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Moderate preventative effect with intraperitoneal liraglutide injection in high-fat diet induced C57BL/6J obese mouse model

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ABSTRACT

Background: Although glucagon-like peptide-1 (GLP-1) based drugs including liraglutide have been among type 2 diabetes drugs for more than a decade, further studies with them in animal models may help to expand their future implications.

Objective: Here we mainly assessed the potential preventative effect of liraglutide and the C-terminal fragment of GLP-1 (GLP-1_{28-36}) on obesity development in a high-fat-diet (HFD) induced obese mouse model.

Methods: Male C57BL/6J mice were fed with HFD for 10 days (instead of 8 weeks or longer by other studies), followed by liraglutide, or GLP-1_{28-36} administration 6 days per week for 5 weeks. In addition to daily body weight monitoring, oral glucose and intraperitoneal insulin tolerance tests were conducted. Plasma as well as hepatic triglyceride contents were also measured.

Results: In comparison to PBS control, liraglutide but not GLP-1_{28-36} administration, moderately attenuated HFD-induced body weight gain, accompanied by moderately improved glucose disposal and attenuated fasting blood glucose level. Liraglutide administration attenuated HFD induced hepatic and plasma triglyceride (TG) elevation, while GLP-1_{28-36} administration reduced plasma but not hepatic TG level. In HFD fed mice, liraglutide treatment also improved insulin stimulated Akt (Ser473) phosphorylation in the liver. Notably, the daily body weight lowering effect of liraglutide was consistently decreased after the mice were rested for one day.

Conclusions: We conclude that preventative effect of liraglutide on obesity development is moderate in our experimental settings and such an effect was absent for GLP-1_{28-36}. Further investigations are needed for evaluating the weight management effect of both GLP-1-based and incretin-based drugs.

1. Introduction

Type 2 diabetes (T2D) and obesity rates for the past few decades have continued to grow rapidly (Zimmet, 2017). This is especially concerning due to numerous related complications including cardiovascular disease, renal disease, non-traumatic lower limb amputations from diabetic foot ulcers, and even a bidirectional link to developing depressive symptoms in addition to immense economic burden (Papatheodorou et al., 2018). Furthermore, while the inherent risk may be higher for some groups, the general rising rates for obesity have put people of all backgrounds at a greater risk for T2D. Moreover, the costs for treatment has led to difficulty for diabetic subjects to follow through with taking recommended medications in addition to making lifestyle changes.

The incretin hormone glucagon-like peptide-1 (GLP-1) is capable of bringing diabetic subjects to euglycemia without inducing hypoglycemia (Tian and Jin, 2016). Over the years GLP-1 in addition to its incretin effect has also been found to have various extra-pancreatic effects in the brain, stomach, heart, liver, fat tissue, and elsewhere (Baggio and Drucker, 2007; Ban et al., 2008; Chai et al., 2012; Nikolaidis et al., 2005; Prigeon et al., 2003; Meier et al., 2003; Yu and Jin, 2010). Particularly, the satiety inducing effect of GLP-1-based drugs that reduces food intake (Turton et al., 1996) and thus body weight is of interest, as many attempts are currently being made to harness that ability for weight loss drugs to aid T2D treatment. Specifically the GLP-1 derivative liraglutide, in which the 34th lysine residue has been substituted to that of arginine and a palmitic acid chain has been attached to 26th lysine residue for increasing its half-life (Drucker

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et al., 2010), has gained a lot attention in recent years (Garber et al., 2009; Nauck et al., 2009; Marre et al., 2009; Zinman et al., 2009; Russell-Jones et al., 2009). Clinical use of liraglutide (Garber et al., 2009; Nauck et al., 2009; Marre et al., 2009; Zinman et al., 2009; Russell-Jones et al., 2009) has resulted in significant reductions in HbA1c in addition to mild to moderate weight loss. Recently, the 56-week long SCALE trial and the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial for diabetes patients found liraglutide to result in weight loss greater than placebo along with decreased BMI, waist circumference, HbA1c, fasting plasma glucose, postprandial glucose increment, glucagon and proinsulin levels, systolic blood pressure, and cardiovascular event related-deaths (Mehta et al., 2017). More importantly the SCALE trial conducted for prediabetes and obesity had similar results suggesting that liraglutide may be an effective prevention method against the development of T2D. Nevertheless, potential preventative effect for liraglutide on obesity development has not been thoroughly assessed in animal models.

Here we assessed the effect of liraglutide in “preventing” body-weight gain and improving glucose homeostasis in high fat diet (HFD) fed mice. We included in this study the C-terminal fragment GLP-$1_{28\text{-}36}$, which was shown by our team and by others to possess body weight lowering effect in HFD fed obese mice (Ip et al., 2013; Tomas et al., 2011a, 2011b; Liu et al., 2012).

2. Materials and methods

Reagents. Liraglutide was obtained from Novo Nordisk Canada (Cooksville, ON) while HPLC and mass spectrometry validated synthetic nonapeptide GLP-$1_{28\text{-}36}$ amide (GLP-$1_{28\text{-}36}$) was produced by Biomatik (Wilmington, DE) (Ip et al., 2013; Shao et al., 2013).

Animals. Thirty-two male C57BL/6J mice (6-week old) were bought from Jackson Laboratory (Bar Harbor, Maine) and kept on light-dark cycle of 12 to 12 h under ambient conditions with food and water supplies made readily obtainable for mice. After a 2-week acclimation period, a quarter of these 8-week old mice were placed on low fat diet (LFD) (Harlan Tekland, Madison, WI) and the remaining on HFD with 60% energy from fat (Bio-Serv, Frenchtown, NJ) in group-housed cages. Mice were continued on respective diets for 10 days prior to being separated into four groups: control low fat diet (LFD) group, HFD group, HFD with liraglutide (HFD/L) and HFD with GLP-$1_{28\text{-}36}$ (HFD/G). All groups were given i.p. injections 6 days per week for 34 days between 1 and 3 p.m., during which PBS injection was conducted for mice in LFD and HFD groups. Daily i.p. injection dosage for liraglutide and GLP-$1_{28\text{-}36}$ was 20μg/kg body weight and 18.5 nM/kg body weight respectively. Body weight was monitored daily per mouse, while food intake was monitored weekly per cage of mice. Food intake was converted into energy intake (kcal) using the conversion factor 3.1 kcal/g for LFD and 5.49 kcal/g for HFD. At the end of the study, mice were euthanized and post-mortem body, liver, epididymal white adipose tissue, inguinal white adipose tissue, and intrascapular brown adipose tissue weights were measured. Metabolic tolerance tests were conducted at indicated time in Fig. 1a. All animal experiments in this study followed the *Guide for Care and Use of Experimental Animals* (University Health Network, Toronto, ON, Canada) and the Animal Use Protocol was approved by the Institutional Animal Care and Use Committee of University Health Network.

Intraperitoneal insulin and oral glucose tolerance tests. Mice were fasted for 6 h for intraperitoneal insulin tolerance test (IPITT) and fasted overnight for oral glucose tolerance test (OGTT), as we have presented previously (Ip et al., 2013).

Liver and plasma triglyceride (TG), and plasma free fatty acid (FFA) measurement. To measure TG content, approximately 30 mg liver tissue samples were homogenized in 5% NP-40/H2O, followed by centrifugation for supernatant collection. The TG content in the liver supernatant and mouse serum (10 μl) was determined by the utilization of Serum Triglyceride Determination Kit (TR0100, Sigma Aldrich, Canada). FFA contents were quantified in the serum (2 μl) using the Free Fatty Acid Quantification Kit (Sigma-Aldrich, Canada), following the instructions of the manufacturer.

RNA extraction and Real Time RT-PCR analysis. Total RNA was extracted from the liver tissue using the TRI reagent. Complementary DNA (cDNA) was generated using a complimentary DNA reverse transcription kit (Applied Biosystems, USA). Real time PCR was conducted using Taq DNA polymerase (New England BioLabs, USA) and the S1000 Thermal Cycler (Bio-Rad), with PCR primers listed in Table 1.

Western Blotting and Antibodies. Lysed liver tissue samples were utilized for Western blotting. The AKT (Cat. 9272) and phospho-Akt (Ser473) (Cat. 293125) antibodies were purchased from Cell Signalling Technology (Danvers, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Methods for Western blotting have been described previously (Ip et al., 2013).

Statistical Analyses. Results were expressed as means and standard error of the mean. Student's T-Test and One-way ANOVA along with Bonferroni's Post-Hoc tests were used where appropriate, with significance determined at $P < 0.05$.

3. Results

3.1. Liraglutide but not GLP-$1_{28\text{-}36}$ treatment attenuates body weight increase in HFD-fed mice-

The “therapeutic” body weight lowering effect of liraglutide has been successfully demonstrated in obese mouse models with HFD feeding for 12 weeks or longer by previous investigations (Buganova et al., 2017; Li et al., 2017). To assess the potential “preventative” effect on obesity development by liraglutide and the C-terminal fragment of GLP-1, GLP-$1_{28\text{-}36}$, we fed the C57BL/6J mice with HFD for only 10 days, followed by daily liraglutide or GLP-$1_{28\text{-}36}$ administration, as illustrated in Fig. 1A. Fig. 1B–C shows the daily body weight and cumulative body weight gain monitory results in the four groups of mice. Notably, when compared with LFD feeding, HFD consumption increased body weight gain during the whole experimental period, while liraglutide but not GLP-$1_{28\text{-}36}$ administration attenuated HFD induced body weight gain increase (Fig. 1B–C). Such attenuation effect of liraglutide, however, was not associated with an appreciable influence on weekly energy intake in HFD fed mice, although mice in all the three HFD-fed groups showed increased energy intake, when compared with that of LFD fed mice (Fig. 1D). Previous studies by our group and by others have shown the body weight lowering “therapeutic” effect of GLP-$1_{28\text{-}36}$ in diet induced obese (DIO) mice (Ip et al., 2013; Tomas et al., 2011b). Here the lack of the “preventive” effect of GLP-$1_{28\text{-}36}$ as compared to liraglutide on body weight could be due to the different mechanisms of the two reagents. It is quite possible that the effect of GLP-$1_{28\text{-}36}$ is GLP-1R independent (Ip et al., 2013), making the effect on the brain much weaker, especially in this experimental setting which is testing the potential “preventative” effect.

Fig. 2A–E shows the final day body weight of the mice and the post-mortem organ or tissue weight. When compared with LFD feeding, HFD feeding increased final day mouse body weight significantly (Fig. 2D), associated with increased post-mortem epididymal fat, inguinal fat, and interscapular fat weights (Fig. 2C–E), but not the liver weight (Fig. 2B). Liraglutide administration reduced HFD induced final day body weight (Fig. 2A). Liraglutide administration showed trend effects on reducing the weight of the three post-mortem fat tissues, although none of them reached statistical significance (Fig. 2C–E). We, however, did not find the difference on liver weight in the four groups of mice (2B).

3.2. Liraglutide moderately improves glucose disposal in mice upon HFD feeding-

We then assessed the effect of liraglutide and GLP-$1_{28\text{-}36}$ treatment

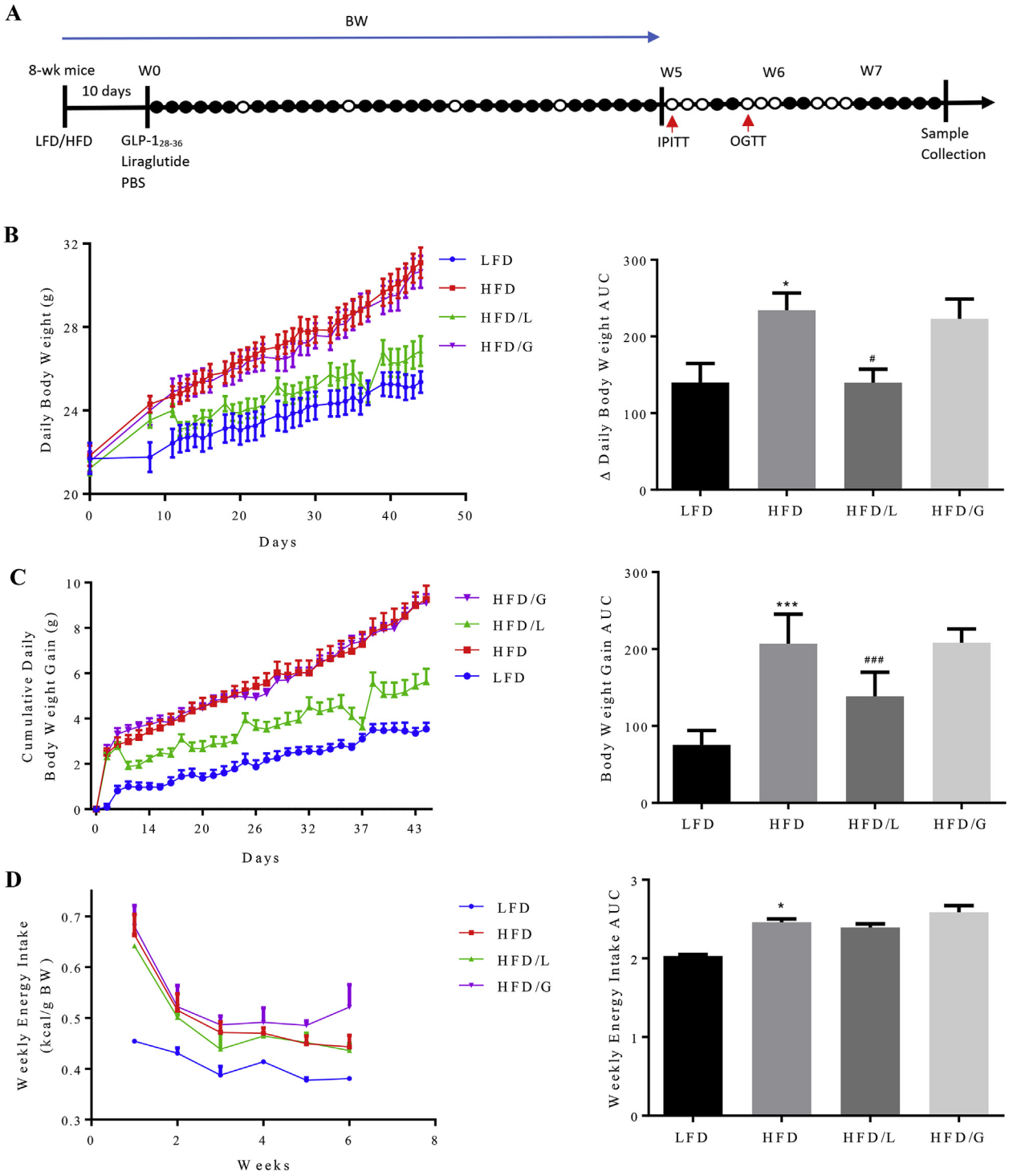

Fig. 1. ***Liraglutide but not GLP-1$_{28-36}$ treatment attenuates body weight increase in HFD-fed mice-*** A) Illustration of the mouse experiments. Close circle and open circle indicate the day that i.p. injection [PBS, (LFD or HFD); liraglutide, (HFD/L); or GLP-1$_{28-36}$ (HFD/G)] was or was not conducted, respectively. Time for IPITT or OGTT was indicated by the arrow. B) Body weight and C) Cumulative body weight gain in the four groups of mice, with area under the curve (AUC) presented at the right. D) Weekly energy intake in the four groups of mice, with AUC presented at the right. N = / > 6 per each of the 4 groups of mice. * and ** ($P < 0.05$ and 0.01, respectively), values were compared between LFD and HFD. # ($P < 0.05$), values were compared between HFD and HFD with an indicated treatment.

on insulin tolerance and glucose disposal in the four groups of mice. Our IPITT result (assessed on week 5) shows that HFD feeding impaired insulin tolerance and the impairment was partially corrected by liraglutide administration but not by GLP-1$_{28-36}$ administration (Fig. 3A). In contrast, HFD feeding impaired oral glucose intolerance (assessed on week 6) was not attenuated by either liraglutide or GLP-1$_{28-36}$ administration (Fig. 3B), although both treatments reduced fasting blood glucose (Fig. 3D) but not random blood glucose levels (Fig. 3C). The improvement effect of liraglutide treatment on insulin signaling sensitization was also verified by Western blotting on insulin stimulated Akt (Ser473) phosphorylation in the mouse liver (Fig. 3E).

Table 1
Nucleotide Sequences of Primers used in the Study.

Name	DNA Sequence
ChREBP	F: 5′-ACCGGGGTGCCCATCACACA-3′ R: 5′-CTGCCCGTGTGGCTTGCTCA-3′
SREBP-1	F: 5′-TAGAGCATATCCCCCAGGTG-3′ R: 5′-GGTACGGGCCACAAGAAGTA-3′
β-Actin	F: 5′-TCATGAAGTGTGACGTTGACA-3′ R: 5′-CCTAGAAGCATTGCGGTG-3′

3.3. Liraglutide treatment reduces hepatic and plasma triglyceride (TG) and free fatty acid (FFA) levels-

We then initiated the assessment on the potential beneficial effect of liraglutide and GLP-1_{28-36} on lipid metabolism in this "preventative" mouse model. As shown, HFD feeding increased both hepatic and plasma TG levels. The elevation in the liver was attenuated by liraglutide treatment but not by GLP-1_{28-36} treatment, while the elevation in the blood was attenuated by both liraglutide and GLP-1_{28-36} treatment (Fig. 4A–B). HFD feeding also increased plasma FFA level, which was attenuated by liraglutide treatment but not by GLP-1_{28-36} treatment (Fig. 4C).

The liver is the central organ for lipogenesis. Carbohydrate-responsive element-binding protein (ChREBP), also known as MLX-interacting protein-like (MLXIPL); and sterol regulatory element-binding protein 1 (SREBP-1) are the two key hepatic lipogenic transcription factors. We then assessed hepatic mRNA expression of these two lipogenic transcription factors in our experimental animals. As shown, when compared with LFD fed mice, expression of both *CHREBP* and *SREBP*-1 mRNA were elevated in HFD fed mice. The elevation for both of them was effectively attenuated in HFD fed mice with liraglutide administration, though only significantly for CHREBP (Fig. 4D–E). Such an effect was not observed in HFD fed mice with GLP-1_{28-36} treatment (Fig. 4D–E).

4. Discussion

The weight management effect of GLP-1 based drugs including liraglutide has been demonstrated in clinical investigations (Garber et al., 2009; Nauck et al., 2009; Marre et al., 2009; Russell-Jones et al., 2009;

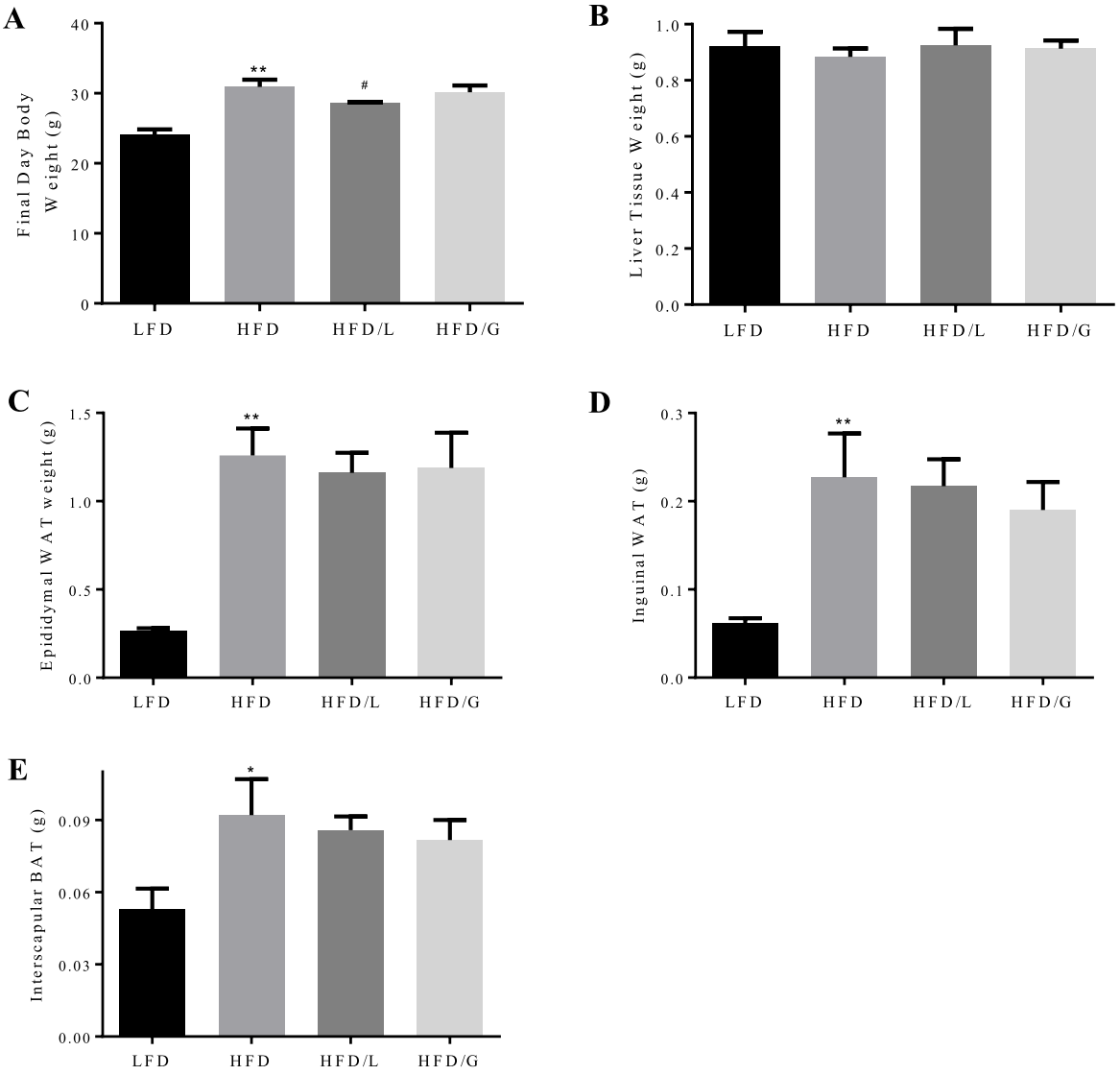

Fig. 2. ***Liraglutide administration attenuates body weight gain in HFD-fed mice-*** A) Final day body weight in the four groups of mice. B) Post-mortem liver weight in the four groups of mice. C-E) Post-mortem fat tissue weight in the four groups of mice. N = / > 6 per each of the 4 groups of mice. * and **, values were compared between LFD and HFD. #, values were compared between HFD and HFD with an indicated treatment.

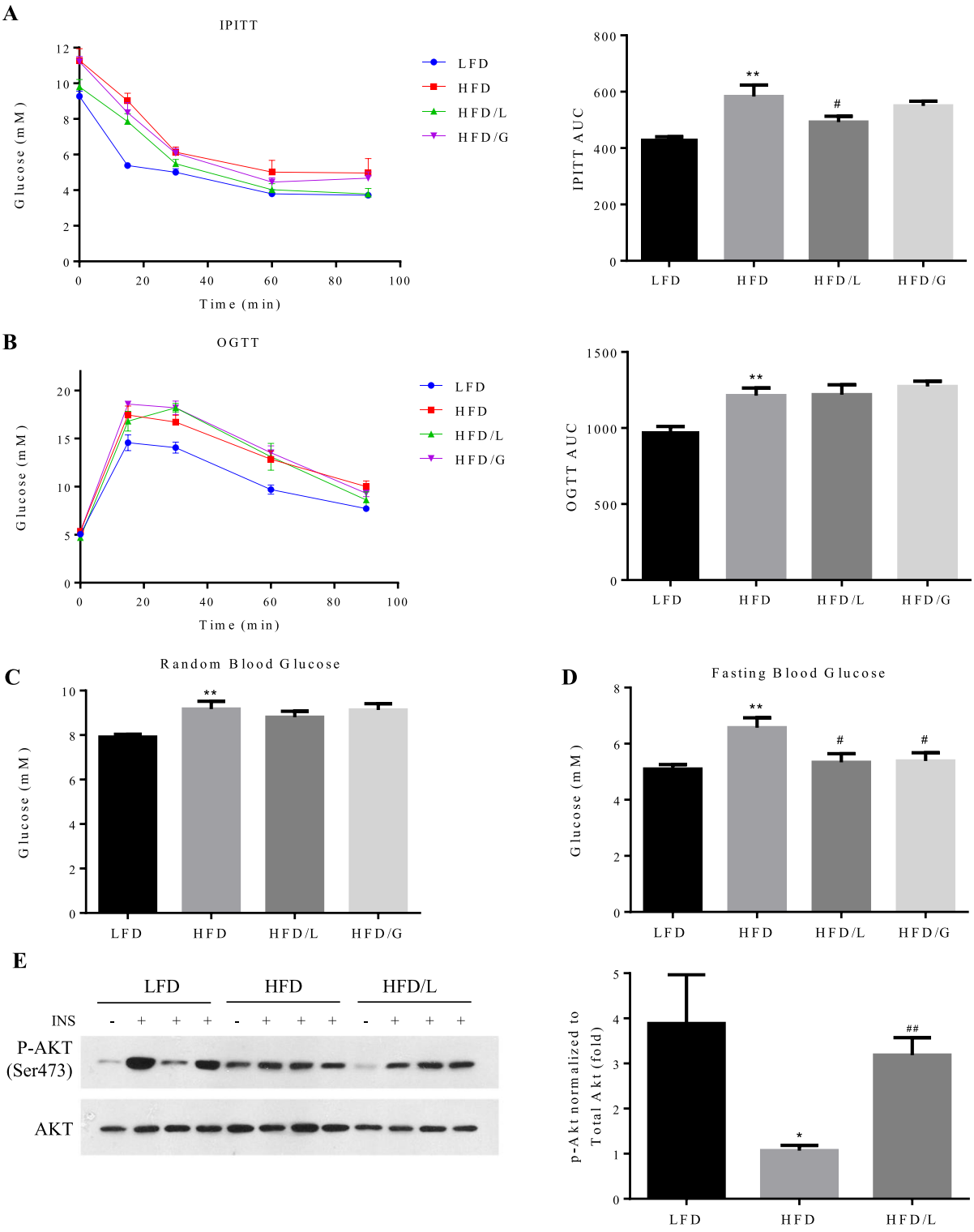

Fig. 3. *Liraglutide administration moderately improves glucose disposal in mice upon HFD feeding*-**A)** Results of IPITT of the four groups of mice, with AUC presented at the right. **B)** Results of OGTT of the four groups of mice, with AUC presented at the right. **C-D)** Random (C) and fasting (D) blood glucose levels in the indicated group of mice. **E)** Western blotting shows the detection of Akt Ser473 phosphorylation in response to insulin injection in the mouse liver tissue for three mice in comparison to liver tissue from a PBS injected control mouse from the same group, with the densitometric analysis data on the right. Insulin (INS, 0.075 U/mL) or PBS were i.p. injected in mice 15 min before they were euthanized. * and **, values were compared between LFD and HFD. # and ##, values were compared between HFD and HFD with an indicated treatment. N = 6 for panels A–D.

Pi-Sunyer et al., 2015a, 2015b; Astrup et al., 2009). Larsen and colleagues have also shown in a monosodium glutamate (MSG) induced obese rat model as well as in normal control rats, that subcutaneous injection of GLP-1 derivative NN2211 for 10 days inhibited both food intake and body weight (Astrup et al., 2009). Here we aim to assess the potential "preventative" effect of liraglutide in our HFD mouse model. We included GLP-$1_{28\text{-}36}$, as this C-terminal fragment of GLP-1 was shown by our group to reduce body weight gain in obese mice

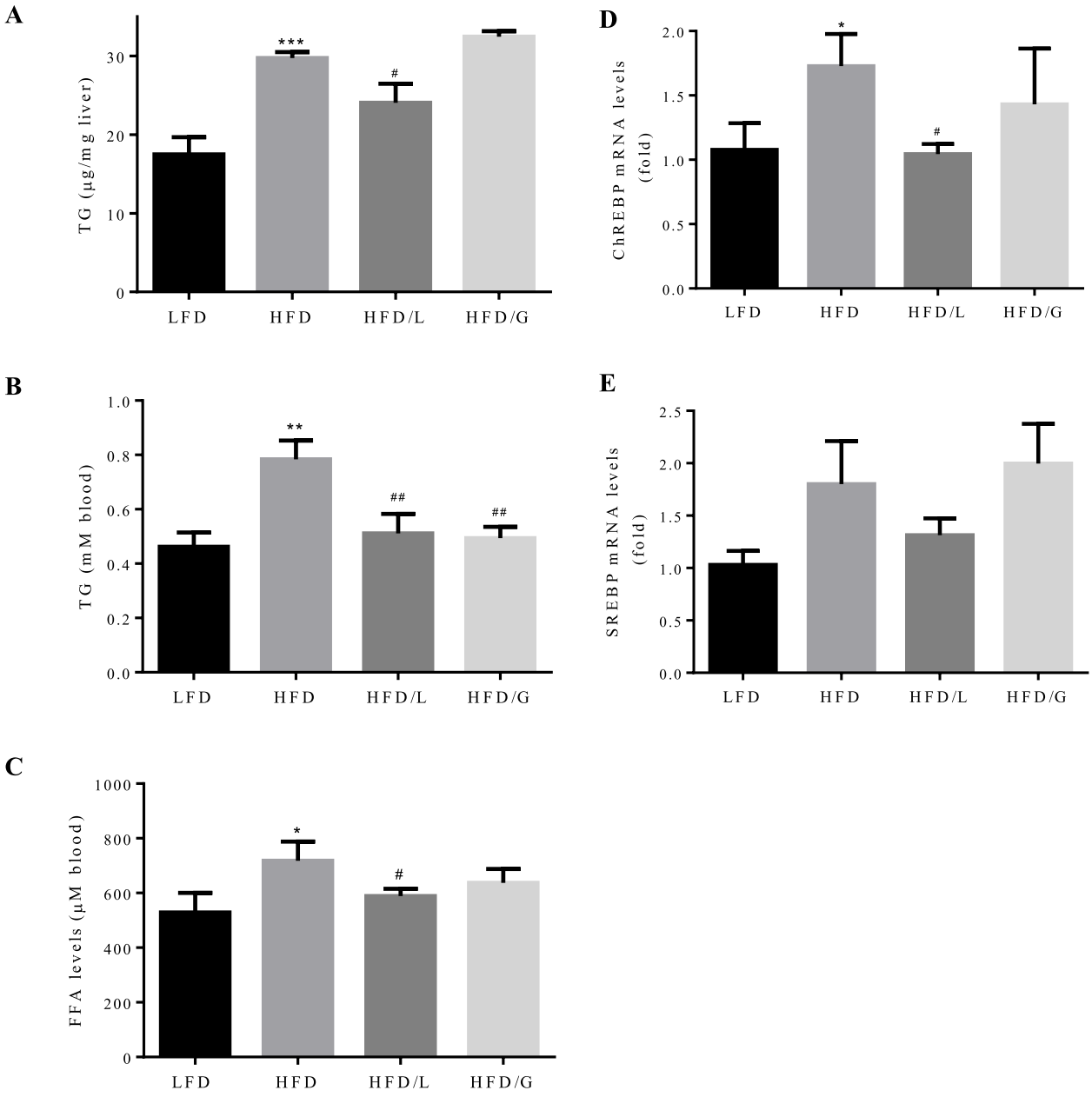

Fig. 4. ***Liraglutide treatment reduces hepatic and plasma triglyceride (TG) and free fatty acid (FFA) levels*****- A-B)** Hepatic (A) and plasma (B) TG levels in indicated group of mice. **C)** Plasma FFA levels in indicated group of mice. **D-E)** Hepatic *ChREBP* (D) and *SREBP-1* (E) mRNA levels in each group of mice. * and **, values were compared between LFD and HFD. # and ##, values were compared between HFD and HFD with an indicated treatment. N = 6 for each of the five panels.

generated with 13 week HFD feeding (Ip et al., 2013). Metabolic beneficial effects of this GLP-1 C-terminal short peptide targeting the liver as well as pancreatic β-cells were also demonstrated by other investigations (Tomas et al., 2011a; Shao et al., 2013). In our current model with HFD feeding for only 10 days, we found that liraglutide but not GLP-$1_{28\text{-}36}$ administration moderately attenuated body weight gain, associated with moderate effect on improving glucose disposal and insulin sensitivity. Unexpectedly, GLP-$1_{28\text{-}36}$ showed no effect on body weight in this model, although both liraglutide and GLP-$1_{28\text{-}36}$ had the effect on reducing fasting glucose levels and plasma TG levels. These observations collectively suggest that at least in our current experimental settings, the "preventative" effect of liraglutide is not highly appreciable when compared with its "therapeutic" effect or other GLP-1 based drugs demonstrated by previous investigations with HFD feeding for 12 weeks or longer (Ip et al., 2013; Buganova et al., 2017; Li et al., 2017; Cox et al., 2017).

In an obese mouse model with 13-week HFD feeding, Buganova et al. found that daily liraglutide injection for 2 weeks decreased body weight as well as fat pad weight along with improved glucose and lipid homeostasis (Buganova et al., 2017). Another study by Li et al. showed that twice-daily liraglutide injection reduced body weight, fasting blood glucose levels, as well as homeostatic model assessment of insulin resistance (HOMA-IR) in mice fed with HFD for 12 weeks (Li et al., 2017). These investigations clearly demonstrated the "therapeutic" effect of liraglutide in obese subjects. The limited body weight lowering effect observed in our current study suggest that the "preventative" effect of liraglutide or GLP-1 based drugs might be limited. This could be due to the fact that target organs, such as the brain, in obese subjects are more sensitive to this category of drugs. Certainly, we also did not observe the effect of either liraglutide or GLP-$1_{28\text{-}36}$ on daily energy intake in our current study. Similarly, the age of mice could be an explanation for the limited effect on energy intake and body weight, as by virtue of our

study testing the "preventative" effect mice were not placed on HFD for a prolonged period that would cause them to be older at the start point of liraglutide administration. In addition, we cannot exclude the experimental limitations in our current study. First of all, the dosage of liraglutide utilized in this study is within the low range (20 μg/kg body weight/day, 6 days per week for 5 weeks), while 1 mg/kg body weight twice per day for 8 weeks was utilized by Li et al. and 200 μg/kg/body weight twice per day for 2 weeks was utilized by Buganova and colleagues (Buganova et al., 2017; Li et al., 2017). Thus, the greater dose or longer duration of liraglutide administration may result in a stronger effect on body weight loss. It is also worth mentioning that daily i.p. injection prevented the mice from becoming obese, even though the control PBS was injected in HFD fed mice. Indeed, in our current study, body weight of the HFD control group only reached ~30 g at the end of the experimental period. This created the "technical" difficulty in demonstrating the body weight lowering effect. Nevertheless, the other beneficial effects, including insulin tolerance as well as OGTT were also modest. These, along with the lack of effect on energy intake, support our speculation that liraglutide might be less effective for its target organs in "normal" subjects compared with that of obese subjects. It is important to note that in the consideration of possible sex differences. Most clinical trials have shown little or non-significant differences for the different genders with liraglutide treatment, though LEADER did show a greater benefit for males than females for cardiovascular outcomes (Mehta et al., 2017). However, studies on pancreatic islet biology have shown certain differences based on gender, particularly with respect to estrogen which increases the production of GLP-1 in mice and inhibits food intake (Gannon et al., 2018). Thus, for our future examinations, it would be valuable to include both genders.

Although GLP-1 based drugs have been utilized for T2D subjects for over a decade, little attention has been given to whether drug resistance can be developed with their usage. A recent study demonstrated that when human islets were implanted into the anterior chamber of the eye in mice with streptozotocin (STZ) induced type 1 diabetes, long-term liraglutide treatment showed gradually reduced human insulin C-peptide release, associated with perturbed glucose homeostasis (Abdulreda et al., 2016). In our current study, we observed that the daily body weight of mice injected with liraglutide increased sharply in a single day upon skipping of the injection one day per week, while liraglutide re-administration on the following day led to a lower drop in body weight. This trend continued over the span of the treatment with the drop becoming lower each week, suggesting that drug resistance could be induced with its continued usage. It is of worth to mention that back in 2014, Roed et al. demonstrated that the GLP-1R could be internalized following liraglutide treatment in pancreatic β-cells (Roed et al., 2014).

As obesity has high comorbidity with diabetes, body weight management becomes more and more important in T2D treatment and its health care. Incretin-based drugs include not only the injectable drugs that are GLP-1 analogues, but also DPP-4 inhibitors, which prevent endogenous incretin hormone degradation (Jin and Weng, 2016). In a previous study, the DPP-4 inhibitor sitagliptin was shown to reduce body weight in an 18-week HFD fed mouse model. As an orally given drug, the technical challenge for testing its "preventative" body weight attenuation effect in HFD fed mouse model will be less harsh. Recent approval for an oral formulation of semaglutide, a GLP-1R agonist like liraglutide, offers a more similar alternative to liraglutide that avoids daily injections (Knudsen and Lau, 2019). In addition, whether the orally taken drug can be combined with nutritional or dietary intervention for body weight management in mouse models needs to be assessed in the future.

Declaration of competing interest

The authors claim that there is no conflict of interest on this line of study.

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