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A semi-quantitative RT-PCR method to measure the *in vivo* effect of dietary conjugated linoleic acid on porcine muscle PPAR gene expression

W.J. Meadus[1*]

[1]Meat Research Section, Agriculture and Agri-Food Canada, Lacombe Research Center, 6000 C&E Trail, Lacombe, Alberta, Canada T4l 1W1.

*To whom correspondence should be addressed. E-mail: meadusj@em.agr.ca

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ABSTRACT

Conjugated linoleic acid (CLA) can activate (*in vitro*) the nuclear transcription factors known as the peroxisome proliferators activated receptors (PPAR). CLA was fed at 11 g CLA/kg of feed for 45d to castrated male pigs (barrows) to better understand long term effects of PPAR activation *in vivo*. The barrows fed CLA had lean muscle increased by 3.5% and overall fat reduced by 9.2% but intramuscular fat (IMF %) was increased by 14% ($P < 0.05$). To measure the effect of long term feeding of CLA on porcine muscle gene expression, a semi-quantitative RT-PCR method was developed using cDNA normalized against the housekeeping genes cyclophilin and β-actin. This method does not require radioactivity or expensive PCR instruments with real-time fluorescent detection. PPARγ and the PPAR responsive gene AFABP but not PPARα were significantly increased ($P < 0.05$) in the CLA fed pig's muscle. PPARα and PPARγ were also quantitatively tested for large differences in gene expression by western blot analysis but no significant difference was detected at this level. Although large differences in gene expression of the PPAR transcriptional factors could not be confirmed by western blotting techniques. The increased expression of AFABP gene, which is responsive to PPAR transcriptional factors, confirmed that dietary CLA can induce a detectable increase in basal PPAR transcriptional activity in the live animal.

INTRODUCTION

In 1997, Dugan and coworkers (1) reported that Conjugated Linoleic acid (CLA) acted as effective fat to lean "repartitioning" agent in swine. Adding CLA to 2% of the feed, increased lean muscle by 2.3% while reducing overall subcutaneous fat 6.8% in genetically lean, Large White pigs. *In vitro* studies using cell cultures have demonstrated that CLA acts as an agonist of the peroxisome proliferator activated receptor family (PPAR). PPARs are DNA binding transcription factors that bind the peroxisome proliferator repeat element (PPRE) (2,3,4) as a heterodimer with the retinoic acid receptor (RxR), which is activated by 9-cis-retinoic acid (vitamin A) (5,6). The PPAR family is currently divided into three subgroups, α, β and γ. PPARβ is expressed throughout the body and is involved in embryo development (7,8). PPARα has a 1000-fold higher affinity for CLA than PPARγ and both are expressed in hepatic, muscle, pancreatic, and adipose tissue.

Total RNA was prepared from pig muscle samples to examine if the CLA induced physiological changes were by differences in gene expression. The mRNA amount of a transcriptional factor such as PPARγ is often relatively very low and difficult to detect using standard Northern blot procedures. As a result, Northern blot methods usually require additional time consuming selective chromatography to isolate poly A+ mRNA and radioactive labeled probes to generate a measurable signal to quantify. Therefore, in order to reduce labor, a semi-quantitative RT-PCR method was developed. RT-PCR is the most sensitive method for detecting gene transcription products; however, it can be highly variable and may not accurately reflect gene activity (9). Subsequently, western blotting was used to quantitatively measure total PPAR protein. Ultimately however, even protein levels may not reflect actual gene activity due to post-translational modifications; therefore, the PPRE responsive genes, adipocyte fatty acid binding protein (AFBAP) and acyl Co A oxidase (ACO), were measured to confirm PPAR activity with its transcription rate.

Often a direct link cannot be made between a physiological response and a genetic marker due to missing biochemical

information. Some studies in swine have found that dietary CLA increased fasting levels of serum insulin (10). Insulin increases muscle nutrient absorption. Glutamine:Fructose aminotransferase (GFAT) is the *rate-limiting* enzyme in the hexosamine pathway, which is involved in producing glycosylation substrates (11). GFAT mRNA expression is increased when intercellular concentrations of glucose or fatty acids are high (12). Therefore the expression of GFAT was measured to examine if the increase lean mass in pigs fed CLA was due to more available energy inside the muscle cells.

MATERIALS AND METHODS

Animal treatments

Muscle samples from 20 castrated male Landrace X Large White pigs (barrows) were used in this study (1). The pigs were fed either a CLA supplemented diet containing 2%-conjugated linoleic acid (n = 10) or a Control diet containing 2% sunflower oil (n = 10). The isomeric composition of the CLA feed has been previously reported (13). The diets were formulated to meet nutrient requirements as outlined by the National Research Council (14). 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 in Dugan (1). The animals were slaughtered at the Lacombe Research Center in accordance with the principles and guidelines set by the Canadian Council on Animal Care (15). Animal carcass evaluations for lean muscle (Lean) and subcutaneous fat (SQ) levels were calculated from in-depth dissections according to the procedures described by Martin (16). Intramuscular fat % (IMF %) was estimated from dried ground *longissimus thoracis* (LT) muscle (Method 39.1.05: AOAC 1995) (17).

RNA Isolation

Total RNA was isolated from 100 mg of *longissimus thoracis* (LT) muscle cores using the guanidine isothiocyanate based TRIzol solution (GIBCO-BRL, Burlington, ON, Canada) according to the manufacturer's specifications (18). The RNA samples were resuspended in 100% formamide and quantified spectrophotometrically at 260*nm*. All RNA isolates had an OD_{260}:OD_{280} between 1.8 and 2.0, indicating clean RNA isolates. The RNA quality was also checked by 1.0% agarose gel electrophoresis, stained with 1 ug/ml ethidium bromide. Genomic DNA was isolated from the meat samples using the guanidium based DNAzol solution (GIBCO-BRL, Burlington, ON, Canada) as outlined in Meadus and MacInnis (19).

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 LT muscle samples at the time of slaughter. Oligo-$(dT)_{10n}$ (InVitrogen, Burlington ON) 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 H_20 and preheated at 65°C for 2 min to denature secondary structures. The mixture was then cooled rapidly to 20°C and then 10 μl 5X RT Buffer, 10 mM DTT and 200 U MMLV Reverse Transcriptase (Sigma-Aldrich, Oakville, ON, Canada) was added for a total volume of 50 μl. The RT mix was incubated at 37°C for 90 min. then 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 house-keeping gene cyclophilin [Genebank accession #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 (20) was performed to measure gene expression of PPARα, PPARγ, AFABP, GFAT, ACO, and m-calpain mRNAs. Primer sequences and optimal PCR annealing temperatures (ta) and are listed in Table 1. To insure 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 was available. In addition, all PCR primer combinations were tested using porcine genomic DNA as a negative control. Polymerase chain reactions were performed on a PTC-200 PCR machine (MJ Research Inc, MA, USA) using ~100 ng of cDNA, 5 pmoles each oligonucleotide primer, 200 μM of each dNTP, 1 unit of REDTaq Polymerase (Sigma-Aldrich, Oakville, ON, Canada) and 1X REDTaq polymerase buffer in a 20 μl volume. The PCR program initially started with a 95°C denaturation for 5 min, followed by 28 to 38 cycles of 95°C/1 min, ta °C /1 min, 72°C/1 min. Linear amplification range for each gene was tested on the adjusted cDNA. The less expressed transcripts of PPARα, and PPARγ required >32 cycles of PCR for detection. B-actin primers were added when 20 cycles were remaining in the specified gene's linear amplification range. The PCR samples were electrophoresed on 8% polyacrylamide gels (8 X 10 cm) or 2.5% agarose gels in TBE buffer [89mM Tris-base pH 7.6, 89mM boric acid, 2mM EDTA]. The gels were stained with ethidium bromide [10 μg/ml] and photographed on top of a 280 nm UV light box. The gel images were digitally captured with a CCD camera and analyzed 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 the One-Dscan software (Scanalytics, Fairfax, VA). RT-PCR values are presented as a ratio of the specified gene's signal in the selected linear amplification cycle divided by the β-actin signal (Fig. 1).

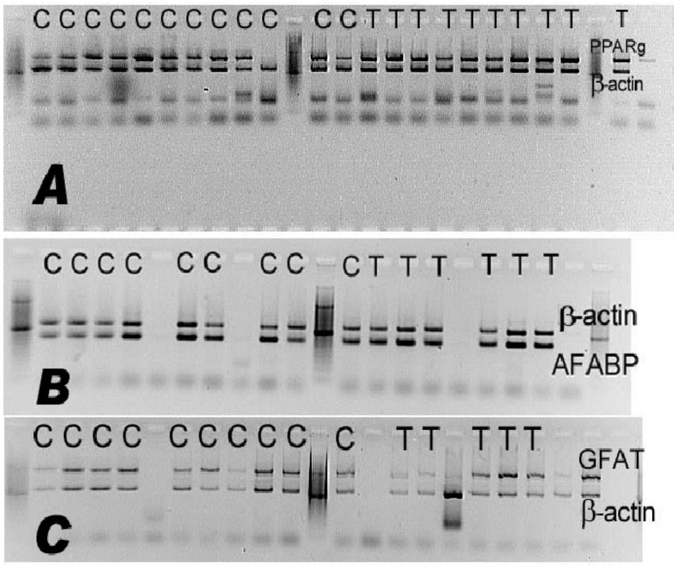

Fig. 1: Gel image of relative RT-PCR for A) PPARγ (381bp) at 34 cycles with β-actin (233bp) added at cycle 14 to act as the internal control. B) GFAT (420bp) at 34 cycles with β-actin added at cycle 14. C) AFABP (147bp) at cycle 26 with β-actin added at cycle 4. C; control fed animals. T: CLA fed animals.

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 dithiothreitol, 2 mM phenymethylsulfonyl fluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl, and 0.1% Triton X-100. The homogenate was then centrifuged at 1000 *xg* for 5 min to pellet large cellular debris. Protein concentrations were measured using the BCA method (Pierce, Rockford, IL). Samples were diluted to 1 ug/ul with extract buffer and then 20 ug 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^{o}C for 5 min then loaded onto 10% SDS-PAGE for electrophoresis in TBE. To perform the Western analysis, the separated proteins were electroblotted to Protran nitrocellulose (Schleicher, Schuell, Keene, NH) and then probed with rabbit anti-rat m-calpain (Sigma, St. Louis, MO), rabbit anti-synthetic PPAR (Sigma, St. Louis, MO), and mouse anti-rabbit GAPDH (Chemicon Intl. Inc, Temecula, CA) which acts as an internal standard between samples. The primary antibodies were detected with secondary anti-rabbit or anti-mouse IgG conjugated with alkaline phosphatase to generate a NBT/BCIP signal (Roche Diagnostics, Laval, PQ). To help in identification, prestained SDS-PAGE protein broad range standards (BioRad, Hercules, CA) were included on the 10% SDS-PAGE. The protein standards were visible on the Protran western blots. The antibody labeled m-calpain (58kDa), PPARα (~54 kDa), PPARγ (~55 kDa) or GADPH (~35kDa) polypeptide bands on the developed blots were digitally captured with a HP Scanjet 5100C scanner (Helwett Packard Co., Greeley, CO) and band intensities were measured using the One-D Scan program (Scanalytics, Fairfax, VA). Western blotting gives a more direct quantitative of a gene's expression level but depends on the availability of specific antibodies and is less sensitive than RT-PCR. Protein quantity also doesn't necessarily reflect gene product activity, since the protein or enzyme may still undergo post-translational modifications.

Statistical analysis

RT-PCR signals were averaged from at least 3 replicates using 2 quantities of cDNA from 10 CLA and 10 Control samples. The RT-PCR ratio values were analyzed using GLM Frequency and Correlation procedures (21). Western blot data was generated from 3 replicate runs using 7 CLA samples and 7 Control samples. Significance was calculated using Student's t-test.

RESULTS AND DISCUSSION

In this study, barrows fed 2% CLA enriched oil for 45d had a significant reduction in subcutaneous fat (SQ) compared to pigs fed diets containing 2% sunflower oil. However, 2% CLA also significantly increased IMF%. The changes in physiology are believed to be due to CLA activating the nuclear transcription factor PPAR (2). The relationship between IMF and dietary CLA are explained in greater detail in previously published work of Meadus *et al*. (2002) (22). The relative gene expression level in LT muscle from pigs fed either the control diet or CLA diet is shown in Fig. 2. In the LT muscle of animals fed CLA; GFAT, AFABP, and PPARγ mRNAs were significantly ($P < 0.05$) increased 36.1%, 20.4% and 26.0% respectively. However, Western blotting analysis (Fig. 3) could not detect a difference in LT muscle PPARγ protein content between Control and CLA treatments.

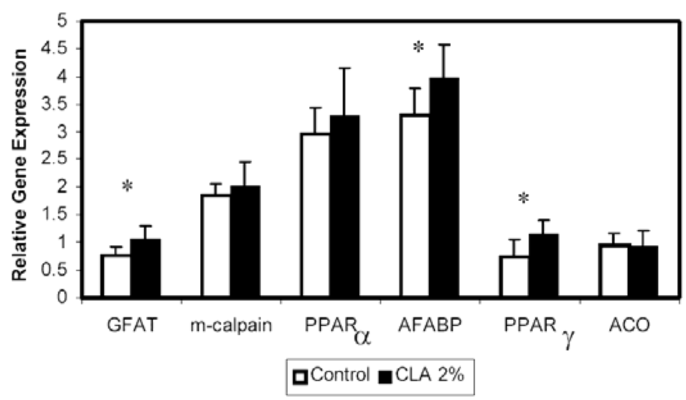

Fig. 2: Adult muscle gene expression in barrows fed CLA (n = 10) relative to the Control sunflower diet (n = 10). Gene transcript quantity was measured by relative RT-PCR using the internal standard β-actin RT-PCR signal as the denominator as described in the materials and methods section. Error bars represent standard deviation. Significance is represented as (*) $P<0.05$, (**) $P<0.01$.

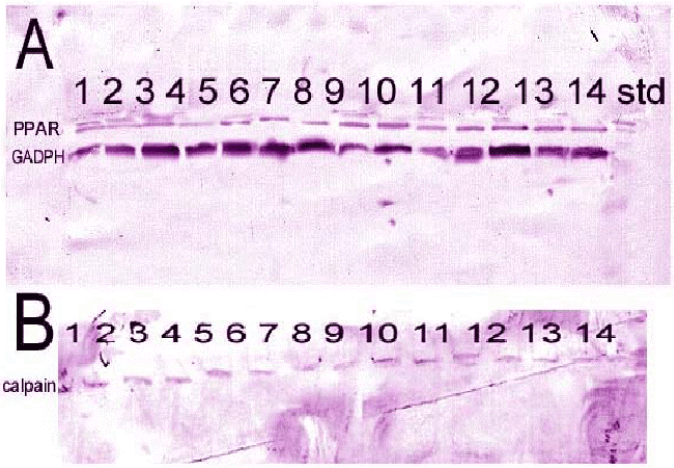

Fig. 3: Western blot of muscle calpain, PPARα (53 kDa) and GAPDH (35kDa) polypeptides labeled with conjugated antibodies from pigs fed control and CLA supplemented diets. The proteins were isolated from muscle homogenates, separated on 10% SDS-PAGE and electroblotted to nitrocellulose membrane as mentioned in the material and methods section. Blots were colormetrically labeled using alkaline phosphatase to generate a NBT/BCIP signal. Western blot A) labeled PPARα and GADPH signal B) m-calpain signal. Lanes 1-7 control diet, Lanes 8-14 CLA diet, std, SDS-PAGE prestained protein standard.

The adipocyte fatty acid binding protein (AFABP) is known to contain PPRE binding sites in its upstream promoter region (23,24) and 2% CLA did increase AFABP gene expression (Fig. 2) in this trial by 26% ($P < 0.05$). Acyl-CoA oxidase (ACO) also contains a PPRE in it promoter and is expressed in both muscle and liver cells. However in rat studies (25), ACO mRNA is increased only in liver but not in muscle, by a CLA diet. The pig muscle ACO also did not show a significant response to dietary CLA. Since IMF adipose was not dissected from the lean muscle tissue for individual testing of RNA, it was possible that the observed increase in PPARγ expression was an artifact of the increased IMF% in the LT muscle from the CLA fed group. To investigate the possibility of sampling variance, a muscle to fat ratio was calculated using the gene markers, m-calpain (muscle), and AFABP (adipocytes). There was still a significant increase in GFAT and PPARγ mRNA expression in the 2% CLA fed animals after adjusting for the relative level of fat in each muscle sample.

Recent pig studies have shown that prolonged dietary treatment with 3% CLA increases the plasma fasted levels of both free fatty acids and insulin (10,26). One of the effects from increased insulin sensitivity is increased protein retention due to reduced skeletal muscle catabolism (27). The protease m-calpain mRNA was measured to determine if dietary CLA reduced protein catabolism. The mRNA and protein level of m-calpain was not significantly reduced by CLA in this trial. Increased insulin activity also increases in the number of insulin receptors and glucose transporters (GLUT) on the plasma membrane of skeletal muscle (28). The net effect is increased intercellular glucose and reduced protein catabolism needed to supply glycogenic amino acids (29). Expression of GFAT mRNA in the hexosamine pathway is increased when an excess amount of intercellular glucose is available. GFAT mRNA expression was significantly increased in the CLA fed pigs. Antibodies for porcine GFAT protein were not available for comparison and plasma insulin levels were not measured in this trial. However, the increased GFAT mRNA expression did indicate that intercellular glucose and possibly plasma insulin was increased by the dietary CLA. This supports the hypothesis that feeding CLA to the pigs increased muscle insulin sensitivity causing an increase in muscle intercellular glucose, GFAT expression, and the hexosamine pathway (12).

To conclude, the semi-quantitative RT-PCR results show that prolonged dietary CLA increased gene transcription of PPARγ, GFAT and AFABP in LT muscle core samples. The increased rate of gene transcription was not detected using western blotting techniques for these same genes but since the PPAR inducible AFABP gene was increased by CLA, it demonstrates that PPAR functional activity was increased in the muscle. Lean muscle mass and GFAT mRNA levels were also increased indicating that the intercellular supply of glucose was improved in the muscle of pigs fed CLA.

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Table 1: Sequence, expected fragment size and annealing temperature (ta) of primers used in the semi-quantitative RT-PCR analysis of mRNA levels of the genes expressed in porcine LT muscle.

Gene	Sequences	Expected fragment size (bp)	ta (^{o}C)	GenBank accession number
PPAR α	Sense: 5'-tta cag act acc agt act tag g-3' Antisense: 5'-agg ctt tgt ccc cac ag-3'	176	58	AF175309
PPAR γ	Sense: 5'-tga cca tgg ttg aca ccg-3' Antisense: 5'-aag cat gaa ctc cat agt gg-3'	381	58	SSC6756
AFABP (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	56	SSY16039
Cyclophilin	Sense: 5'-acc gtc ttc ttc gac atc g-3' Antisense: 5'-caa cca ctc agt ctt ggc-3'	330	62	AY008846
Calpain 1 Exon 1→2	Sense: 5'-tgg aag gac tgg agc ttt gt-3' Antisense: 5'-gac tcc agg gca tca gct g-3'	152	60	SSU23954
ACO	Sense: 5'-ccg gag ctg ctt aca cac at-3' Antisense: 5'-ggt cat acg tgg ctg tgg tt-3'	427	60	AF185048
GFAT	Sense 5'-aac cca gtc ctg tca ata gcc-3' Antisense 5'-acg aga gag att gca gct tcc-3'	420	60	AF441244
β-actin	Sense: 5'-gga ctt cga gca gga gat gg-3' Antisense: 5'-gca ccg tgt tgg cgt aga gg-3'	233	61	SSU07786

PROTOCOLS

Relative Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) analysis

Isolation and preparation of total RNA

1. Total RNA was isolated from animal tissue frozen at -80°C. The tissue was collected immediately after slaughter in the Lacombe Research Centre meat abattoir according CCAC guidelines.
2. Each sample of RNA was collected from 100 mg of tissue in 1000 ul of TRIzol reagent (GIBCO-BRL, Burlington, ON, Canada). The tissue was thawed for 15min then homogenized using a Tissue Tearor (Biospec Products, Bartleville, OK) for 15 s at 30,000 rpm.
3. The homogenized samples were then mixed with 300ul of chloroform, vortexed for 10 s then centrifuged at 12,000xg for 10 min.
4. The aqueous phase was withdrawn to a new tube and mixed with 600 ul isopropanol, and allowed to precipitate at -20°C for > 60 min.
5. The RNA preciptitate was pelleted by centrfugation at 12,000xg for 10 min.
6. The RNA pellet was resuspended in 500 ul Trizol for a second round of purification, mixing with 150 ul chloroform, centrifugation at 12,000 xg for 10 min, collecting the aqueous phase and mixing with 1000 ul ethanol. Precipitating at -20°C for > 60min.
7. The RNA was again pelleted 12,000 xg for 10 min and resuspended in 20 ul of 100% deionized formamide. The formamide solution was heated at 85°C for 10min to dissolve the pellet completely.
8. The quality of RNA was examined by runnning 2ul of the formamide solution on 1.5% agarose gels in TBE buffer [89mM Tris-base pH 7.6 , 89mM boric acid, 2mM EDTA] stained with 10ug/ml ethidium bromide. RNA quality was assessed by the sharpness of the 28S and 18S rRNA bands migrating at the 1000bp and 500bp molecular ladder standards range as visualized on a 330nm UV-transilluminator. Good quality RNA has a sharp 28S rRNA band with approximately 2-fold the intensity of the 18S band and a 260nm /280nm absorbance ratio close to 2.0.
9. The yield of RNA was determined relative to the molecular ladder standard on the 1.5% agarose gel and by absorbance at 260 nm on a spectrophotometer. RNA concentration is estimated by the dilution factor X 50 ug/ml X OD260 nm.

Generation of cDNA

1. Complementary DNA was generated using appoximately 2 ug of total RNA in < 2.0 uls of 100% formamide with 200 U of MMLV-reverse transcritiptase (Bio Basic Inc, Markham, ON), 10U Rnase inhibitor (Sigma, Oakville, ON), 10mM DTT, 40 mM KCl, 6mM $MgCl_2$, 50mM Tris-HCl pH 8.3, 0.5 mM each of dTTP, dCTP, dGTP, and dATP, 400 mM oligo $(dT)_{12\text{-}18}$, and 200 mM random nonamers in a 20ul reaction volume.
2. Preheating the RNA in the RT reaction buffer without the enzyme is not required if it was heated in the 100% formamide.
3. The RT reaction is first incubated at 20°C for 10 min to allow annealling of the random hexamers then incubated at 42°C for 60 min.
4. The RT reaction is stopped by incubating at 95°C for 5 min. Store at -20°C.

Normalizing the cDNA stock samples

1. To evaluate the quality and equalize the amount of cDNA from each sample, a PCR trial was made using the internal standards cyclophilin or β-actin.
2. Using 2.0 ul of the 20 ul RT reaction, primers [5 uM] for β-actin or cyclophilin were mixed with 4 U of REDTaq DNA polymerase (Sigma, Oakville, ON), 0.2mM each of dTTP, dCTP, dGTP, and dATP, into a total volume of 40 ul then divided into 4 tubes of 10 ul each. The PCR program initially started with a 95°C denaturation for 5 min, followed by 16 to 22 cycles of 95°C/1 min, ta °C /1 min, 72°C/1 min. Polymerase chain reactions were performed on a PTC-200 PCR machine (MJ Research Inc, MA, USA)
3. The 10 ul PCR for β-actin or cyclophilin were amplified for 16, 18, 20, and 22 cycles, collecting one tube at each of the specified cycles at the end of the 72°C extention phase.
4. The PCR samples were run on either 2% agarose or 8% native polyacrylamide gels in TBE gels then stained with ethidium bromide for visualization on a UV transilluminator. The β-actin and cyclophilin primers also produce larger PCR products from genomic DNA and therefore could also be used to assess the amount of contaminating DNA in the cDNA preparation.

5. The UV illuminated gel images were digitally captured with a CCD-camera (Coho, Japan) using the Scion/NIH Imager program (Scion Corp. MA). The quantity of cyclophilin or β-actin PCR DNA fragments were estimated according to band intesity using the One-Dscan software (Scanalytics, Fairfax, VA).
6. Samples densities from the 16 to 22 cycles were plotted to find the linear amplification range, which was typically in between 18 and 22 cycles. A minimal signal density for 20 cycles is then chosen as the baseline and then the cDNAs are adjusted to all give the same signal strength for β-actin when amplifying 1 ul of the adjusted cDNA stock.
7. Standardization of the cDNAs is also double checked using cyclophilin as the internal control.

Preparation of primers for RT-PCR analysis

1. Oligonucleotide primers are designed to amplify across intron regions so only the RT prepared cDNA wil give PCR products. The primers are tested on genomic DNA templates to guarantee against false PCR signals from psuedogenes and possible contaminating DNA in the RT cDNA samples.
2. Intial PCR products for each gene were cloned into pCR2.1 plasmids using the TA cloning kit (Invitrogen Corp., Carlsbad, CA). The cloned products were sequenced using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster city, CA) and compared against GenBank (NIH, Bethesda., MD) using the BLAST program (30) to confirm identity. New porcine sequences were considered identified if they were >85% homologous against human or mouse sequences and had an expected false positive probability value $P < 0.0001$.
3. The optimum annealing temperature and linear amplification range for each gene specific primer pair was predetermined using 1 ul of standardized cDNA per 20 ul PCR assay. Some of the lower expressed genes like PPARα and PPARγ required > 32 cycles of PCR amplification to be detected on the ethidium bromide stained 8% polyacrylamide gels.

Relative semi-quantitative PCR

1. To quantitate the relative amount of gene transcription for each treatement, 1 ul of the standardized cDNA was used in a 20 ul PCR solution and cycled to within the predetermined linear amplification range. At 20 cycles before the end of the last cycle, primers for the housekeeping genes b-actin or cyclophlin were added at the end of the 72C extention phase. This would then generate PCR products for the target gene and the housekeeping gene both within their linear amplification range.
2. The PCR products were separated and visualized on either 2% agarose or 8% PAGE and digitally recorded as previously described.
3. Gene expression was determined relative to the internal standard PCR signal. Comparison of target gene expression between individuals was adjusted according to the internal standards which were previously normalized between samples.
4. The proceedure was repeated and the values combined to determine averages and statistical differences.

Protein isolation and analysis

1. Proteins were extracted 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 dithiothreitol, 2 mM phenymethylsulfonyl flouride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl, and 0.1% Triton X-100.
2. The homogenate was then centrifuged at 1000 *xg* for 5 min to pellet large cellular debris. Protein concentrations were measured at A562nm using the rapid Bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein concentrations are estimated relative to a 2 mg/ml BSA generated standard curve.
3. Samples were diluted to 1 μg/ul with extract buffer and then 20 μg 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 90C for 5 min then loaded onto 10% SDS-PAGE (10 X 15 cm) with a 4% polyacrylamide stacker layer for 3 hours at 70v in TBE buffer. SDS-PAGE prestained protein standards (BioRad, Hercules, CA) were included to monitor electrophoresis.
4. The separated proteins were Western blotted from the 10%PAGE to Protran nitrocellulose for 12 hr at 0.1 amps (Schleicher, Schuell, Keene, NH) using a Trans-blot electrophoretic transfer cell in electroblot buffer [192 mM glycine, 25 mM Tris, 20% methanol, 0.1% SDS]. Electrophoretic transfer efficiency was determined by the amount prestained SDS-PAGE standards on the Protran blot after transfer.
5. The PPAR, m-calpain, and GADPH proteins were labeled using rabbit anti-PPARγ [5.0 ug/ml] (Sigma, St Louis, MO), goat polyclonal anti-PPARα [2.0 ug/ml] (Santa Cruz biotechnology, Inc., Santa Cruz, CA), rabbit anti-m-calpain [3.0 ug/ml] (Sigma, St Louis, MO), or mouse anti-GAPDH [2.0 ug/ml] (Chemicon Intl. Inc, Temecula, CA). The primary antibodies were diluted in TBS hybridization buffer [10mM Tris, 0.9% NaCl, 0.05% Tween 20] plus 10% carnation instant powdered skim milk adjusted to pH 7.5] using approximately 10 ml per 20 X 20 cm blot.
6. Primary antibody hybridizations were incubated at 20^{o}C for > 12 hrs.

7. The blots were washed 6 times with TBS for 5 min each wash.
8. Primary antibodies were tagged with goat anti- rabbit IgG (Chemicon Intl. Inc, Temecula, CA), rabbit anti-goat IgG (Sigma, St Louis, MO), or chicken anti-mouse IgG antibodies (Chemicon Intl. Inc, Temecula, CA) conjugated with alkaline phosphatase. The anti-IgG antibodies were diluted 1000X in TBS hybridization buffer and incubated with the blots at 20^{o}C for 1 hr.
9. The blots were then washed 6X in TBS and then soaked in Detection buffer [100mM Tris pH 9.5, 100mM NaCl, 50mM $MgCl_2$] for 5 min.
10. Blots were incubated overnight at 20^{o}C in 10 ml detection buffer + NBT/BCIP [0.4mg/ml nitroblue tetrazolium chloride + 0.19 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toludine-salt] to develop blue colored bands of antibody bound proteins. Colour development was stopped by rinsing with deionized water.
11. Proteins labeled by the alkaline phosphatase colour precipitation were digitally captured and quantitated under white light using a Hewlett-Packard 5100c scanner (Palo Alto, CA) and NIH imager program as previously described in the "Standardizing the cDNA stock samples" section. Polypeptide identification was also confirmed by molecular mass by comparing with the SDS-PAGE protein standards on the same blots.