal/g and 5.24 kcal/g, respectively. Following the 10 weeks of feeding, HF fed mice were randomly assigned to one of three groups (n = 12/group): HF diet, weight-loss by diet only (WL) group, or weight-loss by diet and voluntary wheel running (WL + VWR) group for 8 weeks. All mice were housed n = 2 per cage except the mice that were assigned to cages with running wheels. The mice with access to running wheels were housed individually so that running distance per mouse could be monitored for the 8-weeks. The diet used for weight-loss was the normal diet. Mice on the ND continued to be fed the normal diet for the remaining 8 weeks of the study. Mice in the WL + VWR group had unrestricted access to the in-cage running wheels daily for the 8 weeks. Total running wheel activity data was monitored daily with the Vital View Data Acquisition System and daily distance travelled was recorded for data analyses (Mini-Mitter, Bend, OR).

Animal weights and average food intake for each mouse were recorded weekly. Mice were fasted for 6 h prior to the collection of blood and tissue samples. To prevent the acute effects of physical activity, running wheels were removed 24 h prior to the collection of blood and tissue samples. At the end of the experiment, mice were euthanized by overdose of isoflurane inhalation and then exsanguinated by cardiac puncture. Collected whole blood was treated with EDTA, centrifuged at 1500*g* for 15 min, and plasma was collected and stored at −80 °C until analysis. Following the collection of blood, mice were perfused with ice-cold 0.9% (w/v) NaCl solution and the liver was removed, blotted dry, weighed, and snap frozen in liquid nitrogen.

2.2. Plasma chemistry

Plasma total cholesterol, triglyceride, and glucose concentrations were measured enzymatically using colorimetric assay kits (ThermoFisher, Middletown, VA). Plasma insulin (Mercodia, Winston Salem, NC) and adiponectin (ALPCO, Salem, NH) concentrations were determined using ELISA kits. Plasma concentrations of inflammatory markers IL-1β, IL-6, and TNFα were determined by ELISA (eBioscience, ThermoFisher, Middletown, VA). HOMA-IR was calculated from fasting glucose and insulin levels (glucose, mg/dL x insulin, mU/L ÷ 450).

2.3. Analyses of hepatic lipids

Hepatic lipids were determined by using a modified Hara and Radin protocol (Hara and Radin, 1978). Briefly, 75 mg of hepatic tissue was

Table 1
Diet composition of the low-fat and high-fat diets.

Variable	ND - D12450J	HF - D12492
Kcal/g	3.85	5.24
Protein (% kcal)	20.0	20.0
Casein (g)	200.0	200.0
L-Cystine (g)	3.0	3.0
0.5		
Carbohydrate (% kcal)	70.0	20.0
Corn starch (g)	506.2	0.0
Maltodextrin 10 (g)	125.0	125.0
Sucrose (g)	68.8	68.8
0.5		
Fat (% kcal)	10.0	60.0
Soybean oil (g)	25.0	25.0
Lard (g)	20.0	245.0
Cholesterol (mg)	13.6	279.6
Saturated (%)	22.7	32.1
Monounsaturated (%)	29.9	35.9
Polyunsaturated (%)	47.4	32.0

homogenized in 1 ml of hexane:isopropanol (3:2) with 0.01% (w/v) of butylated hydroxytoluene to prevent lipid peroxidation. Following the addition of 300 μl of 0.47 M Na_2SO_4, samples were centrifuged for 20 min at 7500*g* and the upper organic phase was transferred to a clean tube and evaporated under nitrogen. To solubilize the lipid, 500 μl hexane containing 0.1% Triton-X was added and mixed gently for 20 min. The hexane was evaporated under nitrogen and 500 μl of dH_2O was added to the tube. Prior to assaying for triglyceride and cholesterol levels, samples were incubated at 37 °C for 15 min. Triglyceride and cholesterol concentrations in lipid extracts were determined enzymatically with colorimetric assay kits (ThermoFisher Scientific, Middletown, VA).

2.4. Quantitative RT-PCR

Total RNA was isolated from approximately 50 mg of liver tissue using Trizol RNA isolation reagent (Life Technologies, Grand Island, NY). RNA concentration was quantified using the Qubit 2.0 fluorometer (Life Technologies). Prior to reverse transcription of the RNA, samples were digested with DNase I (Life Technologies) followed by reverse transcription to cDNA (Life Technologies). Briefly, equal volumes of 1 μg RNA and 2X reverse transcriptase and random primers reaction mix were combined and placed in a thermal cycler under the following conditions: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Subsequently, real-time quantitative PCR (qPCR) was performed on the resulting cDNA template in a final volume of 20 μL, which contained 10 ng of reverse-transcribed cDNA, 10 μL of qPCR Master Mix (GeneCopoeia, Rockville, MD), 3.9 μL of DEPC-treated dH_2O, 150 nM ROX dye, and 0.2 μM of reverse and forward assay primers (Integrated DNA Technologies, Coralville, IA) for the detection of genes that regulate *lipid metabolism* (sterol response element binding protein 1c, SREPB1c; peroxisome proliferator activated receptor α, PPARα; PPARγ; carnitine palmitoyltransferase 1a, CPT1a; fatty acid synthase, FASN; acetyl-CoA carboxylase 1, ACC-1; and cluster of differentiation 36, CD36), *inflammatory signaling* (toll like receptor 4, TLR4; CD14; myeloid differentiation primary response gene 88, MyD88; transforming growth factor β1, TGFβ1; and BMP and activin membrane bound inhibitor, BAMBI), and cytokine expression (tumor necrosis factor α, TNFα; interleukin-1β, IL-1β; and IL-6). β-actin served as the reference housekeeping gene. Forward and reverse primer sequences are shown in Table 2. The reaction conditions were initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s. Melting curve analyses were performed immediately following the last cycle to identify the presence of primer-dimer products. The mRNA expression for each gene and sample was calculated using the -ΔΔCt method, in which the ND group served as the referent group. All reactions were carried out in duplicate using the 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA).

2.5. Western blot and ELISA analyses

Frozen hepatic tissue samples (75 mg) were sonicated, and soluble proteins were extracted for immunoblotting with tissue lysis buffer supplemented with protease and phosphatase inhibitors (137 mM NaCl, 20 mM Tris, 1.0% NP-40, 10% glycol, 1 mM PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 0.5 mM sodium orthovanadate, pH = 8.0). Liver extracts were centrifuged at 13,000 rpm and the supernatant was collected and protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Hepatic cytokine protein concentrations were determined using ELISA plates for the determination of IL-1β, IL-6, and TNFα (eBioscience, ThermoFisher, Middletown, VA). For Western blots, proteins were separated on a 4–20% TGX precast gel and transferred to 0.2 μm PVDF membrane (Bio-Rad, Hercules, CA). Ponceau *S* staining (#P7170; Sigma Aldrich, St. Louis, MO) was performed to confirm equal amounts of protein were transferred to each protein. All bands were normalized to the total protein as a loading control. Membranes were blocked with 5% w/v dry non-fat milk-TBST for 1 h and incubated with the following antibodies from Cell Signaling Technology (Danvers, MA) unless otherwise noted: SREBP1c (sc-13551; 1:200; Santa Cruz Biotechnology, Dallas, TX), ACC-1 (#3662; 1:1000), phospho^{Ser79}-ACC-1 (pACC-1; #3661; 1:1000), SCD1 (#2794; 1:1000), FASN (#3189; 1:1000), AMPKα (#2532; 1:1000), phospho^{Thr172}-AMPKα (pAMPKα; #2531; 1:1000), TGFβ1 (sc-130348; 1:200; Santa Cruz Biotechnology), BAMBI (PA5-38027; 1:1000; ThermoFisher), NF-κB p65 (#8242; 1:1000), and phospho^{Ser536}-NF-κB p65 (pNF-κB p65; #3033; 1:1000). Blots were developed with Bio-Rad Clarity Max ECL substrate and visualized on a ChemiDoc MP Imaging System. Images were analyzed using a using Image Lab v.5.2.1 (Bio-Rad, Hercules, CA).

Table 2
List of forward and reverse primer sequences for genes examined following weight-loss.

Target (*Gene name*)	Forward (5′-3′) Reverse (5′-3′)	Exon location	Accession number
ACC1 (*Acaca*)	5′GTCCAACAGAACATCGCTGA-3′ 5′-AACATCCCCACGCTAAACAG-3′	28–29	NM_133360
BAMBI (*Bambi*)	5′-GAGCAGCATCACAGTAGCAT-3′ 5′-GCGAGGCGTCAATGGAT-3′	1–2	NM_026505
CD14 (*Cd14*)	5′CAATCTGGCTTCGGATCTGAG-3′ 5′-AACTTTCAGAATCTACCGACCA-3′	1–2	NM_009841
CD36 (*Cd36*)	5′-CAGCGTAGATAGACCTGCAAA-3′ 5′-GCGACATGATTAATGGCACAG-3′	10–12	NM_001159555
CPT1a (*Cpt1a*)	5′-CTGCTTAGGGATGTCTCTATGAC-3′ 5′ACTCAGAGGATGGACACTGTA-3′	13–14	NM_013495
FASN (*Fasn*)	5′GATGCCTCTGAACCACTCCA-3′ 5′-TGTTGGAAGTCAGCTATGAAGC-3′	3–4	NM_007988
IL-1β (*Il1b*)	5′-CTCTTGTTGATGTGCTGCTG-3′ 5′-GACCTGTTCTTTGAAGTTGACG-3′	3–4	NM_008361
IL-6 (*Il6*)	5′-TCCTTAGCCACTCCTTCTGT-3′ 5′-AGCCAGAGTCCTTCAGAGA-3′	4–5	NM_031168
MyD88 (*Myd88*)	5′-CTGTAAAGGCTTCTCGGACTC-3′ 5′-GTGAGGATATACTGAAGGAGCTG-3′	1–2	NM_010851
PPARα (*Ppara*)	5′TGCAACTTCTCAATGTAGCCT-3′ 5′AATGCCTTAGAACTGGATGACA-3′	8–9	NM_011144
PPARγ (*Pparg*)	5′-TGCAGGTTCTACTTTGATCGC-3′ 5′-CTGCTCCACACTATGAAGACAT-3′	4–5	NM_001127330
SREBP1c (*Srebf1*)	5′GTCACTGTCTTGGTTGTTGATG-3′ 5′-CGAGATGTGCGAACTGGAC-3′	1–2	NM_011480
TGFβ1 (*Tgfb1*)	5′-GCGGACTACTATGCTAAAGAGG-3′ 5′-CCGAATGTCTGACGTATTGAAGA-3′	1–2	NM_011577
TLR4 (*Tlr4*)	5′-AGCTCAGATCTATGTTCTTGGTTG-3′ 5′-GAAGCTTGAATCCCTGCATAG-3′	1–2	NM_021297
TNFα (*Tnfa*)	5′TCTTTGAGATCCATGCCGTTG-3′ 5′-AGACCCTCACACTCAGATCA-3′	2–4	NM_013693
β-Actin (*Actb*)	5′-GTACGACCAGAGGCATACAG-3′ 5′-CTGAACCCTAAGGCCAACC-3′	3–4	NM_007393

2.6. Statistical analyses

Descriptive data are presented as mean ± standard error of the mean (SEM). All analyses were performed using SPSS v24.0 software package (IBM Corp., Armonk, NY). Differences between treatment groups were

identified by one-way ANOVA followed by the Bonferroni's test for *post hoc* comparisons. For all analyses, statistical significance was set at $p < 0.05$.

3. Results

3.1. Voluntary wheel running during weight-loss reduced body mass and liver cholesterol mass more than diet alone

Fig. 1A shows the weekly growth curve of the mice per group. At the conclusion of the study, HF mice were heavier (Fig. 1B) than the ND ($p < 0.001$), WL ($p < 0.001$), and WL + VWR ($p < 0.001$) groups, whereas the WL + VWR body mass was lower than the ND ($p = 0.001$), HF ($p < 0.001$), and WL ($p = 0.001$) groups. Fig. 1C and D shows the average caloric intake of the mice after 10 weeks and again at 18 weeks, respectively. At 10 weeks, the caloric intake was not different between the HF, WL, and WL + VWR groups, but all these groups showed higher ($p < 0.05$) caloric consumption than ND. At 10 weeks, the body mass of HF, WL, and WL + VWR were greater ($p < 0.001$) than ND. At the end of 18 weeks, HF and WL + VWR caloric intakes were greater ($p < 0.005$) than ND and WL. In addition, no difference in caloric intake was observed between HF and WL + VWR. Caloric expenditure for the mice was not measured in this study; however, the average running wheel activity of mice in the WL + VWR was 8.8 ± 0.5 km/day per mouse. With exception of the first week of access to the running wheels (daily average = 4.0 ± 0.9 km/day), mice averaged between 8.2 and 9.5 km/day of wheel running each week thereafter. HF mice had greater ($p < 0.01$) hepatic mass, hepatic:body mass ratio, and perigonadal fat pad mass (Fig. 1E–G) when compared to the other experimental groups. The hepatic mass and hepatic:body mass ratio in WL and WL + VWR groups were not different than the ND group, but perigonadal fat pad mass in both WL ($p = 0.034$) and WL + VWR ($p < 0.001$) groups were lower than the ND group.

Similar to body mass and hepatic mass, hepatic total lipid mass, and hepatic triglyceride (Fig. 1H and I) were greater ($p < 0.001$) in the HF group when compared to the ND. Following weight-loss, both WL and WL + VWR groups had lower ($p < 0.001$) total hepatic lipid mass and hepatic triglyceride than the HF group. In addition, hepatic triglyceride in the WL + VWR group was lower ($p = 0.002$) than ND. Hepatic triglyceride in the WL + VWR was 39.2% lower than WL; however, this difference did not reach the level of statistical significance ($p = 0.129$). Hepatic cholesterol (Fig. 1J) was not different between ND, HF, and WL groups. In the WL + VWR group, hepatic cholesterol was lower than both HF ($p = 0.002$) and WL ($p = 0.026$) groups. Hepatic cholesterol was 33.9% higher in HF when compared to ND, but this difference did not meet the criteria for statistical significance ($p = 0.177$).

3.2. Voluntary wheel running during weight-loss favorably changed plasma adiponectin concentrations

HF mice had elevated ($p < 0.001$) fasting (6 h) plasma glucose, insulin, and HOMA-IR values (Table 3) when compared to the other experimental groups. Weight-loss in both WL and WL + VWR normalized plasma glucose, insulin, and HOMA-IR values comparable to ND; however, no differences between WL and WL + VWR groups were observed following weight-loss. Plasma triglyceride concentration was not different between groups, but plasma cholesterol concentration was higher ($p < 0.001$) in the HF when compared to the other experimental groups. Weight-loss in the WL and WL + VWR groups resulted in plasma total cholesterol concentration similar to ND.

The evaluation of plasma adiponectin concentration revealed no difference in the HF group when compared to ND and WL groups. In the WL + VWR, plasma adiponectin concentration was higher ($p < 0.01$) than the other experimental groups. Circulating IL-1β values were not presented due to most samples being undetectable with our ELISA assay. No difference between experimental groups was observed for plasma IL-6 concentration. Plasma TNFα concentration was the only proinflammatory marker higher ($p = 0.033$) in the HF group when compared to ND. Following weight-loss, TNFα concentration remained elevated in both WL ($p = 0.001$) and WL + VWR ($p < 0.001$) greater than ND.

3.3. Voluntary wheel running during weight-loss did not modify markers of lipid metabolism more than diet alone

Following weight-loss, we observed an increase in the mRNA expression of hepatic genes associated with *de novo* lipogenesis only in the WL + VWR group (Fig. 2A). In the WL + VWR group, the mRNA expression of SREBP1c, FASN, and ACC-1 were higher ($p < 0.01$) than ND and HF groups. In addition, the mRNA expression of FASN and ACC-1 were higher ($p < 0.001$) in the WL + VWR when compared to WL. In the WL + VWR, the elevated mRNA expression did not translate to similar changes in protein levels for SREBP1c and FASN (Fig. 2B and C). Following weight-loss, the protein levels of SREPB1c in WL and WL + VWR were similar to HF, but lower ($p < 0.05$) than ND. No differences were observed between groups for FASN protein levels. In WL + VWR, ACC-1 ($p < 0.001$) and pACC-1 ($p = 0.027$) protein levels were significantly lower than ND (Fig. 2D and E). In addition, ACC-1 was significantly ($p = 0.012$) different between WL and WL + VWR, but WL + VWR trended ($p = 0.065$) to be lower than HF. The pACC-1:ACC-1 ratio, was lower in both WL ($p = 0.046$) and WL + VWR ($p = 0.007$) when compared to HF (Fig. 2F). In WL + VWR, the pACC-1:ACC-1 ratio was lower ($p = 0.027$) than ND. In WL and WL + VWR, AMPKα protein levels were lower ($p < 0.01$) than HF, but similar to ND (Fig. 2G). No significant effect was observed for pAMPKα (Fig. 2H). When compared to HF, WL showed an increase ($p = 0.028$) in the ratio of pAMPKα:AMPKα ratio (Fig. 2I). The pAMPKα:AMPKα ratio was 58.9% higher in WL + VWR when compared to HF, but it did not reach the criteria for statistical significance ($p = 0.143$).

Hepatic PPARα mRNA expression in WL ($p = 0.032$) and WL + VWR ($p = 0.001$) were elevated when compared to ND (Fig. 2A). Similar to PPARα mRNA expression, CPT1a mRNA expression of WL ($p = 0.005$) and WL + VWR ($p = 0.018$) were elevated when compared to ND. The mRNA expression of CPT1a in HF trended towards being ($p = 0.058$) higher than ND. The mRNA expression of PPARγ was significantly ($p < 0.001$) higher in HF when compared to ND. Following weight-loss, mRNA expression of PPARγ was lower in WL ($p < 0.001$) and WL + VWR ($p < 0.001$) when compared to HF. The CD36 mRNA expression was higher ($p < 0.001$) in HF when compared to ND. When compared to HF, CD36 mRNA expression was lower in WL ($p < 0.001$) and WL + VWR ($p < 0.001$).

3.4. Weight-loss independent of voluntary wheel running normalized NF-κB p65 activity

At the end of 18 weeks, the mRNA expression of plasma membrane bound inflammatory signaling proteins TLR4, CD14, and MyD88 were all elevated in WL + VWR when compared to ND (Fig. 3A). The TLR4 mRNA expression in WL + VWR was elevated ($p < 0.001$) when compared to ND, but did not meet the criteria for statistical significance when compared to either HF ($p = 0.054$) or WL ($p = 0.168$). No significant difference for TLR4 mRNA expression was observed between ND, HD, and WL groups. When compared to ND, CD14 mRNA expression was lower than HF ($p = 0.008$), WL ($p = 0.031$), and WL + VWR ($p = 0.039$). No significant difference in CD14 mRNA expression was reported between HF, WL, and WL + VWR groups. MyD88 mRNA expression in ND when compared to HF was not statistically ($p = 0.122$) different. In contrast, MyD88 mRNA expression in ND was lower than WL ($p = 0.027$) and WL + VWR ($p = 0.032$).

At the end of 18 weeks, no significant effect was observed for NF-κB p65 (Fig. 3B) or pNF-κB p65 (Fig. 3C) protein levels. The pNF-κB p65:NF-κB p65 ratio (Fig. 3D), a measure of NF-κB p65 activity, resulted in a significant main effect ($p = 0.0394$); however, pNF-κB p65:NF-κB p65

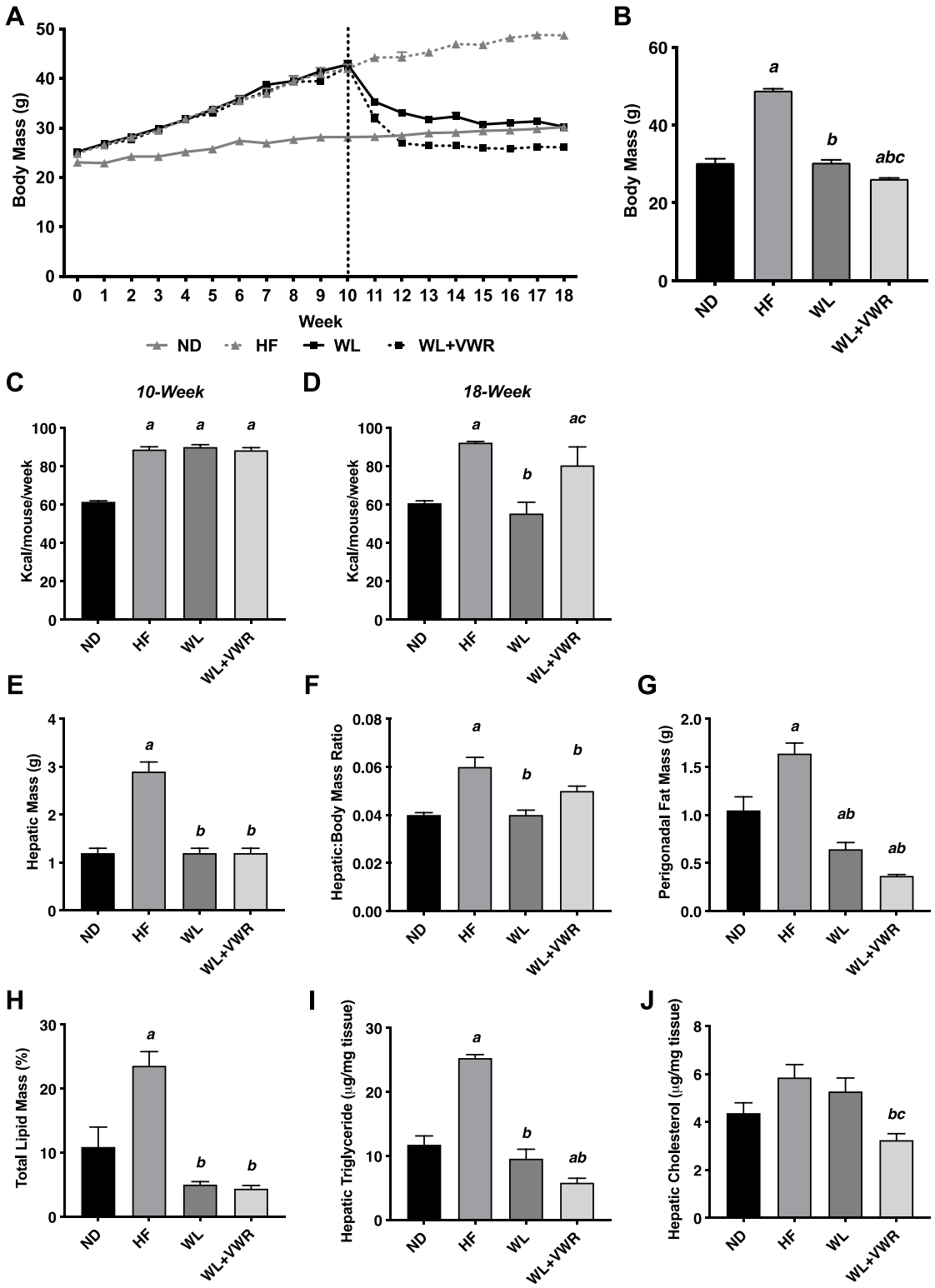

Fig. 1. Changes in body, hepatic, and perigonadal fat masses and total hepatic lipid mass and lipid composition after the intervention. Growth curves (**A**) and final body mass (**B**); average caloric consumption after 10 weeks (**C**) and 18 weeks (**D**); hepatic mass (**E**), hepatic:body mass ratio (**F**), and perigonadal fat mass (**G**); and hepatic total lipid mass (**H**), hepatic triglyceride (**I**) and hepatic cholesterol (**J**). Data are presented as mean ± SEM of n = 12 per group for each measured variable. [a]Significantly different than ND ($p < 0.05$), [b]significantly different than HF ($p < 0.05$), and [c]significantly different than WL ($p < 0.05$).

Table 3
Effects of weight-loss on plasma metabolic and inflammatory markers.

	Experimental Groups			
Variables	ND	HF	WL	WL + VWR
Glucose (mg/dL)	199.9 ± 15.1	349.5 ± 19.6[a]	230.0 ± 19.7[b]	232.9 ± 11.3[b]
Insulin (mU/L)	44.6 ± 5.7	219.9 ± 11.9[a]	32.4 ± 7.8[b]	42.9 ± 5.1[b]
HOMA-IR	22.8 ± 4.2	187.3 ± 11.7[a]	19.4 ± 5.7[b]	25.3 ± 3.8[b]
Triglyceride (mg/dL)	99.4 ± 8.7	96.7 ± 5.5	88.3 ± 6.1	88.4 ± 4.8
Cholesterol (mg/dL)	153.5 ± 10.5	246.0 ± 8.7[a]	148.2 ± 15.5[b]	127.6 ± 4.7[b]
Adiponectin (μg/mL)	5.7 ± 0.5	5.3 ± 0.3	6.7 ± 1.1	10.3 ± 0.7[abc]
IL-6 (pg/mL)	6.4 ± 0.7	6.2 ± 1.1	5.9 ± 0.9	6.3 ± 0.9
TNFα (pg/mL)	30.8 ± 6.6	60.6 ± 5.5[a]	73.9 ± 8.1[a]	82.5 ± 7.7[a]

Data are presented as mean ± SEM of n = 12 per group for each measured variable. [a] Significantly different than ND ($p < 0.05$), [b] significantly different than HF ($p < 0.05$), and [c] significantly different than WL ($p < 0.05$).

ratio in the HF group only trended to be higher than ND ($p = 0.0895$), WL ($p = 0.0970$), and WL + VWR ($p = 0.0579$). No significant difference between ND, WL, and WL + VWR was observed for pNF-κB p65:NF-κB p65 ratio. The mRNA expression of hepatic cytokines IL-1β, IL-6, and TNFα are presented in Fig. 3A. There was no main effect ($p = 0.077$) for hepatic IL-1β mRNA expression. The IL-6 mRNA expression in WL + VWR was lower ($p = 0.005$) than ND and trended ($p = 0.094$) to be lower than WL. The TNFα mRNA expression in WL + VWR was greater ($p = 0.002$) than ND but was not different than HF or WL. When evaluating the cytokine protein levels (Fig. 3E–G), no significant differences between groups were observed for IL-1β, IL-6 or TNFα.

3.5. Voluntary wheel running during weight-loss reduces BAMBI expression

No differences in TGFβ1 mRNA (Fig. 3H) or protein levels (Fig. 3I) were observed between groups. The mRNA expression of BAMBI (Fig. 3H) was elevated ($p < 0.005$) in WL + VWR when compared to the other experimental groups. No difference was observed between ND, HF, or WL groups. Despite for over a 4-fold change in mRNA expression for WL + VWR over the other experimental groups, BAMBI protein levels (Fig. 3J) in the WL + VWR was lower ($p = 0.025$) than HF only. The BAMBI protein levels in HF trended ($p = 0.066$) towards being greater than ND.

4. Discussion

Lifestyle interventions that encourage weight-loss and increased physical activity or exercise are cornerstones for treatment and management of obesity. This includes the reduction of ectopic lipid deposition in organs such as the liver; however, a lack of understanding continues to exist with the effectiveness of similar lifestyle interventions on biomarkers that underlie hepatic inflammation. In part, this is due to differences in the type of physical activity or exercise employed in various research designs, but to a greater extent this is due to the complexity of obesity-induced hepatic inflammation. In this study, we sought to determine the effect of exercise by voluntary wheel running during weight-loss on markers of hepatic lipid metabolism and inflammation in C57Bl/6J mice. We hypothesized that the addition of voluntary wheel running during weight-loss would result in lower hepatic lipids than the weight-loss by diet alone. In addition, the physically active weight-loss mice would have lower measures of hepatic and systemic inflammation. Following weight-loss, the addition of voluntary wheel running in the WL + VWR mice resulted in greater weight-loss and lower hepatic cholesterol and triglyceride levels than WL mice. Despite that hepatic triglyceride did not reach statistical significance due to being underpowered (effect size = 0.956), the 39.2% lower hepatic triglyceride in the WL + VWR when compared to WL was physiologically significant. The reduction in hepatic cholesterol and triglyceride were matched by a greater plasma adiponectin concentration. These metabolic changes occurred despite that WL + VWR mice had a caloric intake similar to HF mice and greater than WL mice. The inclusion of running wheel exercise during weight-loss produces greater reductions in body energy stores than compared to weight-loss by diet alone (Doucet et al., 2018; Riou et al., 2015). As result of lower energy stores produced by running wheel exercise during weight-loss, increased dietary intake was observed to compensate for energy depletion. The effects of running wheel exercise on increased energy compensation through dietary intake have been observed in previous wheel running studies (Copes et al., 2015; Mifune et al., 2020). In contrast, when examining measures of inflammation following weight-loss, the addition of voluntary wheel running did not provide any observable benefits when compared to weight-loss by diet alone.

Following weight-loss in mice performing voluntary wheel running, we observed a significant increase in the mRNA expression of the genes SREBP-1c, FASN, and ACC-1 that are associated with *de novo* lipogenesis; however, this did not translate to an increase in protein levels. Due to the small amount of variation in both mRNA expression and protein abundance, the lack of correlation between mRNA and protein levels is due to post-translational regulation of proteins (Greenbaum et al., 2003). Transcriptional control of many genes in lipid metabolism are regulated by SREBP (Horton et al., 2002). It is well characterized that SREBP-1c overexpression increases FASN mRNA expression; however, less is known about the post-translational regulation of FASN activity (Jensen-Urstad and Semenkovich, 2012). Translational regulation of FASN and other proteins associated with *de novo* lipogenesis are sensitive to energy balance, while at the same time are fairly stable, buffering sudden changes due to increased transcription, preventing rapid translation (Jensen-Urstad and Semenkovich, 2012). Protein levels of SREBP-1c in the WL and WL + VWR remained significantly lower than ND and were similar to HF at the end of 18 weeks. The reduction in SREBP-1c was matched by a reduced pACC-1:ACC-1 ratio in WL and WL + VWR and the pACC-1:ACC-1 ratio in WL + VWR was lower than both ND and HF groups. The reduction in pACC-1:ACC-1 ratio was matched by an increase in pAMPK:AMPK ratio, an inhibitor of ACC-1 phosphorylation and activity (Zhang et al., 2009), in both the WL and WL + VWR groups. Despite no difference in pAMPK:AMPK ratio between WL and WL + VWR, voluntary wheel running did result in an increase in circulating plasma adiponectin. Adiponectin regulates whole body lipid partitioning, promoting adipose tissue lipid deposition, sparing the liver of excess lipid accumulation (Kim et al., 2007). Based on our data, it is unclear what degree that the exercise-induced increase in adiponectin played in reducing hepatic lipid during weight-loss. In order to normalize total hepatic lipid levels; however, weight-loss independent of voluntary wheel running may result in a reduced synthesis of malonyl-CoA and metabolically shift to greater mitochondrial fat oxidation (Wakil and Abu-Elheiga, 2009).

The pathogenesis of chronic liver diseases are mechanistically linked to the activation of proinflammatory cytoplasmic multi-protein complexes by factors that include lipotoxicity, oxidative stress, and adipose tissue derived proinflammatory metabolites such as IL-1β, TNFα, and IL-6 (Buzzetti et al., 2016). During the activation of hepatic inflammation, saturated fatty acids and proinflammatory adipokines sensitize hepatocytes to the endotoxin lipopolysaccharide (LPS), promoting the release of IL-1β in a caspase-dependent manner (Buzzetti et al., 2016; Heymann and Tacke, 2016). Elevated LPS-mediated TLR4-CD14 signaling activates Kupffer cells, monocyte-derived macrophages, and hepatic stellate cells propagating liver diseases such as liver fibrosis and hepatocellular carcinoma (Rivera et al., 2007).

In the present study, the high-fat diet increased CD14 mRNA expression, but not TLR4 or MyD88 mRNA expression. CD14 is a

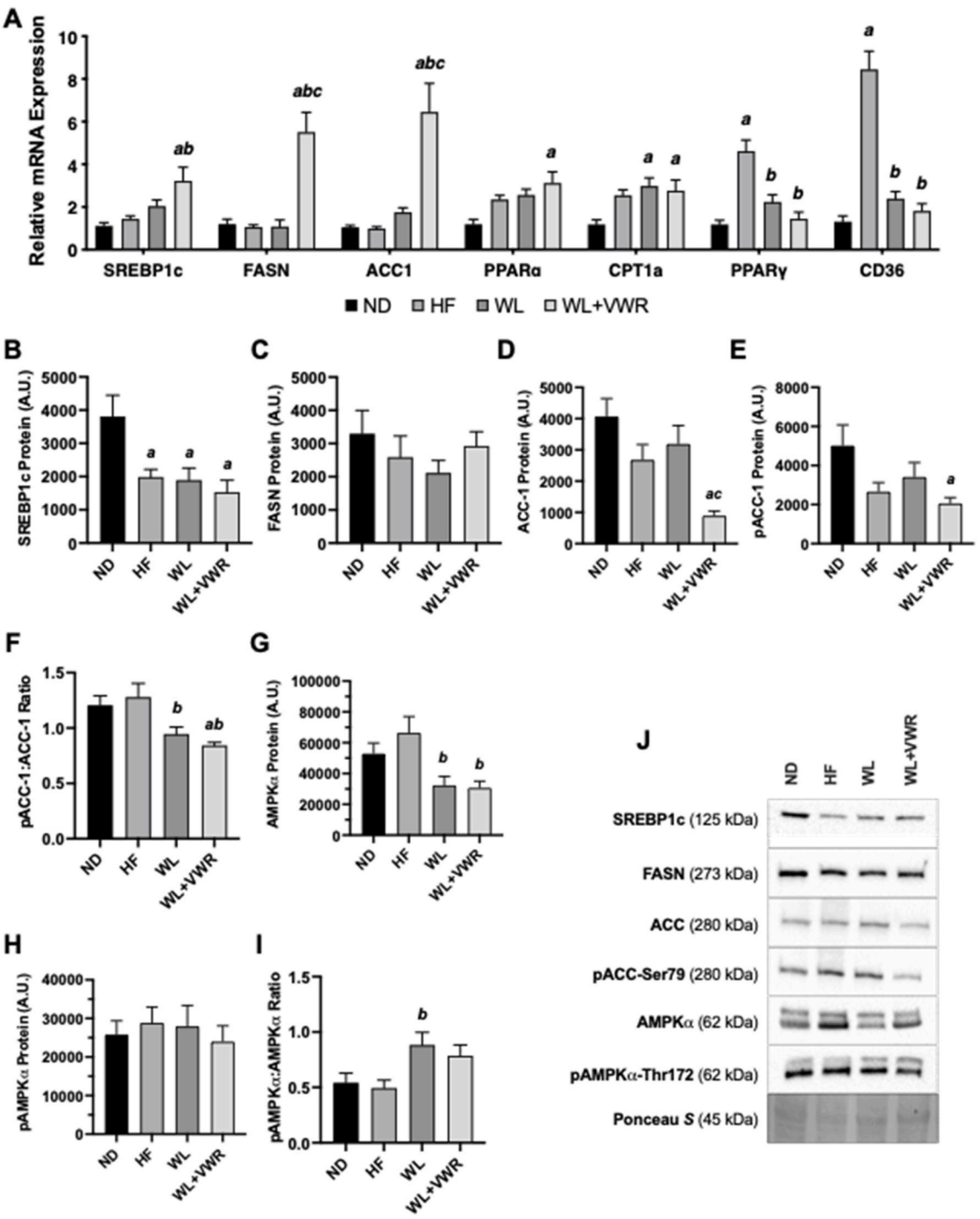

Fig. 2. The effects of exercise during weight-loss on markers of hepatic lipid metabolism. mRNA expression for markers of *de novo* lipogenesis, lipid oxidation, and lipid uptake (**A**); protein levels for SREBP1c (**B**), FASN (**C**), ACC-1 (**D**), pACC-1 (**E**), pACC-1:ACC-1 ratio (**F**), AMPKα (**G**), pAMPKα (**H**), and pAMPK:AMPK ratio (**I**); and representative Western blots (**J**). Data are presented as mean ± SEM of n = 12 per group for each measured variable. [a]Significantly different than ND ($p < 0.05$), [b]significantly different than HF ($p < 0.05$), and [c]significantly different than WL ($p < 0.05$).

glycoprotein that recognizes gram negative bacterial LPS and when activated transfers LPS to the TLR4 protein complex (Palsson-McDermott and O'Neill, 2004). Activation of the TLR4-MyD88 acute phase complex leads to the phosphorylation and activation of NF-κB p65 and has been linked to increased synthesis of proinflammatory cytokines and chemokines (Rivera et al., 2007). In the HF mice, the pNF-κB p65:NF-κB p65 ratio trended towards being greater than the other groups. Despite the greater pNF-κB p65:NF-κB p65 ratio in the HF mice, we did not observe a significant difference between the experimental groups for hepatic IL-1β, IL-6, or TNFα protein levels. Overall, our data showed that the addition of exercise during weight-loss did not affect the state of hepatic inflammation more than diet alone. This may be due to either the diet or duration of feeding on hepatic inflammation. In future studies, we would encourage the use of LPS-induced inflammatory responses with either *in vivo* or *in situ* models to determine the effects of exercise on hepatic inflammation.

Chronic hepatic inflammation paired with cell death serves as the trigger for the pathogenesis of liver fibrosis. Orchestrated events

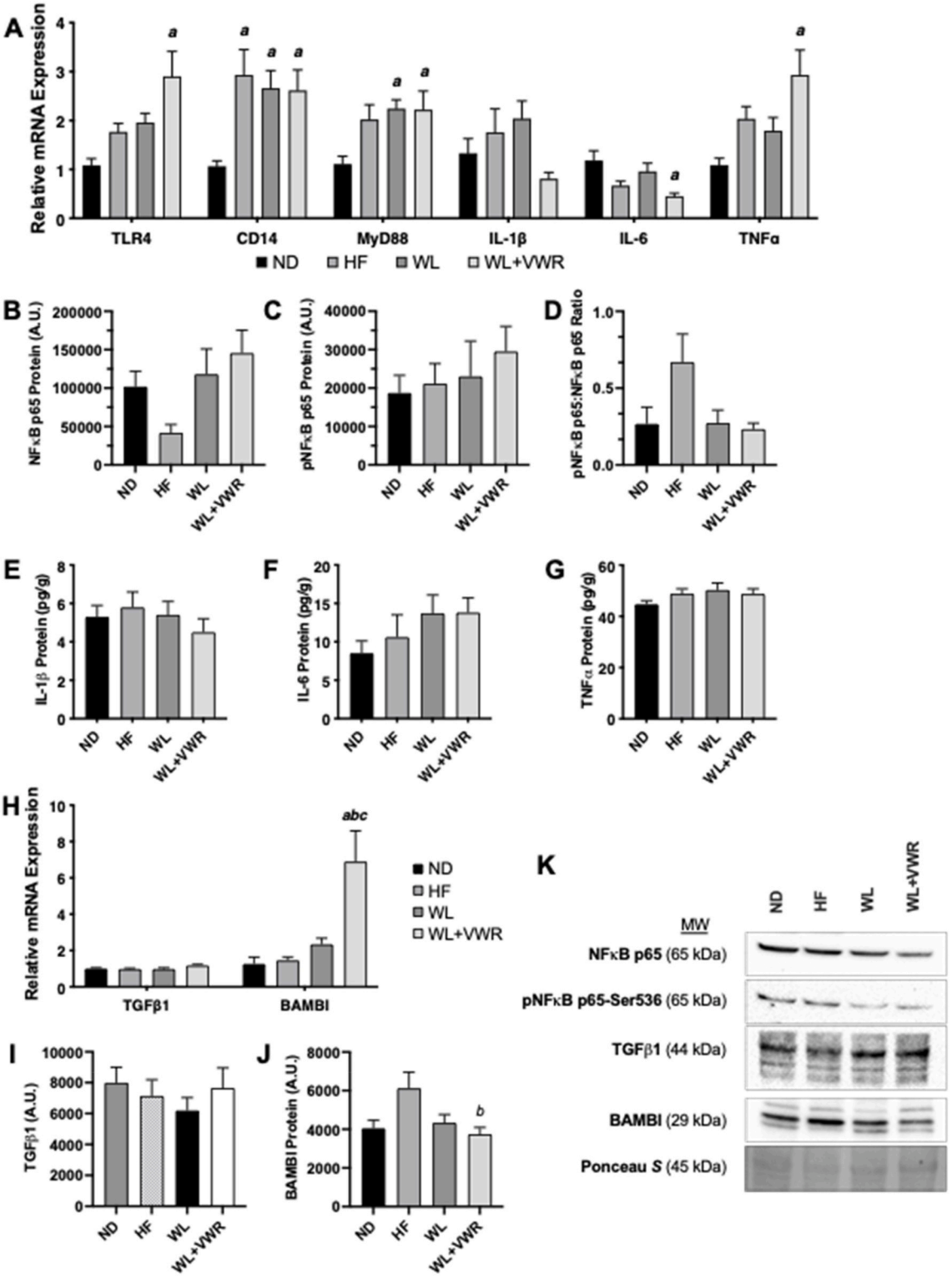

Fig. 3. Biomarkers of hepatic inflammation and TGFβ1 and BAMBI protein levels. mRNA expression of markers for inflammatory signaling and proinflammatory cytokines (**A**); protein levels for NF-κB p65 (**B**), pNF-κB p65 (**C**), pNF-κB p65:NF-κB p65 ratio (**D**); IL-1β (**E**), IL-6 (**F**), and TNFα (**G**); mRNA expression of TGFβ1 and BAMBI (**H**); protein levels of TGFβ1 (**I**) and BAMBI (**J**); and representative Western blots (**K**). Data are presented as mean ± SEM of n = 12 per group for each measured variable. [a]Significantly different than ND ($p < 0.05$), [b]significantly different than HF ($p < 0.05$), and [c]significantly different than WL ($p < 0.05$).

involving hepatic and peripheral macrophages, hepatic inflammation and immunity, and activation of the TGF-β signaling pathway in hepatic stellate cells contributes to the formation of fibrogenic populations in the liver (Yang et al., 2014). In the present study, we did not observe a significant difference between groups for TGFβ1 mRNA or protein levels. This may be due to the lack of chronic inflammation to induce a fibrogenic response. Despite no changes in TGFβ1, we did observe a trend to an increase in BAMBI protein levels ($p = 0.066$) in HF when compared to ND. BAMBI functions as a pseudoreceptor for TGFβ1 and acts as an inhibitor of hepatic stellate cell activation and subsequent hepatic

fibrogenesis (Seki et al., 2007). The lack of increase in TGFβ1 in HF may be due to the increase in BAMBI. Following weight-loss, we observed a reduction in BAMBI protein levels, which reached the level of significance in the WL + VWR group only. These findings are novel in that this is the first study to our knowledge that has evaluated the effects of exercise on BAMBI.

One of the limitations of our study was that the exercise performed was not standardized but was performed *ad libitum* on running wheels. We chose running wheels versus forced exercise to prevent behavioral stress-induced changes in metabolism and body mass. In addition, regular physical activity has been shown to be more beneficial for weight-loss related health benefits versus a single bout of exercise followed by being sedentary for the remaining day in both animal and human studies (Duvivier et al., 2013, 2017). Another limitation of our study was that we did not observe significant changes in hepatic inflammation or biomarkers of fibrosis following high-fat feeding. This is likely due to factors such as the duration of dietary feeding, not using diets supplemented with fructose or cholesterol, or using a methionine-choline deficient diet which would increase the likelihood of fibrogenic development (Machado et al., 2015; Savard et al., 2013). In addition, the lack of hepatic inflammation could be due to the lack of activity of either pattern recognition receptors (PRRs) or damage-associated molecular patterns (DAMPs) to translate and active proteins post-translational. PRRs recognize molecules found in pathogens or molecules released from DAMPs. DAMPs are endogenous danger molecules released from damaged or dying cells activating innate immunity through interactions with PRRs. Inflammatory protein translation and abundance and post-translational regulation of protein function and activity are tightly regulated by both PRRs and DAMPs. PRRs and DAMPs prevent uncontrolled innate inflammatory responses in cells by targeting innate sensors and downstream signaling molecules, including receptors, enzymes and transcriptional factors (Deribe et al., 2010; Liu et al., 2016; Mowen and David, 2014). The development of obesity in the mice may have produced a low-grade inflammatory response that did not active PRRs or DAMPs (Grant and Dixit, 2015).

In conclusion, the addition of voluntary wheel running during weight-loss resulted in greater improvements in hepatic cholesterol and triglyceride levels than weight-loss by diet alone. Despite for no additional benefits derived from exercise for circulating glucose and lipid concentrations, voluntary wheel running caused an increase in plasma adiponectin concentration. These findings are interesting given that the mice in WL + VWR group had caloric intake similar to HF-fed mice. When examining measures of inflammation following weight-loss, the addition of voluntary wheel running did not provide any significant benefits when compared to weight-loss by diet alone; however, the effect of exercise on BAMBI and hepatic fibrogenesis warrants further investigation.

CRediT authorship contribution statement

Joshua S. Wooten: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Funding acquisition. **Kaylee E. Poole:** Investigation. **Matthew P. Harris:** Investigation. **Brianne L. Guilford:** Resources, Supervision, Writing – review & editing. **Megan L. Schaller:** Investigation. **David Umbaugh:** Investigation. **Andrew Seija:** Software, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the Seed Grants for Translational and Exploratory Projects (J.S.W.) and the Undergraduate Creative Arts and Research Program (K.E.P. and A.S.) at Southern Illinois University Edwardsville, Edwardsville, IL.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2022.01.003.

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