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ORIGINAL ARTICLE

Liraglutide induces beige fat development and promotes mitochondrial function in diet induced obesity mice partially through AMPK-SIRT-1-PGC1-α cell signaling pathway

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Abstract

Purpose Glucagon like peptide-1 (GLP-1) is produced to induce postprandial insulin secretion. Liraglutide, a full agonist of the GLP-1 receptor, has a protective effect on weight gain in obese subjects. Brown adipose tissue plays a major role in the control of energy balance and is known to be involved in the weight loss regulated by liraglutide. The putative anti-obesity properties of liraglutide and the cell signaling pathways involved were examined.

Methods Four groups of C57/BL6 mice fed with chow or HFHS diet were injected with either liraglutide or vehicle for four weeks. Western blotting was used to analyze protein expression.

Results Liraglutide significantly attenuated the weight gain in mice fed with HFHS diet and was associated with significant reductions of epididymal fat and inguinal fat mass. Furthermore, liraglutide significantly upregulated the expression of brown adipose-specific markers in perigonadal fat in association with upregulation of AMPK-SIRT-1-PGC1-α cell signaling. However, elevation of brown fat markers in skeletal muscle was only observed in HFHS diet fed mice after liraglutide treatment, and AMPK-SIRT-1 cell signaling is not involved in this process.

Conclusions the anti-obesity effect of liraglutide occurs through adaptive thermogenesis and may act through different cell signaling pathways in fat and skeletal muscle tissue. Liraglutide induces beige fat development partially through the AMPK-SIRT-1-PGC1-α cell signaling pathway. Therefore, liraglutide is a potential medication for obesity prevention and in targeting pre-diabetics.

Keywords Liraglutide · Insulin resistance · Beige fat · Diet induced obesity

Introduction

Obesity is currently one of the most prominent health epidemics in the U.S., affecting 36% of U.S. adults following an exponential increase in prevalence nationally over the past two decades [1]. Obesity is a risk factor for a variety of diseases including type 2 diabetes (T2D), coronary heart disease (CHD), stroke, and some cancers [2]. Due to its rapid increase in prevalence and multitude of associated health risks, obesity has become a global health concern. Encouragingly, studies have shown that losing even a small amount of weight may significantly reduce a patient's risk of developing obesity associated disease [3]. A mere 5% to 10% loss of initial body weight reduces the risk of developing cardiovascular disease (CVD), prevents or delays the development of T2D, and improves other health consequences of obesity [3, 4]. However, lifestyle and behavioral modifications to reduce body weight are often insufficient in sustaining reduced weight, and the side effects of medications for the treating obesity have barred the rapid development of safe and effective drugs [4, 5].

GLP-1 is released in response to nutrient ingestion and induces post-prandial insulin secretion (accounting for up to

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70%) [5]. Furthermore, recent studies indicate its beneficial effects in subjects with vascular disease, heart failure, and obesity with metabolic syndrome [6]. GLP-1 has a short half-life (1–2 min) however, due to rapid enzymatic degradation by dipeptidyl peptidase-IV (DPP4) and renal clearance [5]. Liraglutide is a full agonist of the GLP-1 receptor (GLP-1R) and shares a similar (97%) amino acid sequence to human GLP-1. With its amino acid addition/substitution and acylation properties, liraglutide, which is a once-daily GLP-1 derivative, has long-lasting effects and increases resistance to enzymatic degradation [7]. Liraglutide has been reported to enhance glucose-dependent insulin secretion, inhibit postprandial glucagon secretion, and is one of the recently FDA-approved GLP-1R agonists used to treat T2D [8].

Interestingly, recent reports indicate that GLP-1R agonists induce moderate weight loss [9, 10]. A recent study showed that continuous GLP-1 infusion for 6-weeks in patients with T2D led to significant weight loss and a reduction in food consumption compared to placebo-infused patients [11]. Furthermore, this study showed infusion of each of the long-acting GLP-1R agonists significantly decreased body weight in a dose-dependent manner [11]. An additional study found that GLP-1 agonist treatment reduced body weight in obese subjects with or without T2D suggesting that GLP-1R agonists may have significant therapeutic value as a weight management medication [12]. Studies have also found that the onset of obesity, hyperinsulinemia, and hyperglycemia have been delayed by liraglutide treatment [13, 14]. Taken together, these findings suggest that GLP-1 R agonist treatment may be a promising therapy for reducing or preventing obesity and metabolic syndrome. However, the underlying mechanisms for these effects are unclear.

For decades brown adipose tissue (BAT) was believed to exist only in rodents and human infants for the purpose of sustaining body temperature through heat generation mediated by activation of uncoupling protein-1 (UCP-1). However, recent studies have detected functionally active regions of BAT in human adults and BAT was inversely associated with body mass index (BMI) [15]. To date, two forms of brown fat cells have been identified. The first is the classical brown fat (iBAT) which is found in the interscapular and peri-renal depots, and the second is beige fat which is composed of UCP1-positive cells in white adipose tissue (WAT) depots [16]. It has been documented that BAT thermogenesis is a major mechanism in maintaining energy balance so expansion and/or activation of either classic BAT or beige fat thermogenesis may be a promising approach to "wasting" excess ingested energy that could be used to prevent or treat obesity and obesity-related metabolic disorders [17].

Liraglutide has been shown to stimulate BAT thermogenesis [18]; suggesting BAT thermogenesis may play a major role in GLP-1 mediated body-weight loss. Recent evidence also indicates that liraglutide increases energy expenditure by inducing adaptive thermogenesis and browning of WAT [18]. However, whether liraglutide can prevent the development of HFHS diet-induced obesity, and through which mechanisms and cell signaling pathways, has not been defined. This study is therefore designed to explore the possibility of liraglutide mediated expansion and promotion of BAT activity and to explore the regulatory role of liraglutide on adipogenesis, as well as its underlying mechanisms.

Materials and methods

Chemicals and antibodies

Liraglutide was purchased from Bachem (Torrance, CA). All other chemicals were purchased from Sigma (St. Louis, MO). Antibody resources and dilutions are listed in Table 1.

Table 1 Antibody list

Description	Species	Company	Catalog no	Dilutions
Anti-PPARγ	Rabbit	Cell signaling	2435S	1:1000
Anti-AMPKα	Rabbit	Cell signaling	5831	1:1000
Anti-Phospho-AMPKα (Thr172)	Rabbit	Cell signaling	2535P	1:1000
Anti-ACC	Rabbit	Cell signaling	3676P	1:1000
Anti-Phospho-ACC (Ser79)	Rabbit	Cell signaling	11818P	1:1000
Anti-β-Tubulin	Rabbit	Cell signaling	2146S	1:1000
Anti-β-Actin	Rabbit	Cell signaling	4967S	1:2000
Anti-LCAD	Rabbit	Cell signaling	7076P2	1:1000
Anti-Sirt-1	Mouse	Cell signaling	8469S	1:1000
Anti-AMPKβ	Rabbit	Cell signaling	4150	1:1000
Anti-Phospho-AMPKβ1 (Ser108)	Rabbit	Cell Signaling	4181P	1:1000
Anti-UCP-1	Rabbit	Cell Signaling	ab23841	1:1000
Anti-CPT-1	Rabbit	Santa Cruz	Sc-20669	1:1000
Anti-CIDE A	Rabbit	Abcam	ab8402	1:1000
Anti-PGC1α	Rabbit	Abcam	ab54481	1:1000
Anti-PRDM16	Rabbit	Abcam	ab106410	1:1000
Anti-PPAR α	Rabbit	Abcam	ab24509	1:1000
Anti-C/EBP β	Rabbit	Santa Cruz biotech	sc150	1:1000
Anti-C/EBP α	Rabbit	Santa Cruz biotech	sc61	1:1000

Animal experiments

Male C57BL/6 mice were purchased from Charles River Laboratories (Colbert, GA). Mice were housed in conventional cages in a temperature controlled room (22 ± 2 °C) on a 12-h light/dark cycle in groups of three per cage and provided ad libitum water and either global rodent chow diet or a high fat high sucrose diet (HFHS) (60% kcals from fat, 36% fat and 35.7% carbohydrate) (Harlan Teklad). Starting at 8 weeks of age, mice were randomly divided into four different groups (two groups of mice were fed with chow diet, and two groups of mice were fed with high fat high sucrose (HFHS) diet. Chow and HFHS-diet fed mice were administered intraperitoneal either vehicle (PBS) or liraglutide (0.1 mg/kg) daily for 4 weeks. Body weight, food intake, and blood glucose were measured weekly. At the end of the fourth week of treatment, body temperature was measured with a rectal probe connected to a digital thermometer (Physitemp TCAT-2LV Controller) 2 and 12 h after liraglutide injection. Mice were fasted for 12 h prior to sacrifice. Tissues collected were either immediately flash frozen in liquid nitrogen after dissection and stored at −80 °C until further processing or were fixed in 4% Paraformaldehyde (PFA) solution in PBS for 48 h before embedded in paraffin. All experimental procedures used were reviewed and approved by the Animal Care Committee at Central Michigan University.

Western blot analysis

Homogenized tissue samples from liver, skeletal muscle, and perigonadal fat were lysed in radioimmune precipitation assay (RIPA) buffer containing protease inhibitor cocktail, phenylmethynylsulfonyl fluoride, and sodium orthovanadate. Protein concentrations were measured by Bradford protein assay. Western blotting were performed following standard laboratory protocol as described previously [19]. The protein lysate was separated in SDS-PAGE and electroblotted to PVDF membrane. The membranes were then blocked and incubated with various primary and secondary HRP-linked antibodies. Signal intensity were measured by chemiluminescence. Equal protein loading was confirmed by quantifying anti-β actin or anti-β tubulin.

Blood glucose and IGTT

Intraperitoneal glucose tolerance test (IGTT) was performed in mice after overnight fasting. Mice were weighed and injected with 10% glucose (Trutol 100; Thermo Scientific, Auburn, AL) at 1 g/kg body weight intraperitoneally. The glucose concentration was measured from tail vein blood immediately before and 15, 30, 60, and 120 min post-glucose injection using a OneTouch Ultra 2 glucometer (Lifescan Inc, Milipitas CA).

Histology

Mouse tissues were fixed in 4% PFA and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) according to standard protocol. An Axiocam 506 color camera captured images with a Carl Zeiss Axio Imager M2 microscope at the magnification indicated in the figure legends. Images were compiled with the Carl Zeiss Zen 2 (blue edition) program running on Windows 10 Pro. Average adipocyte size in the adipose tissue sections (expressed as the average cross-section area per cell) was determined by using Image J software and 250–300 cells were measured in each section.

Statistical analysis

The results are expressed as the Mean ± SEM. Two-way ANOVA was used to assess drug and diet effect and interactions. *Post hoc* Tukey test was used to measure multiple group comparisons. All analyses were performed using GraphPad Prism v7.0 software (GraphPad Software Inc., La Jolla, CA). A p value of <0.05 was considered to be statistically significant.

Results

Liraglutide treated mice are resistant to HFHS diet-induced obesity

Liraglutide treatment did not attenuate the weight gain in mice fed with chow diet. However, liraglutide treated mice were resistant to HFHS diet-induced obesity. Specifically, HFHS diet-induced weight gain was attenuated by 23% ($p < 0.005$) and mice maintained similar weights as the chow diet vehicle group (Fig. 1a). Food intake was not significantly affected during four weeks of liraglutide treatment (Fig. 1b).

Liver weight and brown fat mass were not affected by four weeks of HFHS feeding or liraglutide treatment (Fig. 1c, f). However, perigonadal fat mass of HFHS diet mice injected with PBS increased 6-fold compared to chow diet PBS controls after 4 weeks of HFHS diet feeding. Liraglutide treatment maintained perigonadal fat mass and inguinal fat mass the same as the chow diet group after HFHS diet feeding (Fig. 1d, e). Perigonadal fat mass was not significantly affected by liraglutide treatment in chow diet fed mice (Fig. 1e). Similar results were also observed in inguinal fat (Fig. 1d). Detection of fat droplets in

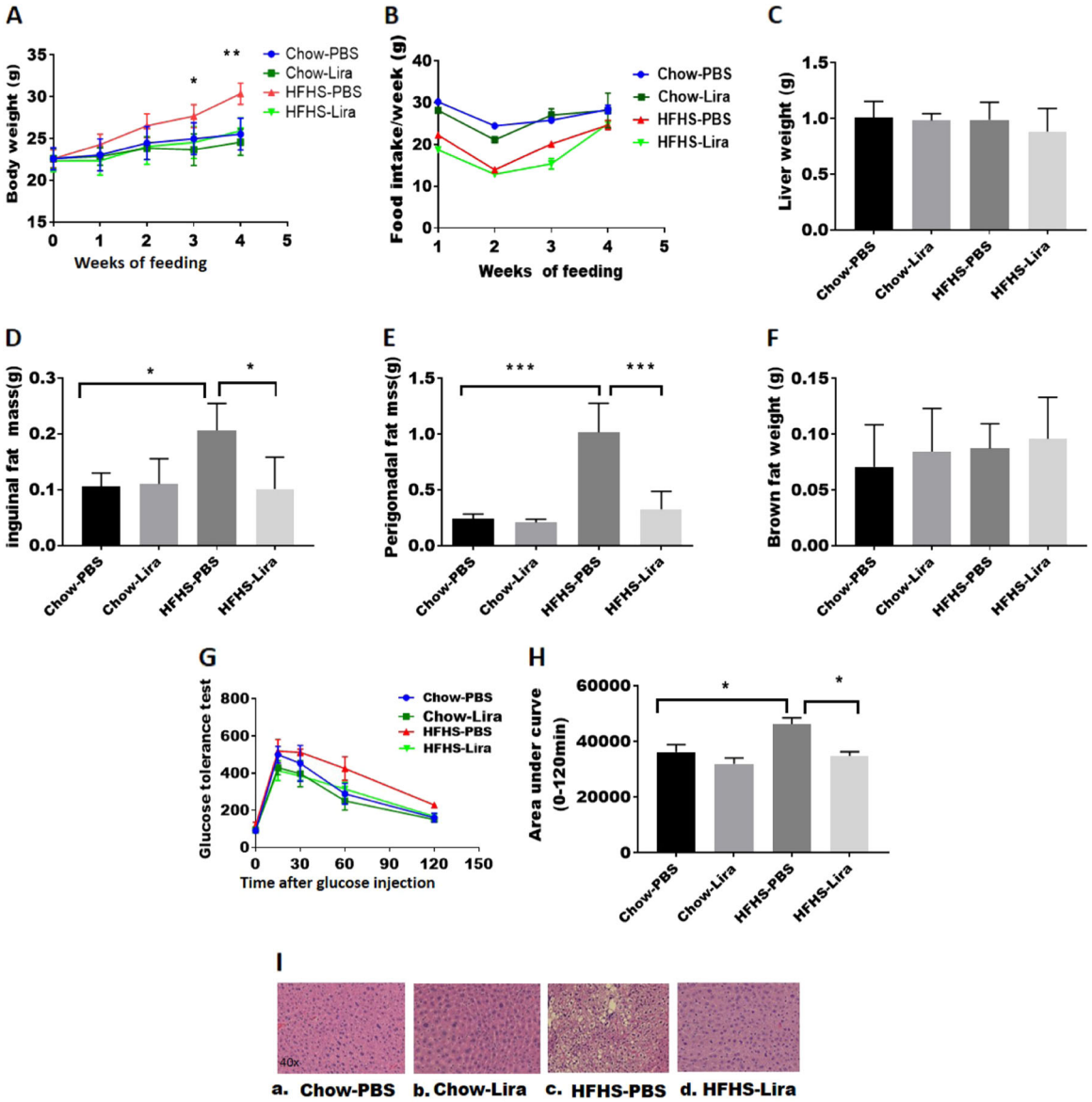


Fig. 1 Liraglutide attenuates HFHS diet-induced obesity and insulin resistance. **a** Average body weight of mice ($n = 8$); **b** Food intake (0-4 weeks post-injection) ($n = 5$); **c** Liver weight ($n = 8$); **d** Inguinal fat mass ($n = 4$), (liraglutide: $P = 0.04$, diet: $P = 0.06$, diet × liraglutide: $P = 0.03$); **e** Perigonadal fat mass ($n = 8$), (liraglutide: $P = 0.001$, diet: $P = 0.002$, diet × liraglutide: $P = 0.0022$,); **f** Brown fat mass ($n = 8$); **g** Glucose tolerance test ($n = 4$); **h** Area under curve ($n = 4$) (liraglutide: $P = 0.001$, diet: $P = 0.002$, diet × liraglutide: $P = 0.1$). I. Representative of H&E staining of mice liver tissue ($n = 4$). Data is analyzed using two-way ANOVA followed by post-hoc turkey test. Data is expressed as Mean ± SEM $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$

hepatocytes was an indication of non-alcohol fatty liver disease (NAFLD). NAFLD was developed in mice after four weeks of HFHS diet feeding, and liraglutide treatment prevents the development of NAFLD (Fig. 1, Ic-d.). After 4 weeks of HFHS diet consumption, mice developed insulin resistance (Fig. 1g, h). Liraglutide treatment significantly improved glucose tolerance test results and insulin resistance. (Fig. 1g, h). Together, these results suggest that liraglutide attenuates HFHS diet induced weight gain, improves insulin sensitivity, and prevents the development of NAFLD.

Liraglutide induces beige fat development

BAT is characterized by a high content of mitochondria that contain UCP-1. UCP-1, when activated, uncouples respiration. Recent evidence suggests white to brown fat conversion, known as “beige fat”, can occur by trans differentiation of preexisting WAT [20]. Like brown fat, beige fat also features clusters of UCP-1 expressing adipocytes and are defined by their multi-locular lipid droplet and high mitochondrial content, and expression of several brown fat-specific genes, such as cell death-inducing DNA

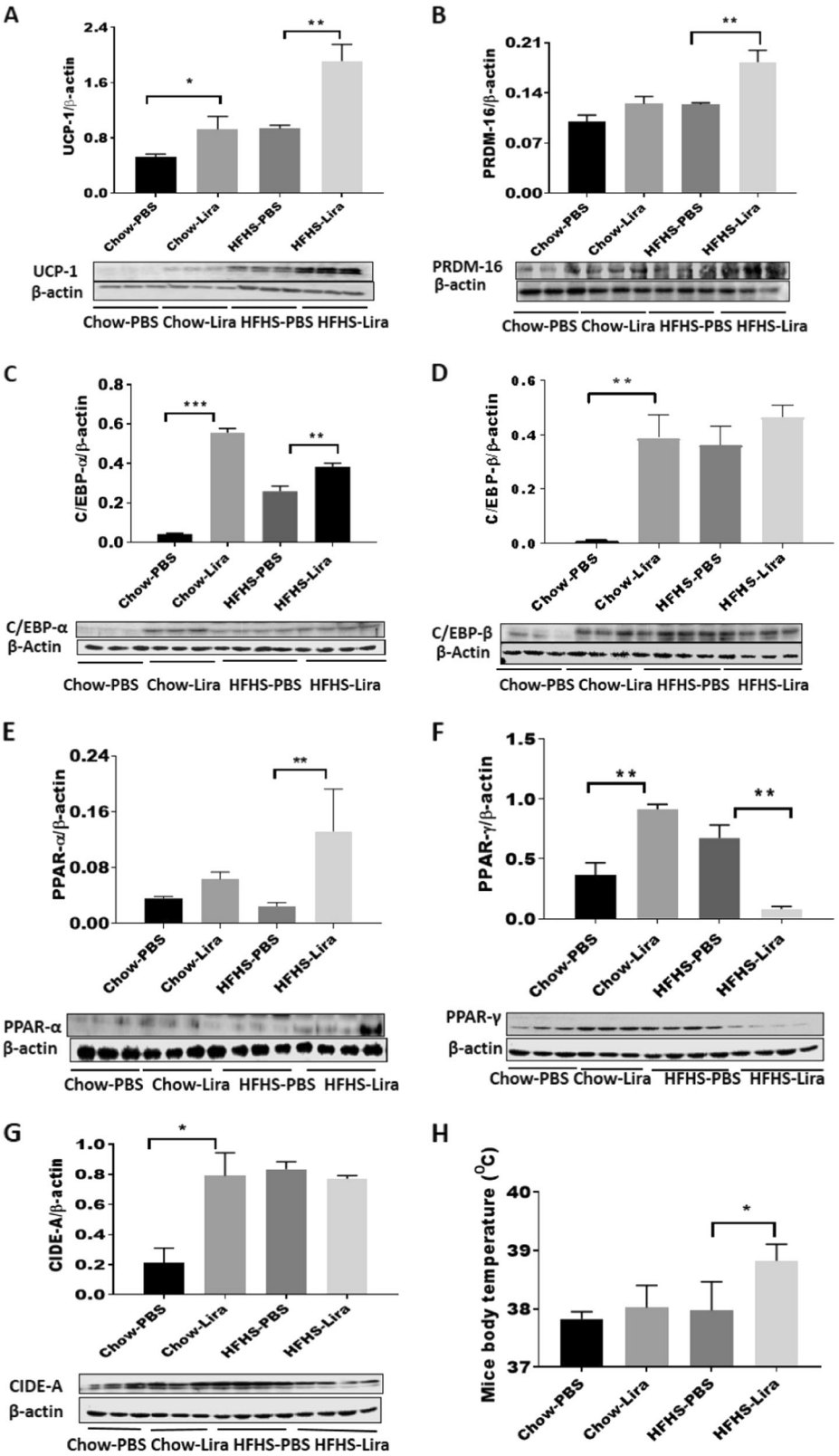
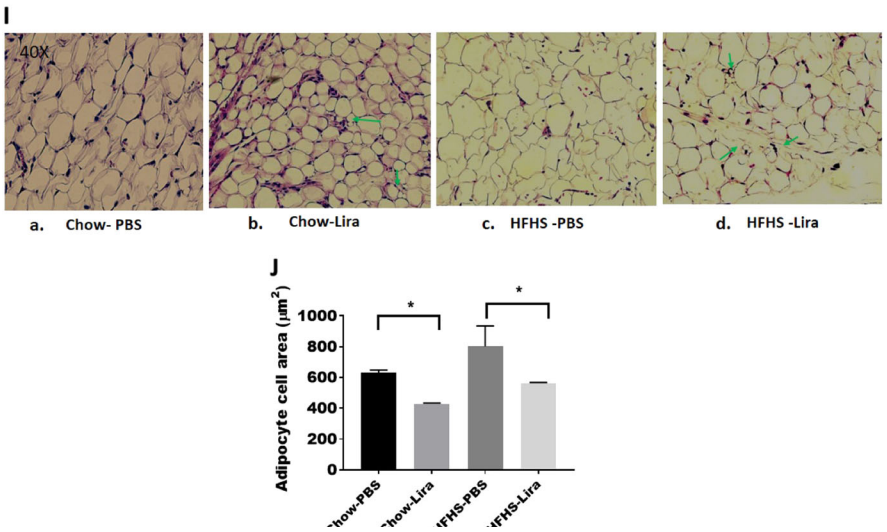


Fig. 2 Liraglutide induces beige fat development. Western blotting with β-actin as loading control assessed protein levels. Protein expression level of **a**. UCP-1 ($n = 3$ per treatment group, liraglutide: $P < 0.0001$, diet: $P < 0.0001$, diet × liraglutide: $P = 0.009$); **b** PRDM-16 ($n = 3$ per treatment group, liraglutide: $P = 0.0001$, diet: $P = 0.0002$,: diet × liraglutide, $P = 0.0262$); **c** C/EBP-α ($n = 3$ per chow diet group, $n = 4$ per HFHS diet group, liraglutide: $P < 0.0001$, diet: $P = 0.3094$, diet × liraglutide: $P < 0.0001$); **d** C/EBP-β ($n = 3$ per chow diet group, $n = 4$ per HFHS diet group, liraglutide: $P < 0.0001$, diet: $P < 0.0001$, diet × liraglutide: $P = 0.0018$); **e** PPAR-α ($n = 3$ chow diet group, $n = 4$ per HFHS diet group, liraglutide: $P = 0.0040$, diet: $P = 0.1408$, diet × liraglutide: $P = 0.05$), **f** PPAR-γ ($n = 3$ per chow diet group, $n = 4$ per HFHS diet group, liraglutide: $P = 0.8108$, diet: $P = 0.0090$, diet × liraglutide: $P < 0.0001$) **g** CIDE-A ($n = 3$ chow diet group, $n = 4$, HFHS diet group, liraglutide: $P = 0.6258$, diet: $P = 0.7596$, diet × liraglutide: $P = 0.0166$) **h** Mice body temperature after 12 h liraglutide injection ($n = 4$ per treatment group,, liraglutide: $P = 0.0630$, diet: $P = 0.0972$, diet × liraglutide: $P = 0.3147$); **i** Representative of H&E staining of mice perigonadal fat tissue ($n = 4$ per treatment group). **j** Average cell size ($n = 4$ per treatment group). Data is expressed as Mean ± SEM and is analyzed using two-way ANOVA followed by *post-hoc* Turkey test, $*p < 0.05$, $**p < 0.005$

Fig. 2 (Continued)



fragmentation factor α-like effector A (CIDE-A) and PR domain containing 16 (PRDM-1), in addition to UCP-1 [21, 22]. To examine if liraglutide induces beige fat, we measured the expression of BAT markers in perigonadal fat. As shown in Fig. 2a, b, liraglutide significantly induced the expression of both UCP-1 (by 49%, $p < 0.005$) and PRDM-16 (by 25%, $P < 0.05$) in chow diet mice, as well as in HFHS diet mice (elevated by 104% for UCP-1, and 48% for PRDM 16, respectively, $p < 0.005$). The induction of beige fat in chow and HFHS diet mice after liraglutide treatment was also detected in H & E staining (Fig 2Ib, Id).

Two transcription factors, CCAAT/enhancer-binding protein (C/EBP) family and peroxisome proliferator-activated receptors (PPARs) work sequentially and co-operatively in adipogenesis [23]. We quantified the expression of both transcription factors in order to analyze the mechanism by which liraglutide regulates BAT adipogenesis. As shown in Fig. 2c, C/EBP-α and C/EBP-β protein expression were upregulated in response to HFHS diet. Furthermore, liraglutide treatment significantly elevated C/EBP-α (by 1079 % in chow, $p < 0.001$, and 52% in HFHS diet, $P < 0.005$) and C/EBP-β (by 420% in chow diet, 27% in HFHS diet, $p < 0.005$) protein levels in both chow and HFHS diet mice (Fig. 2c, d). In a similar fashion, liraglutide significantly upregulated PPAR α by 152% in chow diet and by 50% ($p < 0.005$) in HFHS diet mice (Fig. 2e). In contrast, liraglutide treatment significantly inhibited PPARγ expression by 87.7% in mice fed with HFHS diet ($p < 0.005$), although PPARγ expression in WAT was significantly elevated in chow diet mice (Fig. 2f). CIDE-A is highly expressed in BAT and is induced in WAT after cold exposure [24]. Therefore CIDE-A is used as a distinct marker to distinguish BAT from WAT [25], although its precise role is not fully understood. CIDE-A expression was significantly increased in chow diet mice after liraglutide treatment, however, the expression of CIDE-A protein in HFHS diet mice was not changed (Fig. 2g). Taken together, these data suggest that liraglutide promotes brown adipogenesis in WAT in both chow and HFHS diet mice. Liraglutide inhibited lipogenesis when challenged with HFHS diet.

Adipocyte hypertrophy in visceral adipocyte tissue (VAT) is closely associated with systemic insulin resistance and metabolic syndrome in humans [27]. Specifically, hypertrophic adipocytes with large unilocular lipid droplets exhibit impaired insulin-dependent glucose uptake, closely associated with insulin resistance, a primary determinant of adipose tissue insulin resistance in early stage of obesity [26]. The cell size of each adipocyte was therefore measured and HFHS control mice had enlarged cell size compare to the PBS injected chow diet mice. Liraglutide treatment reduced cell size by 29% ($p < 0.05$) in HFHS fed mice (Fig. 2j).

Similar to classical brown fat cells, beige fat development is also associated with highly activated metabolism and thermogenesis [27]. Mouse body temperature was measured 2 h and 12 h after liraglutide injection. No changes of rectal temperature were observed 2 h post injection however, an elevation of body temperature (0.90 °C) was observed in liraglutide treated HFHS diet mice 12 h after liraglutide injection (Fig. 2h, $n = 4$, $p < 0.05$, unpaired t-test). These results indicate that liraglutide elevated basal metabolic rate in HFHS diet mice.

Liraglutide promotes mitochondrial function in WAT partially though AMPK-SIRT-1-PGC1α pathway

Since mitochondrial biogenesis is a hallmark of WAT differentiation to beige adipose tissue, we next investigated the effect of liraglutide on mitochondrial biogenesis in WAT by

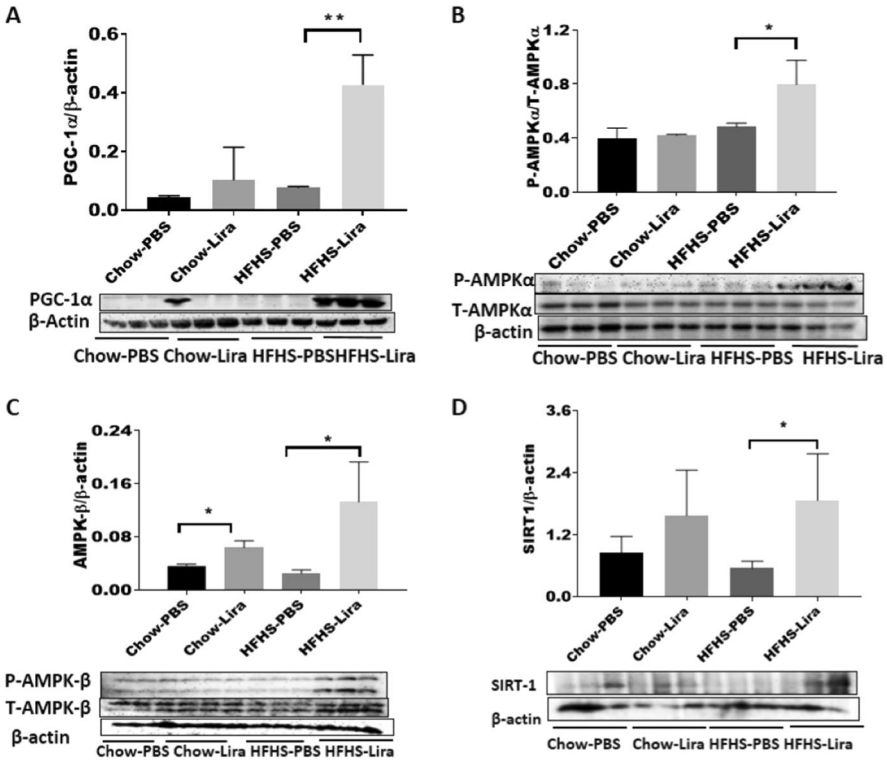


Fig. 3 Liraglutide activates AMPK-SIRT-1-PGC-1α cell signaling pathway in WAT. Protein level was assessed with western blot analyses with β-actin as loading control. Protein expression level of **a**. PGC-1α (liraglutide: $P = 0.0015$, diet: $P = 0.0034$, diet × liraglutide: $P = 0.0097$); **b** AMPK-α (liraglutide: $P = 0.018$, diet: $P = 0.0035$, diet × liraglutide: $P = 0.034$) **c** AMPK-β liraglutdie: $P = 0.004$, diet: $P = 0.1272$, diet × liraglutide $P = 0.053$); **d** SIRT-1 (liraglutide: $P = 0.004$, diet: $P = 0.977$, diet × liraglutide: $P = 0.298$). Data is expressed as Mean ± SEM values. $N = 3$ per chow diet group, $n = 4$ per HFHS diet group. Data is analyzed using two-way ANOVA followed by *post-hoc* Turkey test, $*p < 0.05$, $**p < 0.005$

measuring expression of mitochondrial biogenesis markers. PPARγ coactivator-1α (PGC-1α) is a master regulator of mitochondrial biogenesis and oxidative phosphorylation induced mitochondrial biogenesis through interactions with, and co-activation of, several different transcription factors including PPARs [28]. As shown in Fig. 3a, expression of PGC1-α was significantly elevated (5.56-fold, $p < 0.005$) following liraglutide treatment in HFHS diet mice compared to HFHS diet control mice, suggesting an improvement of mitochondrial biogenesis induced by liraglutide and its crucial role in preventing weight gain when challenged with HFHS diet.

To further explore the regulatory role of liraglutide on mitochondrial function and related cell signaling pathways in WAT, AMP-Activated Protein Kinase (AMPK) and Sirtuin-1 (SIRT-1), two key regulators of mitochondrial metabolism and biogenesis, were also examined. Liraglutide significantly induced the phosphorylation of AMPK α (increased by 65.39% $p < 0.05$) (Fig. 3b), AMPK-β (increased by 35.35% $p < 0.005$) (Fig. 3c) and SIRT-1 (increased by 152.8%, $p < 0.05$) (Fig. 3d) in HFHS diet mice. However, HFHS diet alone did not significantly affect the expression of these three genes in perigonadal fat. Taken together, lirglutide promotes mitochondrial biosynthesis and improves mitochondrial function partially through AMPK-SIRT-1- PGC-1α cell signaling pathway in WAT in response to HFHS diet challenge.

Liraglutide promotes fatty acid uptake in mitochondria in response to HFHS diet feeding in WAT

The carnitine palmitoyltransferase-1 (CPT-1) is a rate-limiting enzyme that shuttles fatty acid and promotes the uptake of fatty acids to mitochondrial matrix for β-oxidation [29]. Long chain Acyl CoA dehydrogenase (LCAD) is a key enzyme in the mitochondria responsible for β-oxidation of unsaturated fatty acid [30]. Similarly, Acetyl-CoA carboxylase (ACC) is a key enzyme for fatty acid biosynthesis and degradations. Deficiency in either CPT-1 or LCAD have been linked to diet induced obesity and insulin resistance [19]. We therefore examined the ability of liraglutide to regulate the fatty acid oxidation enzymes. Interestingly, as shown in Fig. 4a, liraglutide treatment reduced CPT-1 protein levels (by 42%, $p < 0.05$) in WAT of chow diet mice when compared to chow-PBS control. However, CPT-1 expression was significantly enhanced (by 24%, $p < 0.005$) in HFHS diet mice following liraglutide treatment. HFHS diet alone induced the expression of LCAD (Fig. 4b). Liraglutide elevated expression of LCAD in chow diet mice but had no significant effect on LCAD in HFHS diet mice (Fig. 4b). Similarly, phosphorylation of ACC was elevated in chow diet mice after liraglutide treatment, but no change was observed in HFHS diet mice group. These results suggest that liraglutide favors lipogenesis in WAT of chow

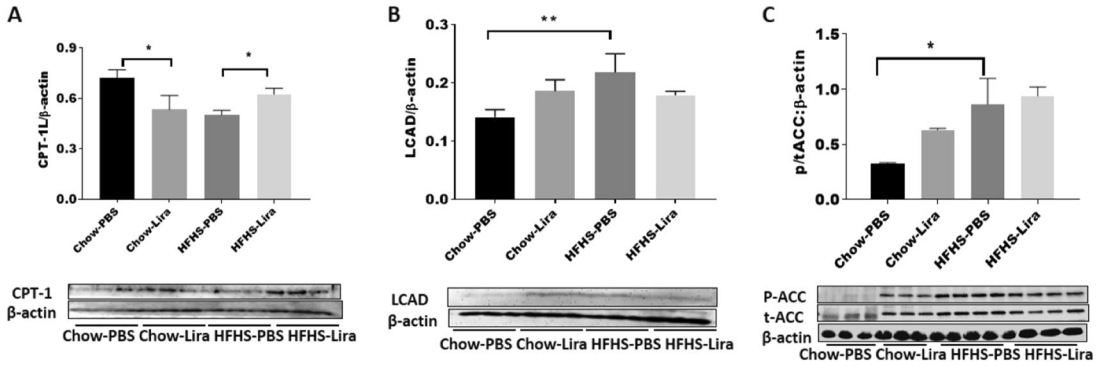


Fig. 4 Effect of liraglutide on fatty acid oxidation in WAT. Western blot analysis was used to assess the protein level with β-actin as loading control. Protein expression level of **a**. CPT-1 (liraglutide: $P = 0.060$, diet: $P = 0.204$, diet × liraglutide: $P = 0.012$). **b** LCAD (liraglutide: $P = 0.8136$, diet: $P = 0.015$, diet × liraglutide: $P = 0.006$). **c** ACC. (liraglutide: $P = 0.027$, diet: $P = 0.0002$, diet × liraglutide: $P = 0.150$). $N = 3$ per chow diet group, $n = 4$ per HFHS diet group. Data is expressed as Mean ± SEM and is analyzed using two-way ANOVA followed by *post-hoc* Turkey test. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$

diet animals but promotes fatty acid uptake in mitochondria in response to HFHS diet feeding, however it had no significant effect on fatty acid oxidation when challenged with HFHS diet.

Liraglutide induces brown fat differentiation in murine skeletal muscle

BAT has the remarkable ability to dissipate excess energy as heat through adaptive thermogenesis, and is activated in response to high fat diet or cold [31]. Hence, it has reemerged as a potential therapeutic target for the prevention and control of diabetes [32]. Since liraglutide improves mitochondrial biogenesis and induced brown fat adipogenesis in WAT in our study, we next investigated its role on brown fat differentiation in skeletal muscle tissue. PRDM16 is a transcriptional regulator that controls the bidirectional cell fate between skeletal myoblasts and brown fat cells [33]. Loss of PRDM16 from brown fat precursors causes a loss of brown fat characteristics and promotes muscle differentiation. Conversely, ectopic expression of PRDM16 in skeletal muscle and white fat precursor cells induces their differentiation into thermogenic, UCP-1 containing, adipocytes [33]. As shown in Fig. 5a, the basal level of PRDM16 is very low in chow diet mice and liraglutide did not affect the expression of PRDM 16 in chow diet skeletal muscle, but significantly induced expression of PRDM-16 (increased by 52.5%, $p < 0.05$) in skeletal muscle of HFHS diet mice. Similarly, UCP-1 expression was upregulated by 21.1% ($p < 0.05$) in response to HFHS feeding (Fig. 5b). Liraglutide treatment further significantly elevated the expression of UCP-1 by 20.4% ($p < 0.05$) in skeletal muscle (Fig. 5b). In contrast to these findings in WAT, neither PRDM-16 nor UCP-1 were induced by liraglutide when mice were fed with chow diet. H & E staining also indicates the brown adipogenesis in skeletal muscle following liraglutide treatment in mice fed with HFHS diet (Fig. 5G-d).

Furthermore, liraglutide elevates PPARα by 23.1% in HFHS mice but not in chow diet mice (Fig. 5c), while PPAR-γ is upregulated by 130% in response to HFHS diet in comparison to the chow-PBS control. Interestingly, liraglutide treatment also upregulated PPAR-γ (by 50%) in the skeletal muscle of chow diet mice but not in HFHS mice (Fig. 5d). Similar to the findings in WAT, liraglutide significantly reduced PPARγ by 31.7% when mice were fed with HFHS diet (Fig. 5d). In addition, liraglutide slightly increased both C/EBP-α (by 18.3%) and C/EBP-β in skeletal muscle when mice were fed HFHS diet in comparison to chow-PBS diet fed mice (Fig. 5e, f). These results indicate liraglutide significantly induced BAT development program genes in skeletal muscle when mice were challenged with HFHS diet.

Liraglutide promotes mitochondrial function in skeletal muscle

Expression of PGC-1α and CIDE-A, regulators of mitochondrial biogenesis, were also measured in skeletal muscle tissue. Liraglutide upregulates PGC-1α in chow diet mice skeletal muscle (by 83%, $p < 0.05$). The expression of PGC-1α in skeletal muscle of HFHS diet fed mice was significantly elevated by 204% ($p < 0.005$) in the presence of liraglutide compared to HFHS diet PBS controls (Fig. 6a). Furthermore, liraglutide elevated CIDE-A (by 182%) in HFHS mice but not in chow diet mice (Fig. 6b). The AMPK-SIRT-1 pathways in skeletal muscle were not affected after liraglutide treatment (data not shown). Our findings indicate that liraglutide induced skeletal muscle mitochondrial biosynthesis is not mediated through the

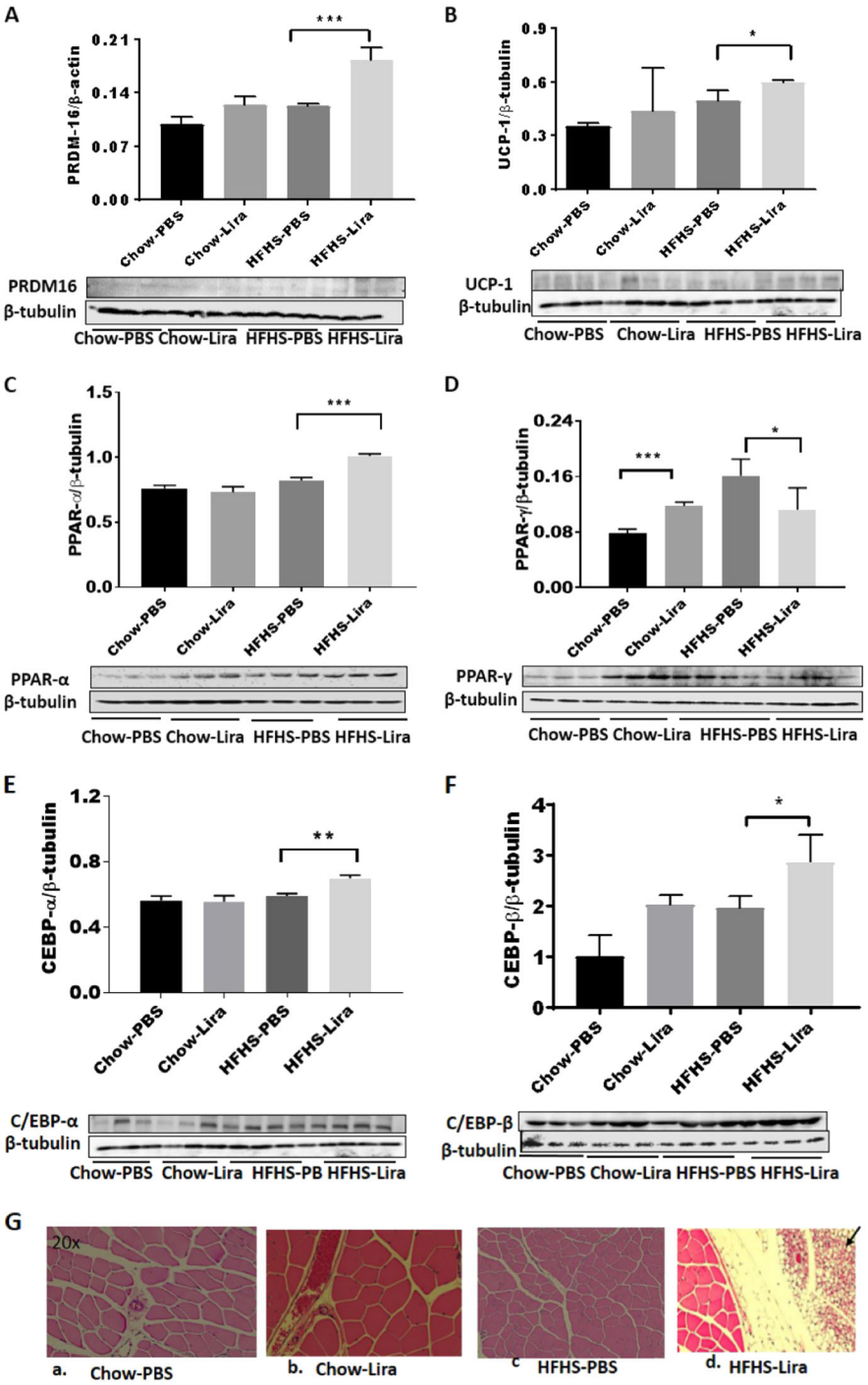


Fig. 5 Liraglutide induces brown fat differentiation in skeletal muscle. Western blot was used to analysis protein level with β-tubulin as loading control **a**. PRDM-16 (liraglutide: $P = 0.0001$, diet: $P = 0.0002$, diet × liraglutide: $P = 0.026$); **b** UCP-1($n = 3$ per chow diet, $n = 5$ per HFHS diet group) (liraglutide: $P = 0.0213$, diet: $P = 0.0814$, diet × liraglutide: $P = 0.2941$); **c**. PPAR-α ($n = 3$ per treatment group, liraglutide: $P = 0.0011$, diet: $P < 0.0001$, diet × liraglutide: $P = 0.0001$); **d** PPAR-γ ($n = 3$ per chow diet group, $n = 4$ per HFHS diet group, liraglutide: $P > 0.9999$, diet: $P = 0.0002$, diet × liraglutide: $P < 0.0001$); **e** C/EBP-α ($n = 3$, per chow diet group, $n = 4$ per HFHS diet group. Liraglutide: $P = 0.0044$, diet: $P = 0.0580$, diet × liraglutide $P = 0.0378$) **f**. C/EBP- β ($n = 3$ per chow diet group, $n = 4$ per HFHS diet group. Liraglutide: $P = 0.0007$, diet: $P = 0.0012$, diet × liraglutide: $P = 0.7845$); **g** Representative of H&E staining of mice skeletal muscle tissue ($n = 4$). Data is expressed as Mean ± SEM and is analyzed using two-way ANOVA followed by *post-hoc* Turkey test $^{*}p < 0.05$, $^{**}p < 0.005$; $^{***}p < 0.001$

AMPK-SIRT-1 cell signaling pathway; different cell signaling pathways might be involved.

Discussion

T2D is a multifactorial disease characterized by insulin resistance in peripheral tissues. Obesity is a major risk factor for the onset of T2D and often presents with pre-diabetic insulin resistance. Liraglutide has been observed to reduce body weight in T2D patients and in overweight or obese adults without T2D, in addition to reducing the prevalence of prediabetes and delaying the onset of T2D [34]. However, very few studies have been performed to elucidate the underlying mechanism of liraglutide in reducing body weight in non-diabetic obese patients. One study suggests liraglutide induces weight loss in obese non-diabetic patients is mediated through reduced food intake

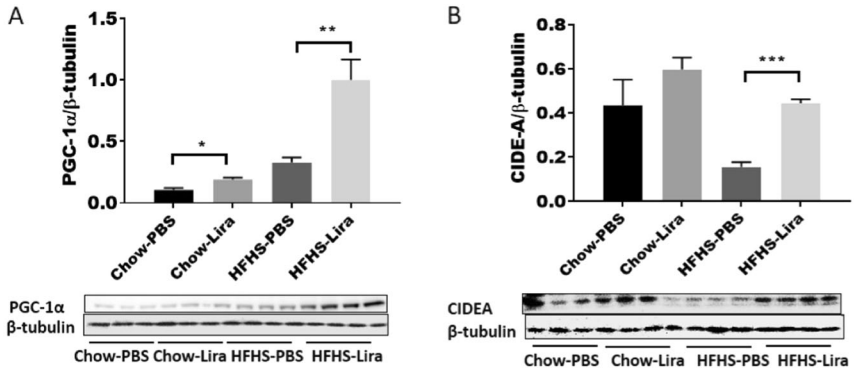


Fig. 6 Liraglutide improves mitochondria function in skeletal muscle. Western blot was used to analysis protein level with β-tubulin as loading control Protein expression level of **a**. PGC-1 α (liraglutide: $P = 0.0039$, diet: $P = 0.0005$, diet × liraglutide: $P = 0.0161$); **b** CIDE-A (liraglutide: $P = 0.0024$, diet: $P = 0.0031$, diet × liraglutide $P = 0.2806$). $N = 3$ per chow diet group, $n = 4$ HFHS diet group. Data is expressed as Mean ± SEM and is analyzed using two-way ANOVA followed by *post-hoc* Turkey test $^{*}p < 0.05$, $^{**}p < 0.005$. $^{***}p < 0.001$

rather than elevation of energy expenditure (EE) [35, 36]. Conversely, another study indicates that liraglutide-induced weight loss is mediated through an increase of EE rather than inhibition of appetite or food intake [37]. Furthermore, reduced subcutaneous but not visceral fat has been observed to contribute to the weight-loss effects of liraglutide [34]. However, reports have also indicated liraglutide reduces both subcutaneous and visceral fat [38].

The differentiation of BAT and WAT share common transcriptional pathways and therefore, common transcriptional markers [39]. Adipogenic markers such as PPAR-γ and C/EBP-α are involved in both lineages while UCP-1, PPARα specifically, are involved in BAT [40]. Importantly, PRDM16 stimulates brown adipogenesis by activating various transcription factors such as C/EBP-β, PPAR-α, and PGC1-α [33]. PRDM-16 is induced by liraglutide in both fat and skeletal muscle tissue in diet-induced diabetes (DIO) mice, accompanied by upregulation of UCP-1, PPARα and CIDE-A in this current study.

Although the weight reducing effect of GLP-1 agonists have been studied on diabetes or diet induced obesity models, the potential role of liraglutide on obesity and/or diabetes prevention has not been studied. Liraglutide treated mice are resistant to diet-induced obesity in our study and liraglutide induced weight loss is accompanied by reduced perigonadal fat mass, inguinal fat mass and improved insulin sensitivity. Furthermore, our data strongly suggests that liraglutide induced activation of BAT other than reduction of food intake plays a significant role in weight loss and improved insulin sensitivity. Recent studies reveal liraglutide increases energy expenditure and suggests BAT thermogenesis and beige fat development to play a prominent role in the weight reducing effects of liraglutide [21]. Our study provides further evidence that liraglutide induces BAT differentiation in both WAT and skeletal muscle tissue when challenged with HFHS diet.

Mice epididymal WAT are relatively resistant to browning, in contrast to inguinal WAT, which are highly prone to browning [27]. Most studies therefore used inguinal fat depot to examine the beige fat development in mice. However, visceral fat correlates strongly with metabolic syndrome in humans than subcutaneous adiposity. In addition, mouse epididymal fat mimics some of the pathogenic properties of human visceral fat [22]. Furthermore, the cell signaling involved in beige fat cell induction in epididymal fat is different from that seen in inguinal fat [41]. Our study observed liraglutide induces beige fat development in epididymal fat in mice fed with HFHS diet. Furthermore, our findings also indicate that liraglutide induces both beige fat development and skeletal muscle BAT differentiation. The regulation role of liraglutide on adaptive thermogenesis contributes to its protective effect against HFHS diet-induced obesity. Interestingly, liraglutide fails to induce BAT differentiation in skeletal muscle when mice fed with chow diet, indicating the anti-obesity effect of liraglutide works differently in physiological and pathophysiological situations in skeletal muscle.

The cell signaling pathway in liraglutide induced adaptive thermogenesis is not clear. Among the possible pathways, GLP-1 is known to have protective effect in coronary artery disease and liver steatosis through GLP-1R [42]. Although endogenous GLP-1R signaling contributes to increased BAT thermogenesis, this pathway does not play a significant role in BW lowering effect of liraglutide in DIO mice [15]. PGC1-α is regulated by AMPK and SIRT-1 according to cellular energy requirements and works as a regulator of mitochondrial biogenesis [43]. Notably, our study showed that liraglutide elevates the activity of SIRT-1, AMPKα, and AMPK-β in perigonadal fat of HFHS diet mice. Although the role of AMPK activation in GLP-1 mediated brown adipose tissue thermogenesis has been documented [18], to our knowledge, this is the first study to

report that liraglutide activates SIRT-1 and AMPK in WAT in response to HFHS feeding. Liraglutide appears to induce adaptive thermogenesis via the AMPK-SIRT-1-PGC-1α cell signaling pathway in WAT when mice are challenged with HFHS diet, however, this induced adaptive thermogenesis does not act through the same cell signaling pathway in skeletal muscle.

Adaptive thermogenesis in BAT occurs through an increase in mitochondrial mass to accommodate increased metabolic demands for respiratory uncoupling. PGC-1 α regulates the oxidation of fatty acids by increasing mitochondrial capacity and function [44]. Liraglutide increases PGC-1 α in both WAT and skeletal muscle tissue in physiological conditions. Importantly, liraglutide induces a marked elevation of PGC-1 α in skeletal muscle tissues in HFHS diet mice. This indicates liraglutide improves WAT and skeletal muscle mitochondrial capacity when mice are challenged with HFHS diet.

PPARγ is a nuclear receptor involved in the regulation of adipogenesis, energy balance, and lipid biosynthesis under normal physiological conditions [45–47]. Improvement of metabolic profile and systemic insulin sensitivity was observed in DIO mice when adipocyte PPARγ was knocked down [48]. We found liraglutide increases PPARγ in murine adipose tissue and skeletal muscle tissue under normal physiological conditions but this expression is reduced with an HFHS diet. Our findings that liraglutide reduced PPARγ under insulin resistance states are consistent with previous findings reported in rats [49] and in db/db mice [50]. Reduced expression of PPARγ in HFHS diet mice after liraglutide treatment may results in impaired adipocyte uptake of FFAs, making them less likely to become hypertrophic [48], which is reflected by reduced adipocyte size. Liraglutide therefore reduces lipogenesis in insulin-resistant states.

CPT-1 is a rate-limiting enzyme in the transport of fatty acids into mitochondria for β-oxidation. The level of CPT-1 was reduced after HFHS diet challenge in WAT, however, liraglutide significantly reversed the HFHS diet-induced inhibition of CPT-1 expression in our study. This finding is consistent with another report that observed these results with exendin-4 treatment [51]. AMPK has been reported to mediate inactivation of lipogenic enzymes by inducing phosphorylation of ACC [52]. Furthermore, AMPK limits fatty acid efflux from adipocytes upon activation and favors local fatty acid oxidation, although ACC and LCAD were not significantly affected by liraglutide treatment in WAT in this study. Our data suggests that liraglutide increases the influx of fatty acids into mitochondria for β-oxidation in WAT, but increased oxidation of fatty acids may require a longer period of liraglutide treatment.

Most of the human and animal studies to date focused on the therapeutic effects of liraglutide in diabetic or DIO models. Very few studies have evaluated the effect of liraglutide in individuals without diabetes [53–55]. Recent clinical studies have suggested liraglutide (3.0 mg/day), as an adjunct to calorie restriction and elevation of physical activity, significantly improves weight loss and insulin resistance and delays the onset of T2D in overweight/obese or prediabetes populations who are at high risk for developing T2D and CVD [14, 56]. However, the cost of this therapy is high, with expenses of around $1000 each month for treatment. Our study is the first to report that chronic administration of a low dosage of liraglutide prevents the development of HFHS diet-induced obesity or insulin resistance in mice, thus, providing evidence for the clinical use of a lower dosage of liraglutide in humans to protect those individuals who have a genetic disposition for obesity, or who have failed life-style interventions to prevent obesity and insulin resistance.

In summary, adaptive thermogenesis is associated with activation of multiple pathways, such as glucose uptake, fatty acid oxidation, brown adipocyte differentiation, and mitochondrial biogenesis [40]. We report that chronic peripheral liraglutide injection directly activates BAT differentiation and mitochondrial biogenesis in HFHS diet mice and leads to improved insulin sensitivity. Furthermore, liraglutide induces the AMPK-SIRT-1-PGC-1α cell-signaling pathway, leading to beige fat development. Our results therefore elucidate mechanisms of liraglutide- induced weight reduction and may provide evidence for the clinical use of liraglutide in treating obesity, metabolic syndrome, and pre-diabetes, specifically as a weight management medication to prevent the onset of obesity and insulin resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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