Analysis of the liver lipidome reveals insights into the protective effect of exercise on high-fat diet-induced hepatosteatosis in mice

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Jordy AB, Kraakman MJ, Gardner T, Estevez E, Kammoun HL, Weir JM, Kiens B, Meikle PJ, Febbraio MA, Henstridge DC. Analysis of the liver lipidome reveals insights into the protective effect of exercise on high-fat diet-induced hepatosteatosis in mice. Am J Physiol Endocrinol Metab 308: E778–E791, 2015. First published February 24, 2015; doi:10.1152/ajpendo.00547.2014.—The accumulation of lipid at ectopic sites, including the skeletal muscle and liver, is a common consequence of obesity and is associated with tissue-specific and whole body insulin resistance. Exercise is well known to improve insulin resistance by mechanisms not completely understood. We performed lipidomic profiling via mass spectrometry in liver and skeletal muscle samples from exercise-trained mice to decipher the lipid changes associated with exercise-induced improvements in whole body glucose metabolism. Obesity and insulin resistance were induced in C57BL/6J mice by high-fat feeding for 4 wk. Mice then underwent an exercise training program (treadmill running) 5 days/wk (Ex) for 4 wk or remained sedentary (Sed). Compared with Sed, Ex displayed improved (P < 0.01) whole body metabolism as measured via an oral glucose tolerance test. Deleterious lipid species such as diacylglycerol (P < 0.05) and cholesterol esters (P < 0.01) that accumulate with high-fat feeding were decreased in the liver of trained mice. Furthermore, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) (the PC/PE ratio), which is associated with membrane integrity and linked to hepatic disease progression, was increased by training (P < 0.05). These findings occurred without corresponding changes in the skeletal muscle lipidome. A concomitant decrease (P < 0.05) was observed for the fatty acid transporters CD36 and FATP4 in the liver, suggesting that exercise stimulates a coordinated reduction in fatty acid entry into hepatocytes. Given the important role of the liver in the regulation of whole body glucose homeostasis, hepatic lipid regression may be a key component by which exercise can improve metabolism.

hepatic steatosis; exercise; lipids; fatty acid transporters; skeletal muscle; insulin resistance

IT IS NOW RECOGNIZED that the current worldwide obesity epidemic has resulted in the increased prevalence of “metabolic disease clusters,” including type 2 Diabetes, fatty liver disease, and certain cancers (21). As body weight increases, expanded adipose tissue depots are no longer able to optimally utilize or store excess lipid, resulting in the accumulation of lipid metabolites at ectopic sites, including the skeletal muscle and liver. This peripheral intramyocellular and intrahepatic (IHL) lipid accumulation is associated with tissue-specific and whole body insulin resistance and, in the case of the liver, is referred to as hepatosteatosis or nonalcoholic fatty liver disease (NAFLD).

NAFLD has become the most common liver disorder in the 21st century and now affects one-third of adults and an increasing number of children (9). NAFLD has a progressive disease spectrum initiated by simple steatosis that arises from an imbalance between triglyceride accumulation and removal via either VLDL export into blood or fatty acid oxidation within the liver (9). If NAFLD continues to remain unchecked, it may result in nonalcoholic steatohepatitis (NASH), cirrhosis, and eventually hepatocellular carcinoma (38). Genetic risk factors that predispose individuals to development of NAFLD have been identified, most notably a missense mutation in patatin-like phospholipase domain-containing 3 gene (PNPLA3) (36). Independent of known genetic risk factors, obesity in itself dramatically increases the risk of IHL accumulation and subsequently the progression of NAFLD. Demonstrating the power of weight loss on liver health, a meta-analysis reported that weight loss surgery leads to improvement and/or resolution of NAFLD in ~80% of patients (30). Current therapeutic strategies to treat NAFLD include prescription of insulin-sensitizing drugs such as metformin (6), thiazolidinediones (1, 2), and glucagon-like peptide-1 (GLP-1) receptor agonists (10), which have been shown to be effective in treating NAFLD, although some patients with this disorder do not present with insulin resistance. Although regular exercise is known to reduce obesity largely by increasing energy expenditure and lipid oxidation in contracting skeletal muscle, it may also be effective as a treatment for NAFLD independent of weight loss. Studies in patient cohorts have demonstrated an inverse association between physical activity and fitness and NAFLD (8, 31), whereas training interventions (17, 23, 40) can impact on NAFLD in a positive manner. Similar findings have been made using various rodent models (3, 35).

Despite the clear nexus between obesity, liver fat, and liver disease, many questions remain regarding the pathogenesis and treatment of this disorder. For example, some patients with NAFLD progress to NASH and cirrhosis, whereas others do not. One factor that likely influences NAFLD progression is the specific lipid species that accumulate in response to nutrient overload. For example, alterations in specific lipid species without changes in overall IHL accumulation protect against high-fat diet (HFD)-induced hepatic insulin resistance in a mouse lacking a long-chain fatty acid elongase, i.e., elongation of long-chain fatty acids family member-6 (Elov6) (28). This suggests that the ratio of certain lipid species may be more important than the total amount that has accumulated (5). In addition, not only the individual lipid species but also the relative ratio of certain lipids may play a role in liver pathol-
ology. Accordingly, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) is associated with membrane integrity and liver failure (25).

Because lipid species of different fatty acid compositions may be important regulators of cellular proliferation (12), inflammation (27), and ER stress-induced apoptosis (4), it is important to delineate the specific lipid signature of NAFLD, as it could provide insights into the signaling pathways relevant in the progression of the condition. In a comparison of murine with human liver tissue samples, mice maintained on a HFD provide a reproducible model of human NAFLD in regard to the specificity of diacylglycerol (DAG) and other lipid species in the liver (14). Currently, an analysis of the precise lipid composition at the species level in the skeletal muscle and liver following exercise training has not been described. Accordingly, to thoroughly investigate the impact of both a HFD and exercise training on IHL comprehensive lipodomic analysis on a broad array of lipid classes in mice. Of significance, we show that a HFD increases a wide array of liver triacylglycerol (TAG), DAG, and cholesterol ester species that are reversed by exercise training. Moreover, although the HFD decreased the PC/PE ratio, this was also reversed by exercise training.

MATERIALS AND METHODS

Mouse Studies

All experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee, and animals were provided humane care in line with the “Principles of Laboratory Care” (NIH publication no. 85-23, revised 1985) and in accordance with the National Health and Medical Research Council of Australia guidelines on Animal Experimentation. Animals were administered their prescribed diet and water ad libitum and housed in a temperature-controlled environment (~22°C) with a 12-h light-dark cycle. Eight-week-old male mice (C57Bl/6J background) were fed either a regular normal chow diet (NC; 14.3 MJ/kg, 76% of kJ from carbohydrate, 5% fat, and 19% protein; Specialty Feeds, Glen Forrest, Western Australia, Australia) or, for the fat-feeding studies, a HFD (19 MJ/kg, 36% of kJ from carbohydrate, 43% fat (42.9% saturated, 35.24% monounsaturated, and 21.86% polyunsaturated fatty acids), and 21% protein; Specialty Feeds; Sigma-Aldrich, St. Louis, MO). Protein quantification was normalized to 100 µg total protein (Coomassie stain).

Exercise Training

Mice were trained on the treadmill using the progressive overload principle 5 days/wk for 30, 40, 45, 50, and 60 min respectively. In week 1, the speed of the treadmill was set at 50% of the maximum speed that the mice reached in their exercise test. In week 2 this was increased to 60%, in week 3 it was 70%, and in week 4 it was 80%. The mice in the Sed group had their cages removed from their holding room and were placed next to the treadmill while the Ex groups trained. All mice were able to complete the exercise program, except for two mice (1 on a chow diet and 1 on a HFD) that could not complete the 4th wk at 80% and were instead trained for a 2nd wk at 70%. To ensure no acute effects of exercise on our measurements, training was withheld on the days of testing and euthanization.

Metabolic Testing

Body composition analysis. Fat and lean mass were determined using the EchoMRI 4-in-1 (Echo Medical Systems, Houston, TX).

Oral glucose tolerance tests. Oral glucose tolerance tests (OGTT) were performed in 5-h-fasted mice. Blood glucose was measured using a glucometer (Accu-Check; Roche, Castle Hill, New South Wales, Australia) at 0, 15, 30, 45, 60, 90, and 120 min after oral administration of glucose (2 g/kg lean body mass) on blood obtained from the tail tip.

Biochemical Analysis

Plasma analysis. Liver function enzymes, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by enzymatic reaction (Australian Specialized Animal Pathology, Mulgrave, Victoria, Australia). Plasma insulin was measured using an ALPCO Insulin ELISA (ALPCO Diagnostics, Salem, MA). Intraassay variation was 3.1–9.3 % coefficient of variation (%CV), and interassay variation was 5.55–11.49 %CV.

Western blotting. Muscle and liver samples were lyzed, and protein concentration was determined and resolved by SDS page, as described previously (19). Immunoblotting was performed using the following primary antibodies: anti-SCD1 (stearoyl-CoA desaturase 1), anti-phosphorylated (p)-AMPK (AMP-activated protein kinase) Thr172, anti-p-ACC1/2 (acyl-CoA carboxylase 1/2) Ser79, total (t)-ACC (streptavidin-horseradish peroxidase), anti-NF-κB p65, anti-p-JNK1/2 Thr183/Tyr185, anti-p-IKB1/2, anti-SREBP1c, anti-CD36, anti-NLRP3, and IL-1β (R & D Systems, Minneapolis, MN), anti-FATP4 (fatty acid transport protein 4; kindly donated by Prof. Joachim Füllekrug, University Hospital of Heidelberg, Heidelberg, Germany), anti-AMPKα2 (kindly donated by Dr. Graham HARDIE, University of Dundee, Scotland, UK), anti-DGAT1 (diacylglycerol O-acyltransferase 1; Novus Biologicals, Littleton, CO), anti-CPT1A (carnitine palmitoyltransferase 1A; Alpha Diagnostics International, San Antonio, TX), anti-caspase 1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma-Aldrich, St. Louis, MO). Protein quantification was normalized to GAPDH, β-actin, or total protein (Coomassie stain).

RNA extraction and real-time quantitative PCR. Total RNA was isolated from liver with TRI Reagent (Sigma-Aldrich) and reverse transcribed to cDNA with the use of random hexamers. Real-time PCR was performed on a 7500 Fast Sequence detector (Applied Biosystems, Foster City, CA). Each assay included a no-template control and a no-reverse transcriptase control. Oligos for glucagon receptor (GCGR; Mm01208835_m1), fatty acid-binding protein 1 (FABP1; Mm00443440_m1), sterol regulatory element-binding factor-1 (SREBF1; Mm01138338), glucokinase (GCK; Mm00439129), fatty acid synthase (FASN; Mm01253292), carbohydrate-responsive element-binding protein (Mlxipl; Mm02342723), CPT1A (Mm00515643_m1), SCD1 (Mm00772290_m1), CPT1A (Mm001550438_m1), CPT1A (Mm00550438_m1), and GAPDH (Mm01265734_m1) were used. Aliquots were used to confirm the identities of specific primers. All qPCR reactions were run in triplicate and normalized to the housekeeping gene GAPDH, as described in the National Institutes of Health publication 85-23, revised 1985, and in accordance with the National Health and Medical Research Council of Australia guidelines on Animal Experimentation.

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Lipidomics. Lipid content was determined in the skeletal muscle or liver using previously described methods (19, 29, 41). Briefly, samples (20–30 mg wet wt) were homogenized in 100 μl of PBS buffer, pH 7.47. Lipids were extracted from 25 (liver) to 50 (muscle) g of protein using 20 volumes of chloroform-methanol (2:1) in a single-phase extraction process, recovering all lipids in a single phase suitable for liquid chromatography-mass spectrometry analysis. Lipid analyses were performed by liquid chromatography-electrospray ionization-tandem mass spectrometry using an HP 1200 liquid chromatography system combined with a PE Sciex API 4000 QTRAP mass spectrometer with a turbo-ionspray source (350°C) and Analyst 1.5 data system. Lipid classes investigated included TAG, DAG, ceramide, sphingomyelin, cholesterol ester, PC, PE, and cardiolipin. All individual lipid species of each class of lipid (8 classes) were summed to give the total pool for each lipid class. Because of the large number of species detected, not all of these individual species are presented in the figures.

Fig. 1. Characteristics of sedentary (Sed) and exercise-trained (Ex) mice fed a normal chow (NC) or high-fat diet (HFD). A–C: body weight after 4 wk on NC or HFD (A), body weight after 8 wk (including 4 wk of training; B), and change in body weight during training period (C). D–F: fat mass after 4 wk on NC or HFD (D), fat mass after 8 wk (including 4 wk of training; E), and change in fat mass during training period (F). G–I: lean mass after 4 wk of NC or HFD, lean mass after 8 wk of NC or HFD (including 4 wk of training; H), and change in lean mass during training period (I). J–L: %body fat after 4 wk of NC or HFD (J), %body fat after 8 wk of NC or HFD (including 4 wk of training; K), and change in %body fat during training period (L; n = 8–10/group. *P < 0.05, **P < 0.01, and ***P < 0.001 for dietary effect; †P < 0.05, ††P < 0.01, and †††P < 0.001 for exercise effect (Sed vs. Ex). Straight horizontal lines represent a main effect, whereas lines with ticks represent an interaction between those 2 groups. Data are means ± SE.
**Oxidative enzymes.** β-Hydroxyacyl-CoA dehydrogenase (β-HAD) and citrate synthase (CS) activity was measured in 5–10 mg of skeletal muscle, as reported previously (7, 18).

**Data and Statistics**

Data were analyzed by two-way analysis of variance with Tukey post hoc tests (SigmaStat version 3.5; Systat Software), where all four groups were included for analysis at once (NC-Sed, NC-Ex, HFD-Sed, and HFD-Ex). Logarithmic transformation was applied to end points not meeting normality assumptions. All data are presented as means ± SE. Statistical significance was set at \( P < 0.05 \).

**RESULTS**

**Exercise Training Limits the HFD-Induced Increase in Adipose Tissue Mass**

We characterized the body composition of mice before and after completion of the training or sedentary protocols. As expected, after 4 wk of dietary intervention, the mice fed a HFD were heavier and had greater fat mass, less lean mass, and a higher body fat percentage (Fig. 1, A, D, G, and \( J \)). Following the completion of the 4-wk exercise intervention, weights were analyzed as a change between the 4- and 8-wk time points. NC-Ex accumulated less fat mass and consequently had a lower body fat percentage than NC-Sed (Fig. 1, \( F \) and \( L \)). HFD-Ex had lower body mass, fat mass, and body fat percentage compared with HFD-Sed (Fig. 1, C, \( F \), and \( L \)), whereas there was a small difference in lean mass between the NC-Ex and HFD-Ex groups (Fig. 1I). The lower weight and fat mass were not due to alterations in eating behavior (Fig. 2A).

**Exercise Training Improves Exercise Capacity, Muscle Oxidative Capacity, and HFD-Induced Glucose Intolerance and Hyperinsulinemia**

To examine the effect of our training protocol on exercise capacity, we performed acute exercise tests before and after
training. Prior to training, mice fed a NC had similar exercise capacity to those on the HFD, indicating that the 4 wk of high-fat feeding did not impact exercise capacity (Fig. 2B). Although training increased the exercise capacity of mice on both NC and HFD, the mice on the NC tended to gain a greater benefit from the training protocol with a 35% improvement in exercise capacity compared with 17% with the HFD (Fig. 2B). To assess a biochemical marker of exercise adaptation, we measured the maximal activities of two important mitochondrial oxidative enzymes in mixed gastrocnemius muscle. The activities of $\beta$-HAD and CS were elevated in the Ex groups independent of diet (Fig. 2, C and D). Together, the functional improvement in exercise capacity and the biochemical alterations in these oxidative enzymes suggested that the exercise protocol was of sufficient duration and intensity to derive a training effect.

To assess the impact of diet and exercise on whole body glucose homeostasis, we performed an OGTT. The HFD promoted glucose intolerance (Fig. 2, E and F) compared with NC. However, the HFD-Ex group displayed improved glucose tolerance compared with the HFD-Sed mice (Fig. 2, E and F). Furthermore, HFD-induced hyperinsulinaemia was attenuated in the HFD-Ex group (Fig. 2G). Because previous observations have emphasized the importance of defective hepatic glucose metabolism in the development of impaired glucose tolerance and insulin resistance (24), we measured plasma markers of liver function. ALT and AST, two markers of liver damage, were increased by the HFD (Fig. 2, H and I). However, the HFD-Ex group had reduced levels of these markers compared with their respective controls (Fig. 2, H and I). Taken together, the exercise protocol led to a large improvement in exercise capacity in the NC-fed mice, with minimal impact on body composition and glucose homeostasis. However, although the mice on a HFD had less improvement in exercise capacity, the exercise training was largely protective against the deleterious effects of high-fat feeding with regard to fat accumulation and glucose intolerance.

Fig. 3. Lipidomic analysis of skeletal muscle in Sed or Ex mice. A: skeletal muscle total triacylglycerol (TAG) levels relative to NC Sed = 1. B: skeletal muscle total diacylglycerol (DAG) levels relative to NC Sed = 1. C: skeletal muscle total ceramide levels relative to NC Sed = 1. D: individual TAG molecular lipid species relative to NC Sed = 1. E: individual DAG molecular lipid species relative to NC Sed = 1; n = 7–10/group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ for dietary effect (NC vs. HFD); †$P < 0.05$ and ††$P < 0.01$ for exercise effect (Sed vs. Ex). Straight lines represent a main effect, whereas the lines with ticks represent an interaction between those 2 groups. Data are means ± SE.
Exercise Training Does Not Affect HFD-Induced Lipid Accretion in Skeletal Muscle

Because skeletal muscle is the organ responsible for locomotion and energy utilization during exercise, we hypothesized that this would be the organ most affected in terms of lipid accumulation with training. Accordingly, we first performed lipidomic analysis on skeletal muscle from all groups of mice for eight classes of lipids: TAG, DAG, ceramide, sphingomyelin, cholesterol ester, PC, PE, and cardiolipin. Our analysis revealed a significant increase in total TAG, DAG, and ceramide with the HFD (Fig. 3, A–C) with multiple individual lipid species altered (Figs. 3, D and E, and 4A). Contrary to our hypothesis, this increase was not attenuated by exercise training (Figs. 3, A–E, and 4A); however, the TAG in the NC Ex group was lower than that in the NC Sed group predominantly because of changes in the TG 16:1_16:1_18:1, TG 16:1_18:1_18:1, and TG 18:1_18:1_18:1 species (Fig. 3, A and D). Sphingomyelin, cholesterol ester, and PC were not affected by either HFD or exercise training (Figs. 4, B, C, and E, and 5, A and B), whereas there was a trend for the HFD to increase PE levels (Fig. 4D) and cardiolipin levels (Fig. 5, C and D) and decrease the PC/PE ratio (Fig. 4F). Analysis of skeletal muscle fatty acid transporters demonstrated an increase in response to the HFD for slc27a1 (FATP1) and a trend for an increase with diet for cd36 and got2 (FABP-pm) (Fig. 5E), whereas exercise training reduced the HFD-induced expression of slc27a1. Together, these data demonstrate that the improvements in metabolic homeostasis observed with the HFD-Ex group are unlikely to be due to alterations in intramyocellular lipids.

Analysis of the Liver Lipidome Reveals Differential Regulation of Lipid Species By Diet and Exercise Training

We have demonstrated previously that lipid accumulation in the liver closely relates to alterations in insulin sensitivity during high-fat feeding and that hepatosteatosis and liver insulin resistance occurs within 1 wk of such a dietary intervention in C57Bl/6 mice (39). In addition, relatively little is known regarding the effect of high-fat feeding and/or exercise training on the liver lipidome, liver inflammation, or function.
Accordingly, we next performed a comprehensive liver lipidomic analysis on tissues from our mice enrolled in the exercise training program. In contrast with the pattern observed in skeletal muscle, TG levels were elevated in the HFD-Sed mice, which was indicative of hepatosteatosis, but this was attenuated with exercise (Fig. 6A). The same general pattern was observed for total liver DAG (Fig. 6B). Analysis of the molecular species of TAG demonstrated a generalized significant increase in all species with HFD (Fig. 6D). These all tended to be decreased in the HFD-Ex group, but none reached statistical significance (Fig. 6D). Whereas TAGs are thought to be generally inert lipids, DGs are thought to be a more deleterious lipid class by acting as second messengers to disrupt signal transduction processes (37) and by activating inflammatory signaling cascades via activation of protein kinase C (PKC) isoforms (15). Numerous DG species were increased in the HFD-Sed group but decreased in the HFD-Ex group (Fig. 6E). Ceramides are thought to be among the most pathogenic of all lipid species because they promote inflammatory signaling cascades and impair vital cell signaling processes (20). However, somewhat unexpectedly, total liver ceramide accumulation was decreased with the HFD and was not impacted by exercise (Fig. 6C). Further analysis of the ceramide species revealed an increase in the ceramide 18:0 and ceramide 20:0 species but a decrease with the longer-chain ceramide 24:0 and ceramide 24:1 species with HFD (Fig. 6F). Because the longer-chain species contributed more to the total pool of ceramide in these samples, this resulted in a decrease in total ceramide levels in response to HFD. Much like the ceramide data, sphingomyelin levels were lower with the HFD (Fig. 7A), but closer inspection of the species data (Fig. 7B) revealed that some species were elevated (sphingomyelin 32:1) and others decreased (sphingomyelin 38:2). There was no major effect of exercise training on hepatic sphingomyelin levels. As discussed, maintaining the PC/PE ratio is important for liver NASH progression and liver membrane integrity (25). In the current study, the hepatic PC/PE ratio decreased with high-fat feeding (Fig. 7E) due to a decrease in PC levels (Fig. 7D) rather than an increase in PE levels (Fig. 7C). Importantly, exercise training in HFD mice resulted in a small but significant increase in the PC/PE ratio compared with HFD-Sed mice (Fig. 7E). Finally, cholesterol ester levels were robustly elevated with HFD and significantly decreased with exercise training (Fig. 8, A and C), whereas total liver cardiolipin levels were decreased with HFD (Fig. 8B).
were not altered by diet or exercise (Fig. 8B), although some species were elevated or decreased with HFD (Fig. 8D).

Exercise Training Decreases the Expression of Fatty Acid Transporters in the Liver

Lipid storage in hepatocytes is determined by the balance between hepatocyte fatty acid uptake, lipid synthesis, oxidation, and exportation. Hence, we next examined these pathways to ascertain a possible mechanism to the protective effects of exercise training on IHL accumulation. We first performed RT-PCR analysis of a number of genes involved in these different processes. High-fat feeding increased expression of the Gcgr, the fatty acid transporter Cd36 [cluster of differentiation 36; also known as FAT (fatty acid translocase)], Srebf1, Gck, and Cpt1A, whereas Mxipl, Scd1, and Ppara were decreased (Fig. 9A). Exercise training decreased the expression of Gcgr, Cd36, Gck, Dgat1, and Cpt1A, whereas there was a tendency for exercise training to increase Fabp1 expression (P = 0.05). The effect of exercise was most notable in its ability to decrease HFD-induced Cd36 and Gck expression (Fig. 9A).

To further characterize potential mechanisms of the phenotype, we also investigated various markers at the protein level. Analysis of CD36 protein levels validated the mRNA findings...
with a significant reduction in protein expression with exercise (Fig. 9, B and C). Quantification of another fatty acid transporter (FATP4) also demonstrated a decrease with exercise (Fig. 9, B and D), indicating that there may be a generalized decrease in hepatic fatty acid transporters with exercise training. DGAT1 protein expression levels were not different (Fig. 9, B and E) and did not align with findings at the mRNA level (Fig. 9A), indicating either a lag time between transcription and translation or nontranscriptional control of these proteins (i.e., posttranslational modifications). Hepatic SCD1 protein levels did correspond tightly with the mRNA data, with a significant decrease with HFD, and a trend ($P = 0.06$) for a further decrease with exercise training on the HFD (Fig. 9, A and F). Analysis of proteins linked to hepatic β-oxidation demonstrated an increase in the ratio of p-ACC to t-ACC (Fig. 9, G and H), which was due to a reduction in total ACC rather than an increase in the phosphorylation status per se (Fig. 9, I and J). Similarly to studies in rats that had exercised voluntarily (34, 35), these changes in ACC were independent of changes in AMPK, a key kinase regulator of ACC (Fig. 9, G and K), whereas no effect was seen for diet or exercise for CPT1A protein expression (Fig. 9, G and L).

**Exercise Training Dampens the Expression of Inflammatory Mediators in the Liver**

Because inflammation has been linked to ectopic lipid-induced impairments in the insulin-signaling cascade in insulin-sensitive tissues such as the liver and skeletal muscle, we analyzed important inflammatory markers in the liver samples. Although there was no dietary effect (there was a trend for an increase with HFD for Tnfα), our analysis revealed a main effect for a decrease in gene expression of Cd11c, Il-1β, Il-6, and Tnfα with exercise, whereas F4/80 expression was not different (Fig. 10A). The phosphorylation of c-Jun NH2-terminal kinases (JNK) was increased with HFD (Fig. 10, B and C) and tended to be decreased with exercise ($P = 0.058$). However, IκB kinase (IKK) phosphorylation (Fig. 10, B and D) and nuclear factor-κ light-chain enhancer of activated B cells (NF-κB) protein expression (Fig. 10, B and E) were unaltered by HFD or exercise. Because IL-1β mRNA levels were decreased with exercise, we investigated protein markers of inflammasome activation. Caspase 1, NLRP3, and IL-1β themselves were unaltered by either diet or exercise (Fig. 10, F and G). Together, although there was only a modest inflammatory phenotype in the hepatocytes of this mouse model, the results suggest an ability of exercise to dampen hepatic inflammation pathways.

**DISCUSSION**

In this study, we demonstrate that specific hepatic lipid species accumulation is ameliorated by exercise training. Moreover, exercise training resulted in a generalized decrease in markers of inflammation and cell damage in high-fat fed animals. Interestingly, these effects were independent of any corresponding changes in intramuscular lipid levels. Together, our data provide evidence that exercise training may be a viable therapeutic intervention to prevent or at least slow the progression of NAFLD associated with nutrient overload.

An important finding from the present study was the increase observed in the PC/PE ratio in the liver that accompanied exercise training. PC and PE are major phospholipids in mammalian membranes and contribute to the regulation of membrane fluidity. This ratio is a key regulator of cell membrane integrity, and a decrease in the ratio plays a role in the progression of hepatic steatosis to steatohepatitis (25). It is likely that abnormally high or low levels of the PC/PE ratio can be detrimental in the setting of NAFLD and that maintenance of a balance between these metabolites is potentially a key factor in preventing the incidence of NAFLD and preventing deterioration to NASH (22). We observed a HFD-induced decrease in this ratio that was offset by an increase in the exercise groups. Consequently, exercise may be one way in which a PC/PE balance may be restored.
Numerous DAG species were elevated in the liver with the HFD and improved with exercise (Fig. 6E). The particular species that were altered coincide with the findings of Gorden et al. (14), who analyzed the DAG species in the livers of C57Bl/6 mice fed a HFD for 12 wk and the DAG species from biopsies obtained from patients with hepatic steatosis. All seven DAG species (similar fatty acid composition) that we present to be elevated in the current study were also found to be elevated in the high-fat fed livers in this previous study (14). Furthermore, four of these seven species were also found to be significantly elevated in the human biopsy samples, indicating that a HFD provides a reproducible model of NAFLD with regard to DAG species accumulation (14). Therefore, the improvements observed with exercise training may have a significant relation to the human condition. Interestingly, exercise training may also impact upon cholesterol homeostasis in the liver, as there was a decrease in accumulated cholesterol esters, which have previously been shown to be increased with high-fat feeding (3). Together, these changes in hepatic lipid levels correlated with the overall health of the liver given the marked improvement in liver function as measured by AST and ALT levels (Fig. 2, H and I).

In the current study, we observed no effect of exercise on skeletal muscle HFD-induced lipid accumulation. This could be due to a number of possibilities. The myocytes could be having their lipid replenished due to a 1:1 replacement of lipid oxidized during exercise, with lipid replaced via fatty acid uptake due to the ample availability of the lipid supplied in the HFD. Although the lipid levels in the skeletal muscle were not altered, an increase in lipid flux through the muscle may be the

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**Fig. 8. Lipidomic analysis of hepatic cholesterol ester and cardiolipin in Sed or Ex mice.** A–D: hepatic total cholesterol ester (A), hepatic total cardiolipin (B), individual hepatic cholesterol ester molecular lipid species (C), and individual hepatic cardiolipin molecular lipid species (D) (all relative to NC Sed = 1); n = 7–10/group. *P < 0.05, **P < 0.01, and ***P < 0.001 for dietary effect; †P < 0.05 and ††P < 0.01 for exercise effect. Data are means ± SE.
mechanism via which the liver is protected. The muscle may simply act as a lipid sink, sequestering circulating fatty acids and glucose away from the liver. In this respect, improved muscle insulin sensitivity may also be playing a role. The reversal of muscle insulin resistance with one bout of exercise has been shown to decrease postprandial hepatic de

Alternatively, another possibility exists whereby the contraction of the skeletal muscle may be initiating tissue cross-talk, where a secreted substance from the muscle, a “myokine,” may be released and then act directly on the liver. For instance, the myokine may signal to the hepatocytes to decrease the levels of the fatty acid transporters CD36 and FATP4 to provide more availability of fat to the skeletal muscle or stimulate a coordinated reduction in inflammatory gene profile. Recently, a liver-muscle axis was described whereby a hepatic de novo lipogenesis-derived lipid metabolite (PC 18:0_18:1) mediates interorgan communications between the liver and the skeletal muscle (26). This PPARδ-dependent hepatic pathway utilizes a circulating lipid (PC 18:0_18:1) to modulate fat use by the muscle and thus coordinates metabolic function between the organs (26). Given these findings of communication from the liver to the skeletal muscle, it is plausible that the exercising skeletal muscle may also communicate to the liver via a similar axis. Additional specific studies would be needed to “capture” the release of such a myokine or lipokine to prove this hypothesis.

Lipid accumulation in hepatocytes increases the liver’s susceptibility to injury by oxidative stress and inflammatory cytokines (11). Indeed, the “two-hit” hypothesis was coined to describe the temporal changes associated with the development of NASH. The first “hit,” the development of liver steatosis,
increases susceptibility of the liver to injury mediated by “second hits” such as cytokines and mitochondrial dysfunction (13). Although it must be noted that our HFD intervention was not of sufficient duration to increase hepatic inflammatory markers, there was an exercise-induced general decrease in many of these markers. Thus, the possibility exists that in disease conditions where inflammatory pathways are elevated in the liver, exercise may be useful in decreasing inflammatory tone.

A limitation of the current study is that only one type (running) and intensity (50–80% of max) of exercise was examined and for a short duration of time (4 wk). Whether different exercise or exercise of less intensity exerts similar effects over time (such as resistance exercise or walking) is unknown. Encouragingly, resistance exercise training in a group of patients with NAFLD reduced total liver fat independent of weight loss (16), suggesting that multiple exercise types may be beneficial in the setting of NAFLD.

In conclusion, our data suggest that exercise training is an important component of the treatment regime for the prevention of NAFLD progression. This is important considering that NAFLD has been described as “the next global epidemic” (33). Future studies may wish to investigate various exercise training principles to elucidate the most effective types of exercise in terms of duration, intensity, and frequency in relation to its impact on hepatic lipid levels. Furthermore, because exercise compliance is the limiting factor to exercise prescription, the effectiveness of each intervention and method of delivery should be studied to ensure that the exercise training will be conducted frequently enough to benefit the liver.

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