

The β_3 Adrenergic Receptor Agonist CL316243 Ameliorates the Metabolic Abnormalities of High-Fat Diet-Fed Rats by Activating AMPK/PGC-1 α Signaling in Skeletal Muscle

This article was published in the following Dove Press journal:
Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy

Li-Na Ding^{1,*}
Ya Cheng^{1,*}
Lu-Yao Xu¹
Le-Quan Zhou^{1,2}
Li Guan^{1,2}
Hai-Mei Liu^{1,2}
Ya-Xing Zhang^{1,2}
Run-Mei Li¹
Jin-Wen Xu^{1,2}

¹The Research Center of Basic Integrative Medicine, Basic Medical College, Guangzhou University of Chinese Medicine, University Town, Guangzhou, 510006, People's Republic of China; ²Department of Physiology, Basic Medical College, Guangzhou University of Chinese Medicine, University Town, Guangzhou, 510006, People's Republic of China

*These authors contributed equally to this work

Purpose: Skeletal muscle has a major influence on whole-body metabolic homeostasis. In the present study, we aimed to determine the metabolic effects of the β_3 adrenergic receptor agonist CL316243 (CL) in the skeletal muscle of high-fat diet-fed rats.

Methods: Sprague-Dawley rats were randomly allocated to three groups, which were fed a control diet (C) or a high-fat diet (HF), and half of the latter were administered 1 mg/kg CL by gavage once weekly (HF+CL), for 12 weeks. At the end of this period, the serum lipid profile and glucose tolerance of the rats were evaluated. In addition, the phosphorylation and protein and mRNA expression of AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α , and carnitine palmitoyl transferase (CPT)-1b in skeletal muscle were measured by Western blot analysis and qPCR. The direct effects of CL on the phosphorylation (p-) and expression of AMPK, PGC-1 α , and CPT-1b were also evaluated by Western blotting and immunofluorescence in L6 myotubes.

Results: CL administration ameliorated the abnormal lipid profile and glucose tolerance of the high-fat diet-fed rats. In addition, the expression of p-AMPK, PGC-1 α , and CPT-1b in the soleus muscle was significantly increased by CL. CL (1 μ M) also increased the protein expression of p-AMPK, PGC-1 α , and CPT-1b in L6 myotubes. However, the effect of CL on PGC-1 α protein expression was blocked by the AMPK antagonist compound C, which suggests that CL increases PGC-1 α protein expression via AMPK.

Conclusion: Activation of the β_3 adrenergic receptor in skeletal muscle ameliorates the metabolic abnormalities of high-fat diet-fed rats, at least in part via activation of the AMPK/PGC-1 α pathway.

Keywords: CL316243, AMPK, PGC-1 α , L6 myotubes, carnitine palmitoyl transferase

Introduction

Skeletal muscle accounts for 40% of body mass and plays an important role in homeostasis.^{1,2} It is quantitatively the most significant site of peripheral insulin resistance in obesity and diabetes, which are associated with a higher risk of cardiovascular disease.^{3,4} Furthermore, the loss of skeletal muscle mass and function can contribute to metabolic syndrome,^{5,6} while the exercise-induced restoration of muscle function is paralleled by an improvement in skeletal muscle insulin sensitivity.^{7,8} Skeletal muscle insulin resistance is a predictor of the development of type 2 diabetes, and the

Correspondence: Jin-Wen Xu
Guangzhou University of Chinese Medicine, University Town, Waihuan East Road 232, Guangzhou, 510006, People's Republic of China
Tel +86-20-39358028
Fax +86-20-39358020
Email xujinwen@gzucm.edu.cn

maintenance of appropriate muscle glucose and lipid disposal helps prevent obesity, diabetes, and metabolic syndrome.⁹

The β -adrenergic receptor (AR) is one of the key regulators of skeletal muscle mass and function,¹⁰ and β -AR agonists may represent a means of preventing or ameliorating the muscle wasting that is associated with aging.^{11,12} Knowledge of the role of β -AR signaling in skeletal muscle is mainly based on the results of studies of the effects of β 2-AR agonists because the β 2-AR is the predominant subtype in skeletal muscle.¹³ However, β 3-ARs are also expressed in human and rodent skeletal muscles,^{14,15} and the selective activation of β 3-ARs has been shown to induce muscle hypertrophy and inhibit protein breakdown in rat skeletal muscle.^{16,17} Although the role of β 3-ARs in the regulation of skeletal muscle structure and function are recognized, less is known regarding the metabolic effects of β 3-AR activation in skeletal muscle.

UCP is one of a protein groups participating thermogenesis and there are 5 main well-known isoforms including UCP-1 to UCP-5.¹⁸ UCP-3, which is the predominant isoform expressed in skeletal muscle, increases metabolic rate in skeletal muscle.^{19,20} Administration of the β 3-adrenergic agonist CL316243 (CL) has been shown to increase the expression of uncoupling proteins 3 (UCP-3) in the skeletal muscle of mice.^{21,22} This suggests that the activation of the β 3-AR in skeletal muscle might have beneficial metabolic effects and help maintain energy homeostasis. In the present study, we first aimed to determine whether the administration of the β 3-AR agonist CL ameliorates the metabolic abnormalities in high-fat diet-fed rats. Then, to gain more insight into the direct effects of β 3-AR in skeletal muscle, we aimed to determine whether CL activates the AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 α pathway in L6 myotubes.

Materials and Methods

Reagents

CL was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's-Modified Eagle's Medium (DMEM), Opti-MEM, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade and obtained from Guangzhou Chemical Reagents (Guangzhou, China).

Animals

All the experimental procedures were performed in accordance with the Guidelines for Animal Experiments of the Committee of Medical Ethics, National Health Department of China. The animal experiments were approved by the Animal Research Center of Guangzhou University of Chinese Medicine. The ethical approval certificate number is 2,016,139. Male Sprague-Dawley rats (220–250 g) were obtained from the Laboratory Animal Center at Guangzhou University of Chinese Medicine. The rats were randomly allocated to three groups, which were fed a control diet and orally administered physiological saline (C group; n=10); fed a high-fat diet (60% hydrogenated coconut oil; D12492; Research Diets, Inc; New Brunswick, NJ, USA) and orally administered physiological saline (HF group; n=10); or fed the high-fat diet and orally administered CL (1 mg/kg once weekly; HF+CL, n=10) for 12 weeks. CL was dissolved in dimethyl sulfoxide (DMSO) and then diluted in physiological saline.

Intraperitoneal Glucose Tolerance Testing

Intraperitoneal glucose tolerance testing was performed as previously described.²³ Briefly, rats were administered 25% w/v glucose solution intraperitoneally at a dose of 1 g/kg body mass. Tail vein blood samples were collected immediately and 30, 60, 90, and 120 min later. Blood glucose concentrations were measured using a glucose meter (Johnson & Johnson).

Serum and Tissue Collection

Trunk blood was then obtained, left at room temperature for 30 min, and then centrifuged at 4500 \times g and 4°C for 15 min. The lipid concentrations of the serum obtained were measured using a Hitachi clinical analyzer.

Histological Analysis

Tissue sections (5 mm) were obtained from adipose tissue samples that had been fixed and embedded in paraffin. These were then deparaffinized in xylene, rehydrated, and washed in phosphate-buffered saline, prior to hematoxylin and eosin (HE) staining. The diameter of the adipocyte in the HF and HF+CL groups was normalized to that of the C group.

Cell Culture

The L6 cell line was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). For differentiation into myotubes, L6 myoblasts were cultured

at 37 °C with 95% humidity in DMEM supplemented with 2% horse serum, which was changed every other day. Myotube formation was achieved after 5 days of incubation, at which time the cells were used in the experiments.

Immunoblotting

Immunoblotting was performed as previously described.²³ Briefly, tissue lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to membranes. The primary antibodies used were as follows: anti-PGC-1 α (PA5-22,958; Thermo), anti-AMPK (5831; Cell Signaling Technology), anti-p-AMPK (2535; Cell Signaling Technology), anti-CPT-1b (ab134988, Abcam), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5174; Cell Signaling Technology). Membranes were incubated with primary and secondary antibodies using standard techniques and the detection of specific protein bands was accomplished using enhanced chemiluminescence. Images of the bands were acquired and analyzed using a quantitative digital imaging system (Quantity One; Bio-Rad), avoiding saturation.

Real-Time Reverse Transcription-PCR

Real-time PCR was performed as previously described.²⁴ cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. For PGC1- α , the primers were 5'-CAACAATGAGCCTGCGAACA-3' (forward) and 5'-TGAGGACCGCTAGCAAGTTTG-3' (reverse), resulting in a 71-bp RT-PCR product. For CPT-1b, the primers were 5'-GGCTGCCGTGGGACATT-3' (forward) and 5'-TGCCCTGGCTACTTGGTACGA-3' (reverse), resulting in a 100-bp RT-PCR product. For GAPDH, the primers were 5'-AGA CAGCCGCATCTTCTTG-3' (forward) and 5'-CTTG CCGTGGGTAGAGTCAT-3' (reverse), resulting in a 207-bp RT-PCR product. RT-PCR reactions were performed using a CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) in final volumes of 20 μ L, which contained 10 μ L of FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland), 1 μ L of each primer (10 μ M), 2 μ L of cDNA, and 7 μ L of PCR-grade water. The RT-PCR products underwent melting point analysis and were quantified using the $\Delta\Delta$ CT method, with GAPDH as the reference gene. The expression in the HF and HF+CL groups was normalized to that of the C group.

Immunofluorescence

L6 myotubes were grown on coverslips, treated as described below, then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X. The cells were then blocked using 3% normal serum for 20 min and incubated with an antibody against p-AMPK (BD Transduction Laboratories) and a FITC-conjugated secondary antibody (K00018968, Dako North America Inc., Dako, Denmark). After washing, the nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma). Immunofluorescence was visualized using an Olympus BX41 microscope (Tokyo, Japan) and images were obtained using a high-resolution DP70 Olympus digital camera.

Statistical Analysis

Data are presented as mean \pm standard deviation (SD) and represent the results of at least three independent experiments. Statistical comparisons were made using Student's *t*-test or one-way analysis of variance, followed by Tukey's test where applicable, to identify significant differences between mean values. $P < 0.05$ was considered to represent statistical significance.

Results

CL Administration Reduces the Body Mass and Ameliorates the Serum Lipid Abnormalities and Glucose Intolerance of High-Fat Diet-Fed Rats

As expected, the body masses of the HF rats were significantly higher than those of the C rats, but CL administration reduced the body mass of the HF rats (Figure 1A). The HF rats had significantly higher total serum cholesterol, triglyceride, and low-density lipoprotein (LDL)-cholesterol concentrations than the C group, whereas the high-density lipoprotein (HDL)-cholesterol concentration in the HF rats was significantly lower. However, CL administration improved the lipid profile of the HF rats (Figure 1B). Intraperitoneal glucose tolerance testing showed the area under the glucose curve (AUC) was significantly larger in HF rats than C rats, and this difference was also eliminated by concurrent CL administration (Figure 1C and D).

CL Administration Reduces the Peri-Epididymal and Inguinal Fat Depot Masses of HFD-Fed Rats

HE staining of tissue sections showed that HF rats had larger lipid droplets in their peri-epididymal and inguinal

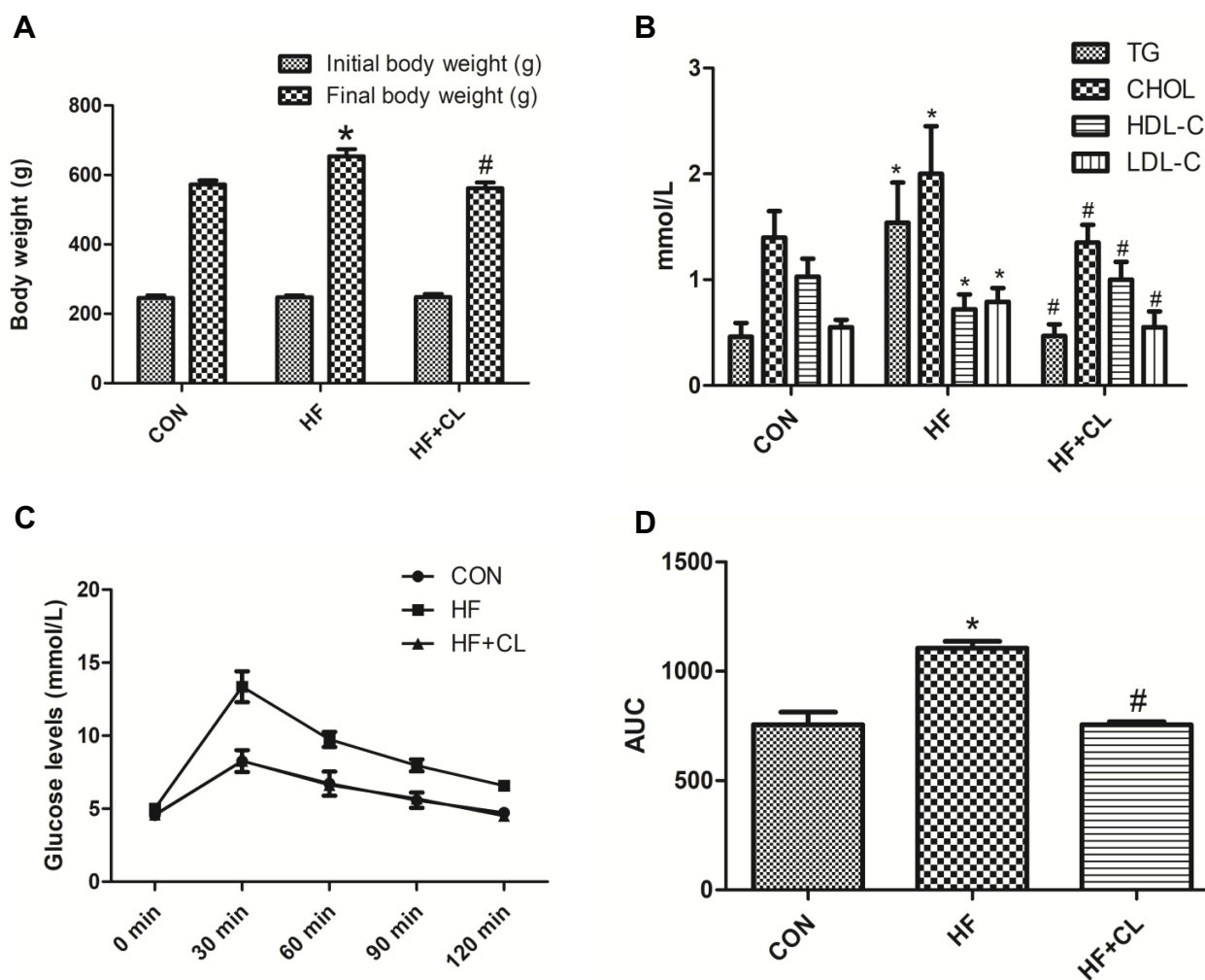


Figure 1 CL administration reduces the body mass, and improves the serum lipid profile and glucose tolerance, of high-fat diet-fed rats. The body mass (A), serum lipid concentrations (B), glucose tolerance curves (C), and AUCs (D) of C, HF, and HF+CL mice. * $p < 0.05$ versus C, # $p < 0.05$ versus HF. Data are mean and SD, $n = 8$. **Abbreviations:** AUC, area under the curve; C, control group; HF, high-fat diet-fed group; HF+CL, high-fat diet-fed and CL-treated group.

adipocytes (Figure 2A and B). Concurrent CL administration eliminated these differences.

CL Administration Increases the Expression of p-AMPK, PGC-1 α , and CPT-1b in the Soleus Muscles of HF Rats

Immunoblotting showed that the protein expression levels of AMPK, p-AMPK, PGC-1 α , and CPT-1b in the soleus muscles of HF rats were significantly lower than those of C rats, and CL administration eliminated these differences (Figure 3A and B). Consistent with this, CL administration increased PGC-1 α mRNA expression in the soleus muscles of HF rats (Figure 3C).

CL Treatment Increases AMPK, PGC-1 α , and CPT-1b Expression in L6 Myotubes

Immunoblotting showed that CL increased PGC-1 α and CPT-1b protein expression levels in a dose-dependent manner in L6 myotubes (Figure 4A). Treatment with CL (1 μ M) for 4 h also increased AMPK phosphorylation, as demonstrated using Western blotting and immunofluorescence (Figure 4B and C). 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR/acadesine, a cell-permeable activator of AMPK, 0.5 mM/L) increased PGC-1 α expression, whereas the co-treatment of L6 myotubes with compound C (an inhibitor of AMPK, 10 μ M/L) alongside CL significantly reduced PGC-1 α expression (Figure 4D).

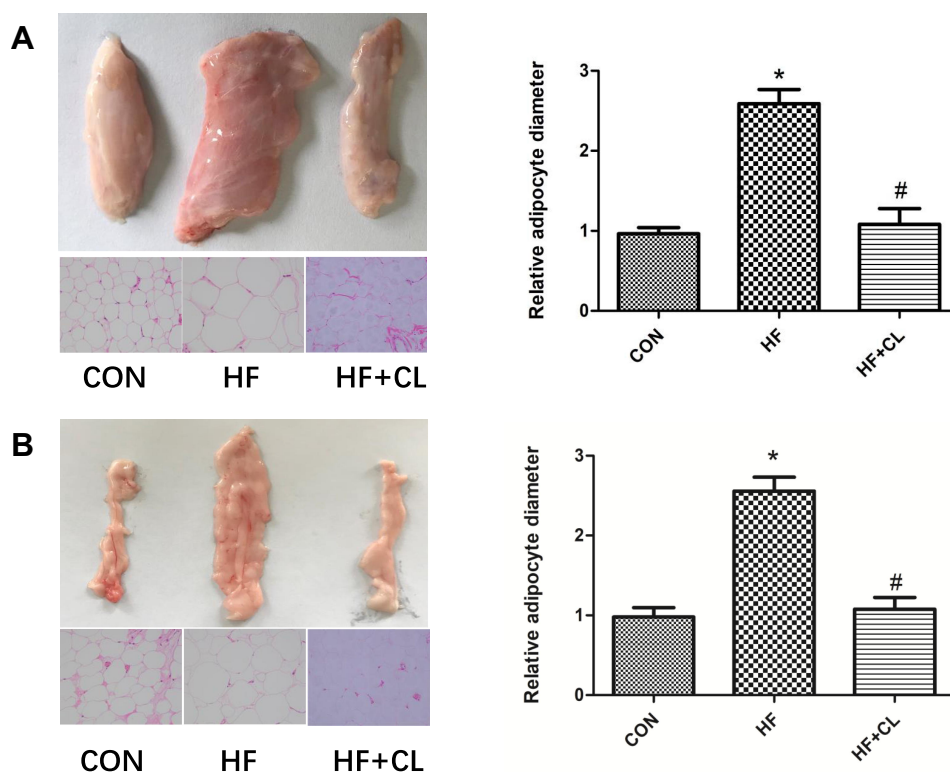


Figure 2 CL administration reduces the peri-epididymal and inguinal adipose tissue depot masses of high-fat diet-fed rats. Representative images of peri-epididymal adipose tissue (A) and inguinal adipose tissue (B) are shown. Magnification 400 \times . * $p < 0.05$ versus CON, # $p < 0.05$ versus HF. Data are mean and SD, $n = 8$.

Abbreviations: EAT, peri-epididymal adipose tissue; SAT, subcutaneous inguinal adipose tissue; C, control group; HF, high-fat diet-fed group; HF+CL, high-fat diet-fed and CL-treated group.

Discussion

Skeletal muscle plays an important role in the regulation of whole-body energy homeostasis, and energy production depends on mitochondrial function. The dysfunction or loss of skeletal muscle mass has been shown to contribute to obesity and type 2 diabetes.^{5,6} Mitochondrial dysfunction and/or a reduction in mitochondrial content has been reported in skeletal muscle and adipose tissue from obese and diabetic subjects.^{25,26} PGC-1 α is a major transcriptional regulator of mitochondrial function in skeletal muscle,^{27,28} and AMPK acts as a key sensor of energy status in skeletal muscle and regulates glucose and fatty acid metabolism.²⁹ The activation of AMPK regulates the expression and activity of PGC-1 α in skeletal muscle,³⁰ and high-fat diet-feeding has been shown to reduce the expression and activity of PGC-1 and AMPK and downstream signaling in skeletal muscle.^{31,32} Consistent with this, in the present study, high-fat diet-feeding induced metabolic abnormalities that were associated with lower expression of PGC-1 α and p-AMPK in skeletal muscle (Figure 3).

The β 3-AR is an established therapeutic target for obesity and metabolic disease.^{33,34} CL, a highly selective systemic β 3-AR agonist, induces expression of UCP-1 in adipose tissue and has anti-obesity effects.^{35–37} Although β 3-AR is principally expressed in adipose tissue, it is also expressed in human and rodent skeletal muscles.^{14,15} Recently, the β 3-AR agonist mirabegron has been shown to improve glucose homeostasis and increase the expression of PGC-1 α in the skeletal muscle of obese humans.³⁸ Consistent with this, in the present study, we have shown that selective activation of β 3-AR by CL in high-fat diet-fed rats reduces their body mass and ameliorates their glucose intolerance, as well as increasing the protein expression of PGC-1 α and p-AMPK in skeletal muscle.

Long-chain fatty acids (LCFAs) are a major energy source in muscle. CPT-1 is a rate-limiting enzyme in the mitochondrial β -oxidation of LCFAs.³⁹ The muscle isoform, CPT-1b, is mainly expressed in brown and white adipose tissue, the heart, and skeletal muscle.^{40,41} The activity of CPT-1b is lower in obese skeletal muscle, which contributes to the lower fatty acid oxidation.⁴² Furthermore, mice with CPT-1b deficiency become

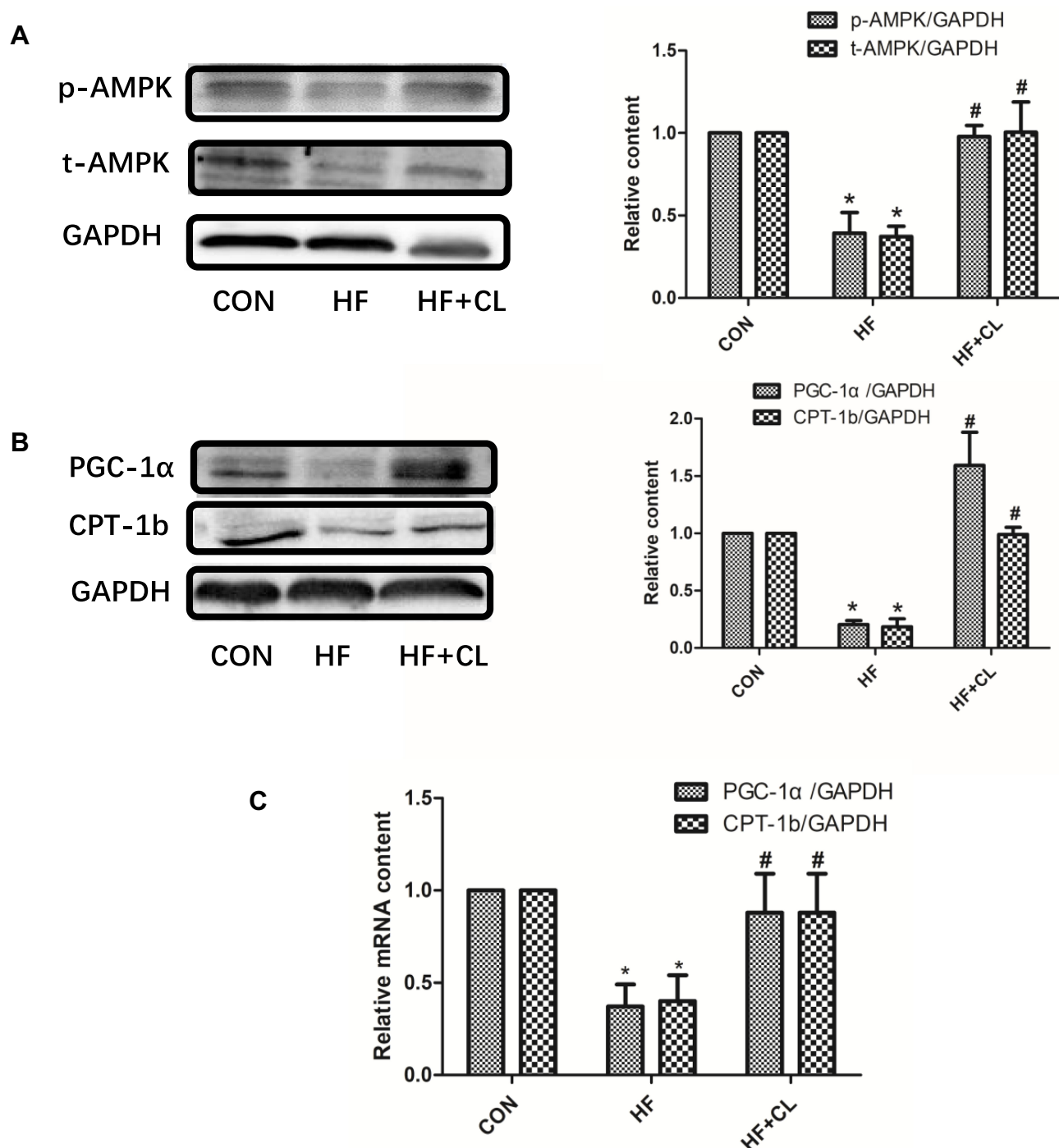


Figure 3 CL administration increases the expression of p-AMPK, PGC-1 α , and CPT-1b in the soleus muscles of high-fat diet-fed rats. **(A)** CL increased the p-AMPK and AMPK protein expression levels in the soleus muscle of HF rats. Bars represent SD, $n = 4$. **(B)** CL increased PGC-1 α and CPT-1b protein expression levels in the soleus muscle of HF rats, according to Western blot analysis. **(C)** CL increased PGC-1 α and CPT-1b mRNA expression in the soleus muscle of HF rats, according to qPCR. The expression of each target protein/mRNA was normalized to that of GAPDH, then to that of the control samples. * $p < 0.01$ versus CON, # $p < 0.05$ versus HF. Data are mean and SD, $n = 4-5$.

Abbreviations: C, control group; HF, high-fat diet-fed group; HF+CL, high-fat diet-fed and CL-treated group.

severely insulin resistant after 7 months of high-fat diet-feeding.⁴³ Consistently, we found that high-fat diet-feeding reduced the expression of CPT-1b in skeletal muscle and CL administration increased the protein and mRNA expression

of this enzyme. These results suggest that the beneficial effect of CL on the lipid profile of HF rats is mediated through higher protein and mRNA expression of CPT-1b in skeletal muscle.

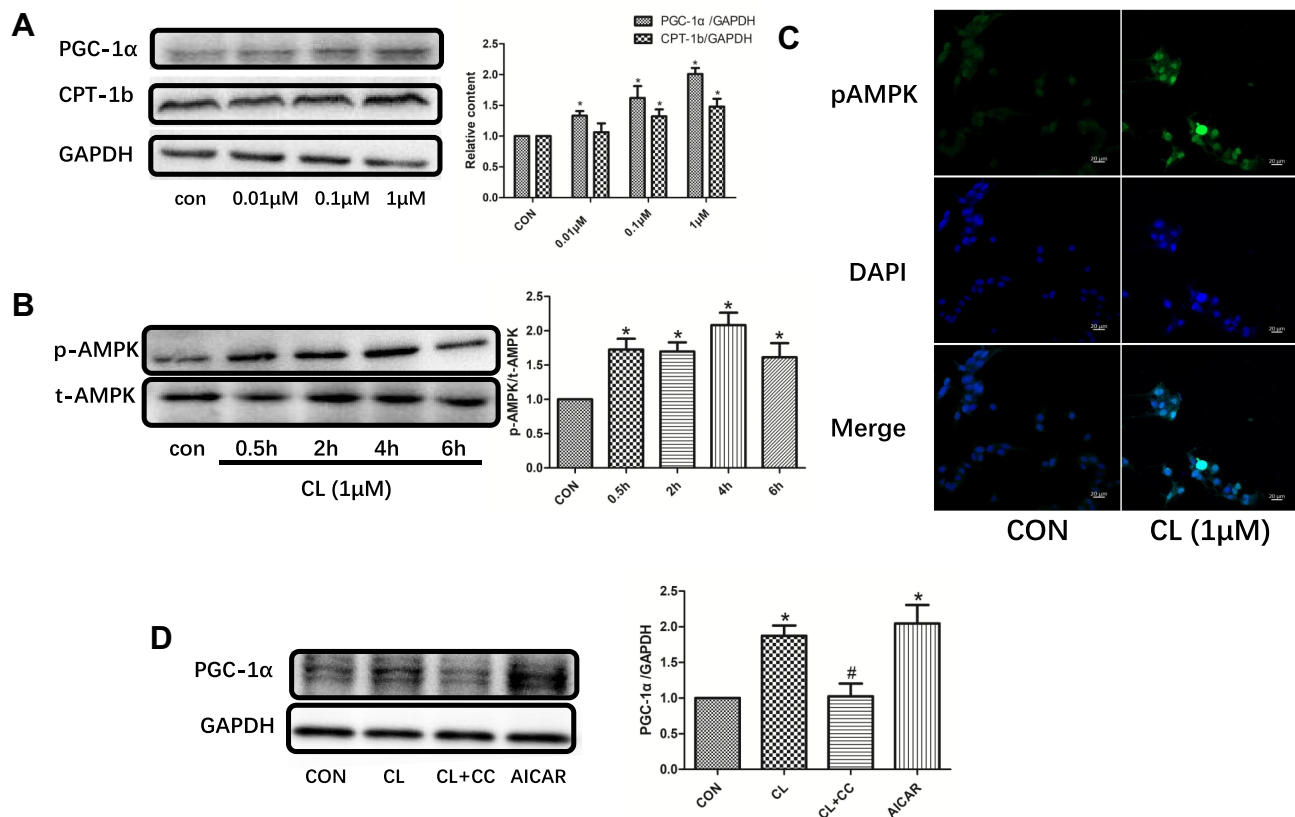


Figure 4 CL increases the expression of AMPK, PGC-1 α , and CPT-1b in L6 myotubes. **(A)** CL treatment for 24 h dose-dependently increased PGC-1 α and CPT-1b protein expression in L6 myotubes, according to Western blot analysis. The expression of each target protein was normalized to that of GAPDH, then to that of the control samples. **(B)** Effects of the treatment of L6 myotubes with CL (1 μ M) for the indicated times on the phosphorylation (p-AMPK) and expression of AMPK, according to Western blot analysis. p-AMPK band intensity was normalized to that of total AMPK, then to that of the control samples. **(C)** Effects of the treatment of L6 myotubes with CL (1 μ M) for 4 h on AMPK phosphorylation in immunofluorescence-stained L6 myotubes. Anti-p-AMPK conjugated to FITC and DAPI were used. Magnification 400 \times . **(D)** Effects of treatment with CL (1 μ M), acadesine (AICAR, a cell-permeable activator of AMPK, 0.5 mM/L), and/or compound C (CC, an inhibitor of AMPK, 10 μ M/L) on the expression of PGC-1 α . The expression of PGC-1 α was normalized to that of GAPDH, then to that of the control samples. * p < 0.01 versus C, # p < 0.01 versus CL. Data are mean and SD, n = 4–6.

Abbreviation: C, control group.

Adipose tissue communicates with skeletal muscle through the secretion of adipokines, such as adiponectin, leptin, omentin, osteopontin, and cardiotrophin-1; and batokines, such as bone morphogenetic protein 8b, fibroblast growth factor-21, endothelin-1, and interleukin-6.^{44,45} Adiponectin activates the AMPK/PGC-1 α pathway and stimulates fatty acid oxidation.^{46,47} Furthermore, a 7-day infusion of CL316243 increases adiponectin expression in white adipose tissue and the serum concentration of adiponectin.⁴⁸ Therefore, the effects of CL on the skeletal muscle of the mice in the present study might have been secondary to effects on adipose tissue. However, the in vitro data confirm that CL has a direct dose-dependent effect to increase PGC-1 α protein expression in muscle cells. Because PGC-1 α expression is regulated by AMPK,^{49,50} we next determined whether AMPK inhibition would prevent the CL-induced activation of PGC-1 α in L6 myotubes. Indeed, CL (1 μ M) treatment for 4 h increased

AMPK phosphorylation, and compound C, an AMPK inhibitor, reduced PGC-1 α protein expression in CL-treated L6 myotubes. These results suggest that CL increases PGC-1 α protein expression by activating AMPK.

In addition to adipose tissue and skeletal muscle, β 3-AR are also found in the brain, in areas such as the brain stem and the hypothalamus.⁵¹ Acute central injection of CL potentially reduced food intake and increased hypothalamic neuronal activity in rats.⁵² One limitation of our study is that we did not detect the food intake of the rats. Chronic effect of CL on the animal feeding habits needs to be further studied.

Conclusion

Our study suggests that CL improves glucose tolerance and lowers lipid profiles in the high-fat diet-feeding of rats by activating the AMPK/PGC-1 α pathway. This finding

supports the development of novel anti-obesity therapies that involve the activation of β 3-ARs in skeletal muscle.

Acknowledgments

We thank Mark Cleasby, PhD, from Liwen Bianji, Edanz Group China, for editing the English text of a draft of this manuscript. We also thank the rats sacrificed in this study.

Funding

This study was supported by the National Natural Science Foundation of China, the Natural Science Foundation of Guangdong Province, and the Guangzhou University of Chinese Medicine (grant numbers 81774107, 81673717, and QNYC20170101).

Disclosure

The authors declare that they have no conflicts of interest relevant to this work.

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