Conjugated linoleic acid induces hepatic expression of fibroblast growth factor 21 through PPAR-α

Jie Yu1,2, Bing Yu1,2, Honglin Jiang3 and Daiwen Chen1,2*

1Animal Nutrition Institute, Sichuan Agricultural University, Ya’an, Sichuan 625014, China
2Key Laboratory of Animal Disease-Resistance Nutrition, Ministry of Education, Sichuan Agricultural University, Ya’an, Sichuan 625014, China
3Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

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Abstract
Fibroblast growth factor 21 (FGF21) is a PPAR-α-regulated metabolic regulator that plays critical roles in glucose homoeostasis, lipid metabolism, insulin sensitivity and obesity. Conjugated linoleic acids (CLA), especially trans-10 (t-10), cis-12 (c-12), have shown anti-obesity properties. In addition, CLA is reported as a high-affinity ligand and activator of PPAR-α. This raises the possibility that FGF21 might be involved in the anti-obesity effect of CLA. In the present study, we tested the hypothesis that FGF21 expression in the liver could be induced by t-10, c-12-CLA through PPAR-α. HepG2 cells were treated with 100 μM-bovine serum albumin, 10 μM-10, c-12-CLA or 100 μM-t-10, c-12-CLA for 8 h. A total of ten adult C57BL/6J mice were fed with the diets containing 1 % soya oil or 1 % t-10, c-12-CLA for 5 d. t-10, c-12-CLA stimulated hepatic FGF21 mRNA abundance as determined by real-time RT-PCR. t-10, c-12-CLA also increased serum FGF21 concentrations as measured by an ELISA. Co-transfection analysis indicated that reporter gene expression from the mouse FGF21 promoter was induced by PPAR-α. This FGF21 and PPAR-α linkage may provide another potential explanation for the anti-obesity effect of t-10, c-12-CLA.

Key words: Conjugated linoleic acid; Fibroblast growth factor 21; PPAR-α; Liver

Fibroblast growth factor 21 (FGF21), whose expression can be induced by PPAR-α activation in the liver1–3, is a novel metabolic regulator that plays critical roles in glucose homoeostasis, lipid metabolism, insulin sensitivity and obesity4,5. Transgenic mice overexpressing FGF21 are resistant to diet-induced obesity5,6. Systemic administration of FGF21 to obese mice also reduced serum TAG levels, liver steatosis, as well as body weight and adiposity6. Conjugated linoleic acids (CLA) are positional and geometric conjugated dienoic isomers of linoleic acid. The cis-9, trans-11-CLA and trans-10 (t-10), cis-12 (c-12)-CLA possess biological activity7. Many studies8–10 have shown that CLA has anti-obesity effects, and t-10, c-12-CLA is specifically responsible for the anti-obesity benefit11,12. Although much attention has been focused on the anti-obesity properties of CLA, the underlying mechanism still remains elusive. CLA is now recognised as a high-affinity ligand and activator of PPAR-α13,14. This notion raises the possibility that FGF21 might be involved in the anti-obesity effect of CLA.

In the present study, we tested the hypothesis that FGF21 expression in the liver is induced by t-10, c-12-CLA. We also investigated the role of PPAR-α in the t-10, c-12-CLA induction of FGF21 expression.

Experimental methods
Preparation of conjugated linoleic acid–bovine serum albumin complexes
Fatty acid-free bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St Louis, MO, USA). CLA–BSA complexes were prepared as reported15. Briefly, 10 μmol t-10, c-12-CLA (Natural Lipids Limited, Hovdebygda, Norway) were dissolved in 0.1 M-KOH solution together with 10 μmol BSA solution in PBS, and then incubated overnight at 4°C.

Abbreviations: BSA, bovine serum albumin; c-12, cis-12, CLA, conjugated linoleic acid; FGF21, fibroblast growth factor 21; t-10, trans-10.

* Corresponding author: Professor D. Chen, fax +86 835 2885106, email daiwenc@yahoo.com
The pH and volume were subsequently adjusted to 7.2 and 5.0 ml, respectively. After filter sterilisation, these complexes were ready to use.

**Cell culture**

The HepG2 cell line was maintained in minimum essential medium (MEM, Sigma-Aldrich) plus 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM of L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Mediatech Inc., Manassas, VA, USA) at 37°C in a 5% CO₂ humidified atmosphere. When the cells were 70% confluent, they were plated into six-well plates at 5 × 10⁵ per well and cultured. After 24 h incubation, the cells were serum starved for 16 h; the medium was then replaced by MEM plus 100 μM-BSA, 10 μM-t-10, c-12-CLA or 100 μM-t-10, c-12-CLA for 8 h.

**Animal experiment**

The animal-related protocols were approved by Sichuan Agricultural University Institutional Animal Care and Use Committee. A total of ten 12-week-old male C57BL/6J mice were housed at 22°C on a 12 h light–12 h dark cycles and had free access to diets and water. The mice were blocked by initial body weight and assigned into two treatment diets. The mice (n = 5) were fed with a control diet with 1% soya oil or a diet containing 1% t-10, c-12-CLA. After 5 d of the treatment, postprandial mice were anaesthetised and bled between 07.00 and 08.00 hours. The livers were collected and frozen for RNA extraction. Serum samples were prepared and stored at −20°C for future measurements.

**RNA extraction and real-time RT-PCR**

Total RNA from HepG2 cells and mouse liver tissue were extracted using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. Total RNA from HepG2 cells and mouse liver tissue were extracted using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. The RNA extraction and real-time RT-PCR were performed using random primers and RT (Promega, Madison, WI, USA). Real-time quantitative PCR were performed using Power SYBR Green PCR master mix (Applied Biosystems, WI, USA). The forward and reverse cycles of 95°C for 15 s and 60°C for 1 min. The real-time PCR of each sample were performed in duplicate. The data were analysed using the cycle threshold (2⁻¹⁰ΔCt) method, as recommended by Applied Biosystems. The forward and reverse primers used were as follows: 5'-ACCTGGGATCATCGGAG-GAT-3' and 5'-AGTGGGAGCAGCTACATA-3' for human FGF21, 5'-ACCTGGGATCATCGGAG-GAT-3' and 5'-GGCTCTCC-AGCAGCAGTTTC-3' for mouse FGF21, 5'-AGAGCTACGAGCTCGGTAG-3' and 5'-AGCAGTCTGTTGCGGTAG-3' for human β-actin, 5'-CGCGGTTCATTGTTGTTGTTG-3' and 5'-AGCGCATGTTATGGTC-3' for mouse 18S rRNA.

**Fibroblast growth factor 21 measurement**

Serum FGF21 concentrations were measured using a specific mouse ELISA kit (BioVendor, Candler, NC, USA).

**Plasmid construction**

The FGF21 promoter construct – 1821/+10, which contains two putative PPAR-α binding sites (16), was amplified from mouse genomic DNA by PCR with sequence-specific primers containing KpnI and XhoI restriction sites at their 5’-end (forward 5’-ATGGTACCTCTCAGTTTCTATGCAGTTC-3’ and reverse 5’-ATCTCGAGAAGGCTGTCTGTTGGAACGCA-3’). The PCR product was digested with restriction enzymes KpnI and XhoI, and cloned into the promoter-less luciferase reporter vector pGL2-basic (Promega) to generate the plasmid pGL2B-mFGF21P. The insert for PPAR-α over-expression was amplified from mouse liver cDNA by PCR using forward primer (5’-ATGCTTAGCCAAACATGTTGGACACAGAG-3’) containing NheI restriction site at the 5’-end and reverse primer (5’-ATCTCGAGACCTGGCATCTCAGGAAAGAT-3’) with XhoI restriction site at the 5’-end. The PCR product was digested with restriction enzymes NheI and XhoI, and cloned into expression vector pcDNA3.1 to generate PPAR-α expression plasmid pcDNA3.1-mPPAR-α. All inserts of the plasmids were verified by DNA sequencing (Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA, USA).

**Transfections and luciferase assays**

The Chinese hamster ovary cell line cells were grown in MEM as described previously. The cells were seeded in twenty-four-well plates at a density of 5 × 10⁴ cells per well and cultured for 24 h. Then the cells were transected with 500 ng of mouse FGF21 promoter construct pGL2B-mFGF21P, 500 ng of mouse PPAR-α expression plasmid pcDNA3.1-mPPAR-α and 1 ng of pRL-CMV (Promega) per well, using FuGENE6 (Roche Applied Science, Indianapolis, IN, USA). After 24 h, the transfection, the medium was replaced by serum-free MEM, and the cells were further cultured for 8 h. Subsequently, the cells were treated with 100 μM-t-10, c-12-CLA or BSA for 16 h. Cell lysis and dual-luciferase assay were performed using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions. The luciferase activity expressed from a promoter construct was divided by that from pRL-CMV in the same well to normalise the variation in transfection efficiency.

**Statistical analyses**

All statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). Comparisons between the two means were analysed using the t test. Multiple means were compared using ANOVA followed by Tukey’s test. The data are expressed as means with their standard errors. P values < 0.05 were considered significant.
indicated that the relative abundance of FGF21 mRNA was significantly induced by 100 μM-t-10, c-12-CLA in vitro (P<0.05; Fig. 1(a)). CLA at a low dosage (10 μM) also improved FGF21 mRNA levels (Fig. 1(a)), but no statistical significance was observed (P=0.26). The animal experiment was then conducted to examine the effects of t-10, c-12-CLA on hepatic FGF21 expression in vivo. Dietary t-10, c-12-CLA significantly stimulated the relative FGF21 mRNA expression in the mouse livers (P<0.01; Fig. 1(b)). ELISA measurement showed that t-10, c-12-CLA administration significantly increased serum concentrations of FGF21 protein (P<0.05; Fig. 1(c)).

The trans-10, cis-12-conjugated linoleic acid activated the fibroblast growth factor 21 promoter in a PPAR-α-dependent manner

To determine whether t-10, c-12-CLA activates the mouse FGF21 promoter, a co-transfection analysis was applied. The administration of t-10, c-12-CLA (100 μM) significantly increased the luciferase activity expressed from the transfected mouse FGF21 promoter in the Chinese hamster ovary cells (P<0.05; Fig. 2). To investigate whether PPAR-α contributes to the response of the FGF21 promoter to t-10, c-12-CLA treatment, an empty pcDNA3.1 vector was used in the co-transfection analysis instead of PPAR-α expression plasmid pcDNA3.1-mPPAR-α. The absence of PPAR-α abolished the response of the FGF21 promoter to t-10, c-12-CLA (P<0.05; Fig. 2).

Results

The trans-10, cis-12-conjugated linoleic acid induced hepatic fibroblast growth factor 21 expression

To understand whether t-10, c-12-CLA treatment induces hepatic FGF21 expression, HepG2 cells were cultured with t-10, c-12-CLA (10 μM or 100 μM). Real-time RT-PCR analysis

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![Fig. 1. Effects of trans-10 (t-10), cis-12 (c-12)-conjugated linoleic acid (CLA) on hepatic fibroblast growth factor 21 (FGF21) expression. (a) Real-time RT-PCR analysis of FGF21 mRNA expression in HepG2 cells incubated with 100 μM bovine serum albumin (BSA), 10 μM-t-10, c-12-CLA or 100 μM-t-10, c-12-CLA for 8 h (n 3). Relative mRNA expression of FGF21 (b) and ELISA-determined serum FGF21 levels (c) in the liver of male C57Bl/6J mice fed with control diets or diets containing 1 % t-10, c-12-CLA for 5 d (n 5). Values are means, with their standard errors represented by vertical bars. Mean values were significantly different from those of the BSA or control group: *P<0.05, **P<0.01.](https://www.cambridge.org/core/doi/10.1017/S0007114511003205)
Discussion

Since obesity is becoming more prevalent these days, people are increasingly interested in the strategies of reducing body weight. Research during the past decades has reported that supplementation with either a CLA mixture or t-10, c-12-CLA alone decreases body fat mass and body weight in various animal models and in some human studies\(^{(17–19)}\). Thus, CLA, especially t-10, c-12-CLA, is perceived as a potential therapeutic candidate for obesity reduction. The possible mechanisms by which t-10, c-12-CLA reduces adiposity include (1) decreasing energy intake by suppressing appetite; (2) increasing energy expenditure in white adipose tissue, muscle and liver tissue; (3) decreasing lipogenesis and increasing lipolysis; and (4) inducing adipocytes apoptosis via endoplasmic reticulum stress, inflammation and/or insulin resistance\(^{(20)}\). The present study showed that t-10, c-12-CLA up-regulated hepatic FGF21 expression (Fig. 1). FGF21 is known as a regulating hormone related to adiposity control\(^{(5)}\). FGF21 can increase energy expenditure in diet-induced obese mice\(^{(6)}\) and FGF21 knockout results in impaired lipolysis in white adipose tissue\(^{(21)}\). However, recent studies in human subjects showed that circulating FGF21 concentrations exhibit a circadian rhythm and are associated with hepatic steatosis and TAG\(^{(22–24)}\). Overfeeding that caused liver steatosis also increases hepatic FGF21 expression in mice\(^{(25)}\). Interestingly, FGF21 has been shown to reverse hepatic steatosis\(^{(60)}\). Therefore, feedback regulation between steatosis and FGF21 might exist. It is probable that FGF21 is a biomarker for fatty liver, whereas increased FGF21 could reverse hepatic steatosis. Taken together, although the exact role of FGF21 in CLA correction of obesity remains unclear, the present results suggested that FGF21 might be a downstream regulator that was involved in the anti-obesity effect of CLA.

Feed intakes and body weight changes in mice in the animal experiment were not monitored because of the short experiment period. We initiated the present study for repeating the induction of t-10, c-12-CLA on the hepatic FGF21 expression in vivo. These data provided us new insight into the anti-obesity effect of CLA. However, further studies are still required to understand more about the anti-obesity mechanisms.

Treatment of t-10, c-12-CLA activated the FGF21 promoter, whereas the absence of PPAR-\(\alpha\) abolished the response of the FGF21 promoter to CLA (Fig. 2). This finding demonstrated that CLA regulates FGF21 transcription through PPAR-\(\alpha\) in the liver. Although t-10, c-12-CLA is proved as a ligand of PPAR-\(\alpha\) and PPAR-\(\beta/\delta\)\(^{(15)}\), t-10, c-12-CLA decreases the expression and activity of PPAR-\(\gamma\) in the adipose tissue\(^{(12,20)}\). FGF21 is one of the PPAR-\(\alpha\) target genes in the liver\(^{(2)}\), but adipose FGF21 is regulated by PPAR-\(\gamma\)\(^{(27)}\). Therefore, FGF21 may act in an endocrine manner to mediate the anti-obesity effects of CLA.

In conclusion, the present results indicate that t-10, c-12-CLA induces hepatic FGF21 expression through PPAR-\(\alpha\). The linkage between FGF21 and PPAR-\(\alpha\) may provide another potential explanation for the anti-obesity effect of t-10, c-12-CLA and warrants further investigation.

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References


