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Metabolism of CLA isomers, *c*9,*t*11- & *t*10,*c*12 in adipocyte cultures and their effect on delta-6 desaturase expression.

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ABSTRACT: Commercial conjugated linoleic acid (CLA) dietary supplements of contain equal mixtures of the C18:2 isomers, cis-9,trans-11 and trans-10,cis-12. CLA-c9t11 occurs naturally in meat and dairy products, whereas the CLA-t10,c12, occurs mainly through chemical synthesis. Biological activity of these 2 isomers differ, CLA-c9,t11 has anti-inflammatory properties, while the t10,c12 CLA inhibits lipid accumulation. CLAs, t10c12 and c9t11, were added to 3T3 adipocyte cultures to study their effect on gene transcription and identify their metabolites. In mature adipocytes, CLA- t10,c12 decreased fatty acid synthase (FAS) expression and increased carnitine palmitoyltransferase 1 (CPT1) expression, which increased fatty acid oxidation. The CLA-t10,c12 was not readily elongated to C20 products but was delta-6 desaturated (FADs2) to C18:3–c6,t10,c12. In contrast, the CLA- c9,t11 did not significantly alter gene expression but was significantly desaturated and elongated (Elov5) and to C20:3 and C22:3 metabolites, indicating potential eicosanoid production and reduced lipid accumulation.
Keywords: CLA, gene expression, adipocyte.

Introduction

Conjugated linoleic acids (CLA) are a family linoleic acid isomers containing a conjugated instead of a methylene interrupted double bond system, and are found naturally in meat and dairy products derived from ruminants. Mixed isomers of CLA are marketed as a dietary supplement on the basis of its supposed health benefits. Feeding synthetic CLAs which are a 1:1 mixture of CLA-c9,t11 and CLA-t10,c12 to swine, has been shown to reduce fat accumulation and increase lean muscle mass (Dugan et al. 1997). The main natural isomer of CLA is *c*9,*t*11-18:2 (rumenic acid), accounting for over 75% of total CLA (Kramer et al. 1997). The CLA-t10,c12 isomer does not accumulate in dairy or meat products, and its principle source is chemically synthesized dietary supplements containing equal mixtures of CLA-c9,t11 and -t10,c12 isomers.

The genetic effects of dietary CLA are thought to act through the peroxisome proliferator activated receptor (PPAR) family of transcription factors. Biological activity of CLA-*c*9,*t*11 is associated with reduced inflammation and anti-carcinogenic effects, while CLA-*t*10,*c*12 is associated with substantial lipid oxidation which may in part be induced by inflammatory signalling (Benjamin and Spener 2009; Reynolds and Roche 2010). In mouse liver (Rasooly et al. 2007) and human adipocytes, CLA-c9t11 stimulates fat synthesis enzymes including acetyl coa carboxylase (ACC), fatty acid synthase (FAS) and steroyl co a delta-9 desaturase (SCD1) while the PPAR levels are unchanged, while CLA-t10c12 reduced the fat metabolic enzymes and reduced PPAR levels and also increased the inflammation markers tumor necrosis factor alpha (TNFa) and cyclooxygenase-2 gene (COX-2)(Reardon et al. 2012). This divergent effect of the two major CLA isomers could be due to effects of their actions as ligands for the PPARs nuclear receptors or to their respective metabolic products acting downstream to influence expression. The objective of the present study was to investigate CLA-c9,t11 and CLA-t10,c12 interactions with the PPAR transcriptional protein and to study the effects of the two isomers of dietary CLA on gene expression and their effect on the rate-limiting enzyme, delta-6 desaturase (FADs2) and elongase (Elov5) which are precursor for the eicosanoid pathway(Stoffel et al. 2008). We also identified new C16 metabolites of the CLA isomers.

Materials & Methods

In vitro competitive binding studies with PPARg. PPARg competitive binding assays were performed in vitro using LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) method (Invitrogen, Carlsbad, CA). The fluormone Pan-PPAR green ligand competed with unlabelled CLA-c9,t11, CLA-t10,c12, linoleic acid (LA ~18:2-cis9, cis12) and GW1929, which is a known PPARg agonist, for the binding site of a recombinant human PPARg – GST protein which was monitored with a terbium-labelled anti-GST antibody. The half-maximal inhibitory concentration (IC50) value of each test compound was assessed using a sigmoidal dose-response equation (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA).

Cell culture. 3T3-L1 cells (Zen-Bio Inc., NC, USA) were used to characterise the effects of c9,t11-CLA, t10,c12-CLA and linoleic acid (18:2-c9c12) on gene expression and lipid metabolism. Fatty acids, linoleic acid, CLA-c9,t11, CLA-t10,c12 (98%+ pure, Nu-Chek Prep Inc., Elysian, MN, USA) were complexed to 7.5% fatty acid free bovine serum albumin (BSA) at 4mM to provide 35 μM and 70 μM concentrations. The 3T3-L1 cells in their pre-adipocyte stage were grown in DMEM + 10% FBS +1%

antibodies. Treatment began after the cultures reached >90% confluence. Mature adipocyte were terminally differentiated by treatment with 10 µg/ml insulin + 0.1 µM IBMX and 0.5 mM dexamethasone in DMEM + 10% fcs for 48h, and then maintained in by DMEM + 10% FBS + 10 µg/ml insulin. Mature adipocyte cultures were visually assessed by the appearance of lipid droplets in the cells. Cell viability was assessed using 3-4,5-Dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide (TBTB-MTT) assay (Denizot and Lang 1986).

Gene expression analysis. Total RNA was isolated using TRIzol Reagent (Life Science Technologies, ON, Canada) and purified of using the Aurum Total RNA kit (Bio-Rad Laboratories, Mississauga, ON, Canada) Gene expression levels of: PPARγ, AFABP (aP2), fatty acid synthase (FAS), stearoyl CoA desaturase(SCD1), acyl CoA oxidase(Acox1), delta 6 desaturase (D6D ~ FADS2), and elongase (Elovl5) transcripts, were compared to b-actin as a housekeeping gene (Meadus et al. 2011). Gene expression was calculated by ΔΔCt according to (Schmittgen and Livak 2008).

Fatty acid analysis. To measure fatty acid contents and profiles, c10-17:1 was added to wells as an internal standard, and lipids were extracted with 1 ml methanol followed by 1ml isopropanol. Triglyceride and phospholipid bound fatty acids in the cells were methylated using 0.5M sodium methoxide. Fatty acid methyl esters were analysed by gas chromatography using a CP-Sil88 column (100 m, 25 µm ID, 0.2 µm film thickness) in a CP-3800 gas chromatograph equipped with an 8600-series auto-sampler (Varian Inc., Walnut Creek, CA, USA). Metabolites of CLA were isolated using Ag+-SPE (750mg/6ml, Supelco, Bellefonte, PA, USA) (Kramer et al. 2008), followed by separation using a preparative 250 mm x 10mm Chrompack ChromSpher 5 Lipids Ag+-ion column attached to a Prostar 230 HPLC, equipped with a Prostar 410 autosampler, a Prostar 335 Photo-Diode-Array detector and Prostar 701 series fraction collector (Varian Inc., Walnut Creek, CA, USA). Mean difference between treatments was analysed by harvest point using a one-way ANOVA with the mixed procedure of SAS v 9.3 (SAS Institute, Cary, IN, USA). Significance was set at the $P<0.05$ level.

Results and Discussion.

3T3 adipocytes cultured with fatty acid treatments exhibited different rates of fatty acid accumulation. Cells cultured with CLA-*c*9,*t*11 led to the highest contents of total fatty acids. Culturing with 70 uM linoleic acid (18:2-c9,c12) a omega-6 fatty acid, reduced total fatty acids by 9.3%, and culturing with 35 uM and 70 uM CLA-t10,c12 reduced total fatty acid contents by 35% and 46%, respectively ($P<0.001$). Incorporation of the fatty acid treatments was greatest for linoleic acid followed by dose dependant accumulations of CLA- *c*9,*t*11 followed by CLA-*t*10,*c*12 ($P<0.001$). The differential accumulation of CLA isomers has been demonstrated *in vivo* in pigs (Kramer et al. 2008).

Accumulation of total CLA metabolites in CLA treated cultures were dose dependant and were 2-3 fold greater for CLA-t10,c12. Of the chain shortened products, the 16:2 metabolites predominated, and we believe this is the first report of conjugated 16:2 in 3T3 adipocytes. Conjugated 16:3 were greater in the CLA-c9,t11 treated cultures compared to CLA-t10,c12 treatments and are likely the result of β-oxidation of conjugated 18:3.

Delipidation effects of CLA-t10,c12 was also observed by the reduced PPARγ gene expression and a nearly two-fold reduction in FAS and SCD expression and a two-fold increase in CPT1 expression. Delta 6 desaturase (D6D~ Fads2) activity, which added a cis-6 double bond the CLA isomers to make 18:3 isomers, was observed in the 48h and 144h mature adipocytes and this was reflected in the gene expression assays. Delta 6 desaturase (D6D) activity appeared stronger with CLA-t10,c12 accounting for over 40% of metabolites but this could have been due to CLA-c6,t10c12 not being further metabolized to C16 or C20 products. Elongase (ELOV5) activity on CLA isomers, forming 20:2 and 22:2 products, was greater with the CLA-c9,t11 treatment according to lipid profiles but this was not reflected by the FADs2 and ELov5 gene expression. It appears that once the cells absorbed, both CLA-t10,c12 or CLA-c9,t11 isomer are actively metabolized by FADs2, but the natural isomer CLA-c9,t11, is better converted by Elov5.

The promoter of FADs2 contains a peroxisome proliferator receptor element (PPRE) but also a SREBP binding site (Lattka et al. 2010). However, according to the TR-FRET PPAR-gamma receptor competitive binding assay, the CLA-c9,t11 bound at 5.4 uM which was less than either the CLA-t10,c12 at 1.3 uM or the linoleic acid (18:2-c9,c12) with an IC50 of 1.2 uM. This was in comparison to the strong synthetic PPAR-gamma agonist GW1929, which bound at IC50 of 0.09 uM. Evidence presented in this report suggests that SREBP may have a greater influence on FADs2 expression than PPAR.

Conclusion

Delipidation effects of CLA-t10,c12 were apparent after 48h treatment of pre-adipocytes and mature adipocytes, and sustained for up to 144h. In contrast, CLA-c9,t11 did not affect lipogenesis in comparison with linoleic acid (18:2-c9,c12). The different CLA treatments formed unique metabolites and the CLA-c9,t11 isomer was preferentially converted by delta-6 desaturation (FADs2) followed by elongase (Elov5) to a conjugated 20:3 -c8,c11,t13. Alternatively, the CLA-t10,c12 was only desaturated to conjugated 18:3- c6,t10,c12, possibly being an unsuitable substrate for further elongation. The results presented suggest that the rate limiting enzyme FADs2, is not the limiting factor, when adipocytes are exposed to CLA isomers. CLA isomers -c9,t11 and -t10,c12 were readily metabolized in the mature adipocytes but it was the amount of CLA isomer entering the cells that was limiting. Together it suggests that both CLA isomers reduce fat accumulation but once the CLA

isomers are inside the adipocytes, they can be still metabolized into the C20 fatty acids, which can potentially influence eicosanoids production (House et al. 2005). Further work will be necessary to confirm the potential eicosanoid activity of CLA isomer's metabolites in other cell types such as hepatocytes or blood lymphocytes.

References

Benjamin, S. and Spener, F. 2009.. Nutrition & Metabolism 6(1):36.

Denizot, F. and Lang, R. 1986. J Immunol Methods 89(2):271-7.

Dugan, M. E. R., Aalhus, J. L., Schaefer, A. L. and Kramer, J. K. G. 1997.. Canadian Journal of Animal Science 77(4):723-725.

House, R. L., Cassady, J. P., Eisen, E. J., Eling, T. E., Collins, J. B., Grissom, S. F. and Odle, J. 2005.. Physiol Genomics 21(3):351-61.

Kramer, J. K. G., Fellner, V., Dugan, M. E. R., Sauer, F. D., Mossoba, M. M. and Yurawecz, M. P. 1997. Lipids 32(11):1219-1228.

Lattka, E., Eggers, S., Moeller, G., Heim, K., Weber, M., Mehta, D., Prokisch, H., Illig, T. and Adamski, J. 2010.. J Lipid Res 51(1):182-91.

Meadus, W. J., Duff, P., Rolland, D., Aalhus, J. L., Uttaro, B. and Dugan, M. E. R. 2011.. Canadian Journal of Animal Science 91(4):601-612.

Rasooly, R., Kelley, D. S., Greg, J. and Mackey, B. E. 2007.. Br J Nutr 97(1):58-66.

Reardon, M., Gobern, S., Martinez, K., Shen, W., Reid, T. and McIntosh, M. 2012.. Lipids 47(11):1043-51.

Reynolds, C. M. and Roche, H. M. 2010. Prostaglandins Leukotrienes and Essential Fatty Acids 82(4-6):199-204.

Schmittgen, T. D. and Livak, K. J. 2008. Nat Protoc 3(6):1101-8.

Stoffel, W., Holz, B., Jenke, B., Binczek, E., Gunter, R. H., Kiss, C., Karakesisoglou, I., Thevis, M., Weber, A. A., Arnhold, S. and others. 2008. EMBO J 27(17):2281-92.