Prolonged dietary treatment with conjugated linoleic acid stimulates porcine muscle peroxisome proliferator activated receptor γ and glutamine–fructose aminotransferase gene expression *in vivo*

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Abstract

Peroxisome proliferator activated receptors (PPARs) represent a family of DNA binding proteins that are activated by a variety of dietary and endogenous fatty acids. The PPAR proteins are expressed throughout the body and are the target of a variety of lipidaemic and insulin sensitizing drugs. Conjugated linoleic acid (CLA) is a collective name for octadecadienoic acid isomers with conjugated double bonds, which can also act as ligands for some of the PPAR family. To gain better understanding of the long-term effects of PPAR activation, CLA was fed at 11 g/kg of feed for 45 days to castrated male pigs (barrows). These barrows had a significant repartitioning of subcutaneous fat to lean tissue in the carcass: fat was reduced by 9.2% and lean muscle was increased by 3.5%, but intramuscular fat content was also increased by 14% ($P < 0.05$). PPAR γ , glutamine–fructose aminotransferase (GFAT), adipocyte fatty acid binding protein (AFABP), but not PPAR α mRNA levels were significantly increased ($P < 0.05$) in the CLA-fed pigs. The increased expression of PPAR γ and AFABP indicates that CLA induced the development of preadipocytes from stromal-vascular (s-v) stem cells to promote intramuscular fat content. The increase in the expression of GFAT mRNA indicates that the glucose supply of the muscle cells had been increased with the CLA diet, possibly sparing intramuscular fatty acid reserves.

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Introduction

Conjugated linoleic acid (CLA) acts as an agonist of the peroxisome proliferator activated receptor (PPAR) family. PPARs are DNA-binding transcription factors that bind the peroxisome proliferator response element (PPRE) (Isseimann & Green 1990, Forman *et al.* 1995, Oritz *et al.* 1999) as a heterodimer with the retinoic acid receptor RxR, which is activated by 9-*cis*-retinoic acid (vitamin A) (Mandrup & Lane 1997, Mukherjee *et al.* 1997). The PPAR family are currently divided into three subgroups: α , β and γ . PPAR β is expressed throughout the body and is involved in embryo development (Braissant & Wahli 1998, Michalik & Wahli 1999). PPAR α is found in liver, skeletal muscle, spleen, kidney, brown adipose and pancreatic β -islet cells and is primarily responsible for activating genes involved in lipid catabolism

(Kersten *et al.* 2000) or reducing fatty acid synthesis (Munday & Hemingway 1999). PPAR α is activated by fatty acids such as linoleic acid, linolenic acid, fibrates-type lipid catabolic drugs and fasting (Hashimoto *et al.* 2000). PPAR γ is found primarily in brown and white adipose tissue and to a lesser extent in spleen, intestine and skeletal muscle. Its activation increases adipocyte differentiation, fat storage, and insulin sensitivity (Brun & Spiegelman 1997, Valmaseda *et al.* 1999). PPAR γ is activated by 15 deoxy-prostaglandin J₂, the insulin-sensitizing thiazolidinedione drugs, arachidonic acid (Lehmann *et al.* 1995) and linoleic acid (Thoennes *et al.* 2000). PPAR α has a 1000-fold greater affinity for CLA than has PPAR γ , but PPAR α and PPAR γ both bind the same DNA element PPRE.

In 1997, Dugan and coworkers reported that CLA acted as an effective fat-to-lean ‘repartitioning’

agent in swine. Adding 2% CLA to the animals' feed increased lean muscle by 2.3% while reducing subcutaneous fat by 6.8% in genetically lean, Large White pigs (Dugan *et al.* 1997). Surprisingly, however, intramuscular fat content was increased by CLA treatment. The present research was performed to investigate why the two different fat depot sites, subcutaneous and intramuscular, had opposite responses to the CLA diet. Muscle cores were used to prepare total RNA to determine if some of the CLA-induced differences in gene expression reported *in vitro* could be detected in the whole animal.

Materials and methods

Animal treatments

Muscle samples used in this study were from 20 castrated male pigs (barrows) from Landrace boar by Large White sow (Landrace × Large White) matings (Dugan *et al.* 1997, 1999). The animals were slaughtered at the Lacombe Research Centre in accordance with the principles and guidelines set by the Canadian Council on Animal Care (1993). Pigs were fed 2% CLA-enriched oil, equivalent to 11 g CLA per kg of feed ($n=10$) or 2% sunflower oil (controls; $n=10$). The isomeric composition of the CLA feed has been reported previously (Kramer *et al.* 1998). The diets were formulated to meet nutrient requirements as outlined by the National Research Council (1988). The feeding trial commenced when the pigs reached an average weight of 60 kg, and lasted for 45 days. The pigs were slaughtered at an average weight of 105 kg, as outlined by Dugan *et al.* (1997). Animal carcass evaluations for lean muscle and subcutaneous fat contents were calculated from in-depth dissections according to the procedures described by Martin *et al.* (1981). The percentage of intramuscular fat was estimated from dried, ground longissimus thoracis muscle according to Method 39.1.05 of the Association of Official Analytical Chemists (1995).

RNA isolation

Total RNA was isolated from 100 mg of longissimus thoracis muscle cores using guanidine isothiocyanate-based TRIzol solution (Gibco-Brl, Burlington, ON, Canada) according to the manufacturer's specifications (Chomczynski &

Sacchi 1987). The RNA samples were quantified spectrophotometrically at 260 nm. All RNA isolates had an $OD_{260}:OD_{280}$ between 1.8 and 2.0, indicating that they were pure and clean. The quality of RNA was also checked by 1.0% agarose gel electrophoresis and staining with 1 µg/ml ethidium bromide.

Relative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

A two-step semi-quantitative RT-PCR method was used to measure gene expression in the longissimus thoracis muscle samples at the time of slaughter. Oligo-(dT)_{10n} was used as primer in the first step of cDNA synthesis. Total RNA (5 µg) was combined with 0.5 µg oligo-dT, 200 µM dNTPs and H₂O and preheated at 65 °C for 2 min to denature secondary structures. The mixture was then cooled rapidly to 20 °C and 10 µl 5 × RT buffer, 10 mM dithiothreitol (DTT) and 200 U Moloney murine leukaemia virus reverse transcriptase (Sigma-Aldrich, Oakville, ON, Canada) was added to a total volume of 50 µl. The RT mix was incubated at 37 °C for 90 min and the reaction stopped by heating at 95 °C for 5 min. The cDNA stock was stored at −20 °C. The yield of cDNA was measured according to the PCR signal generated from the internal standard housekeeping gene, *cyclophilin* (Genebank accession number AY008846) or β -actin amplified from 18 to 24 cycles starting with 0.1 µl of the cDNA solution. The volume of each cDNA pool was adjusted to give the same exponential-phase PCR signal strength for β -actin after 20 cycles.

Relative RT-PCR (Spencer & Christensen 1999) was performed to measure gene expression of PPAR α , PPAR γ , adipocyte fatty acid binding protein (AFABP), glutamine-fructose aminotransferase (GFAT), acyl-CoA oxidase (ACO) and calpain mRNAs. Primer sequences and optimal PCR annealing temperatures (t_a) are listed in Table 1. To ensure that no false-positive PCR fragments would be generated from pseudogenes in contaminating genomic DNA, primer sequences were designed to span intron regions, when genomic sequence data were available. In addition, all PCR primer combinations were tested using porcine genomic DNA as a negative control. PCRs were performed on a PTC-200 PCR machine (MJ Research Inc., Watertown, MA, USA) using

Table 1 Primers, PCR reaction conditions and expected products used in the semi-quantitative RT-PCR analysis of mRNA levels of the genes expressed in porcine longissimus thoracis muscle

Gene	Primer sequences	PCR product (bp)	GenBank accession no.	ta (°C)
<i>PPARα</i>	Sense: 5'-tta cag act acc agt act tag g-3' Antisense: 5'-agg ctt tgt ccc cac ag-3'	176	AF175309	58
<i>PPARγ</i>	Sense: 5'-tga cca tgg ttg aca ccg-3' Antisense: 5'-aag cat gaa ctc cat agt gg-3'	381	SSC6756	58
<i>AFABP</i> (aP2) Exon 1→2	Sense: 5'-tgg aaa ctt gtc tcc agt g-3' Antisense: 5'-ggt act ttc tga cta atg gtg-3'	147	SSY16039	56
<i>Cyclophilin</i>	Sense: 5'-acc gtc ttc ttc gac atc g-3' Antisense: 5'-caa cca ctc agt ctt ggc-3'	330	AY008846	62
<i>Calpain 1</i> Exon 1→2	Sense: 5'-tgg aag gac tgg agc ttg gt-3' Antisense: 5'-gac tcc agg gca tca gct g-3'	152	SSU23954	60
<i>ACO</i>	Sense: 5'-ccg gag ctg ctt aca cac at-3' Antisense: 5'-ggt cat acg tgg ctg tgg tt-3'	427	AF185048	60
<i>GFAT</i>	Sense: 5'-aac cca gtc ctg tca ata gcc-3' Antisense: 5'-acg aga gag att gca gct tcc-3'	420		60
<i>β-Actin</i>	Sense: 5'-gga ctt cga gca gga gat gg-3' Antisense: 5'-gca ccg tgt tgg cgt aga gg-3'	233	SSU07786	61

ta, optimal PCR annealing temperature.

~100 ng cDNA, 5 pmol each oligonucleotide primer, 200 μ M each dNTP, 1 U REDTaq Polymerase (Sigma-Aldrich, Oakville, ON, Canada) and 1 $\times$ REDTaq polymerase buffer in 20 μ l volume. The PCR program began with a 95 °C denaturation for 5 min, followed by 30–38 cycles of 95 °C/1 min, ta/1 min, 72 °C/1 min. The linear amplification range for each gene was tested on the adjusted cDNA. β -Actin primers were added when 20 cycles were remaining in the PCR cycle according to the linear amplification range of the specified gene. The PCR samples were electrophoresed on 8% polyacrylamide gels (8 $\times$ 10 cm) or 2.5% agarose Tris–borate–ethylene diamine tetraacetic acid (EDTA) gels. The gels were stained with ethidium bromide (10 μ g/ml) and photographed on top of a 280 nm UV light box. The gel images were digitally captured with a charge coupled device (CCD) camera and analysed with the NIH Imager Beta version 2 program. The quantity and base pair size of the PCR-generated DNA fragments were estimated relative to DNA ladder standards. Densitometry values were measured at each cycle sampling using One-Dscan software (Scanalytics, Fairfax, VA, USA). RT-PCR values are presented

as a ratio of the specified gene signal in the selected linear amplification cycle divided by the β -actin signal.

Protein isolation and analysis

Protein extracts were prepared from the thawed muscle samples by homogenizing 100 mg of muscle in 1 ml of extract buffer A (100 mM Tris HCl pH 7.5, 0.5 mM DTT, 2 mM phenylmethylsulphonyl fluoride, 5 mM EDTA, 500 mM NaCl, and 0.1% Triton X-100). The homogenate was then centrifuged at 1000 *g* for 5 min to pellet large cellular debris. Protein concentrations were measured using the bicinchoninic acid assay method (Pierce, Rockford, IL, USA). Samples were diluted to 1 μ g/ μ l with extract buffer and then 20 μ g were mixed with an equal volume of SDS-PAGE loading buffer (50 mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, 0.002% bromophenol blue), heated at 90 °C for 5 min and then loaded onto 10% SDS-PAGE for electrophoresis. To perform the western analysis, the separated proteins were electroblotted to Protran nitrocellulose (Schleicher & Schuell, Keene, NH, USA)

and then probed with rabbit anti-m-calpain (Sigma, St Louis, MO, USA), rabbit anti-PPAR (Sigma), and mouse anti-GAPDH (Chemicon Intl Inc., Temecula, CA, USA). The primary antibodies were detected with secondary anti-rabbit or anti-mouse IgG conjugated with alkaline phosphatase to generate a Nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) signal (Roche Diagnostics, Laval, PQ, Canada). Developed blots were digitally captured with a CCD camera and band intensities were measured as outlined under the RT-PCR methods section.

Statistical analysis

Data were analysed using GLM Frequency and Correlation procedures (Statistical Analysis System Institute 1985). RT-PCR signals were averaged from at least three replicates using two quantities of cDNA from 10 CLA and 10 control samples. Western blot data were generated from three replicate runs using seven CLA samples and seven control samples. Significance was calculated using Student's *t*-test.

Results

Market-weight barrows from the study by Dugan *et al.* (1997) fed with CLA at 2% of total feed for 45 days had their subcutaneous fat reduced by 9.2%, their lean yield increased by 3.5% and their percentage of intramuscular fat increased by 14% (Fig. 1). The diets were isocaloric and differed in their composition of 18:2 isomers. Fatty acids in the sunflower diet contained 59% linoleic acid (18:2n-6), whereas the fatty acids in the CLA diet had 25.4% linoleic acid and 32.85% CLA (mixed isomers) (Dugan *et al.* 1999). Thus both the control and the CLA diets were nearly equivalent with respect to their content of polyunsaturated fatty acids (PUFA; controls 62.5%, compared with CLA 61.21%). The effects on animal physiology of dietary PUFA compared with mono- and saturated fatty acids have been well studied (see review by Clarke 2000). The present research compared the effect of different types of PUFAs, specifically CLAs, which are believed to have a high affinity for the PPAR family of nuclear transcription factors (Moya-Carmarena *et al.* 1999, Thoenes *et al.* 2000).

Figure 1 The effect of feeding 2% conjugated linoleic acid (CLA) for 45 days on the carcass composition of market-weight pigs. Carcass compositions were measured as outlined in Materials and methods. Lean (Lean%) and subcutaneous (SQ%) fat are expressed as percentages per kg of body weight, and percentage intramuscular fat (IMF%) is expressed as grams of lipid per gram of muscle on a dry matter basis. Error bars represent standard deviation. □, Controls; ■, 2% CLA-fed. Significant differences: * $P < 0.05$, ** $P < 0.01$.

Figure 2 Adult muscle gene expression in barrows fed 2% CLA (■, $n=10$) compared with animals fed the control sunflower diet (□, $n=10$). Quantities of gene transcripts were measured by relative RT-PCR using the internal standard β -actin RT-PCR signal as the denominator, as described in Materials and methods. Error bars represent standard deviation. Significant differences: * $P < 0.05$, ** $P < 0.01$.

The relative levels of gene expression in longissimus thoracis muscle from pigs fed either the control diet with 2% sunflower oil or the test diet with 2% CLA-enriched oil are shown in Fig. 2. In the longissimus thoracis muscle of animals fed 2% CLA, GFAT, AFABP, and PPAR γ mRNAs were significantly ($P < 0.05$) increased, by 36.1%, 20.4% and 26.0% respectively (Fig. 3). However, western blotting analysis (data not shown) revealed no difference in PPAR protein contents of longissimus thoracis muscle after control or CLA treatments.

Because intramuscular adipose was not dissected from the lean muscle tissue for individual testing of RNA, it was possible that the observed increase in

Figure 3 Gel image of relative RT-PCR. (a) PPAR γ at 34 cycles with β -actin added at cycle 14 to act as the internal control. (b) GFAT at 34 cycles with β -actin added at cycle 14. (c) AFABP at cycle 26 with β -actin added at cycle 4. C, control animals. T, CLA-fed animals.

PPAR γ expression was an artefact of the increased percentage of intramuscular fat in the longissimus thoracis muscle of the CLA-fed group. To investigate the possibility of sampling variance, a muscle:fat ratio was calculated using the gene markers for m-calpain (muscle) and AFABP (adipocytes). There was still a significant increase in GFAT and PPAR γ mRNA expression in the 2% CLA-fed animals after adjustment was made for the relative content of fat in each muscle sample (Fig. 3).

Calpain was chosen as a muscle-specific gene marker, but was also a potential indicator of muscle protein deposition rates, as it functions as an endogenous protease (Huang & Forsberg 1998). Some studies in swine have found that dietary CLA increased fasting concentrations of serum insulin (Stangl *et al.* 1999), which may have increased lean body mass by reducing muscle catabolism (Rooyackers & Nair 1997), as indicated by increased nitrogen retention in the CLA-fed pigs (Muller *et al.* 2000). One of the known effects of insulin is to reduce muscle protein catabolism, possibly by reducing gene expression of proteases such as calpain (Fernandez & Sainz 1997). The CLA diet did not cause a significant change in calpain mRNA or protein levels as determined by western blot analysis. This was not surprising, because m-calpain does not have a PPRE element in its promoter region, but it does not rule out the

possibility that the gene activity of m-calpain or some other protease was decreased by the CLA treatment.

AFABP is adipocyte-specific and is known to contain active PPRE sites in its upstream promoter region (Houseknecht *et al.* 1998, Gerbens *et al.* 2000) and, as expected, 2% CLA did increase the expression of the *AFABP* gene in this trial, by 26% ($P < 0.05$; Fig. 3). Expression of ACO mRNA was also examined, as it is a known muscle and liver peroxisomal enzyme that is induced during lipid excess and by CLA in liver cells (Moya-Camarena & Belury 1999). ACO mRNA expression in the longissimus thoracis tissue was not significantly affected by the CLA diet.

Expression of the *GFAT* gene was significantly increased in muscle core samples from the CLA-fed animals. GFAT is the rate-limiting enzyme in the hexosamine pathway, which is involved in producing glycosylation substrates during periods of excess available intercellular glucose (Wang *et al.* 1998). Expression of *GFAT* is increased when plasma concentrations of glucose or fatty acids are high (Hawkins *et al.* 1997).

Discussion

In this study, barrows fed 2% CLA-enriched oil for 45 days had a significant reduction in subcutaneous fat compared with pigs fed diets containing 2% sunflower oil. However, 2% CLA also significantly increased the percentage of intramuscular fat. These changes in physiology are believed to be due to CLA activating the nuclear transcription factor, PPAR (Isseman & Green 1990). In this trial, longissimus thoracis expression of PPAR γ mRNA and the PPAR-responsive gene, *AFABP*, were significantly increased by dietary treatment with CLA. CLA treatment reduces subcutaneous fat in rats by increasing carnitine palmitoyltransferase and fatty acid β -oxidation activity in mature adipocytes, but not in skeletal muscle (Martin *et al.* 2000). The conflicting increase in intramuscular fat deposits and associated reduction in subcutaneous fat deposits in CLA-fed animals may be due to differences in PPAR expression and adipocyte cellular development between the two fat locations (Brun & Spiegelman 1997, Grindflek *et al.* 1998, Valmaseda *et al.* 1999). Intramuscular fat and muscle tissue have a higher percentage of s-v stem

cells and preadipocytes than subcutaneous fat (Ramsay *et al.* 1989, May *et al.* 1994). It has also been demonstrated *in vitro* that s-v cells, fibroblasts and newborn mouse muscle stem cells can be induced to differentiate into preadipocytes and to express adipocyte-specific genes such as *AFABP*, by treating them with PPAR γ activators such as thiazolidinedione drugs (Lehmann *et al.* 1995, Teboul *et al.* 1995). Therefore, it was possible that prolonged PPAR stimulation by CLA increased the apparent intramuscular fat content because more stem cells were available for intramuscular fat than for subcutaneous fat locations.

Recent studies in pigs have shown that prolonged dietary treatment with 3% CLA increases the fasting plasma concentrations of both free fatty acids and insulin (Stangl *et al.* 1999). Insulin production and *PPAR α* gene expression in pancreatic β -islet cells are inversely related: *PPAR α* gene expression is inhibited in pancreatic β -cells during glucose and free fatty acid feeding (Roduit *et al.* 2000) and increased during fasting (Kroetz *et al.* 1998, Hashimoto *et al.* 2000). The prolonged high dose of dietary CLA may be suppressing pancreatic *PPAR α* , thus allowing the production of more insulin. One of the effects of increased insulin sensitivity is increased protein retention as a result of reduced skeletal muscle catabolism (Rooyackers & Nair 1997), and CLA has been shown to increase lean muscle mass significantly. The relationship between increased insulin sensitivity and skeletal muscle protein retention is complex. Plasma insulin increases the number of insulin receptors and glucose transporters on the plasma membrane of skeletal muscle (Ezaki 1997, Park *et al.* 1998). The net effect is increased glucose destined for oxidation, with a potential sparing of dietary amino acids for protein synthesis, thereby reducing the need for protein catabolism to supply glycogenic amino acids (Baier *et al.* 2000). The protease, m-calpain, is an indicator for the catabolic enzymes that would theoretically be reduced during high glucose feeding and increased insulin concentrations (Fernandez & Sainz 1997, Wingertzahn *et al.* 1998), although m-calpain was not significantly reduced by CLA in the present trial. However, expression of GFAT mRNA was significantly increased. This supports the hypothesis that, in the CLA-fed pigs, muscle was supplied with greater amounts of energy, as GFAT and the hexosamine pathway are increased when increased

intercellular concentrations of glucose are available. Muscle glucose might have been spared as a result of the availability of more plasma triacylglycerols, which are typically increased by CLA diets (Stangl *et al.* 1999).

Conclusions

The semi-quantitative RT-PCR results show that prolonged dietary administration of CLA increased gene transcription of *PPAR γ* , *GFAT* and the PPAR-inducible *AFABP* gene in longissimus thoracis muscle core samples. Lean muscle, but also intramuscular fat content, were increased in the CLA-fed pigs. This indicates that CLA increased the percentage of intramuscular fat as a result of a combination of local stem cells being recruited into an adipocyte lineage by CLA activation of the nuclear transcription factor, PPAR γ , and increased nutrient supply in and around muscle. The increase in percentage lean muscle mass in CLA-fed pig carcasses may be due to an increase in the basal concentration of insulin, which would increase muscle glucose uptake, as indicated by the increase in GFAT mRNA.

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