RESEARCH

Impact of exenatide on mitochondrial lipid metabolism in mice with nonalcoholic steatohepatitis

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Abstract

Exenatide (Exe) is a glucagon-like peptide (GLP)-1 receptor agonist that enhances insulin secretion and is associated with induction of satiety with weight loss. As mitochondrial dysfunction and lipotoxicity are central features of nonalcoholic steatohepatitis (NASH), we tested whether Exe improved mitochondrial function in this setting. We studied C57BL/6J mice fed for 24 weeks either a control- or high-fructose, high-*trans*-fat (TFD)-diet (i.e., a NASH model previously validated by our laboratory). For the final 8 weeks, mice were treated with Exe (30 µg/kg/day) or vehicle. Mitochondrial metabolism was assessed by infusion of [$^{13}C_3$]propionate, [3,4-$^{13}C_2$]glucose and NMR-based ^{13}C-isotopomer analysis. Exenatide significantly decreased fasting plasma glucose, free fatty acids and triglycerides, as well as adipose tissue insulin resistance. Moreover, Exe reduced 23% hepatic glucose production, 15% tri-carboxylic acid (TCA) cycle flux, 20% anaplerosis and 17% pyruvate cycling resulting in a significant 31% decrease in intrahepatic triglyceride content ($P = 0.02$). Exenatide improved the lipidomic profile and decreased hepatic lipid byproducts associated with insulin resistance and lipotoxicity, such as diacylglycerols (TFD: 111 ± 13 vs Exe: 64 ± 13 µmol/g protein, $P = 0.03$) and ceramides (TFD: 1.6 ± 0.1 vs Exe: 1.3 ± 0.1 µmol/g protein, $P = 0.03$). Exenatide lowered expression of hepatic lipogenic genes (*Srebp1C*, *Cd36*) and genes involved in inflammation and fibrosis (*Tnfa*, *Timp1*). In conclusion, in a diet-induced mouse model of NASH, Exe ameliorates mitochondrial TCA cycle flux and significantly decreases insulin resistance, steatosis and hepatocyte lipotoxicity. This may have significant clinical implications to the potential mechanism of action of GLP-1 receptor agonists in patients with NASH. Future studies should elucidate the relative contribution of direct vs indirect mechanisms at play.

Key Words

- nonalcoholic fatty liver disease
- liver metabolism
- insulin resistance
- lipidomics

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by intrahepatic triglyceride accumulation, hepatic insulin resistance and dysregulated mitochondrial metabolism. About 70% of patients with obesity or type 2 diabetes mellitus (T2DM) have NAFLD (Rinella 2015, Bril & Cusi 2016, Cusi 2016), and as many as ~30–50% may develop the more severe form of the disease known as nonalcoholic steatohepatitis (NASH), characterized by hepatocyte necrosis (ballooning) and lobular inflammation, and often with progressive fibrosis (Cusi 2012). In the setting of obesity or diabetes, NASH is frequently associated with fibrosis and a more rapid progression of fibrosis. Therefore, patients that develop NASH have a high risk for cirrhosis as well as cardiovascular disease from a more unfavorable cardiovascular risk profile (Charlton *et al.* 2011).

Abnormal mitochondrial oxidative metabolism is a central feature for the transition from isolated steatosis to NASH (Sunny *et al.* 2010, Satapati *et al.* 2012). Hepatic insulin resistance and inflammation, two key components of NASH, are closely associated with alterations in mitochondrial oxidative metabolism (β-oxidation, TCA cycle and mitochondrial respiration) in several rodent models of NAFLD (Satapati *et al.* 2012, 2015, Patterson *et al.* 2016, Kalavalapalli *et al.* 2018). In humans, several studies have reported abnormal mitochondrial fatty acid oxidation and ATP generation in NAFLD using different techniques (Sunny *et al.* 2017) and even with complex measurements of TCA cycle activity (Sunny *et al.* 2011) and in direct tissue assessments of mitochondrial function (Koliaki *et al.* 2015). However, the underlying mechanisms by which these alterations in mitochondrial metabolism mediate the progression of the disease are not well understood. It appears that chronic induction of mitochondrial oxidative flux can drive reactive oxygen species (ROS) generation and inflammation in NASH (Satapati *et al.* 2015). As shown earlier by our laboratory, despite elevated TCA cycle activity, lipotoxic intermediates from incomplete fat oxidation (ceramides and diacylglycerols) can accumulate in mice models of NASH (Patterson *et al.* 2016).

Among the many approaches under investigation for NASH, glucagon-like peptide-1 receptor agonists (GLP-1RAs) have shown a significant promise for the treatment of NAFLD (Ding *et al.* 2006, Blonde & Russell-Jones 2009, Cusi 2012, Armstrong *et al.* 2013, 2016*a*,*b*, Van Can *et al.* 2014, Xu *et al.* 2014, Abdul-Ghani *et al.* 2017). Exenatide, a glucagon-like peptide-1 receptor agonist, binds to and activates the GLP-1 receptor to enhance glucose-dependent insulin secretion, glucose-dependent suppression of inappropriately high glucagon secretion, slow of gastric emptying and induce satiety with induction of weight loss (Amori *et al.* 2007, Xu *et al.* 2014, Dutour *et al.* 2016, Dhir & Cusi 2018). They have shown beneficial effects in rodent models of NASH (Trevaskis *et al.* 2012, Xu *et al.* 2014). In a meta-analysis of 6 RCT LEAD (Liraglutide Efficacy and Action in Diabetes) trials, a significant decrease in plasma aminotransferase and hepatic steatosis (assessed by CT scan) was observed at the higher dose of liraglutide (1.8 mg), an effect closely related to the magnitude of weight loss (Armstrong *et al.* 2013). Several small uncontrolled studies have reported benefit with GLP-1RAs in NAFLD (Jendle *et al.* 2009, Eguchi *et al.* 2015, Dong *et al.* 2017). Significant liver histological benefit was observed in patients with biopsy-proven NASH treated with liraglutide for 48 weeks (Armstrong *et al.* 2016*a*). However, the exact mechanisms of GLP-1RAs on liver mitochondrial function and hepatic steatosis remain incompletely understood.

The objective of this study was to examine if exenatide treatment could improve hepatic glucose and mitochondrial metabolism in a high-fructose, high *trans*-fat diet mice model of NASH. We hypothesized that exenatide treatment in mice with NASH will alleviate distinctive hepatocyte mitochondrial defects, such as increased TCA cycle flux and the accumulation of lipotoxic intermediates.

Materials and methods

Materials and reagents

[3,4-$^{13}C_2$]glucose (98%) was purchased from Omicron Biochemicals (South Bend, IN, USA). [U-^{13}C]propionate was purchased from Cambridge Isotopes (Andover, MA, USA). Internal standards, including ceramide (d18:1/17:0) and d5-DG Internal standard mixture I were purchased from Avanti Lipids (Alabaster, AL, USA). Other common chemicals were obtained from Sigma.

Animals and diets

Mouse studies were approved by the Institutional Animal Care and Use Committee at University of Florida. Male C57BL/6 mice were obtained from Jackson Laboratories at ~7 weeks of age and the diets were purchased from Research Diets. Mice were randomly assigned to a control group (C; 10% Kcal fat; Research Diets, Inc. #D09100304; n=8) or a high-fructose high trans-fat diet

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(TFD; 40% Kcal fat, 20% Kcal fructose, 2% cholesterol; Research Diets, Inc.# D09100301; $n=20$). Mice were kept on the above diets for a period of 24 weeks, the period of time needed for the TFD diet-fed mice to develop NASH, as validated earlier by Clapper *et al.* (Trevaskis *et al.* 2012, Clapper *et al.* 2013) and in prior by our laboratory (Patterson *et al.* 2016, Kalavalapalli *et al.* 2018). Eight weeks prior to the metabolic studies with stable isotope infusions, mini-osmotic pumps (ALZET osmotic pumps, Cupertino, CA, USA) were implanted subcutaneously into all animals (C and TFD groups). Control mice received 10% DMSO in saline as vehicle and TFD-fed mice were randomly assigned to either vehicle or exenatide (Exe; 30 µg/kg per day in 10% DMSO) (Mack *et al.* 2006) by continuous infusion for the next 8 weeks. The mini-osmotic pump was replaced at 4 weeks (maximal duration of use) and a second one implanted for the last 4 weeks of the study. After a total of 8 weeks of drug delivery starting at 16 weeks on either diet, stable isotope infusions and metabolic analysis were performed at 24 weeks.

In vivo stable isotope infusion

Five days prior to the hepatic flux analysis, a jugular vein catheter was implanted into the mice for the infusion of stable isotopes. Following an overnight fast (12 h; consistent with our prior studies), mice were infused a mixture containing [$^{13}C_3$]propionate to determine mitochondrial fluxes and [3,4-$^{13}C_2$]glucose to determine endogenous glucose production (EGP) for 90 min. Upon completion of the infusion, blood was collected by exsanguination under anesthesia and processed for ^{13}C-nuclear magnetic resonance (NMR)- based isotopomer analysis (Satapati *et al.* 2012). Tissues were flash frozen in liquid nitrogen and stored at −80°C until further analysis.

Analysis of mitochondrial TCA cycle metabolism and EGP by NMR

Blood glucose was converted to the 1,2-isopropylidene glucofuranose derivative (mono acetone glucose, MAG). MAG was analyzed by ^{13}C isotopomer analysis on a 600-MHz, Agilent NMR spectrometer. Peak areas were analyzed using 1D NMR software ACD/Labs 9.0 before metabolic analysis as reported previously (Satapati *et al.* 2012). The isotopomer analysis of the multiplets arising from ^{13}C labeling of carbon-2 of glucose was used to determine direct functional activity of hepatic TCA cycle metabolism, including gluconeogenesis, mitochondrial anaplerosis and TCA cycle flux. These relative mitochondrial fluxes were converted to absolute fluxes by normalizing with EGP (Satapati *et al.* 2012, Patterson *et al.* 2016).

Analysis of lipids by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Approximately 20–25 mg fine powdered mice liver tissue was used for lipidomic analysis by LC-MS/MS. Relative concentrations of various lipid classes including triacylglycerols (TGs), diacylglycerol (DAGs), ceramides (Cer), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were determined as described previously (Koelmel *et al.* 2017). In brief, liver tissue was homogenized with ceramic beads along with the internal standards, Cer (d18:1/17:0) and d5-DG internal standard mixture I, in chloroform: methanol (2:1, v/v). Lipids were extracted by folch extraction (Folch *et al.* 1957) for the determination of metabolite concentrations. Metabolites were quantified by peak area comparison to their respective or a representative internal standard. Hepatic lipids were identified with heated electrospray ionization probe (HESI II) and a Q-Exactive Orbitrap (Thermo Scientific) was used to acquire mass spectra in full scan mode using data-dependent top5 analysis (ddMS2 -top5) in both positive and negative polarity. Feature processing was done by MZmine 2.0 and lipids were identified using LipidMatch (Pluskal *et al.* 2010, Koelmel *et al.* 2017). Only exact mass of the MS/MS fragments was used for matching. After identification, lipids were quantified using LipidMatch Quant, which uses the closest eluting standard representative of a lipids class for relative quantification of each respective feature. Both LipidMatch and LipidMatch Quant are available at http://secim.ufl.edu/secim-tools/.

Gene expression analysis

As previously reported (Patterson *et al.* 2016), frozen liver was ground to fine powder in liquid nitrogen. Total mRNA was extracted from liver tissue by TRIzol method and converted to cDNA using cDNA Reverse Transcription kit (Bio-Rad, iScript cDNA synthesis kit). 25 ng cDNA was amplified by quantitative real-time PCR (CFX Real-Time system, Bio-Rad, C1000 Touch Thermal Cycler) using SYBR GreenER qPCR SuperMix. Primers were purchased from IDT (Integrated DNA Technologies, Iowa). The comparative threshold method was used to determine the relative mRNA levels. Gene expression was normalized to cyclophilin b (*Ppib*) which was used as the internal control. Primer sequences available upon request.

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Biochemical measurements

Fasting plasma total ketone and free fatty acid (FFA) concentrations were determined using an analytical kit (Wako Chemicals, Richmond, VA, USA). Fasting plasma insulin was measured by enzyme linked immunoassay using the mouse Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA). Plasma and liver triglyceride concentrations were determined using an analytical kit (Sigma). Plasma cytokine concentrations of IL1b and TNFa were determined using mouse cytokine magnetic bead panel kit purchased from Milliplex/Millipore Corporation. All assays were performed according to the manufacturer's instructions.

Statistics

Data were expressed as means ± S.E., and differences between groups were analyzed using one-way ANOVA and *post hoc* analysis adjusted for Bonferroni's multiple comparisons test. Comparisons between two groups were done using an unpaired Student's *t*-test and were considered significantly different at $P \leq 0.05$. Statistical analyses for the lipidomics data were performed in R, version 3.1.2. In order to maximize the separation and understand which variables are responsible for the detected separation between the groups we conducted a multivariate analysis for all classes of lipids using Partial Least Squares Discriminant Analysis (PLS-DA) in MetaboAnalyst 3.0. A quality of prediction (Q^2) value greater than 0.5 and an index of reproducibility of the PLS-DA model (R^2) value greater than 0.6, were considered good for the PLS-DA analysis.

Results

Exenatide administration reduces body weight, fasting plasma glucose and FFAs in mice with NASH

The metabolic characteristics of mice (C and TFD after vehicle or Exe treatment) are presented in Table 1. As expected, 24 weeks of a TFD resulted in significant weight gain. Before the administration of specific diets, body weights were similar between groups (C: 19.6 ± 0.8 g, TFD: 19.6 ± 0.7 g, Exe: 19.2 ± 0.7 g). At 16 weeks before surgery and treatment, weights were equally increased in TFD and Exe mice compared to control animals (C: 28.3 ± 0.7 g, TFD: 37.3 ± 1.1 g, Exe: 39.2 ± 1.3 g). Administration of exenatide for 8 weeks reduced body weight and liver weight significantly. Exenatide lowered fasting plasma glucose levels in mice with NASH. Plasma triglycerides and nonesterified fatty acid levels were significantly lower in exenatide-treated mice. Taken together, these results suggest that exenatide treatment improved glucose metabolism and whole body insulin sensitivity.

Effect of exenatide treatment on hepatic glucose production and TCA cycle activity in TFD mice

We have previously reported that with the onset of NAFLD, both in rodent models (Patterson *et al.* 2016, Kalavalapalli *et al.* 2018) and in human subjects (Sunny *et al.* 2011),

Table 1 Metabolic characteristics of C57Bl/6J mice fed either control (C), high-fructose-high *trans*-fat (TFD) or the TFD mice treated with Exenatide (Exe).

	C	TFD	Exe	ANOVA *P* value
Body weight, g	26.5 ± 1.5	32.7 ± 0.7*	29.8 ± 1.2$^{\#}$	0.002
Fasting plasma glucose, mg/dL	94.0 ± 3.6	100.1 ± 5.5	82.8 ± 4.1$^{\#}$	0.041
Fasting plasma insulin, ng/mL	0.06 ± 0.01	0.19 ± 0.03*	0.16 ± 0.03$^{\$}$	0.004
HIRI, ng/mL × μmol/min	0.3 ± 0.1	1.2 ± 0.2*	0.8 ± 0.2$^{\$}$	0.002
Fasting plasma FFA, mM	0.19 ± 0.02	0.21 ± 0.02	0.15 ± 0.02$^{\#}$	0.095
Adipo-IR$_i$, mM × ng/mL	0.011 ± 0.002	0.041 ± 0.005*	0.022 ± 0.004$^{\#}$	<0.001
Fasting plasma ketones, μM	678 ± 147	1363 ± 68*	1492 ± 159$^{\$}$	<0.001
Fasting plasma triglyceride, mg/mL	0.49 ± 0.03	0.43 ± 0.01	0.37 ± 0.02$^{\#,\$}$	0.009
Plasma IL1β, pg/mL	4.6 ± 2.2	31.4 ± 4.9*	15.8 ± 2.7$^{\#,\$}$	0.001
Plasma TNFα, pg/mL	2.6 ± 0.4	6.9 ± 0.5*	5.2 ± 0.6$^{\#,\$}$	<0.001
Liver weight, g	1.6 ± 0.1	4.5 ± 0.4*	2.6 ± 0.4$^{\#}$	<0.001
% liver weight (per body)	6.3 ± 0.6	13.7 ± 1.1*	7.8 ± 1.3$^{\#}$	<0.001
Liver triglyceride, mg/g liver	145 ± 25	287 ± 23*	198 ± 27$^{\#}$	0.012
Liver protein, mg/whole liver	257 ± 6	155 ± 5*	218 ± 22$^{\#}$	<0.001

Values are mean ± S.E.M. (n = 5–12 per group). Pairwise comparisons as follow: *$P \leq 0.05$ C vs TFD; $^{\#}P \leq 0.05$ TFD vs Exe; $^{\$}P \leq 0.05$ C vs Exe.
Adipo-IR$_i$, adipose tissue insulin resistance index; FFA, free fatty acids; HIRI, hepatic insulin resistance index.

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there are increased rates of hepatic mitochondrial TCA cycle metabolism as well as of EGP which may promote disease progression from simple steatosis to NASH. Hepatic insulin resistance index (Wang *et al.* 2014) calculated as EGP × fasting plasma insulin was not significantly lower in exenatide-treated animals with NASH (Table 1). Utilizing NMR-based isotopomer analysis of plasma glucose, we investigated whether exenatide therapy could alleviate dysfunctional mitochondrial metabolism induced by a TFD diet. Consistent with previous findings (Patterson *et al.* 2016, Kalavalapalli *et al.* 2018), both mitochondrial TCA cycle activity and EGP were elevated in mice with NASH following a 24-week TFD diet (Fig. 1). An overall increase in mitochondrial TCA cycle metabolism in NASH mice was also evident from the concurrent increase in the rates of mitochondrial anaplerosis and pyruvate cycling, both pathways instrumental in fueling TCA cycle metabolism. EGP (Fig. 1A), mitochondrial TCA cycle activity (Fig. 1B), anaplerosis (Fig. 1C) and pyruvate cycling (Fig. 1D) were non-significantly reduced after exenatide treatment compared to TFD-fed animals. Of note, reductions in these parameters were strongly correlated between them (TCA flux and anaplerosis: $r=0.86$, $P<0.0002$).

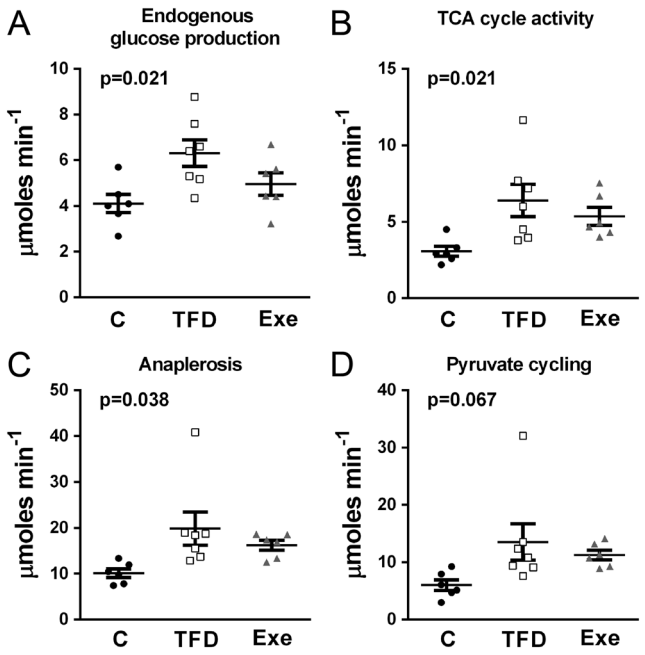

Figure 1
Endogenous glucose production and hepatic mitochondrial TCA cycle activity in mice with NASH treated with exenatide. (A) Endogenous glucose production, (B) TCA cycle flux, (C) anaplerosis and (D) pyruvate cycling were determined using ^{13}C-NMR-based isotopomer analysis. Values in scatter dot plot are represented as mean ± S.E. (n = 5–7 per group). P value is overall F statistics (ANOVA). C, Control mice; TFD, high trans-fat high-fructose fed mice; Exe, mice fed TFD diet treated with exenatide.

Exenatide treatment decreases hepatic triglyceride content and improves hepatocyte lipidomic profile of mice with NASH

In the setting of obesity, insulin-resistant adipose tissue promotes hepatic steatosis and hepatocyte injury from lipotoxicity (Cusi 2012, Lomonaco *et al.* 2012, Bril *et al.* 2017, Sunny *et al.* 2017). As exenatide treatment significantly reduced triglycerides in liver of mice with NASH (Table 1), we determined the levels of various classes of hepatocyte lipids, for example TGs, DAGs, Cer, LPC, PC, PE and PG using high-resolution LC-MS/MS. There was a trend observed in the reduction of total intrahepatic lipid content (Fig. 2A) and total TGs (Fig. 2C). Most importantly, exenatide significantly decreased accumulation of DAGs (Fig. 2E) and Cer (Fig. 2G) in comparison to mice with NASH.

We also conducted a PLS-DA analysis to illustrate the changes in total and multiple classes of lipids. These score plots of individual metabolite classes exhibited a clear separation and allowed us to understand which variables are responsible for the separation between all three groups of mice (C, TFD and Exe). All the PLS-DA models were validated and considered high relevant if the quality of prediction (Q^2) was greater than 0.5 and the reproducibility of the model (R^2) was greater than 0.6. The PLS-DA analysis of total lipids (Fig. 2B), total TGs (Fig. 2D), total DAGs (Fig. 2F) and total Cer (Fig. 2H) showed a clear separation between the groups treated with exenatide versus TFD, which was particularly striking for DAGs.

Targeted metabolic analysis demonstrated a significant reduction of several specific DAGs (Fig. 3B) and some TGs (Fig. 3A) and Cer (Fig. 3C) species following exenatide treatment. There were no significant changes observed in some of the lipid intermediates from other classes including lysophosphatidylcholines, phosphatidylcholines, phosphatidylethanolamines and phosphatidylglycerols with exenatide administration (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Fold change in different sub species of LPC, PC, PE and PG are presented in Supplementary Fig. 2. Taken together, the improvements in the lipidomic profile following exenatide treatment reflect the global decrease in hepatic lipid byproducts, most clearly of DAGs, reflecting improved mitochondrial lipid oxidation.

Exenatide reverses the activation of genes that lead to steatosis and inflammation in mice with NASH

To better understand the mechanism of action of exenatide to reduce intracellular lipid accumulation,

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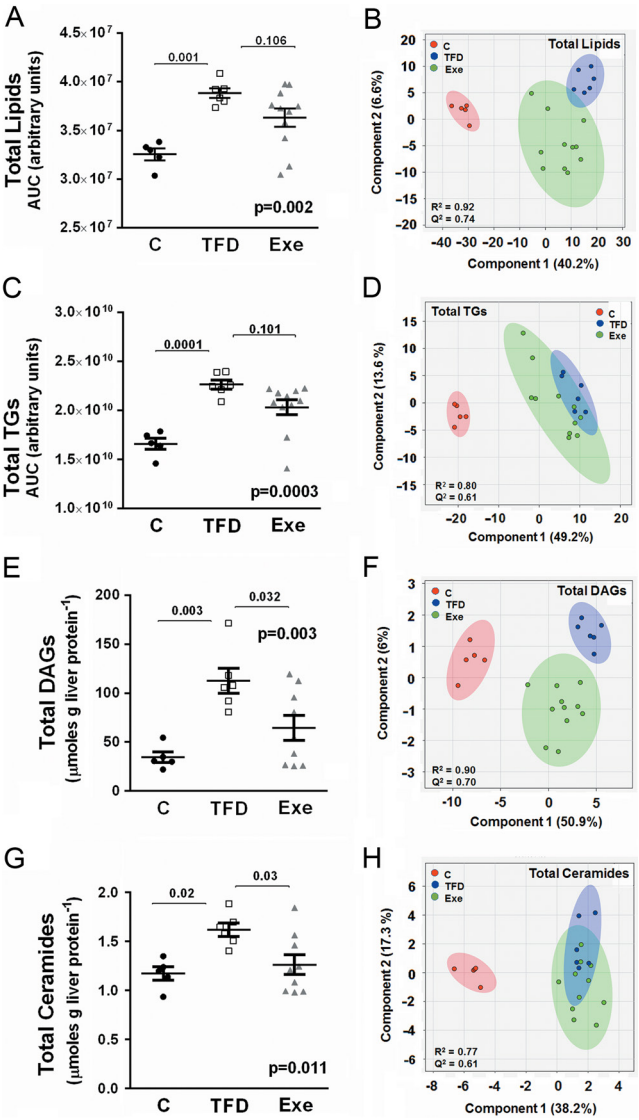

Figure 2

Exenatide administration decreases multiple lipid classes in the liver of mice with NASH. The hepatic content of each lipid class and their respective PLS-DA score plots for the three groups of mice (C ($n = 5$), TFD ($n = 6$) and Exe ($n = 11$)) are presented for (A and B) total lipids, (C and D) total triglycerides, (E and F) total diacylglycerols and (G and H) total ceramides. *P* value is overall F statistics (ANOVA) and *post hoc* analyses adjusted for multiple comparisons. C, control mice; Exe, mice fed TFD diet supplemented with Exenatide; TFD, high trans-fat high-fructose fed mice. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0007.

we measured hepatic mRNA levels of genes related to carbohydrate, lipid and mitochondrial metabolism among the three groups (Fig. 4 and Table 2). Exenatide regulated the expression of genes involved in mitochondrial fatty acid oxidation and mitochondrial biogenesis (Fig. 4A, B and C). Exenatide treatment resulted in the upregulation of peroxisome proliferator activated receptor alpha (*Ppara*) (Fig. 4A). This was associated with a significant reduction in liver triglyceride content ($P=0.02$) and an increase in liver protein content ($P=0.003$), as shown in Table 1. Concurrently, we observed in mice treated with exenatide a significant reduction in hepatocyte cluster of differentiation 36 (*Cd36*) (Fig. 4D) and a non-significant reduction in *Srebp1c* (Fig. 4E) gene expression, genes involved in fatty acid transport and lipogenesis, respectively. There was also an amelioration by exenatide administration in the expression of genes linked to inflammation and fibrosis, as indicated by significantly lower mRNA levels of tumor necrosis factor a (*Tnfa*), tissue inhibitor of metalloproteinase 1 (*Timp1*) and matrix metallopeptidase 13 (*Mmp13*) (Fig. 4G, H and I) as well as a significant reduction in plasma cytokine levels of ILβ and TNFα (Table 1). Exenatide treatment was associated with a non-significant reduction in lobular inflammation (3.0±0.0 vs 2.6±0.2, $P=0.18$) and fibrosis stages (1.8±0.2 vs 1.4±0.2, $P=0.24$) compared to TFD-fed animals. Taken together, these results suggest that exenatide treatment reduces TFD-induced defects like hepatic steatosis, inflammation and fibrosis.

Exenatide treatment reverses defects in adipose tissue gene expression in mice with NASH linked to insulin resistance

Exenatide treatment improved adipose tissue insulin sensitivity and overall function as evidenced by a ~30% reduction in plasma FFA levels and also as indicated by adipose tissue insulin resistance index (Adipo-IR_i), calculated as fasting FFA × insulin ($P=$ 0.001) (Table 1). To confirm this, we examined mRNA levels of key genes involved in mitochondrial fatty acid metabolism and inflammation in adipose tissue of control. As observed in Fig. 5, exenatide administration downregulated gene expression of forkhead box O1 (*Foxo1*), uncoupling protein 2 (*Ucp2*), *Cd36* and a non-significant reduction of long-chain acyl-CoA dehydrogenase (*Lcad*), alpha smooth muscle actin (*aSma*) and Toll-like receptor 4 (*Tlr4*) genes. Taken together, these results indicate that exenatide therapy improved overall adipose tissue function in mice with NASH.

Discussion

The mechanism(s) of action by which GLP-1RAs may reverse steatohepatitis remain incompletely understood. Animal models (Satapati *et al.* 2015, Patterson *et al.* 2016, Kalavalapalli *et al.* 2018) and human studies (Sunny

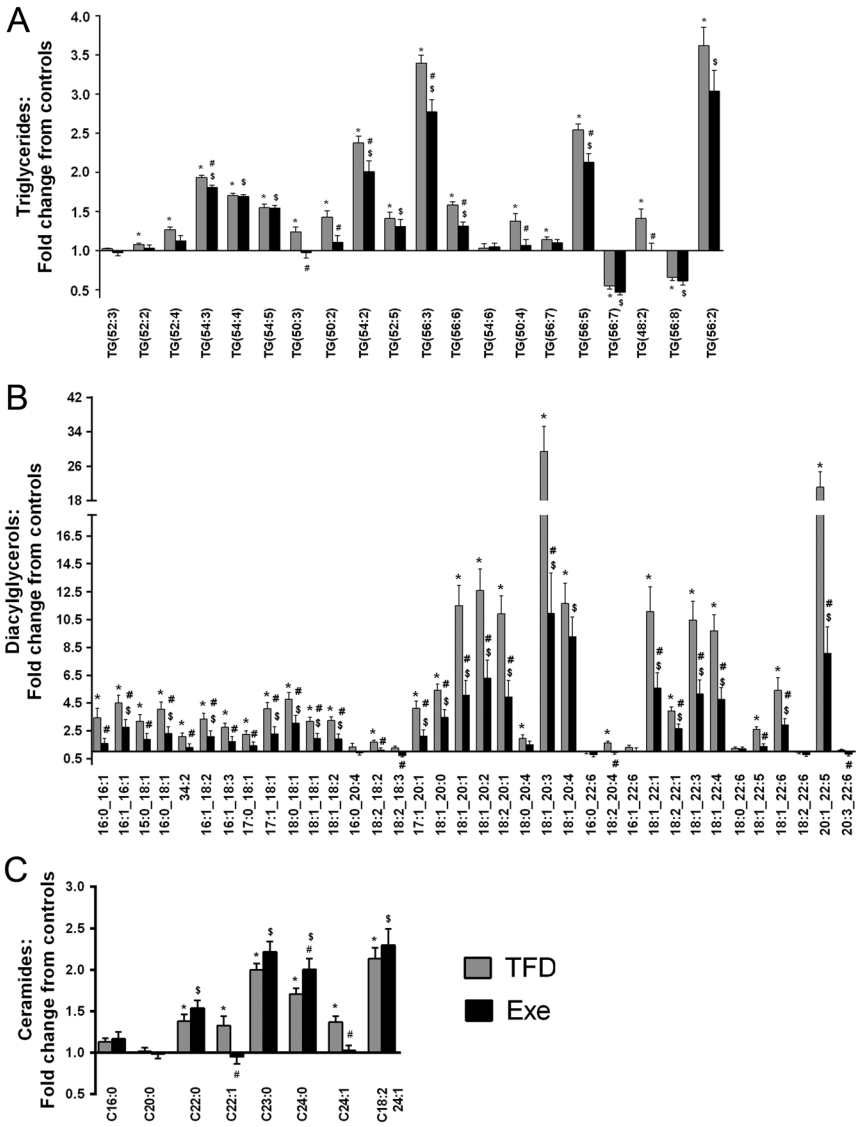

Figure 3
High-resolution LC-MS/MS-based lipidomic profiling of hepatic (A) triglycerides, (B) diacylglycerols and (C) ceramides demonstrate significant changes in several of these lipid intermediates in response to exenatide administration in mice with NASH. Values for each bars are expressed as mean fold change from the control mice average ± S.E. (n = 5–11 per group). Comparisons between the two groups were performed using unpaired Student's t-test. $^{*}P \leq 0.05$ between C and TFD, $^{\#}P \leq 0.05$ between TFD and Exe, $^{\$}P \leq 0.05$ between C and Exe. C, control mice; Exe, mice fed TFD diet supplemented with exenatide; TFD, high trans-fat high-fructose fed mice.

et al. 2011, 2017) suggest that lipotoxicity may trigger inflammation, cell death and activate fibrogenic pathways in NASH by inducing mitochondrial dysfunction. Because some GLP-1RAs have proven to be beneficial in NASH (Van Can *et al.* 2014, Abdul-Ghani *et al.* 2017, Dhir & Cusi 2018), we felt compelled to examine more carefully their role to modulate mitochondrial function in this setting and their effect on the accumulation of toxic lipid metabolites that trigger lipotoxicity. We found that in a validated diet-induced mouse model of NASH (Patterson *et al.* 2016), exenatide administration induced weight loss, improved adipose tissue insulin sensitivity as indicated by Adipo-IR_i, slightly alleviated hepatic mitochondrial oxidative TCA flux and resulted in a reduction of lipotoxic intermediates, in particular DAGs. Taken together, these results expand on the role of GLP-1RAs on liver metabolism in NAFLD and further support their clinical relevance for the management of patients with NASH.

It is well established that in NASH, dysfunctional adipose tissue promotes hepatic steatosis and drives hepatocyte lipotoxicity. Lipotoxicity occurs in parallel with elevated mitochondrial oxidative fluxes promoting high rates of ROS production and inflammation (Satapati *et al.* 2015, Sunny *et al.* 2017, Apostolopoulou *et al.* 2018). The higher rates of triglyceride accretion, inflammation and fibrosis during NASH is accompanied with chronically elevated mitochondrial oxidative flux through TCA cycle which further has the potential to uncouple hepatic TCA cycle activity from mitochondrial respiration by disrupting the mitochondrial electrochemical gradient and to impair ATP synthesis (Koliaki *et al.* 2015, Satapati *et al.* 2015, Apostolopoulou *et al.* 2018). Several animal models have

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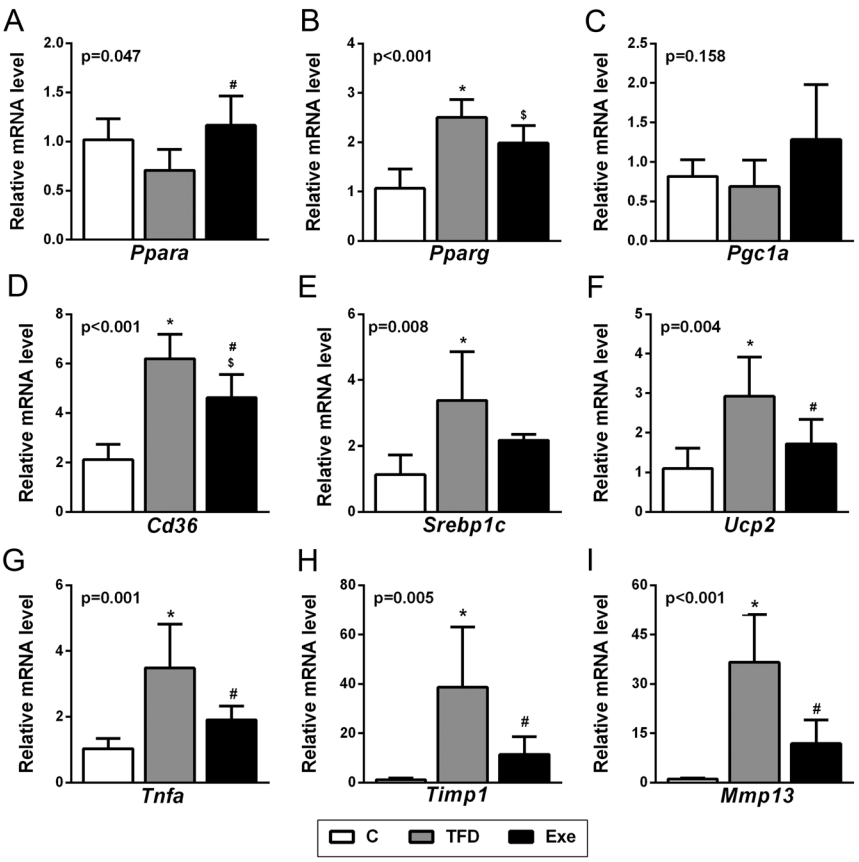

Figure 4
Trans-fat diet (TFD)-induced hepatic steatosis, inflammation and fibrosis are attenuated by exenatide treatment in mice with NASH. Changes in hepatic mRNA levels of genes related to fatty acid and lipid metabolism, inflammation and fibrogenesis in mice with NASH treated with exenatide indicates increased fat oxidation and reduced lipogenesis. (A) *Ppara*, (B) *Pparg*, (C) *Pgc1a*, (D) *Cd36*, (E) *Srebp1c*, (F) *Ucp2*, (G) *Tnfa*, (H) *Timp1* and (I) *Mmp13.* Data are represented as the mean ± S.E. (n = 5–8). P value is overall F statistics (ANOVA) and *post hoc* analyses adjusted for multiple comparisons. $^{*}P \leq 0.05$ between C and TFD, $^{\#}P \leq 0.05$ between TFD and Exe, $^{\$}P \leq 0.05$ between C and Exe. C, control mice; Exe, mice treated with exenatide; TFD, high trans-fat high-fructose fed mice.

reported positive metabolic effects of GLP-1RAs to improve hepatic insulin sensitivity and decrease steatosis, even fibrosis (Trevaskis *et al.* 2012), in animal models of diet-induced obesity (Lee *et al.* 2012, Xu *et al.* 2014, Seo *et al.* 2016). Several different mechanisms of action have been described for GLP-1 agonists, including improved insulin secretion, delayed gastric emptying, decreased appetite by central mechanisms, nausea/vomiting, among others. It is likely that a combination of the above explains the overall metabolic effects observed with these compounds, as evidenced by prior studies (Gu *et al.* 2011, Drucker 2018). It has also been proposed that there may be hepatic GLP-1 receptors that may account for, at least part of, their beneficial action in the liver (Gupta *et al.* 2010, Svegliati-Baroni *et al.* 2011). However, other investigators have been unable to reproduce these findings (Panjwani *et al.* 2013, Pyke *et al.* 2014, Jin & Weng 2016). A lowering of plasma AST/ALT and of hepatic steatosis with GLP-1RAs has been reported in several (Armstrong *et al.* 2013, Eguchi *et al.* 2015, Vanderheiden *et al.* 2016, Petit *et al.* 2017), but not all (Tang *et al.* 2015, Smits *et al.* 2016), studies. Early proof-of-concept studies reported that GLP-RAs could improve hepatic steatosis in obesity and/or diabetes (Jendle *et al.* 2009, Cuthbertson *et al.* 2012, Ohki *et al.* 2012). However, this effect appears more linked to weight loss than a specific effect on the liver. For instance, a recent review examining the effect of liraglutide in NAFLD, weight loss occurred in eight out of nine studies (Cusi 2019). In the only controlled study without weight loss, there was no change in liver steatosis on imaging (Smits *et al.* 2016). The hepatic effects appear related to indirect mechanisms linked to weight loss or changes in plasma insulin and glucagon levels, or improvement of insulin resistance (either induced by weight loss or unclear pathways). However, acute short-term studies giving exenatide that improve hepatic insulin sensitivity. For instance, exenatide infusion lowers hepatic glucose production in humans in whom insulin and glucagon secretion have been kept constant by means of a somatostatin infusion (Prigeon *et al.* 2003, Seghieri *et al.* 2013). Exenatide may also acutely ameliorate hepatic insulin resistance during an OGTT independent of changes of glucagon concentration (Gastaldelli *et al.* 2016). Therefore, we are unable to say whether the changes reported in steatosis, insulin action or TCA cycle activity by exenatide in this study may include mechanisms beyond weight loss.

In an elegant study, Armstrong *et al.* (2016*a*) reported that 48 weeks of treatment with liraglutide of patients with

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Table 2 Expression of genes related to carbohydrate, lipid and mitochondrial metabolism in liver of overnight-fasted TFD and exenatide administered mice compared to control mice.

Gene	C	TFD	Exe
Cpt1a	1.0 ± 0.06	0.60 ± 0.06*	0.80 ± 0.05$
Lcad	1.0 ± 0.10	0.82 ± 0.02	0.95 ± 0.07
Hmgcs2	1.0 ± 0.14	0.63 ± 0.09*	0.64 ± 0.05$
Fgf21	1.0 ± 0.45	1.64 ± 0.54	0.54 ± 0.09
Pepck	1.0 ± 0.17	1.18 ± 0.23	1.85 ± 0.25#,$
Foxo1	1.0 ± 0.44	0.77 ± 0.22	1.07 ± 0.36
Glut1	1.0 ± 0.13	1.52 ± 0.26	1.35 ± 0.19
Cycs	1.0 ± 0.07	0.94 ± 0.18	1.03 ± 0.10
Chrebp	1.0 ± 0.31	0.71 ± 0.16	0.87 ± 0.11
Acc1	1.0 ± 0.30	1.11 ± 0.15	0.87 ± 0.14
Acc2	1.0 ± 0.09	0.94 ± 0.16	0.71 ± 0.11$
Fasn	1.0 ± 0.16	1.15 ± 0.23	1.07 ± 0.09
Il6	1.0 ± 0.25	0.79 ± 0.14	0.79 ± 0.14
Tlr4	1.0 ± 0.64	0.73 ± 0.16	0.67 ± 0.19
PC III	1.0 ± 0.30	12.40 ± 3.37*	5.69 ± 1.65$
αSma	1.0 ± 0.16	0.97 ± 0.15	0.96 ± 0.18
Sirt1	1.0 ± 0.09	1.15 ± 0.08	1.15 ± 0.11
Bckdk	1.0 ± 0.11	0.94 ± 0.04	1.06 ± 0.07
Bckdha	1.0 ± 0.16	0.69 ± 0.08	1.10 ± 0.13#
Bcat2	1.0 ± 0.09	0.81 ± 0.13	1.01 ± 0.09

Values are means ± S.E. (n = 5–6).
*$P \leq 0.1$ C and TFD, #$P \leq 0.1$ TFD and Exe and $$P \leq 0.1$ C and Exe.

biopsy-proven NASH led to a significant improvement in liver histology, and an improvement in insulin sensitivity at the level of the liver and adipose tissue at least in a subset of patients (Armstrong *et al.* 2016*b*). GLP-1RAs reduce hepatic lipogenesis, may increase hepatic glucose uptake/glycogen synthesis and improve VLDL clearance (Ben-Shlomo *et al.* 2011, Armstrong *et al.* 2016*b*, Seo *et al.* 2016). Dutour *et al.* (2016) reported a reduction in liver and epicardial fat accumulation in patients with T2DM. More recently, using a novel dynamic PET technique, Gastaldelli *et al.* (2016) reported that exenatide may improve both hepatic and adipose tissue insulin resistance.

In prior work, we have provided evidence that a TFD diet fed for 24 weeks induces hyperglycemia, hyperinsulinemia, along with hepatic steatosis, inflammation and fibrosis in a validated animal model of NASH (Patterson *et al.* 2016, Kalavalapalli *et al.* 2018). It has also been reported that GLP-1RAs may increase fatty acid oxidation in rodents (Ding *et al.* 2006, Gupta *et al.* 2010, Ben-Shlomo *et al.* 2011, Armstrong *et al.* 2016*b*). To better understand the role of GLP-1RAs in NASH, we applied a unique stable isotope, state-of-the-art technique, to gain insights on how exenatide may work to reduce plasma glucose, FFA and plasma/hepatic triglycerides. We established for the first time that, at least in part, exenatide reverses steatosis and hepatocyte lipotoxicity from the accumulation of toxic byproducts of incomplete fatty acid metabolism (Fig. 3). The hepatic content of some of the lipid classes was reduced, in particular DAGs, as shown in their respective separation by PLS-DA score plots (Fig. 2). Accumulation of DAGs has been clearly linked with impairment of insulin signaling and hepatic insulin resistance (Birkenfeld & Shulman 2014, Szendroedi *et al.* 2014). The reduction of DAGs by exenatide and improvement in hepatic insulin action are consistent with the above and offer a better understanding to the mechanism of action of GLP1-RAs in NAFLD. The trivially decreased TCA flux, in tandem with a reduction in DAGs, appears to link improved mitochondrial function with less accumulation of toxic lipid metabolites. Of note, there was also a reduction in some, but not all, ceramide subspecies (Fig. 3C). Ceramides have been recently implicated as being associated with activation of inflammatory pathways in patients with NASH (Apostolopoulou *et al.* 2018). This may occur by ameliorating excessive rates of TCA activity, anaplerosis and pyruvate cycling, which decreased by 15–20%, although changes did not reach statistical significance. Future studies will more clearly establish the relative contribution of DAGs and ceramides, as well as TCA activity, to the natural history of necroinflammation and fibrosis in NASH.

Elevated mitochondrial TCA cycle activity has the potential to further drive pathways of ROS production and inflammation. Compared to mice with NASH, the lower EGP and hepatic TCA cycle activity induced by

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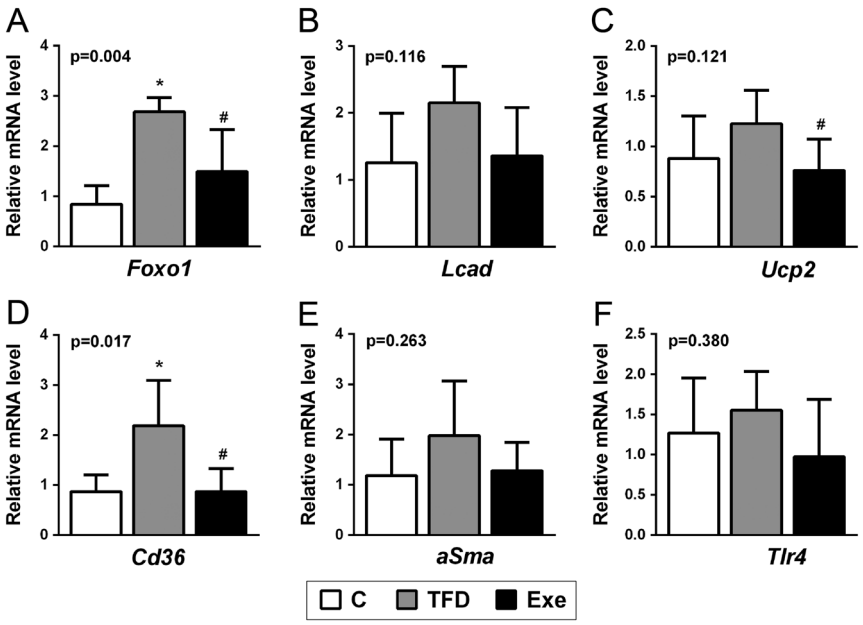

Figure 5
Abnormal adipose tissue gene expression profile is normalized by exenatide treatment in mice with NASH. Treatment with exenatide improves the expression in adipose tissue of genes involved in mitochondrial fatty acid metabolism (A) *Foxo1*, (B) *Lcad*, (C) *Ucp2*, (D) *Cd36*, (E) *aSma*, (F) *Tlr4*. Data are represented as the mean ± S.E. (n = 5–8). P value is overall F statistics (ANOVA) and post-hoc analyses adjusted for multiple comparisons. $^{*}P \leq 0.05$ between C and TFD, $^{\#}P \leq 0.05$ between TFD and Exe, $^{\$}P \leq 0.05$ between C and Exe. C, control mice; Exe, mice treated with exenatide; TFD, high trans-fat high-fructose fed mice.

exenatide was associated with changes in hepatic mRNA levels of genes related to fatty acid and lipid metabolism, inflammation and fibrogenesis (Fig. 4). Previous work in a high-fat-induced obesity mouse model has shown that Exendin-4 may reduce hepatic triglyceride accumulation via sirt1 signaling (Lee *et al.* 2012, Xu *et al.* 2014) or by activating β-catenin (Seo *et al.* 2016). Exenatide administration also significantly down regulated mRNA levels of hepatocyte lipogenic transcription factors like *Srebp1c* and *Cd36*. Finally, whether the effects of exenatide to decrease TCA flux are secondary to improved adipose tissue function with diminished FFA flux to the liver (as indicated by the decrease in plasma FFA concentration, Adipo-IR_i and adipose tissue expression of genes involved in lipid metabolism and inflammation) or rather related to a direct effect on hepatocyte signaling pathways, remains to be established.

In summary, this work indicates that exenatide ameliorated TCA flux and significantly reduced hepatic triglycerides, insulin resistance and lipotoxicity. Whether these changes are exclusively secondary to weight loss, or may be at least in part the result of a direct effect on hepatocyte mitochondrial function or other mechanisms, remains to be established. In any case, the findings led additional credence that GLP-1RAs play an important role in the future management of patients with NASH.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-19-0007.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
S K, F B, N E S and K C conception and design of research; S K, J G, A V, and N E S performed experiments; S K, T J G, and N E S analyzed data; S K, F B, J G, T J G, N E S, and K C interpreted results of experiments; S K, and N E S prepared figures; S K, and K C drafted manuscript; S K, F B, J G, A V, T J G, N E S, and K C edited and revised manuscript; S K, and K C approved the final version of the manuscript.

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