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ORIGINAL RESEARCH

Liraglutide reduces lipogenetic signals in visceral adipose of db/db mice with AMPK activation and Akt suppression

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Abstract: Liraglutide, a glucagon-like peptide-1 analog, has been proved to reduce body weight and visceral adipose tissue (VAT) in human studies. In this study, we aimed at examining lipogenetic signal changes in VAT after weight-loss with liraglutide in db/db mice. The mice were divided into two groups: liraglutide-treated group (n=14, 8-week-old, fasting glucose. >10 mmol/L, liraglutide 300 μg/kg twice a day for 4 weeks) and control group (n=14, saline). We found body weight gain and food intake were reduced after liraglutide treatment ($P<0.05$). Compared to the control group, the VAT weights were significantly lower in the treated group (2.32±0.37 g versus 3.20±0.30 g, $P<0.01$) than that in control group. In VAT, compared with control group, the lipogenetic transcription factors PPARγ and C/EBPα expressions were both reduced with pAMPK and pACC increased 3.5-fold and 2.31-fold respectively, while pAkt and pP38MAPK were reduced 0.38-fold and 0.62-fold respectively ($P<0.01$). In conclusion, VAT was reduced after weight loss with AMPK activation and Akt suppression with liraglutide treatment, which was associated with reduction of lipogenetic process in VAT.

Keywords: liraglutide, visceral adipose tissue, AMP-activated protein kinase, lipogenesis

Introduction

The growing prevalence of obesity constitutes a major health problem worldwide.[1] Associated with obesity, particularly abdominal obesity, metabolic disorders including hyperinsulinemia, impaired glucose tolerance, and dyslipidemia are often observed, which increase the risk for type 2 diabetes mellitus, cancer, and heart disease.[2–6] Indeed, visceral and subcutaneous depots differ considerably from the histological, physiological, and metabolic points of view.[7] Abdominal fat accumulation represents a risk factor per se.[8] To reduce visceral adipose tissue (VAT) is crucial to type 2 diabetes mellitus and cardiovascular disease therapy.

Lipid accumulation increases throughout the adipogenic process, and it is regulated by genetic and growth factors.[9] PPARγ and C/EBPα are two major lipogenetic transcription factors.[10,11] AMPK is a serine/threonine heterotrimeric kinase that acts as an intracellular energy sensor[12,13] or "fuel gauge".[14] In keeping with its energy sensor role, starvation activates AMPK in adipose tissue,[15,16] and AMPK exerts antilipolytic effects,[15–17] as well as inhibiting adipocyte fatty acid synthesis, by phosphorylating ACC[16] and inhibiting insulin-induced glucose uptake.[17] The overall effect of AMPK is to convert adipocytes into lipid oxidizing cells with suppressed lipogenesis and lipolysis.[16]

GLP-1, an insulinotropic gastrointestinal peptide produced mainly from intestinal endocrine L-cells in response to food intake, lowers blood glucose, delays gastric emptying,

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and increases satiety as well as reduces body weight.[18–21] Liraglutide is a GLP-1 analog with 97% amino acid sequence identity to native human GLP-1 and an acyl side-chain attachment, which makes it bind to albumin. These small structural differences prolong the half-life of the analog to 13 hours, making it suitable for once daily administration.[22] Large Phase III clinical studies have consistently shown that liraglutide improves glycemic control, blood pressure, and lipid profiles with weight loss.[23–28] In clinical trials, body weight index and the waist/hip ratio are significantly reduced after liraglutide treatment.[29] The liraglutide effect and action in diabetes for 26 weeks (LEAD-2) and 52 weeks (LEAD-3) studies have shown that reductions in body weight with liraglutide primarily come from reductions in fat mass rather than lean tissue mass.[30] In addition, the computed tomography (CT) assessment from within the LEAD-2 study showed that the mean reductions in tissue area from baseline were greater for VAT (−16.4%) than abdominal subcutaneous adipose tissue (−8.5%).[31] Now, liraglutide 3.0 mg per day has already been approved by the US Food and Drug Administration (FDA) in obesity treatment.

In our study, we aimed at examining the effects of liraglutide on lipogenetic signal changes in VAT.

Materials and methods

Animals and procedures

All experiments were carried out with permits from the Animal Experiments Ethical Committee of Peking University First Hospital. Six-week-old male db/db mice (C57BL/KsJ-db/db) were purchased from Peking University Laboratory Animal Center. All the mice were housed (seven mice/cage) in an air-conditioned room at 22°C±2°C with controlled ambient conditions following a 12-hour light:12-hour dark cycle, with lights on at 8 am. Drinking water and high fat rodent diet with 23% fat content (HFK Bioscience, Beijing, People's Republic of China) were supplied ad libitum.

After a week of adjustable feeding, mice were randomly assigned into the liraglutide-treated group (n=14) and the control group (n=14). When fasting blood glucose was above 10 mmol/L, the nearly 8-week-old mice received subcutaneous injections of liraglutide (300 μg/kg) or 0.9% saline of the equal volume twice a day for 4 weeks. Fasting blood glucose, food intake, and body weight were measured every week.

At the end of the experimental period, all mice were fasted overnight with free access to water and then killed by decapitation. Plasma was collected and frozen and VAT were quickly dissected and weighed. The perirenal, epididymal, and omental adipose tissue, as representative of visceral fat, were pooled.

Ribonucleic acid (RNA) isolation and cDNA synthesis

Total RNA was isolated from mouse islets using TRI reagent. First-strand cDNA was synthesized from total RNA using a reverse transcriptase synthesis system (SuperScript III; Invitrogen, Carlsbad, CA, USA) and random hexamers. Real-time polymerase chain reaction was performed with a sequence detection system (ABI Prism 7500; Thermo Fisher Scientific). The messenger RNA (mRNA) levels were calculated by comparative CT methods ($X_{Test}/X_{GAPDH}=2^{-\Delta\Delta CT}$) with GAPDH as the endogenous reference gene. Primers and probes used in quantitative real-time polymerase chain reaction were designed by Primer Express 3.0 as listed in Table 1.

Western blot analysis

Total protein was extracted and then quantified using a BCA protein quantification kit (Beyotime, Shanghai, People's Republic of China). A total of 50 μg protein from each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) and transferred to a nitrocellulose membrane (0.45 μm pore size; PALL Corporation, Port Washington, NY, USA). The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% bull serum albumin for 1 hour at room temperature. After incubation with the primary antibodies at 4°C overnight, the membranes were washed in TBST for

Table 1 Primer pairs for real-time PCR of genes associated with lipogenesis

Gene name	Forward (5′→3′)	Reverse (5′→3′)	Product size (bp)
PPARγ	AGGGCGATCTTGACAGGAAA	TCTCCCATCATTAAGGAATTCATG	77
C/EBPα	CTGCGAGCACGAGACGTCTA	AAGAGGTCGGCCAGGAACTC	81
FoxO1	AGCTGGGTGTCAGGCTAAGAGT	GCTCCTCAGTTCCTGCTGTCA	81
PI3K	TGGTGGTGCTCTGAAAAATGC	CTGTTGGCAATTGATAGGGACTT	120
AMPKα	TGTAGAGCAATCAAGCAGTTGGA	TCCTTCGTACACGCAAATAATAGG	70
ACC	TGGCTTCTCCAGCAGAATTTG	ACGGATAGATCGCATGCATTT	110
GAPDH	AGCCTCGTCCCGTAGACAAA	GTGACCAGGCGCCCAATAC	121

Abbreviation: PCR, polymerase chain reaction.

15 minutes three times prior to incubation with the secondary anti-rabbit (Abgent Company, San Diego, CA, USA) or anti-mouse (Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, People's Republic of China) horseradish peroxidase-conjugated antibody for 1 hour at room temperature. After the membranes were washed again with TBST for 15 minutes three times, the bands were visualized with enhanced chemiluminescence reagents (EMD Millipore, Billerica, MA, USA). Primary antibodies PPAR-γ, pAkt, Akt, pP38 MAPK, P38 MAPK, pERK1/2, ERK1/2, FoxO1, pAMPK, AMPK, pACC, ACC (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Beijing Zhongshan Gold Bridge Biotechnology Co.) were used.

Statistical analysis

Data were expressed as mean ± standard error. Significant differences among groups were evaluated by one-way analysis of variance and Tukey's multiple comparisons test or by unpaired two-tailed Student's *t*-test by using PRISM software. Groups of data from both were considered to be significantly different if $P<0.05$.

Results

Liraglutide reduced body weight gain and food intake

There was an insignificant difference on baseline body weights and food intake between the treated and control groups. Compared to baseline, the body weights of liraglutide-treated group were significantly decreased after 1-week treatment ($P<0.001$, Figure 1A), whereas body weight of the control group was steadily increased (Figure 1A). An average relative body weight gain was calculated by subtracting the baseline body weight from the body weight measured each week. We found that the body weight gain was markedly lower in liraglutide-treated group than the control group ($P=0.02$, Figure 1B).

After 4-week treatment, food intake of the liraglutide-treated group was decreased from baseline of 5.21±0.56 g/d per mouse to 4.47±0.32 g/d per mouse, while that of the

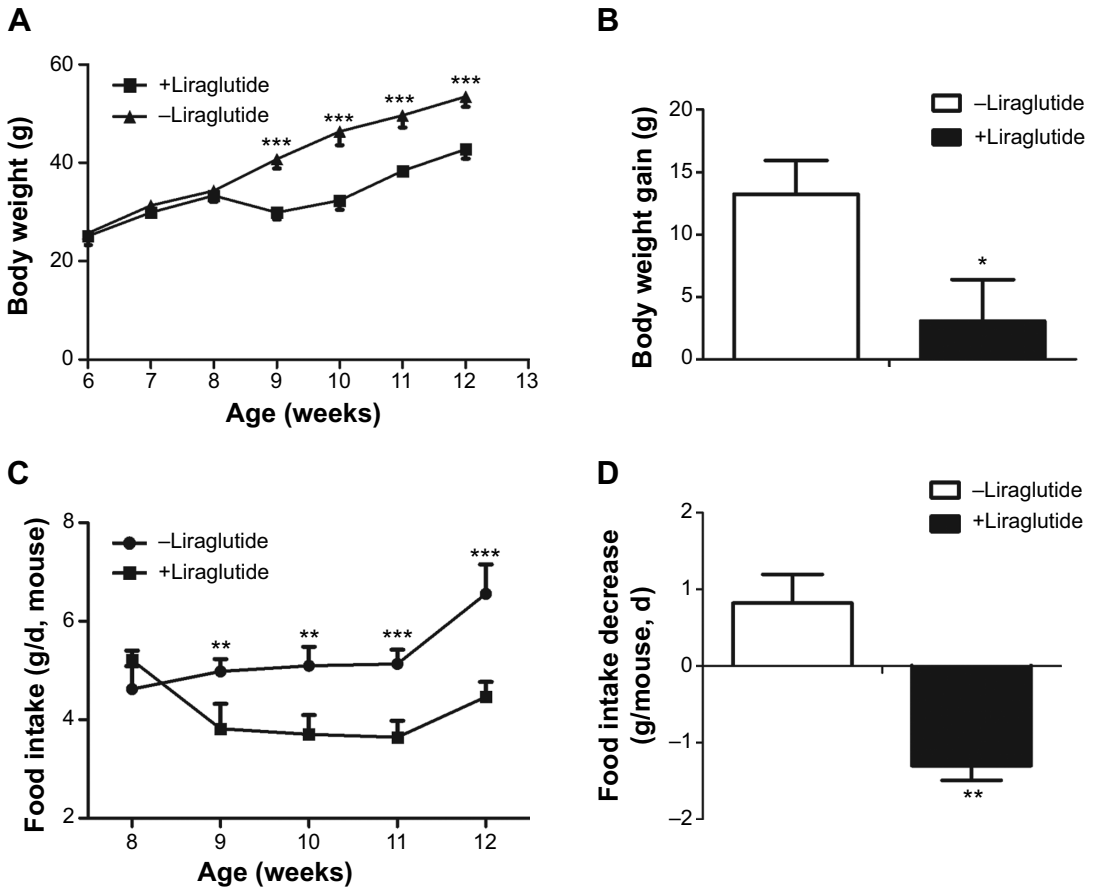

Figure 1 Effects of liraglutide on body weight and food intake.
Notes: The mice were subcutaneously injected liraglutide or saline at 8 weeks old. (**A**) Effect of liraglutide on the body weight of mice. (**B**) An average relative body weight gain was calculated by subtracting the baseline body weight from the body weight measured each week. (**C**) Effect of liraglutide on the food intake of db/db mice. (**D**) Food intake decrease of treated and control group. Data represent the means ± standard deviation. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with the control group.
Abbreviation: d, day.

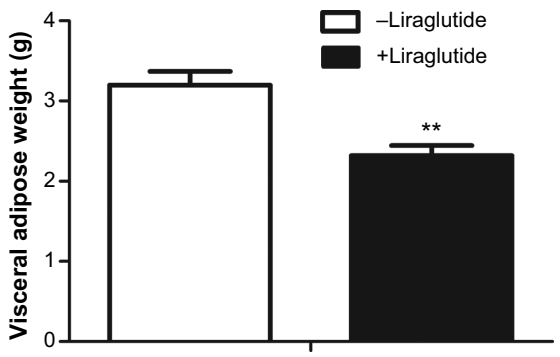

Figure 2 Effects of liraglutide treatment on visceral adipose weight.
Notes: After 4 weeks treatment, the perirenal, epididymal, and omental adipose tissue were pooled and used as visceral adipose tissue. Data represent the means ± standard deviation. $^{**}P<0.01$, compared with the control group.

control group was increased from 4.62±0.47 g/d per mouse to 6.56±0.45 g/d per mouse ($P<0.01$, Figure 1C). The food intake decrease of the treated group was significantly lower than that of the control group ($P=0.002$, Figure 1D).

VAT contents were reduced after liraglutide treatment

We took all the perirenal, epididymal, and omental adipose tissue as VAT. After liraglutide treatment, VAT weights were reduced significantly compared with the control group respectively (2.32±0.37 g versus 3.20±0.30 g, $P<0.01$, Figure 2).

Liraglutide reduced lipogenetic transcription factors expressions in VAT

The mRNA expressions of specific lipogenetic transcription factor PPARγ and C/EBPα were all reduced in VAT compared with the control group ($P<0.01$, Figure 3A). In addition, the protein levels of PPARγ were reduced by 0.703-fold in VAT as assessed by Western blot analysis ($P<0.0001$, Figure 3B).

Effect of liraglutide on Akt signaling pathways

Further, we evaluated the protein expressions of pAkt, pP38 MAPK, and pERK1/2. Protein levels of pAkt, pP38 MAPK were reduced by 0.38-fold and 0.62-fold in VAT compared to the control group ($P<0.0001$, Figure 4A–C). There was no significant difference for pERK1/2 in VAT between the treated and control group. Since a previous study has shown that Akt-mediated inhibition of FoxO1 induces PPARγ and subsequent adipocyte differentiation[32] and PI3K is in the upstream of Akt signaling pathway, we further found that the protein levels of FoxO1 were increased in VAT ($P<0.0001$, Figure 4D). In VAT, the mRNA expression of FoxO1 in the treated group was increased by 1.7-fold and PI3K was reduced by 0.56-fold compared to the control group ($P<0.0001$, Figure 4E).

Effect of liraglutide on AMPK signaling pathways

AMPK has been demonstrated to mediate inactivation of lipogenic enzymes by inducing phosphorylation of ACC.[33] We found that the mRNA levels of AMPK in the treated group were increased by 1.83-fold and that of ACC was reduced by 0.56-fold in VAT compared to the control group ($P<0.01$, Figure 5A). After liraglutide treatment, AMPK signaling pathways were activated in VAT with protein levels of pAMPK increased by 3.5-fold ($P<0.0001$, Figure 5B) and those of pACC increased by 2.31-fold ($P<0.0001$, Figure 5C) compared with that in control group.

Discussion

In this study, we have shown that liraglutide reduced body weight gain and VAT. The expressions of lipogenetic

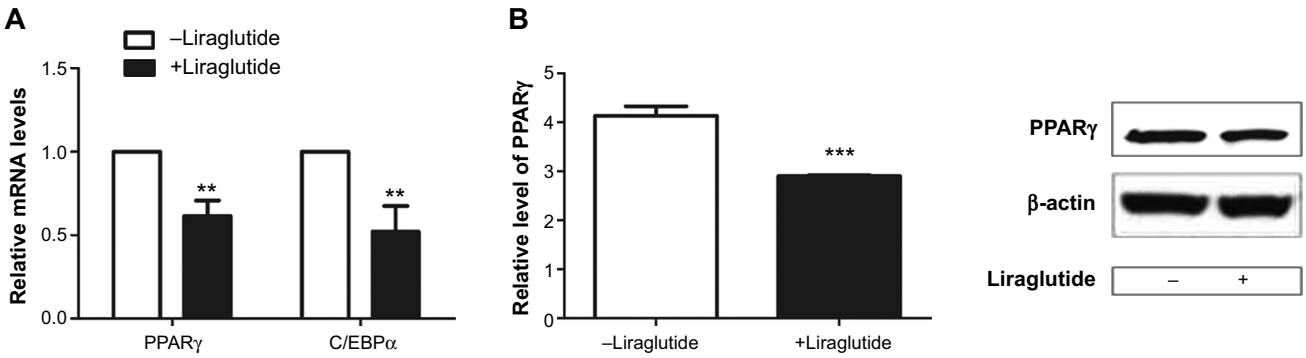

Figure 3 Effects of liraglutide on lipogenesis of subcutaneous and visceral adipose tissue.
Notes: Relative mRNA levels of PPARγ and C/EBPα were analyzed by real-time PCR. (**A**) Relative mRNA levels of PPARγ and C/EBPα in visceral adipose tissue (VAT). (**B**) The protein expression levels of the transcription factors PPARγ were assessed by Western blot analysis. The data shown represent the means ± SD. $^{**}P<0.01$, $^{***}P<0.0001$ versus control.
Abbreviations: mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; SD, standard deviation.

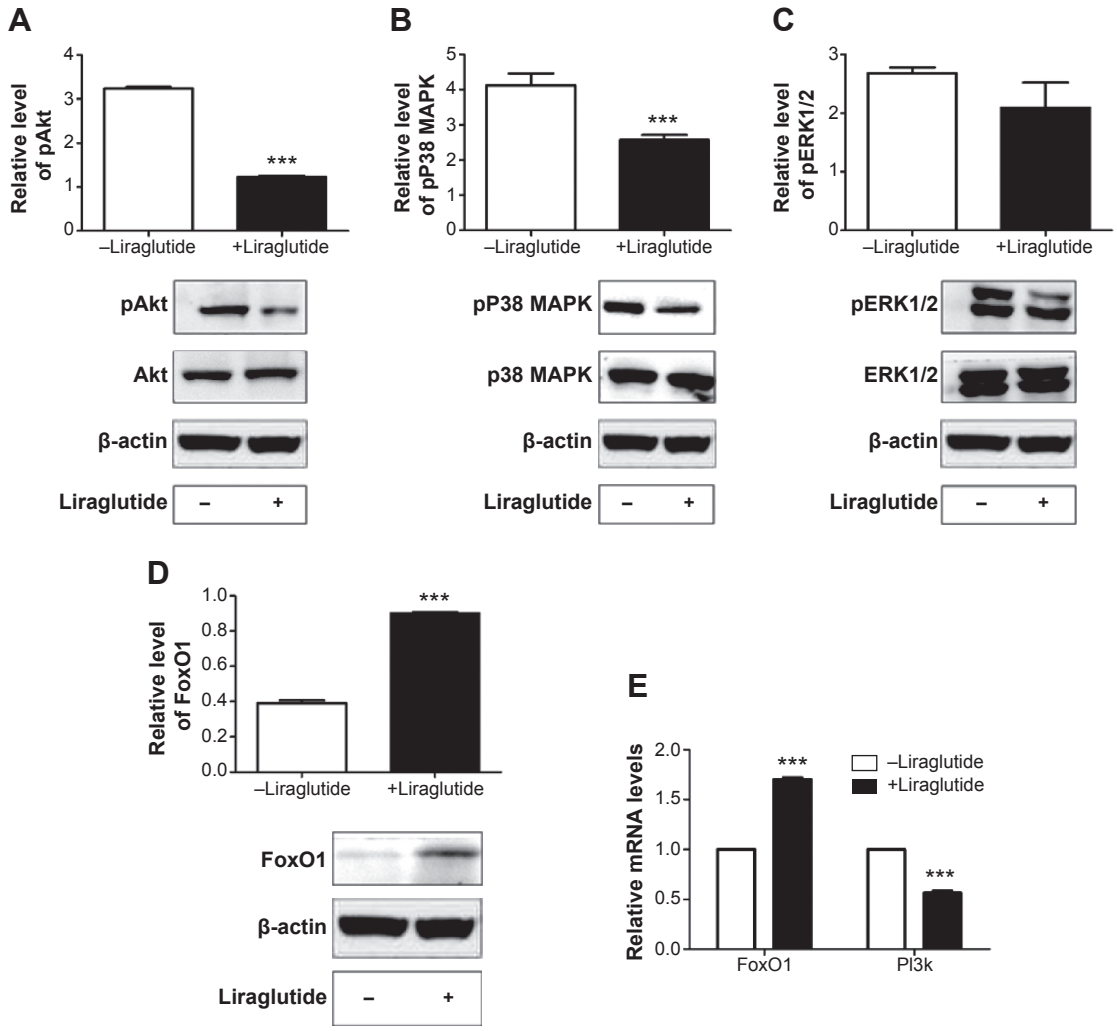

Figure 4 Effects of liraglutide on Akt signaling pathway.
Notes: Western blot showing (**A**) pAkt, Akt, (**B**) pP38 MAPK, P38 MAPK, (**C**) pERK1/2, ERK1/2, and (**D**) FoxO1 in visceral adipose tissue of liraglutide-treated and control group. Real-time PCR showing relative mRNA levels of FoxO1 and PI3K (**E**) in visceral adipose tissue of liraglutide-treated and control group. The data shown represent the means ± SD. ***$P<0.0001$ versus control.
Abbreviations: PCR, polymerase chain reaction; mRNA, messenger ribonucleic acid; SD, standard deviation.

transcription factors PPARγ and C/EBPα in VAT were reduced with activation of AMPK signaling pathway and suppression of Akt signaling pathway.

It is known that activated AMPK is a master regulator of cellular responses to low energy states, which suppresses lipogenesis pathways.[34] It has been reported that the expression of AMPK was reduced in VAT both in obese mice[35] and in morbidly obese patients.[36] Gauthier et al reported that AMPK activity is lower in VAT of morbidly obese individuals who are insulin resistant than in comparably obese people who are insulin sensitive.[37] In the present study, the activation of AMPK in VAT suggested liraglutide partly reversed pathophysiology of visceral obesity.

AMPKs are activated by two distinct signals: a Ca^{2+}-dependent pathway mediated by CaMKKb and an AMP-dependent pathway mediated by LKB1.[38,39] The calcium concentration could be increased either by direct stimulation of sympathetic nerves or the GLP-1 receptor-cAMP-PKA signaling pathway.[40] Central nervous system GLP-1 directly controls adipocyte lipid metabolism and this action seems to be partially modulated through effects on sympathetic outflow, and more specifically by β1- and β2-adrenergic receptor.[41] In adipose tissue, fasting and exercise activate AMPK.[15,17,42] Since both situations are concomitant with adrenergic stimulation, it could be anticipated that β-adrenergic agonists and their second messenger cAMP would stimulate AMPK activity. Nogueiras et al found chronic central infusion of GLP-1 reduced body weight and fat mass with up sympathetic nerve activity independent of food intake.[41] It was indicated that the actions of GLP-1 on VAT should be modulated by sympathetic tone to activate AMPK,

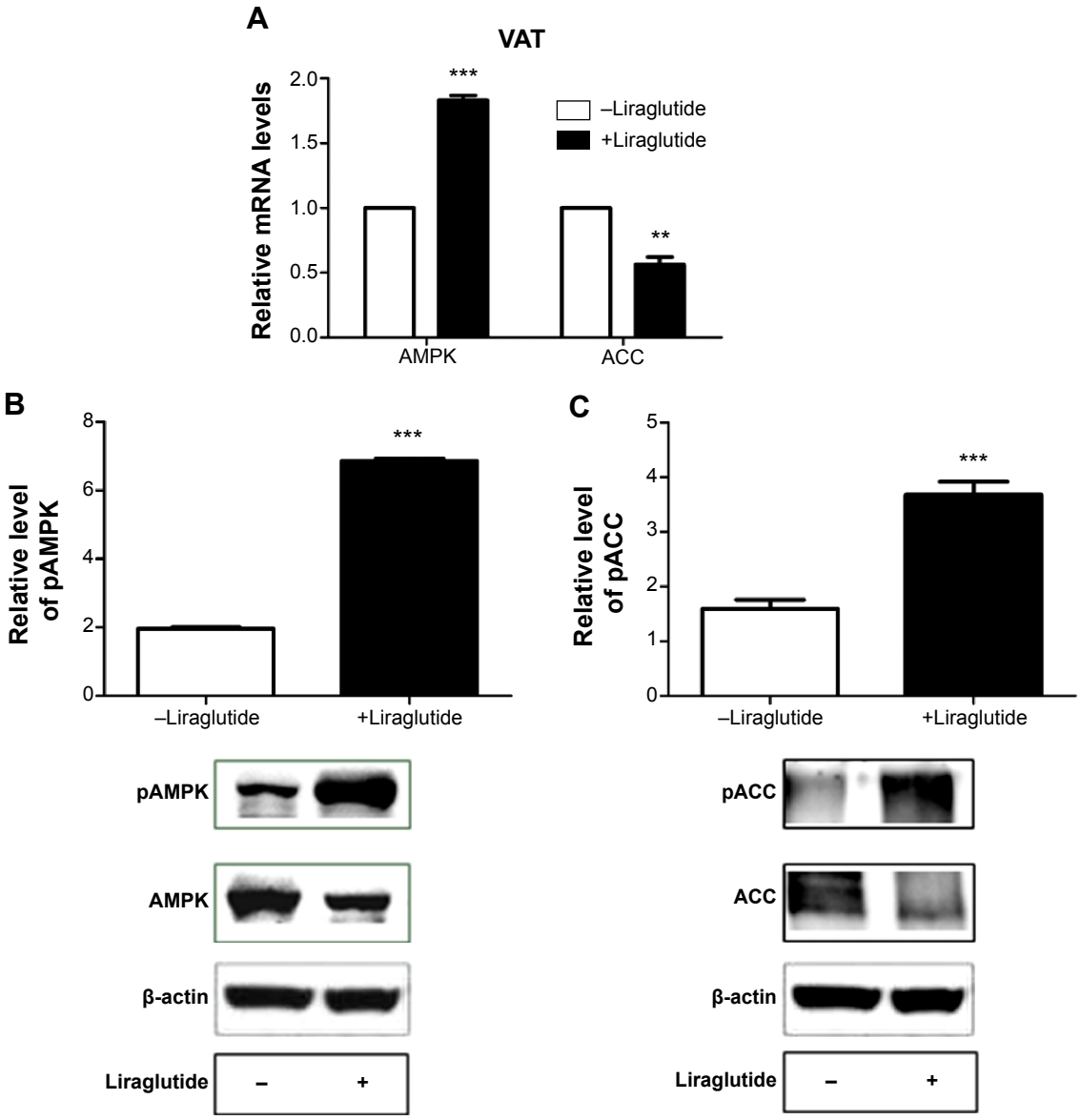

Figure 5 Effects of liraglutide on AMPK signaling pathway.
Notes: Relative mRNA levels of AMPK and ACC were analyzed by real-time PCR. (**A**) Relative mRNA levels of AMPK and ACC in visceral adipose tissue (VAT). (**B**) The protein expression levels of pAMPK and AMPK were assessed by Western blot analysis. (**C**) The protein expression levels of pACC and ACC. The data shown represent the means ± SD. $^{**}P<0.01$, $^{***}P<0.0001$ versus control.
Abbreviations: PCR, polymerase chain reaction; mRNA, messenger ribonucleic acid; SD, standard deviation.

leading to decreased lipogenesis and reduced triglyceride content (Figure 6).

Akt is known to mediate a number of biological responses to insulin. Inhibition of Akt activity resulted in a reduction in de novo lipid synthesis. In our study, AMPK activation and Akt suppression led to increased pACC in VAT after weight loss with GLP-1. Consequently, the lipogenesis in VAT was down-regulated. But how Akt was inactivated and the effects of insulin on it were not clear. Kim et al found AMPK stimulates dephosphorylation of Akt/PKB through PP2A activation in MDA-MB-231 cells.[43] We supposed there were mutual effects between Akt and AMPK, just as shown in Figure 6.

One of the limitations of the present study is that we could not conclude that these were direct effects of liraglutide on VAT because of the present study design. We are going to design an AMPK knockdown experiment to confirm the present results in the future. The other is that the evaluation of body fat content was not conducted by imaging methods due to experimental conditions. Even though the adipose tissue was removed as much as possible, it still could not reflect the whole condition of the body fat. Besides, as an

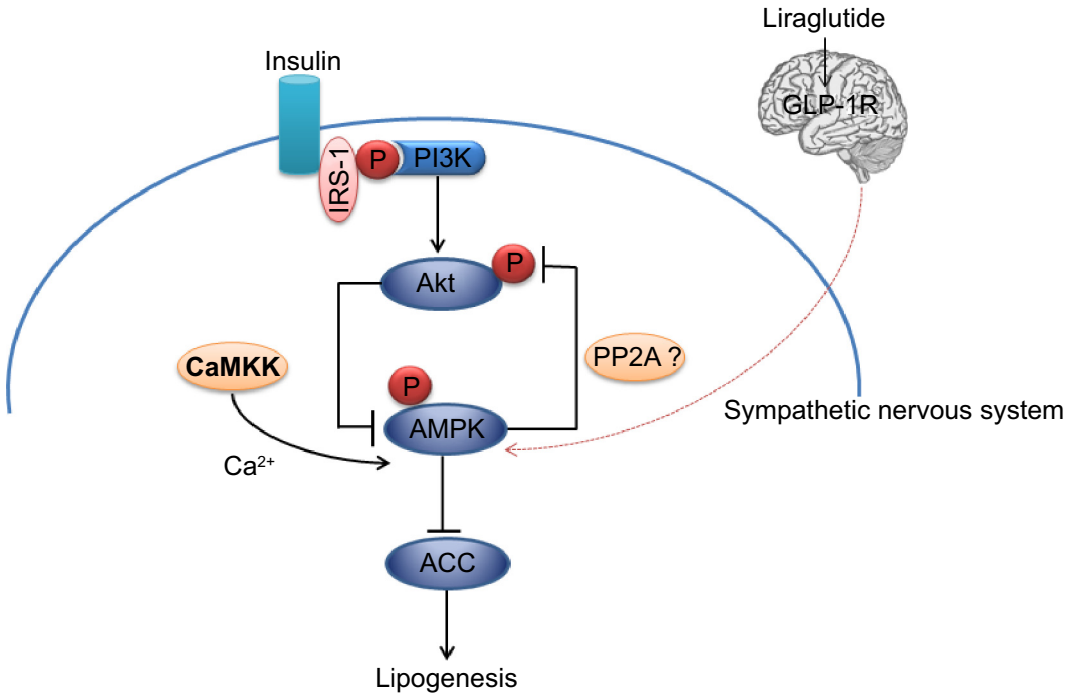

Figure 6 Model of Akt and AMPK signaling network in adipocyte.
Notes: Arrows represent activation, whereas bars represent inhibition.

animal study, caution is advised when applying the results to people.

In summary, we found that VAT was reduced after weight loss with AMPK activation and Akt suppression with liraglutide. It was associated with the reduction of lipogenetic process in VAT.

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Disclosure

The authors declare that they have no conflicts of interest.

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