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The key role of a glucagon-like peptide-1 receptor agonist in body fat redistribution

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Abstract

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are an ideal therapy for type 2 diabetes and, as of recently, for obesity. In contrast to visceral fat, subcutaneous fat appears to be protective against metabolic diseases. Here, we aimed to explore whether liraglutide, a GLP-1RA, could redistribute body fat via regulating lipid metabolism in different fat depots. After being fed a high-fat diet for 8 weeks, 50 male Wistar and Goto-Kakizaki rats were randomly divided into a normal control group, a diabetic control group, low- and high-dose liraglutide-treated groups and a diet-control group. Different doses of liraglutide (400 µg/kg/day or 1200 µg/kg/day) or an equal volume of normal saline were administered to the rats subcutaneously once a day for 12 weeks. Body composition and body fat deposition were measured by dual-energy X-ray absorptiometry and MRI. Isotope tracers were infused to explore lipid metabolism in different fat depots. Quantitative real-time PCR and Western blot analyses were conducted to evaluate the expression of adipose-related genes. The results showed that liraglutide decreased visceral fat and relatively increased subcutaneous fat. Lipogenesis was reduced in visceral white adipose tissue (WAT) but was elevated in subcutaneous WAT. Lipolysis was also attenuated, and fatty acid oxidation was enhanced. The mRNA expression levels of adipose-related genes in different tissues displayed similar trends after liraglutide treatment. In addition, the expression of browning-related genes was upregulated in subcutaneous WAT. Taken together, the results suggested that liraglutide potentially redistributes body fat and promotes browning remodeling in subcutaneous WAT to improve metabolic disorders.

Key Words

- ▶ glucagon-like peptide-1
- ▶ liraglutide
- ▶ lipid metabolism
- ▶ diabetes
- ▶ isotope tracer

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Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder that is often associated with obesity, dyslipidemia, hypertension, microvascular disease and other complications (American Diabetes Association 2016).

All these complications increase the risk for cardiovascular disease (CVD), which is the major cause of morbidity and mortality in T2DM patients. However, obesity, especially visceral fat obesity, is located

upstream of T2DM, dyslipidemia and coronary heart disease (Inoue *et al.* 2014). The distribution of body fat plays an important role in this relationship. The ratio of abdominal to lower-body fat accumulation is a major determinant of adverse metabolic consequences and mortality that is independent of the degree of obesity (McQuaid *et al.* 2010). Over the past several years, it has been revealed that lower-body fat is not only less harmful than abdominal fat but also provides protection against coronary heart disease and T2DM (Manolopoulos *et al.* 2010).

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by gastrointestinal L cells in response to oral nutrient ingestion (Wan *et al.* 2017) and is an ideal therapy for obesity and T2DM (Rajeev & Wilding 2016). However, native GLP-1 has a short circulating half-life ($t_{1/2} < 2$ min) that results mainly from rapid enzymatic inactivation by dipeptidyl peptidase-IV (DPP-IV) and/or from rapid kidney clearance (Wan *et al.* 2017). To prolong the action of GLP-1, DPP-IV inhibitors and GLP-1 receptor agonists (GLP-1RAs) are introduced.

Liraglutide is a GLP-1RA that shares 97% homology to native GLP-1 and extends the circulating half-life of GLP-1 from 1 to 2 min to 13 h. A series of Liraglutide Effect and Action in Diabetes (LEAD) studies showed the significant effect of liraglutide on glycemic control and weight reduction, and its long-term efficacy on weight reduction was demonstrated in a subanalysis of the LEAD study (Garber *et al.* 2011, Nauck *et al.* 2013) and in other studies (Inoue *et al.* 2011, Fujishima *et al.* 2012).

Because GLP-1R activation has been revealed to function in cardiovascular protection (Rizzo *et al.* 2014), GLP-1RAs are being considered as a promising therapy for both micro- and macro-vascular diseases linked to obese T2DM (Sivertsen *et al.* 2012). Glycemic control is known to reduce the risk of microvascular complications; however, the macrovascular benefits are less certain (Marso *et al.* 2016). Thus, GLP-1RAs might protect patients from macrovascular complications by mechanisms other than glycemic control. Many clinical studies have shown that GLP-1RAs improve the lipid profile and blood pressure in patients (Wing *et al.* 2011, Inoue *et al.* 2014). However, few studies have investigated whether GLP-1RAs could change body fat distribution by regulating lipid remobilization and turnover in different fat depots. Because visceral fat accumulation and dysfunction are more closely associated with metabolic disorders than is subcutaneous fat, the body fat distributions between visceral and subcutaneous fat, especially lower-body fat, play an important role in the occurrence of metabolic diseases.

The Goto-Kakizaki (GK) rat line is established by repeated inbreeding from a Wistar rat strain selected at the upper limit of normal distribution for glucose tolerance. GK rats are regarded as one of the best available rodent strains for the study of spontaneous T2DM, since the model can offer sufficient commonalities with human T2DM (Portha *et al.* 2012). Thus, GK rats were chosen in our study to elucidate various types of etiological and pathogenic mechanisms that could also operate in humans.

Based on this information, in the current study, we used liraglutide to explore whether GLP-1RAs could redistribute body fat via regulating lipid metabolism in different fat depots to improve metabolic disorders and macrovascular complications.

Materials and methods

Animals and experimental design

Ten male Wistar rats and thirty male GK rats (3 weeks old) were bred and housed locally under a 12/12-h light/darkness cycle in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) with free access to food and water. After 1 week of acclimation, the animals were all given a high-fat diet (HFD: 40% carbohydrate, 20% protein and 40% fat) (Zhao *et al.* 2017) for 8 weeks. Then, the animals were randomly divided into four groups and given the high-fat diet continuously for 12 weeks. The groups were formed as follows:

Control group (Wistar+normal saline (NS), $n=10$): Wistar rats from the same genetic background as GK rats but with normal glucose levels. The animals were subcutaneously injected with a volume of NS equal to the injection volume in the intervention groups during the 12-week experiment.

Diabetic group (GK+NS, $n=10$): GK rats are spontaneous type 2 diabetic rats. In this group, the animals were also subcutaneously injected with an equal volume of NS for 12 weeks.

Low-dose liraglutide (LIRA, Victoza, Novo Nordisk)-treated group (GK+LIRA 400 $\mu\text{g/kg/day}$, $n=10$): The rats were subcutaneously injected with a low dose of LIRA (400 $\mu\text{g/kg/day}$) for 12 weeks.

High-dose LIRA-treated group (GK+LIRA 1200 $\mu\text{g/kg/day}$, $n=10$): The rats were subcutaneously injected with a high dose of LIRA (1200 $\mu\text{g/kg/day}$) for 12 weeks.

Diet-control group (GK+Diet-control, $n=10$): To explore whether the effects of liraglutide on lipid metabolism in various fat depots were different from

weight change by food restriction, one group of rats was simply food restricted. The rats in this group were fed the same HFD but were restricted to 50% of the spontaneous food intake (Marcelino *et al.* 2013) of weight-matched GK controls for 12 weeks.

Body weights and blood glucose levels were measured every 2 weeks. The glucose levels were measured immediately using an electronic glucometer (Terumo, Tokyo, Japan). Lipid profiles, including total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL), and insulin levels were assayed at the 0th, 4th and 12th weeks using Siemens Dimension MAX (Siemens Healthcare Diagnostics Inc.). Non-esterified fatty acid (NEFA) levels were determined at the 0th, 4th and 12th weeks using a LabAssay NEFA kit (Wako, Japan). Fasting insulin (FINS) levels were also assayed at the 0th, 4th and 12th weeks using an ELISA kit (Shibayaji, Japan). Then, the homeostatic model assessment index (HOMA-IR) and the insulin sensitivity index (ISI) were calculated from the fasting blood glucose (FBG) and FINS levels using the following formulas: $HOMA-IR = FBG \times FINS / 22.5$ and $ISI = \ln(1 / (FBG \times FINS))$ (Zhao *et al.* 2017). After 12 weeks of treatment, leptin and adiponectin levels were also assayed with ELISA kits (Crystal, USA). The above indicators were assayed in all rats. All experimental procedures were conducted in accordance with the ethical principles for animal research adopted by the Department of Laboratory Animal Science and were approved by the Animal Experimental Ethical Committee of JiaoTong University School of Medicine, Shanghai, China.

Determination of body composition and body fat distribution

At the end of the experiment, the rats (10 in each group) from the five groups were anesthetized with chloral hydrate (10%, 5 mL/kg), and body composition was measured by dual-energy X-ray absorptiometry (DXA) (Hologic, Boston, MA, USA). In the DXA results summary, fat mass and total mass were measured, and the ratio of fat to total mass was calculated (Eleftheriades *et al.* 2016). Body fat deposition was scanned by MRI (MesoMR23-060H-I, Suzhou, China).

Isotope infusion

All rats were fasted overnight for 8 h and examined the next morning. For the diet-controlled rats, their fasting time was counted beginning when their food was fully consumed. After local anesthesia with lidocaine, the lateral tail vein was catheterized for the infusion of

tracers, and the tail artery was catheterized for blood sampling using previously described methods (Guo & Zhou 2003). The rats were conscious and relaxed during the whole process of the experiments (Fig. 1A). After the FBG levels returned to baseline (usually within 30 min), $[U-^{13}C]$ -glycerol (0.84 $\mu\text{mol/kg/min}$, Cambridge Isotope, Andover, MA, USA) was constantly infused for 90 min through the tail vein by a Harvard mini infusion pump (Harvard Apparatus, Holliston, MA, USA). $[9,10-^3H]$ -palmitic acid (1 μCi , PerkinElmer) was also injected through the tail vein at 60 min. During the final 10 min, three arterial blood samples (0.5 mL each) used to quantify steady-state glycerol metabolism were collected at 5-min intervals (Fig. 1B). Then, the animals were killed under anesthesia with pentobarbital (50 mg/kg). A strip of gastrocnemius muscle (approximately $13 \times 3 \times 1$ mm, 50 mg) was promptly obtained and cultured *in vitro* to examine the β -oxidation of $[9,10-^3H]$ -palmitic acid (1 μCi). Other tissues were also quickly harvested, immersed in liquid nitrogen and stored at -80°C for further analysis.

Figure 1

The platform and protocol for isotope tracer infusion in rats.

(A) Experimental setup for tracer infusion. The lateral tail vein was catheterized for tracer infusion, and the tail artery for blood collection. To minimize the experimental stress, all rats were conscious and relaxed, with the ability to groom themselves as normal and drink water freely throughout the experiments. (B) This figure shows the process of tracer infusion and blood collection.

Measurement of isotope tracers

The arterial plasma samples were processed with methoxyamine HCl and BSTFA to obtain the trimethylsilyl derivatives of [U-¹³C]-glycerol. Then, the enrichment of the derivatives was gauged by gas chromatography/mass spectrometry (GC-MS, Agilent 5975C, Agilent Technologies). Ions with mass-to-charge ratios (*m/z*) of 218 (unlabeled glycerol) and 321 (labeled glycerol) were monitored. The 221/218 ratio peak area was calculated, and the corresponding enrichment was determined from standard curves.

Lipids were extracted from the liver and fat tissues using the Folch method (Folch *et al.* 1957), and TG concentrations were assayed with an ELISA kit (Jiancheng, Nanjing, China). Then, pure TG was isolated using thin layer chromatography (TLC). In addition, ³H₂O generated from the process of [9,10-³H]-palmitic acid β-oxidation was obtained by removing the lipids with chloroform. Next, ³H radioactivity from both TG and ³H₂O was determined using liquid scintillation counting (LS6500 Multipurpose Scintillation Counter, Beckman) as previously described (Zhao *et al.* 2017).

Calculations

The appearance rates of glycerol (*R_{gly}*) were calculated with the steady-state equation from the respective tracer infusion rates (*F*) and mole percent excess (MPE). Lipidosis and lipogenesis in the liver and different fat depots were calculated by dividing the total concentrations of TG or the corresponding tissue mass by the radioactivity of the corresponding labeled TG, respectively (Shadid *et al.* 2007). Fatty acid β-oxidation ratios were deduced by measuring the specific activity of ³H₂O. All the relative formulas are shown as follows (Vella & Rizza 2009):

Deposition rate of TG (dpm/g) = ³H-TG radioactivity (dpm)/tissue mass (g)

Synthetic rate of TG (dpm × g protein/mmol) = ³H-TG radioactivity (dpm)/total TG concentration (mmol/g protein)

R_{gly} (μmol/kg/min) = *F_{gly}* (μmol/kg/min)/[U-¹³C]-glycerol MPE – *F_{gly}* (μmol/kg/min)

Fatty acid β-oxidation ratio (%) = ³H₂O radioactivity (dpm) × theoretical muscle mass (50 mg)/[9,10-³H]-palmitic acid radioactivity (dpm)/actual muscle mass (mg) × 100%

Tissue histology

Liver and adipose tissues (five in each group) were fixed in 4% paraformaldehyde and sliced after being paraffin embedded on a microtome (SLEE, Germany). Multiple sections were prepared and stained with hematoxylin and eosin (HE) and were analyzed under an optical microscope (CKX41, Olympus) for the morphological changes. Hepatic lipid accumulation was also determined using oil red O (ORO) staining. ORO staining was viewed using an Axiophot I microscope mounted with an Axiocam color charge-coupled device camera. The intramyocellular TG levels were quantified using Image-Pro software. Additionally, the fat cell sizes at 200× magnification in different adipose tissues were measured by using Image-Pro Plus 6.0 software.

Quantitative real-time PCR

Total RNA (5 in each group) was extracted from tissues using TRIzol reagent (TaKaRa) following the manufacturer's instructions. cDNA was synthesized using a cDNA Reverse Transcription Kit (TaKaRa). Quantitative real-time PCR was conducted using a LightCycler 96 system (Roche Applied Science) and SYBR Green I as the dsDNA-specific binding dye for continuous fluorescence monitoring. Gene expression was evaluated using the ΔΔCt method. β-Actin was used as the housekeeping gene. The sequences of the gene primers were as follows: monoacylglycerol acyltransferase (*Mgat*), forward 5'-AGG GCA TCT GTG GAG AGC-3' and reverse 5'-GTG GAG AAA GTG GGG AAG GTA CA-3'; diacylglycerol O-acyltransferase 2 (*Dgat2*), forward 5'-ACA AGC CTA TGC TCA GAC TGG G-3' and reverse 5'-GGA ACA TCC TCA GGG GAA TGC T-3'; hormone-sensitive lipase (*Hsl*), forward 5'-TCC TCT GCT TCT CCC TCT CGT-3' and reverse 5'-GTC CCT GAA TAG GCG CTC ACA-3'; carnitine acyltransferase 1a (*Cpt1a*), forward 5'-TTG CCG ATG ACG GCT ATG GT-3' and reverse 5'-TGA GTC TGT CTC AGG GCT AGA GA-3'; carnitine acyltransferase 1b (*Cpt1b*), forward 5'-TTC GGC AAA GGC CTG ATC A-3' and reverse 5'-TTG CCT TTG TCC CGG AAA TG-3'; uncoupling protein-1 (*Ucp1*), forward 5'-ATC AAA CCC CGC TAC ACT GG-3' and reverse 5'-CAG TAA ATG GCA GGG GAC GT-3'; peroxisome proliferator activated receptor gamma coactivator 1α (*Pgc1α*), forward 5'-CAT GCA AAC CAC ACC CAC AG-3' and reverse 5'-CTG AGC AGG GAC GTC TTT GT-3'; bone morphogenetic protein 4 (*Bmp4*), forward 5'-CAG GGC CAA CAT GTC AGG AT-3' and reverse 5'-TGG CGA CGG CAG TTC TTA TT-3' and

β -actin, forward 5'-GCC CCT CTG AAC CCT AAG-3' and reverse 5'-CAT CAC AAT GCC AGT GGT A-3'.

Western blot analysis

A primary antibody against UCP1 (1:1000) was purchased from Abcam (ab23841), and an antibody against GAPDH (1:1000) was purchased from Biotech Well (WB0197). An HRP-conjugated goat anti-rabbit IgG (H+L) was used as the secondary antibody (1:20,000, Jackson). Protein band densities (4 in each group) were quantified using ImageJ.

Statistical analysis

Data analysis was conducted with IBM SPSS Statistics, version 22 (IBM Corporation). All data were presented as the means $\pm$ standard deviations (s.d.), and statistical significance was assessed by one-way ANOVA (LSD). $P < 0.05$ was considered statistically significant.

Results

Liraglutide-induced weight loss and improved glycemic parameters

After an 8-week HFD, blood glucose levels and insulin resistance were significantly greater in GK rats than in Wistar rats. After the first injection of liraglutide, the anti-hyperglycemic effects and weight-sparing effects were observed within the first 2 weeks. Liraglutide treatment significantly decreased the blood glucose levels in both fasting (Fig. 2A) and postprandial (Fig. 2B) states. Food restriction also decreased the glucose levels to those of Wistar rats. Food intake was acutely suppressed by liraglutide within the first week. (Fig. 2C). However, body weight gain was steadily attenuated from the 2nd week, although food intake was not inhibited by liraglutide as much as in the first week (Fig. 2D). In the GK+Diet-control group, food intake was less than that in the liraglutide-treated groups, resulting in more weight loss. Although FINS levels (Fig. 2E) were not significantly decreased after 4 weeks of intervention, improvement in insulin resistance was observed in the two liraglutide-treated groups and GK+Diet-control group. After 12 weeks of intervention, the two liraglutide-treated groups and GK+Diet-control group all showed clear improvements in FINS concentrations, HOMA-IR (Fig. 2F) and ISI (Fig. 2G), compared with the GK+NS group.

In particular, in the GK+LIRA (1200 μ g/kg/day) group, FINS concentrations and HOMA-IR were similar to those in the Wistar+NS group, while ISI was still lower than that of the Wistar+NS group.

Liraglutide improved lipid profiles, leptin and adiponectin levels

After 4 weeks of intervention, TG and NEFA levels were significantly improved in the liraglutide-treated and GK+Diet-control groups. Compared with the Wistar+NS and GK+NS groups, liraglutide treatment dramatically reduced TG, TC and NEFA levels after 12 weeks, and there were no differences between the two liraglutide-treated groups (all $P < 0.05$). These were also improved in the GK+Diet-control group, which were comparable with those of the GK+LIRA (1200 μ g/kg/day) group (Fig. 3A, B and D). LDL levels were also decreased at the end of the experiment after high-dose liraglutide treatment or food restriction (Fig. 3C). Additionally, a dramatic reduction in leptin levels (Fig. 3E) and an obvious increase in adiponectin levels (Fig. 3F) were observed from the 4th week of liraglutide intervention. Lower leptin levels and higher adiponectin levels were also observed in the GK+Diet-control group, but adiponectin levels were not increased to the same degree as those of the GK+LIRA (1200 μ g/kg/day) group ($P < 0.05$, Fig. 3E and F).

Liraglutide reduced whole-body fat mass, especially visceral fat mass

The weight reducing effects of liraglutide were found to be associated with reductions in body fat mass. At the end of the experiment, the body compositions of all the rats were measured with DXA (Fig. 4A). The results showed that the ratios of body fat to total mass in the GK+LIRA (400 μ g/kg/day) ($22.40 \pm 2.12\%$), GK+LIRA (1200 μ g/kg/day) ($21.78 \pm 2.08\%$) and GK+Diet-control ($24.36 \pm 2.02\%$) groups were significantly lower than those in the Wistar+NS ($52.10 \pm 1.64\%$) and GK+NS ($38.36 \pm 3.36\%$) groups. There were no significant differences between the three intervention groups (Fig. 4B). Similarly, the total fat mass in GK+LIRA (400 μ g/kg/day) (85.4 ± 5.77 g), GK+LIRA (1200 μ g/kg/day) (78.74 ± 11.60 g) and GK+Diet-control (89.96 ± 4.05 g) groups were significantly lower than that in the Wistar+NS (256.1 ± 5.72 g) and GK+NS (158.86 ± 15.31 g) groups. Also, no significant differences existed between the three intervention groups. The MRI results revealed that liraglutide treatment considerably

Figure 2

Liraglutide attenuated glucose levels, food intake, body weight gain and insulin resistance. (A) Dose-dependent improvement of fasting blood glucose (FBG) levels. (B) Dose-independent improvement of postprandial blood glucose (PBG) levels. (C) Dose-independent inhibition of food intake. (D) Dose-independent attenuation of body weight gain. (E) Decreases in fasting insulin (FINS) levels. (F) Improvement of insulin resistance. (G) Elevation of insulin sensitivity. The above indicators were assayed in all rats ($n = 10$ in each group). Data are presented as the mean $\pm$ s.e.m. * $P < 0.05$, vs Wistar + NS group; # $P < 0.05$, vs GK + NS group; & $P < 0.05$, vs GK + LIRA (400 µg/kg/day) group; * $P < 0.05$, vs GK + LIRA (1200 µg/kg/day) group.

decreased the visceral fat accumulation (Fig. 4C), but the subcutaneous fat accumulation appeared to be slightly increased (Fig. 4D). Interestingly, in the GK+Diet-control group, the visceral fat was not obviously decreased, but the subcutaneous fat was relatively decreased compared with GK+NS group (Fig. 4C and D).

Liraglutide improved hepatic steatosis and reduced adipocyte size in fat depots

Liraglutide injections or food restriction drastically reduced hepatic lipid accumulation in GK rats (Fig. 5A and B). The ratios of the oil red O-stained area to the total area in the liver were significantly lower in the GK+LIRA (400 µg/kg/day) ($21.00 \pm 2.58\%$), GK+LIRA (1200 µg/kg/day) ($17.75 \pm 2.75\%$) and GK+Diet-control ($23.00 \pm 2.58\%$) groups than in the Wistar+NS ($33.25 \pm 2.75\%$) and GK+NS groups ($34.00 \pm 2.94\%$) (Fig. 5C, $P < 0.05$). Although there was no difference between the two liraglutide-treated groups, the hepatic lipid accumulation in the GK+Diet-control group was more severe than that in the GK+LIRA (1200 µg/kg/day) group ($P < 0.05$). Figure 5D, E and F

showed the morphological changes of the mesenteric, inguinal and cluneal white adipose tissue (WAT). The cell sizes in all fat depots were smaller in the liraglutide-treated and GK+Diet-control groups (103.33 ± 3.14 , 96.50 ± 5.54 and 98.67 ± 7.76 µm, respectively) than in the controls (Wistar+NS: 118.17 ± 12.59 , 125.67 ± 3.78 , 117.83 ± 3.19 µm; GK+NS: 119.00 ± 8.08 , 112.83 ± 7.49 , 121.00 ± 3.90 µm, $P < 0.05$). There were no differences in cell sizes between the GK+LIRA (400 µg/kg/day) (71.67 ± 3.20 , 84.83 ± 3.87 and 101.33 ± 5.82 µm, respectively) and GK+LIRA (1200 µg/kg/day) (69.17 ± 4.71 , 88.67 ± 5.65 and 95.83 ± 5.23 µm, respectively) groups for all fat depots. However, in the mesenteric WAT and inguinal WAT, the cell sizes were larger in the diet-control group than in the two liraglutide-treated groups ($P < 0.05$).

Liraglutide decreased the deposition and synthetic rates of TG in the liver and mesenteric WAT but increased these rates in the cluneal WAT

Compared with those in the GK+NS group, the deposition and synthetic rates of TG in the liver and mesenteric

Figure 3

Liraglutide improved lipid profiles and leptin and adiponectin levels. (A) Triglyceride (TG) levels. (B) Total cholesterol (TC) levels. (C) Low-density lipoprotein-cholesterol (LDL) levels. (D) Non-esterified fatty acid (NEFA) levels. (E) Leptin levels. (F) Adiponectin levels. The above indicators were assayed in all rats ($n = 10$ in each group). Data are presented as the mean $\pm$ s.e.m. * $P < 0.05$, vs Wistar + NS group; # $P < 0.05$, vs GK + NS group; & $P < 0.05$, vs GK + LIRA (400 $\mu\text{g/kg/day}$) group; * $P < 0.05$, vs GK + LIRA (1200 $\mu\text{g/kg/day}$) group.

WAT were significantly reduced in the two liraglutide-treated groups, and there were no significant differences between these two groups. In the GK+Diet-control group, although the deposition and synthetic rates of TG were also significantly decreased in the liver compared with those in the GK+NS group, the synthetic rates of TG were higher than those in the GK+LIRA (1200 $\mu\text{g/kg/day}$) group ($P < 0.05$). Interestingly, compared with those in the GK+NS group, the deposition and synthetic rates of TG in the cluneal WAT were drastically elevated in the two liraglutide-treated groups but not in GK+Diet-control group (Fig. 6A and C). Correspondingly, the ratios of lipid deposition in different fat depots (cluneal/mesenteric) gradually increased in the liraglutide-treated groups but not in GK+Diet-control group (Fig. 6B).

In the inguinal WAT, the change trends were different from those in cluneal WAT. There were no differences in the synthetic rate of TG in the inguinal WAT among the five groups (Fig. 6C). Moreover, the deposition rate of TG was decreased after liraglutide treatment. Nevertheless, the ratios of lipid deposition in different fat depots (inguinal/mesenteric) were still increased in the liraglutide-treated groups. The deposition rate of TG was also decreased by food restriction in the inguinal WAT, so its ratio to that in mesenteric WAT was not different

from the controls (Fig. 6A and B). The specific data were shown in Table 1.

Liraglutide decreased lipolysis and promoted fatty acid β -oxidation in the skeletal muscle

R_{gly} in the blood can reflect the extent of lipolysis, as the main source of glycerol is peripheral adipose tissue degradation during fasting (Zhao *et al.* 2017). After a 12-week intervention, the two liraglutide-treated groups (0.89 ± 0.13 and $0.63 \pm 0.13 \mu\text{mol/kg/min}$) and GK+Diet-control group ($0.87 \pm 0.03 \mu\text{mol/kg/min}$) all had lower R_{gly} than the Wistar+NS ($1.4 \pm 0.27 \mu\text{mol/kg/min}$) and GK+NS ($1.48 \pm 0.78 \mu\text{mol/kg/min}$) groups (all $P < 0.05$) (Fig. 6D). In contrast, the ratios of fatty acid β -oxidation in the skeletal muscle were dramatically increased in the liraglutide-treated groups ($10.72 \pm 4.81\%$ and $16.30 \pm 1.11\%$) compared with the GK+NS group ($1.33 \pm 0.42\%$, $P < 0.05$), and this effect was drug dose dependent. The ratio of fatty acid β -oxidation in the GK+Diet-control group ($7.85 \pm 1.16\%$) was also augmented compared with that in the GK+NS group, but was lower than that in the Wistar+NS and GK+LIRA (1200 $\mu\text{g/kg/day}$) groups. Relative to the GK+NS group, the Wistar+NS group ($11.48 \pm 3.11\%$) had a higher ratio of fatty acid β -oxidation ($P < 0.05$) (Fig. 6E).

Figure 4

Liraglutide reduced whole-body fat mass, especially visceral fat mass. (A and B) Reduction in whole-body fat mass. Dual-energy X-ray imaging showed that liraglutide significantly reduced the body fat ratios in a dose-independent manner. (C and D) Images of visceral and subcutaneous fat deposition with magnetic resonance imaging (MRI). Liraglutide considerably decreased visceral fat accumulation, while subcutaneous fat accumulation appeared to be slightly increased in a dose-dependent manner. The above indicators were assayed in all rats ($n = 10$ in each group). * $P < 0.05$, vs Wistar + NS group; # $P < 0.05$, vs GK + NS group.

Liraglutide regulated gene expression related to lipid metabolism in the liver and in different fat depots

To further evaluate the effects of liraglutide on lipid metabolism, the mRNA expression levels of adipose-related genes were investigated by qPCR. Liraglutide treatment significantly downregulated the mRNA expression levels of key enzymes (*Mgat* and *Dgat2*) involved in TG synthesis in the liver and mesenteric WAT but upregulated these enzymes in the inguinal and cluneal WAT. Food restriction also downregulated *Mgat* and *Dgat2* expression in the liver but upregulated their expression in inguinal WAT. Besides, *Dgat2* was low-expressed in mesenteric WAT and was over-expressed in cluneal WAT by food restriction, but these effects were weaker than those in the high-dose liraglutide group (Fig. 7A and B).

The mRNA expression levels of a key enzyme (*Hsl*) involved in TG degradation were downregulated by liraglutide and food restriction in both liver and mesenteric WAT. In the mesenteric WAT, the expression of *Hsl* was decreased much more in GK+LIRA (1200µg/kg/day) group than in GK+Diet-control group. For the subcutaneous WAT, the mRNA expression levels of *Hsl* were also downregulated by liraglutide in the cluneal WAT but upregulated in the inguinal WAT. Food restriction also increased the expression of *Hsl* in inguinal WAT, but had a weaker effect than did the high dose of liraglutide (Fig. 7C).

Both *Cpt1a* and *Cpt1b* are key enzymes involved in fatty acid β -oxidation. *Cpt1a* was mainly expressed in the liver and fat tissues, whereas *Cpt1b* was expressed in the skeletal muscle. After liraglutide intervention, *Cpt1a* was over-expressed in the liver, mesenteric and cluneal WAT,

Figure 5

Liraglutide improved hepatic steatosis and reduced adipocyte size in different fat depots. (A) HE staining of liver tissues (magnification $\times 200$). (B) Oil red O staining of liver tissues (magnification $\times 200$). (C) Area ratios of oil red O staining. Liraglutide or food restriction dramatically decreased ectopic lipid accumulation in the liver after intervention for 12 weeks. (D) HE staining of mesenteric white adipose tissue (WAT) (magnification $\times 200$). (E) HE staining of inguinal WAT (magnification $\times 200$). (F) HE staining of cluneal WAT (magnification $\times 200$). Liver or adipose tissues in different fat depots were all randomly chosen from the five groups ($n = 5$ in each group). $^*P < 0.05$, vs Wistar + NS group; $^*P < 0.05$, vs GK + NS group; $^*P < 0.05$, vs GK + LIRA (400 µg/kg/day) group; $^{\#}P < 0.05$, vs GK + LIRA (1200 µg/kg/day) group.

but its expression was reduced in the inguinal WAT. A similar trend in the expression of *Cpt1a* in the liver and different fat depots was also observed in the GK+Diet-control group, but only in the cluneal WAT, there was a significant difference between GK+NS and GK+Diet-control groups (Fig. 7D).

The mRNA expression levels of *Cpt1b* in the skeletal muscle were also considerably upregulated by the high-dose of liraglutide and food restriction. The effect of the high-dose of liraglutide was stronger than that of food restriction (Fig. 7E). *Ucp1* was mainly expressed in brown adipose tissue (BAT) and involved in thermogenesis

Figure 6

Liraglutide redistributed visceral and subcutaneous fat deposition by regulating lipid metabolism in different tissues. (A) Deposition rates of TG in different tissues. (B) Ratio of lipid deposition in the subcutaneous white adipose tissue (WAT) to that in the visceral WAT. (C) Triglyceride (TG) synthetic rates in different tissues. (D) Inhibition of lipolysis. (E) Enhancement of fatty acid β -oxidation in skeletal muscle. The above indicators were assayed in all rats ($n = 10$ in each group). Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$, vs Wistar + NS group; # $P < 0.05$, vs GK + NS group; & $P < 0.05$, vs GK + LIRA (400 $\mu\text{g/kg/day}$) group; & $P < 0.05$, vs GK + LIRA (1200 $\mu\text{g/kg/day}$) group.

activation. *Bmp4* could induce brown fat-like changes in WAT and increase insulin sensitivity (Qian *et al.* 2016). WAT browning could be enhanced through the induction of *Ucp1* expression and/or mitochondrial oxidative metabolism (Mao *et al.* 2018). *Pgc1 α* , a browning-related gene, was known as a significant regulator of mitochondrial biogenesis, oxidative phosphorylation and fatty acid metabolism (Balampanis *et al.* 2018). Both liraglutide and food restriction significantly upregulated the mRNA expression levels of *Ucp1* in the inguinal and cluneal WAT, and the expression of *Ucp1* was increased most by the high-dose of liraglutide (Fig. 7F). However, there were no obvious differences among the five groups in the mesenteric WAT. The expression levels of *Pgc1 α* (Fig. 7G) and *Bmp4* (Fig. 7H) mRNA were in line with those of *Ucp1*.

Liraglutide upregulated UCP1 protein expression in the subcutaneous fat depots

In the BAT, the UCP1 protein expression levels were not significantly different among the five groups after

liraglutide intervention and food restriction (Fig. 8A). There were also no significant differences in the mesenteric WAT among these five groups (Fig. 8B). However, the protein expression levels of UCP1 were significantly upregulated by liraglutide in the inguinal and cluneal WAT, an effect that was dose independent. The UCP1 protein expression levels in the subcutaneous WAT were also upregulated in GK+Diet-control group, and were comparable with those in the two liraglutide-treated groups (Fig. 8C and D). This revealed that liraglutide could promote browning remodeling in subcutaneous WAT, and this effect was similar to that of food restriction.

Discussion

In the current study, we found that liraglutide could decrease the visceral fat deposition but increase subcutaneous (inguinal and cluneal) fat deposition by bi-directionally regulating lipid metabolism in different fat depots, which was revealed by the regulation of the mRNA expression levels of adipose-related genes in

Table 1 Deposition and synthetic rates of TG in different fat depots.

Group	Deposition rates of TG (dpm/g)				Synthetic rates of TG (dpm × Protein/mmol)			
	Liver	Mesenteric WAT	Inguinal WAT	Cluneal WAT	Liver	Mesenteric WAT	Inguinal WAT	Cluneal WAT
Wistar + NS	3.35 ± 1.01	12.63 ± 4.34	9.31 ± 0.37	14.92 ± 2.91	14.79 ± 1.91	5.45 ± 0.87	4.20 ± 0.59	2.67 ± 0.56
GK + NS	5.36 ± 4.00	8.68 ± 2.97*	9.81 ± 1.90	10.71 ± 3.01*	18.62 ± 5.79	6.93 ± 0.84*	4.01 ± 0.79	2.60 ± 0.42
GK + LIRA (400 µg/kg/day)	2.00 ± 1.17#	4.40 ± 1.05*#	5.24 ± 0.37*#	12.42 ± 2.28	10.66 ± 3.48#	4.80 ± 1.43#	4.13 ± 1.79	3.46 ± 0.44*#
GK + LIRA (1200 µg/kg/day)	1.84 ± 1.17#	4.45 ± 0.87*#	4.82 ± 0.48*#	16.91 ± 3.83*#	8.99 ± 1.02*#	4.16 ± 0.96#	5.42 ± 3.37	3.71 ± 0.47*#
GK + Diet-control	2.29 ± 0.49#	7.15 ± 1.41*	5.71 ± 1.27*#	8.93 ± 2.60*#	11.76 ± 0.79# ^q	5.98 ± 1.63 ^q	3.45 ± 0.38	2.84 ± 0.28 ^q

Data are summarized as the mean ± s.d., and statistical significance was assessed by one-way ANOVA (LSD).

**P* < 0.05, vs Wistar + NS group; #*P* < 0.05, vs GK + NS group; **P* < 0.05, vs GK + LIRA (400 µg/kg/day) group; ^q*P* < 0.05, vs GK + LIRA (1200 µg/kg/day) group.

various tissues. Browning remodeling of the inguinal and cluneal WAT was also found. In addition, improvements in weight gain, blood glucose, lipid profiles, leptin and adiponectin levels, and insulin resistance were observed after liraglutide intervention.

Interestingly, this study indicated for the first time that liraglutide could redistribute body fat by decreasing visceral fat and relatively increasing lower-body subcutaneous fat deposition, which could partly be attributed to changes in the mRNA expression levels of corresponding key enzymes for lipid metabolism. The major anatomical fat depots include intra-abdominal (omental and mesenteric depots, also termed visceral fat), lower-body (gluteal, subcutaneous leg and intramuscular fat), and upper-body subcutaneous fat (Tchkonia *et al.* 2013). Upper-body fat (visceral and subcutaneous abdominal) is commonly associated with the unfavorable complications of obesity, while lower-body fat (gluteal-femoral) may be protective (White & Tchoukalova 2014). This is because of their different metabolic and functional properties. Subcutaneous adipocytes were often reported to be smaller than visceral. Dietary fat was stored more efficiently in visceral than in upper- or lower-body subcutaneous fat. Besides, subcutaneous preadipocytes had a greater replicative potential and capacity for adipogenesis. Additionally, visceral fat produced more harmful inflammatory cytokines and adipokines, which would contribute to the metabolic diseases. Genome-wide expression profiles analysis of primary preadipocytes also showed that expression of >500 genes varied significantly visceral and subcutaneous fat (Hilton *et al.* 2015). The effects of GLP-1 analogues on indirectly improving hepatic lipid metabolism have been previously reported most (Parlevliet *et al.* 2012, Mells & Anania 2013, Panjwani *et al.* 2013, Taher *et al.* 2014, Tanaka *et al.* 2014, He *et al.* 2016). Liraglutide has also been shown in many studies to improve hepatic steatosis (Mells & Anania 2013, Panjwani *et al.* 2013, Taher *et al.* 2014) and decrease central fat deposition (Inoue *et al.* 2011, Fujishima *et al.* 2012). Additionally, GLP-1RAs had been reported to have the effects on fat redistribution. Morano *et al.* (2015) reported that a short course of GLP-1RA treatment resulted in a significant decrease in both subcutaneous (peri-umbilical and sub-xiphoid) and visceral (pre-aortic, peri-renal, and epicardial) fat deposits. LEAD-2/3 showed that liraglutide reduced not only visceral adipose tissue but also abdominal subcutaneous adipose tissue (Jendle *et al.* 2009), since abdominal subcutaneous fat was reported to be independently associated with increased glucose and lipid levels (Lebovitz & Banerji 2005); however,

Figure 7

Liraglutide regulated the mRNA expression levels of genes related to lipid metabolism in different tissues. (A and B) mRNA expression levels of key enzymes (*Mgat*, *Dgat2*) involved in triglyceride (TG) synthesis. (C) mRNA expression levels of a key enzyme (*Hsl*) involved in TG degradation. (D and E) mRNA expression levels of key enzymes (*Cpt1a*, *Cpt1b*) for fatty acid β -oxidation. (F) mRNA expression levels of *Ucp1* in different fat depots. (G) mRNA expression levels of *Pgc1 α* in different fat depots. (H) mRNA expression levels of *Bmp4* in different fat depots. Liver, skeletal muscle or adipose tissues in different fat depots were all randomly chosen from the five groups ($n = 5$ in each group). Data are expressed as the mean $\pm$ s.e.m. * $P < 0.05$, vs Wistar + NS group; # $P < 0.05$, vs GK + NS group; % $P < 0.05$, vs GK + LIRA (400 µg/kg/day) group; $^wP < 0.05$, vs GK + LIRA (1200 µg/kg/day) group.

few studies have investigated the effects of liraglutide on lower-body subcutaneous fat. Our study reported for the first time that liraglutide could relatively increase lower-body subcutaneous fat deposition.

Possible mechanisms involved in the reduction of hepatic fat accumulation after GLP-1RAs intervention include an enhancement of hepatic lipophagy, a reduction

in lipid synthesis, VLDL overproduction and lipotoxicity, and a change in hepatocyte enzyme expression levels, which favors a switch in energy utilization from carbohydrate to lipid (Panjwani *et al.* 2013, Taher *et al.* 2014). Induction of autophagy via the AMPK/mTOR pathway (Decara *et al.* 2016) was also reported as one of the possible mechanisms. Other studies demonstrated

Figure 8

Liraglutide upregulated UCP1 protein expression in the subcutaneous fat depots. There was no significant difference in the UCP1 protein expression levels between the four groups in the brown adipose tissue (BAT) (A) and visceral (mesenteric) white adipose tissue (WAT) (B); however, the UCP1 levels were significantly upregulated in the subcutaneous (inguinal and cluneal) WAT (C and D) after liraglutide intervention. Adipose tissues in different fat depots were all randomly chosen from the five groups ($n = 4$ in each group). * $P < 0.05$, vs Wistar + NS group; # $P < 0.05$, vs GK + NS group; % $P < 0.05$, vs GK + LIRA (400 µg/kg/day) group; $^wP < 0.05$, vs GK + LIRA (1200 µg/kg/day) group.

that the decreased hepatic lipid contents in HFD mice were accompanied by a decrease in the expression levels of hepatic lipogenesis genes, while the decreased respiratory exchange ratio was coupled to a reduction in the lipid content in WAT via an upregulation of lipolytic genes (Parlevliet *et al.* 2012, Tanaka *et al.* 2014). Part of these effects might also be mediated by the central nervous system, as centrally administered exendin-4 was found to increase peripheral energy utilization and decrease hepatic lipid synthesis (Taher *et al.* 2014). Since the direct access of liraglutide to the brain is facilitated by the chemical structure of the compound (Lutz & Osto 2016), the central nervous system may play a partial role in the above effects. Previous study had demonstrated that GLP-1RAs administered in the periphery were stable and highly likely to act on the brain through the humoral and neural pathways. Since GLP-1RAs could remain in the brain for several hours, they could exert effects similar to those induced by the brain-derived endogenous GLP-1 (Katsurada & Yada 2016). Secher *et al.* (2014) found that fluorescence-labeled liraglutide administered in the periphery could reach the brain including all circumventricular organs, the zona interna of the median eminence, the area postrema, etc. And liraglutide might exert central effects on increasing feelings of satiety through activating pro-opiomelanocortin neurons and increasing levels of the cocaine- and amphetamine-stimulated transcript neuropeptide messenger ribonucleic acid, and on preventing a hunger through increasing agouti-related peptide and neuropeptide Y neuropeptide messenger ribonucleic acid (Knudsen *et al.* 2016). In our study, the corresponding key enzymes of lipid metabolism in different tissues also changed after liraglutide treatment. In contrast to previous studies, the expression levels of genes related to lipogenesis and lipolysis were downregulated in the liver and visceral WAT but upregulated in the subcutaneous WAT. This may be attributed to the different types of GLP-1RAs, animal species and different lengths of treatment among studies. Further investigations are needed to explore the exact reason.

Another interesting finding was that liraglutide induced subcutaneous WAT browning but not visceral WAT browning. Generally, due to their different effects on metabolic homeostasis, visceral fat is considered 'bad fat' (Bouchi *et al.* 2017), whereas subcutaneous fat and BAT are considered 'good fat' (Hocking *et al.* 2013). WAT is mainly responsible for energy storage, and BAT is mainly responsible for dissipating chemical energy in

the form of heat (Wu *et al.* 2015). Recent studies suggest that WAT can present brown-like features in response to certain stimuli and therefore exert an energy-disposal capacity characterized by upregulation of the BAT-specific gene *Ucp1* (Wu *et al.* 2015, Jeremic *et al.* 2017). A previous study revealed that central GLP-1 administration decreased lipid storage in WAT and stimulated adipocyte browning by modulating AMPK in the ventromedial nucleus (VMH), an effect that was accompanied by substantial weight loss (Beiroa *et al.* 2014). AMPK is considered to be the principal energy sensor in eukaryotic cells and hypothalamic AMPK plays a major role in the central control of food intake by mediating the effects of peripheral nutritional and hormonal stimuli (Lopez *et al.* 2016). Central administration of GLP-1RAs decreases fatty acid accumulation in WAT, stimulates PGC1 α /UCP1-related thermogenesis in BAT and increases the uptake of plasma triacylglycerol-derived fatty acids by BAT and WAT actions mediated by inhibition of hypothalamic AMPK phosphorylation and increases in sympathetic outflow (Lockie *et al.* 2012, Kooijman *et al.* 2015, Lopez *et al.* 2015). However, intraperitoneal injection of a GLP-1RA was found to upregulate the expression of *Ucp1* in subcutaneous WAT but did not significantly change the expression of *Ucp1* in BAT and visceral WAT (Wan *et al.* 2017). Our study showed a similar trend with the latter, although liraglutide was subcutaneously administered. This finding implies that different routes of drug administration can influence the effects of GLP-1RAs on lipid metabolism in different fat depots, which needs further investigation.

It is important to note that liraglutide treatment elicited a general shift to smaller adipocyte sizes in all white adipose depots, which was consistent with the results of a previous report (Wan *et al.* 2017). The size of adipocytes can, to a certain extent, reflect the metabolic function of adipocytes (Wan *et al.* 2017). Although there is controversy about the harmful range of adipocyte sizes (Verhoef *et al.* 2013), it is generally accepted that the conversion of small adipocytes to large ones is closely related to metabolic diseases. In addition, an increase in the number of small adipocytes can promote lipid metabolism and insulin sensitivity (Wan *et al.* 2017). Our study revealed that adipocyte sizes were reduced after intervention, partly explaining the anti-obesity effects of liraglutide.

Liraglutide was found to have extra protective effects on regulating lipid metabolism in different fat depots compared with food restriction. Calorie restriction is one

of the basic ways to improve glucose and lipid metabolism disorders. In our study, food restriction reduced glucose levels and weight gain, and improved insulin resistance and lipid profiles. These effects were all also elicited by liraglutide. In the current study, food restriction also decreased the TG deposition in the liver and inguinal WAT, but did not increase TG deposition in the cluneal WAT. However, TG deposition in the mesenteric WAT was not attenuated by food restriction. Correspondingly, the ratios of fat deposition in the subcutaneous WAT to that in the visceral WAT were not augmented. Advantages of liraglutide included that it not only decreased visceral (liver and mesenteric) fat accumulation, but also increased cluneal fat accumulation. Even in the inguinal WAT, fat deposition was increased relative to that in the mesenteric WAT, after liraglutide treatment. This means that, to some extent, liraglutide affected body fat redistribution.

In conclusion, in the present study, we demonstrated that the long-lasting GLP-1RA liraglutide redistributed body fat through tissue-specific modulation of lipid metabolism in different fat depots, an effect that was different from the effects of simple food restriction on improving lipid metabolism disorders. This effect was associated with the regulation of key enzymes related to lipid metabolism in various tissues. Moreover, liraglutide indirectly regulated fat depot function by reducing the size of adipocytes and stimulating subcutaneous WAT browning. T2DM-related metabolic disorders, which are also risk factors for macrovascular complications, were all improved by liraglutide treatment. These findings provide us with a novel understanding of the anti-obesity action of liraglutide. Liraglutide may protect against macrovascular complications in T2DM patients via a mechanism that is independent of glycemic control.

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

Y L designed the study; L Z, C Z, M L, C C, X N, A B, K Z, Z N, J C and F X performed the experiments; L Z and N W analyzed the data and interpreted the results of the experiments; L Z prepared the figures and wrote the manuscript; Y L, J M and N W edited and revised the manuscript. All authors read and approved the final manuscript.

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