British Journal of Nutrition (2012), **107**, 461–465
doi:10.1017/S0007114511003205

British Journal of Nutrition

Short Communication

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

Jie Yu[1,2], Bing Yu[1,2], Honglin Jiang[3] and Daiwen Chen[1,2]*

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

(Received 17 February 2011 – Revised 27 April 2011 – Accepted 9 May 2011 – First published online 18 July 2011)

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-*t*-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 *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 *t*-10, *c*-12-CLA in a PPAR-α-dependent manner. Taken together, these results suggest that *t*-10, *c*-12-CLA induces hepatic FGF21 expression through 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 liver[1–3], is a novel metabolic regulator that plays critical roles in glucose homoeostasis, lipid metabolism, insulin sensitivity and obesity[4,5]. Transgenic mice overexpressing FGF21 are resistant to diet-induced obesity[5]. Systemic administration of FGF21 to obese mice also reduced serum TAG levels, liver steatosis, as well as body weight and adiposity[6].

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 activity[7]. Many studies[8–10] have shown that CLA has anti-obesity effects, and *t*-10, *c*-12-CLA is specifically responsible for the anti-obesity benefit[11,12]. 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-α[13,14]. 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 reported[15]. 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 % CO_2 humidified atmosphere. When the cells were 70 % confluent, they were plated into six-well plates at 5×10^5 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 timed 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 instruction. RNA concentration and quality were determined by spectrophotometry. The cDNA was synthesised from 1 μg total RNA using random primers and RT (Promega, Madison, WI, USA). Real-time quantitative PCR were performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7500 real-time PCR system. The conditions for these PCR were forty 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 ($2^{-\Delta\Delta CT}$) method, as recommended by Applied Biosystems. The forward and reverse primers used were as follows: 5′-ACCTGGAGATCAGGGAGGAT-3′ and 5′-AGTGGAGCGATCCATACAGG-3′ for human FGF21, 5′-ACCTGGAGATCAGGGAGGAT-3′ and 5′-GTCCTCCAGCAGCAGTTCTC-3′ for mouse FGF21, 5′-AGAGCTACGAGCTGCCTGAC-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′ for human β-actin, 5′-CGCGGTTCTATTTTGTTGGT-3′ and 5′-AGCGGCATCGTTTATGGTC-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 *Kpn*I and *Xho*I restriction sites at their 5′-ends (forward 5′-AT*GGTACC*TCAGGTTCTATGCACGTTCC-3′ and reverse 5′-AT*CTCGAG*AAGGCTGTCTGGTGAACGCA-3′). The PCR product was digested with restriction enzymes *Kpn*I and *Xho*I, 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′-AT*GCTAGC*CCAACATGGTGGACACAGAG-3′) containing *Nhe*I restriction site at the 5′-end and reverse primer (5′-AT*CTCGAG*CCTGCCATCTCAGGAAAGAT-3′) with *Xho*I restriction site at the 5′-end. The PCR product was digested with restriction enzymes *Nhe*I and *Xho*I, 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 5×10^4 cells per well and cultured for 24 h. Then the cells were transfected 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). At 24 h after 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$<0{\cdot}05$ were considered significant.

British Journal of Nutrition

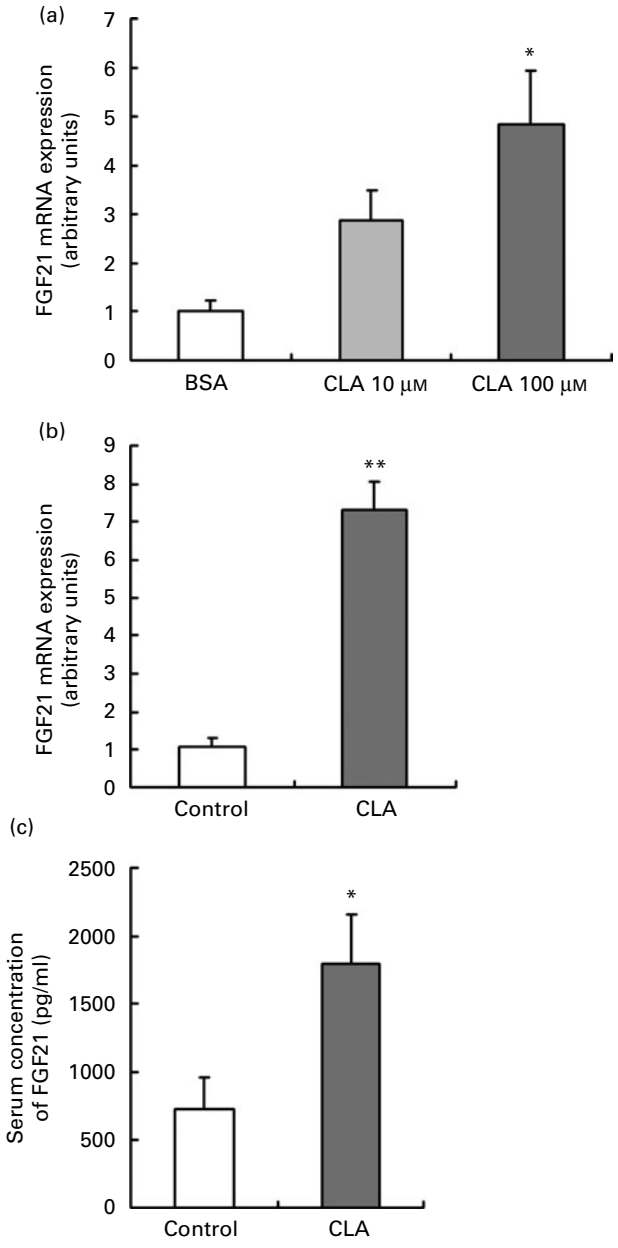

Fig. 1. Effects of *trans*-10 (*t*-10), *cis*-12 (*c*-12)-conjugated linoleic acid (CLA) on hepatic fibroblast growth factor 21 (FGF21) expression. (a) Real-time RT-PCR analysis of FGF21 mRNA expression in HepG2 cells incubated with 100 μM-bovine serum albumin (BSA), 10 μM-*t*-10, *c*-12-CLA or 100 μM-*t*-10, *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: $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$.

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 indicated that the relative abundance of FGF21 mRNA was significantly induced by 100 μM-*t*-10, *c*-12-CLA *in vitro* ($P<0{\cdot}05$; Fig. 1(a)). CLA at a low dosage (10 μM) also improved FGF21 mRNA levels (Fig. 1(a)), but no statistical significance was observed ($P=0{\cdot}26$). 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 ($P<0{\cdot}01$; Fig. 1(b)). ELISA measurement showed that *t*-10, *c*-12-CLA administration significantly increased serum concentrations of FGF21 protein ($P<0{\cdot}05$; 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 ($P<0{\cdot}05$; 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 ($P<0{\cdot}05$; Fig. 2).

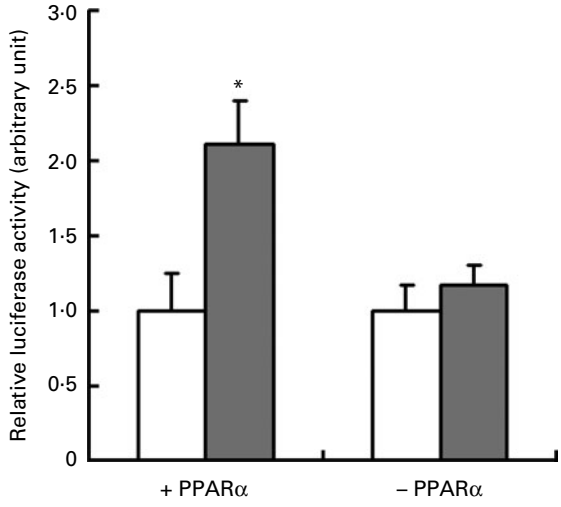

Fig. 2. Effects of *trans*-10 (*t*-10), *cis*-12 (*c*-12)-conjugated linoleic acid (CLA; ■) on reporter gene expression from the mouse fibroblast growth factor 21 (FGF21) promoter and the role of PPARα in this regulation. The mouse PPARα expression plasmid pcDNA3.1-mPPARα or empty pcDNA3.1 vector (indicated by +PPARα or −PPARα, respectively) was co-transfected with the FGF21 promoter plasmid pGL2B-mFGF21P, in which two putative PPARα binding sites were intact, and the transfection efficiency control plasmid pRL-CMV into CHO cells for 24 h. The cells were then serum starved for 8 h and subsequently treated with 100 μM-*t*-10, *c*-12-CLA or bovine serum albumin (BSA; □) for 16 h before dual-luciferase assay. Values are means, with their standard errors represented by vertical bars (n 4). *Mean value was significantly different ($P<0{\cdot}05$).

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 regulative 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 induces hepatic FGF21 expression in mice[25]. Interestingly, FGF21 has been shown to reverse hepatic steatosis[6]. 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-α abolished the response of the FGF21 promoter to CLA (Fig. 2). This finding demonstrated that CLA regulates FGF21 transcription through PPAR-α in the liver. Although *t*-10, *c*-12-CLA is proved as a ligand of PPAR-α and PPAR-β/δ[13], *t*-10, *c*-12-CLA decreases the expression and activity of PPAR-γ in the adipose tissue[12,26]. FGF21 is one of the PPAR-α target genes in the liver[2], but adipose FGF21 is regulated by PPAR-γ[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-α. The linkage between FGF21 and PPAR-α may provide another potential explanation for the anti-obesity effect of *t*-10, *c*-12-CLA and warrants further investigation.

Acknowledgements

The present study was supported by the Program for Changjiang Scholars and Innovative Research Team in University, Ministry of Education of China (IRT0555-5). J. Y. performed the study, analysed the data and wrote the manuscript; B. Y. analysed the data and wrote the manuscript; H. J. provided the research conditions, methodological directions and revised the manuscript; D. C. designed the study and wrote the manuscript. None of authors had conflicts of interest. We are grateful to Dr Benjamin A. Corl from Virginia Tech for help in the preparation of the CLA–BSA complexes.

References

1. Badman MK, Pissios P, Kennedy AR, *et al.* (2007) Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab* **5**, 426–437.
2. Oishi K, Uchida D & Ishida N (2008) Circadian expression of FGF21 is induced by PPARalpha activation in the mouse liver. *FEBS Lett* **582**, 3639–3642.
3. Lundasen T, Hunt MC, Nilsson LM, *et al.* (2007) PPARalpha is a key regulator of hepatic FGF21. *Biochem Biophys Res Commun* **360**, 437–440.
4. Kharitonenkov A, Shiyanova TL, Koester A, *et al.* (2005) FGF-21 as a novel metabolic regulator. *J Clin Invest* **115**, 1627–1635.
5. Coskun T, Bina HA, Schneider MA, *et al.* (2008) Fibroblast growth factor 21 corrects obesity in mice. *Endocrinology* **149**, 6018–6027.
6. Xu J, Lloyd DJ, Hale C, *et al.* (2009) Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes* **58**, 250–259.
7. Pariza MW, Park Y & Cook ME (2001) The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res* **40**, 283–298.
8. Evans M, Lin X, Odle J, *et al.* (2002) *Trans*-10, *cis*-12 conjugated linoleic acid increases fatty acid oxidation in 3T3-L1 preadipocytes. *J Nutr* **132**, 450–455.
9. Gaullier JM, Halse J, Hoye K, *et al.* (2005) Supplementation with conjugated linoleic acid for 24 months is well tolerated by and reduces body fat mass in healthy, overweight humans. *J Nutr* **135**, 778–784.
10. Whigham LD, Watras AC & Schoeller DA (2007) Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. *Am J Clin Nutr* **85**, 1203–1211.
11. Brown JM, Halvorsen YD, Lea-Currie YR, *et al.* (2001) *Trans*-10, *cis*-12, but not *cis*-9, *trans*-11, conjugated linoleic acid attenuates lipogenesis in primary cultures of stromal vascular cells from human adipose tissue. *J Nutr* **131**, 2316–2321.
12. Miller JR, Siripurkpong P, Hawes J, *et al.* (2008) The *trans*-10, *cis*-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPARgamma-dependent and PPARgamma-independent mechanisms. *J Lipid Res* **49**, 550–562.
13. Moya-Camarena SY, Vanden Heuvel JP, Blanchard SG, *et al.* (1999) Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARalpha. *J Lipid Res* **40**, 1426–1433.
14. Konig B, Spielmann J, Haase K, *et al.* (2008) Effects of fish oil and conjugated linoleic acids on expression of target genes of PPAR alpha and sterol regulatory element-binding proteins in the liver of laying hens. *Br J Nutr* **100**, 355–363.

15. Park Y, Albright KJ, Liu W, *et al.* (1997) Effect of conjugated linoleic acid on body composition in mice. *Lipids* **32**, 853–858.
16. Inagaki T, Dutchak P, Zhao G, *et al.* (2007) Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* **5**, 415–425.
17. Corl BA, Mathews Oliver SA, Lin X, *et al.* (2008) Conjugated linoleic acid reduces body fat accretion and lipogenic gene expression in neonatal pigs fed low- or high-fat formulas. *J Nutr* **138**, 449–454.
18. Wang Y & Jones PJ (2004) Dietary conjugated linoleic acid and body composition. *Am J Clin Nutr* **79**, 1153S–1158S.
19. Silveira MB, Carraro R, Monereo S, *et al.* (2007) Conjugated linoleic acid (CLA) and obesity. *Public Health Nutr* **10**, 1181–1186.
20. Kennedy A, Martinez K, Schmidt S, *et al.* (2010) Antiobesity mechanisms of action of conjugated linoleic acid. *J Nutr Biochem* **21**, 171–179.
21. Hotta Y, Nakamura H, Konishi M, *et al.* (2009) Fibroblast growth factor 21 regulates lipolysis in white adipose tissue but is not required for ketogenesis and triglyceride clearance in liver. *Endocrinology* **150**, 4625–4633.
22. Tyynismaa H, Raivio T, Hakkarainen A, *et al.* (2011) Liver fat but not other adiposity measures influence circulating FGF21 levels in healthy young adult twins. *J Clin Endocrinol Metab* **96**, E351–E355.
23. Li H, Fang Q, Gao F, *et al.* (2010) Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *J Hepatol* **53**, 934–940.
24. Yu H, Xia F, Lam KS, *et al.* (2011) Circadian rhythm of circulating fibroblast growth factor 21 (FGF21) is related to the diurnal changes in fatty acids in humans. *Clin Chem* **57**, 691–700.
25. Gaemers IC, Stallen JM, Kunne C, *et al.* (2011) Lipotoxicity and steatohepatitis in an overfed mouse model for non-alcoholic fatty liver disease. *Biochim Biophys Acta* **1812**, 447–458.
26. Kennedy A, Chung S, LaPoint K, *et al.* (2008) Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARgamma activity in primary cultures of human adipocytes. *J Nutr* **138**, 455–461.
27. Muise ES, Azzolina B, Kuo DW, *et al.* (2008) Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor gamma and altered metabolic states. *Mol Pharmacol* **74**, 403–412.

British Journal of Nutrition