Effect of dietary polyunsaturated fatty acids on Stearoyl CoA-Desaturase gene expression in intramuscular lipids of lamb

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Abstract

The effect of replacement of dietary sunflower oil (SO) with linseed oil (LO) on Stearoyl CoA desaturase (SCD) gene expression was investigated. Thirty-six lambs were randomly assigned to four groups and fed with one of the experimental diets, consisting of lucerne pellets with oil (60 g/Kg). The diets varied in the percentage of the oil supplemented and were: 100% SO; 66.6% SO plus 33.3% LO; 33.3% SO plus 66.6% LO and 100% LO. The trial period was of 7 weeks. Longissimus dorsi was separated from each carcass and stored at -80°C until the analysis. Total fatty acids composition of muscles was determined by gas-chromatograph, while SCD mRNA expression was assessed by Real-Time Reverse-Transcription PCR. Replacement of SO with LO decreases significantly the SCD mRNA content with a concomitant increment of polyunsaturated fatty acids (PUFA) n-3. These results are related to the higher level of PUFA n-3 present in linseed than sunflower. Although, there were differences on mRNA level, there was not a simultaneous changes on SCAD activity. In conclusion, PUFA n-3 act on the regulation of mRNA SCD level without affecting the activity of the relative enzyme.

Introduction

Stearoyl-CoA Desaturase enzyme (SCD) (also known as Δ9-desaturase) plays a key role in the lipid metabolism, because it introduces a double bond at the Δ9-position in a large spectrum of fatty acids (FA) (Ntambi, 1995; Ntambi and Miyazaki, 2004). The most important substrates of SCD are acyl-CoA of myristic, palmitic, stearic acid and C18:1 t11 (known as vaccenic acid) which are converted into C14:1 c9, C16:1 c9, C18:1 c9, and C18:2 c9, t11, respectively (Coi et al., 2001), the latter being a conjugated linoleic acid (CLA) isomer of potential interest in human health. In fact, different in vivo and in vitro studies reported anti-diabetic (Belury and Kempa-Steczko, 1997), anti-carcinogenic and anti-atherogenic effects (Scollan et al., 2006) of CLA. Milk and meat from ruminants are the major sources of CLA in the human diet (Ritzenthaler et al., 2001). Oxivine SCD protein is encoded by a gene located on chromosome 22, in a region where Carta et al. (2008) have detected a positional quantitative trait locus (QTL) for the CLA:Vaccenic acid ratio in milk.

SCD gene expression in liver and adipose tissue is regulated by different factors (Dobryzn and Dobryzn, 2006): dietary fat (e.g. polyunsaturated FA, cholesterol, vitamin A), hormonal signals (e.g. insulin, glucagon), environmental factors (temperature, metals, alcohol, thiazolidinediones), peroxisomal proliferators and development processes (Ntambi and Miyazaki, 2004). These factors may act on translational or post-translational regulation, affecting the enzyme expression or activity, respectively. Polyunsaturated FA (PUFA) may inhibit transcription of key genes involved in lipid synthesis, including Acetyl-CoA Carboxylase (ACACA), fatty acids synthase (FASN), SCD, Malic Enzyme, L-pyruvate kinase, adipocyte lipid-binding protein (aP2), resulting in a decrease of de novo lipogenesis (Jump and Clarke, 1999; Al-Hasani and Joost, 2005). PUFA reduce the SCD expression, as well as the other lipogenic genes, by a repression of sterol regulator element binding protein (SREBP-1) activity (Miyazaki and Ntambi, 2003). Moreover, PUFA activate peroxisome proliferator-activated receptor proteins (PPARs) to modulate the gene expression, in response to environmental stimuli (Miyazaki and Ntambi, 2003). In bovine, some studies reported the effect of SREBP-1 and PPAR modulation on SCD gene expression (Graugnard et al., 2009; Waters et al., 2009). In the literature, the effect of dietary PUFA on SCD gene expression or activity in ruminants are related to the use of linseed oil (Bas et al., 2007; Bernard et al., 2005, 2009a, 2009b; Herdman et al., 2010) and/or CLA (Baungard et al., 2002) on beef, lamb and goat. Studies on rodents reported that alpha-linolenic acid inhibited the SCD gene expression with a greater extent than linoleic acid (Sessler et al., 1996), but no data are available about the comparison of dietary PUFA n-3 and n-6 on SCD expression in lamb muscles. In sheep, feeding trials shown that the SCD protein activity in muscle from concentrate fed lambs did not differ to the SCD activity in muscle from forage fed animal (Vasta et al., 2009). On the contrary, Bas et al. (2007) revealed a reduction of SCD activity (expressed as desaturation ratio) only in subcutaneous adipose tissue as a consequence of linseed increment in the diet. The supplementation of ruminant diets with PUFA rich lipids is the most effective approach to decrease saturated FA and promote the enrichment in unsaturated FA of meat and milk (Jeronimo et al., 2009; Mele, 2009; Lanza et al., 2011). However, more knowledge are needed in order to evaluate how different sources of PUFA in the diet may affect the expression of lipogenic genes in animal tissues.

In the present study the effect of dietary substitution of sunflower oil (SO) with linseed oil (LO) was evaluated, in order to investigate the role of dietary PUFA in regulating the SCD expression in skeletal muscle of lambs. The two types of plant oil were chosen as source of 18:2 n-6 (SO) and 18:3 n-3 (LO) to evaluate the effect of PUFA n-6 and PUFA n-3 series on lipid metabolism.

Key words: Lamb, Diet, Gene expression, SCD, Linseed oil.

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**Materials and methods**

**Experimental design**

Animals, diets and experimental design are fully described by Jerónimo et al. (2009). Briefly, animal handling followed the EU directive 86/609/EEC, concerning animal care. The trial tacked place in the Centro de Experimentação do Centro Alentejo (Reguengos de Monsaraz, Portugal), using thirty-six Merino Branco ram lambs. The lambs were reared on pasture with their dams until weaning, at about 90 days of age. The average initial weight of lambs was 22.9±2.78 kg (mean ± SD). Animals were randomly assigned to four groups of nine lambs each and were fed one of the four experimental diets: pelletted dehydrated lucerne with 6% of SO (diet 6S); pelletted dehydrated lucerne with 4% of SO and 2% of LO (diet 4S2L); pelletted dehydrated lucerne with 2% of SO and 4% of LO (diet 2S4L); pelletted dehydrated lucerne with 6% of LO (6L). The target for oil inclusion was 33.3% of SO replaced with LO, %° (6S) (4S2L) (2S4L) (6L).

**Lipid analysis**

Intramuscular lipids were extracted by the method of Folch et al. (1957), using a dichloromethane and methanol (2:1, vol/vol) solution instead of the chloroform and methanol (2:1, vol/vol) solution (Carlson, 1985). The FA were transesterificated with ethidium bromide. Positions of the dominant 16S and 28S rRNA bands are indicated by arrows.

![Figure 1. Total RNA (5 μg) was fractionated on a 1% non-denaturing agarose gel, stained with ethidium bromide. Positions of the dominant 16S and 28S rRNA bands of individual samples subjected to ethidium bromide staining agarose gel electrophoresis (Figure 1). Concentration and purity of RNA were determined by spectrophotometry analysis (Lambda 25 UV/visible spectrometer, PerkinElmer Instruments, Les Ulis, France) at 260, 280, and 320 nm (A260–A320–A280–A320, where A is absorbance). All the RNA samples shown a ratio ranged from 1.807 to 2.072. Genomic DNA contamination was eliminated by DNase treatment, using TURBO DNase kit (Applied Biosystem, Carlsbad, CA, USA), following the manufacturer’s instructions. The absence of genomic DNA was controlled by PCR using a pair of primers (named SCD1genF and SCD1genR, Table 2) from ovine SCD gene, including the 4th intron (1667 bp), that would yield PCR products of either 1913 or 347 bp when starting from genomic DNA or cDNA, respectively. The PCR was performed by GeneAmp PCR 9600 Thermo cycler (Perkin Elmer Life Sciences) according the follow program: the DNA was preheated at 94°C for 5 min and then amplified with 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min. The final cycle had an extension time of 7 min at 72°C. The PCR mix was constituted by 12.5 µL of 2× PCR Master Mix (Fermentas International Inc., Burlington, Canada; Taq DNA polymerase 0.05 units/µL, MgCl₂, 4 mM, and dNTP, 0.4 mM of each), 0.5 µL of 10 pmol/µL primer forward, 0.5 µL of 10 pmol/µL reverse primer, 250 ng of DNA and ddH₂O up to 25 µL. Primers used for PCR were designed with Primer3 software (http://frodo.wi.mit. edu/cgi-bin/primer3/ primer3_www.cgi) with the ovine mammary gland SCD mRNA sequence (NM_001009254; Ward et al., 1998).

Table 1. Chemical composition of the experimental diets.

| Crude protein, g/kg DM | Ether extract, g/kg DM | NDF, g/kg DM | Fatty acids composition (% of total fatty acids) |
|------------------------|------------------------|--------------|-----------------------------------------------|
| 0 (S)                  | 33.3 (4S2L)            | 66.6 (2S4L)  | 100 (6L)                                      |
| 153                    | 153                    | 153          | 153                                          |
| 70                     | 71                     | 72           | 76                                           |
| 500                    | 482                    | 485          | 481                                          |
| 8.2                    | 8.3                    | 7.9          | 7.0                                          |
| 2.4                    | 3.0                    | 3.3          | 3.4                                          |
| 20.4                   | 19.5                   | 17.7         | 15.6                                         |
| 57.1                   | 43.9                   | 30.6         | 18.3                                         |
| 6.3                    | 20.4                   | 33.6         | 48.0                                         |

*Zero percentage of sunflower oil (SO) by linseed oil (LO) replacement – diet 6S; 33.3% of SO with LO replacement – diet 4S2L; 66.6% of SO with LO replacement – diet 2S4L; 100% of SO with LO replacement – diet 6L. NDF, neutral detergent fibre.
Analysis of mRNA using Real-Time Reverse-Transcription PCR

Level of SCD mRNA in *Longissimus dorsi* muscle was quantified by real-time reverse-transcription PCR. Single-strand cDNA was obtained from 4 µg of purified total RNA, using High Quality cDNA Archive Kit (Applied Biosystem) containing the MultiScribe™ Reverse Transcriptase, 50 U/µL, following the manufacturer’s instructions. Quantitative PCR was performed in 100-µL tubes using a Rotorgene 2000k (Corbett Research, Sydney, Australia) thermal cycler with real-time detection of fluorescence. PCR was conducted in a volume of 50 µL using 1X MESA GREEN qPCR MasterMix Plus for SYBR® (including dNTPs, Meteor Taq DNA polymerase, 4mM MgCl₂, SYBR® Green I and stabilizers) (Eurogentec, San Diego, CA, USA) and 0.3 µM of each primer. PCR program consisted of a Meteor Taq DNA polymerase activation at 95 °C for 5 min, followed by 3-steps cycles, repeated 40 times: denaturation for 15 s at 95 °C, annealing for 20 s at 60 °C and extension for 40 s at 72 °C. The fluorescence intensity of SYBR Green I was read and acquired at 72 °C after completion of the extension step of each cycle. PCR conditions for individual primer sets were optimized by varying template cDNA and magnesium ion concentration, in order to obtain amplifications yielding a single product and melt curves with a single uniform peak. Quantification of individual transcripts was performed using the Comparative Quantitation option of the software supplied by Corbett Research for the Rotorgene. This option allows to assess the relative expression of the SCD gene compared to the housekeeping gene. The gene expression intensity was measured as cycle thresholds (Ct). The Ct of SCD gene (Ct1) was normalized according to the transcription level of the housekeeping gene (Ct2), and the relative fold-change (ΔCt) was obtained. Ct1 and Ct2 were calculated for each sample. The mean efficiency of a group of cycling curves is calculated at the point that the cycling curves take off and used to calculate a fold-change according to the formula (Warton *et al.*, 2004):

\[
\text{fold change} = 2^{-\frac{ΔCt}{Ct2}}
\]

where A is the efficiency of amplification reaction (for an efficiency of 100%, A = 2; this data is calculated directly by the software); Ct1 (Ct of SCD-1 gene) and Ct2 (Ct of housekeeping gene) are the take off values of the cycling curves being compared.

Cyclophilin A (*PPIA*) was used as a housekeeping gene for comparison of RNA extraction efficiency. Although, relying on treatment effects as a guide for the selection of reference genes for normalization is not the most optimal, the lack of response of cyclophilin A (*PPIA*) mRNA to nutritional factors in previous studies (Bernard *et al.*, 2006, 2009; Ollier *et al.*, 2009) allowed to use this gene as an internal standard reference gene. Results were expressed as the mRNA copy number of SCD gene relative to cyclophilin. Reactions were repeated three times for each sample. Primers for SCD and cyclophilin A for quantitative RT-PCR were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with the ovine mammary gland SCD (NM_001009254; Ward *et al.*, 1998) and cyclophilin A (AY251270; Etschmann *et al.*, 2004, unpublished) mRNA sequence and are listed in Table 2.

Desaturase activity

The extent of FA desaturation was determined by calculating the ratio c9-unsaturated to c9-unsaturated + saturated for the different FA that SCD catalyzes (Palmquist *et al.*, 2004). The following ratio were calculated:

- c9:14-1 to c9:14+1 = 14:0: desaturation index for C14:0 (DI14);
- c9:16-1 to c9:16+1 = 16:0: desaturation index for C16:0 (DI16);
- c9:18-1 to c9:18+1 = 18:0: desaturation index for C18:0 (DI18);
- c9,11-18:2 to c9,11-18:2 + c11-18:1: desaturation index for Vaccenic acid (RAVA).

Statistical analysis

The effect of dietary replacement of SO with LO was analyzed using the GLM procedure of SAS (1999), using a linear model where dietary oil replacement was included as linear and quadratic effect. Dietary oil replacement was included as linear and quadratic effect.

Results and discussion

In this work, the effect of dietary PUFA on SCD gene expression and activity was investigated. In particular, diets with different n-6/n-3 ratio were used, in order to modulate the content of SO (rich in PUFA n-6) and LO (rich in PUFA n-3).

The effect of experimental diets on FA composition of polar, neutral and total lipid of muscle samples has been previously reported and discussed in Jeronimo *et al.* (2009). Descriptive statistic for the investigated traits is reported in Table 3. In the present paper, only FA ratios, which are a proxy of SCD activity, and total PUFA n-3 content were reported in Table 4 and Figure 2, respectively.

SCD gene showed a higher expression level in the diet 6S and 4S2L (6 and 3 times respectively), while it was slightly lower in the diets 2S4L and 6L (0.88 and 0.68 times, respectively) (Table 4). Data reported in Table 4 showed a significant quadratic decrease of mRNA content when SO was replaced with LO in the diet. The SCD mRNA amount significantly decreased until the 2S4L diet, which represent the 66.6% of replacement of SO with LO, and then stabilized (Table 4). On the other hand, the content of total PUFA n-3 in the intramuscular fat showed an increasing trend with the increment of LO in the diet (Figure 2). Muscle samples from lambs fed 6S diet contained a higher amount of SCD mRNA (8.6 times) and a lower amount of PUFA n-3 than muscle samples from lambs fed 6L diet (Figure 2). This result agreed with Waters *et al.* (2009) and Dervishi *et al.* (2010), which found a negative relationship between SCD mRNA in muscles and the amount of dietary PUFA n-3, in beef and lamb, respectively. In particular, Dervishi *et al.* (2010) shown a higher SCD gene expression in the lambs fed a diet rich in PUFA n-6, similarly to what found in the present study.

Table 2. Primer sequences used and relative gene bank accession number.

| Gene       | Application | Name         | Sequence 5’→3’               | Reference sequence gene bank accession number |
|------------|-------------|--------------|------------------------------|-----------------------------------------------|
| SCD        | Genomic     | SCD1genF     | CCACATCTCCAGGTCCTGCT         | NM_001009254                                 |
|            | contamination | SCD1genR     | ATTCCTCTGGGGGTATTG          |                                               |
| SCD        | Quantitative | SCDHF        | CCGAGAAGGTACCCGTTG          | NM_001009254                                 |
|            | PCR         | SCDIR        | GGGCGGTTGTCATGAGCAA         |                                               |
| Cyclophilin | Quantitative | CYCF         | CAAAAGTCTGGCGCAAG           | AY251270                                      |
|            | PCR         | CYCR         | GCAAAAGAAGAAGTCGGAAC        |                                               |
The modulation of the rate of n-6 and n-3 in the diet plays an important role to define the SCD gene expression through the SREBP-1c in muscle, as reported by Waters et al. (2009). On the contrary, Bernard et al. (2009b) revealed no effect on SCD mRNA abundance in mammary, liver or perirenal adipose tissues of goat after LO treatment.

The product/substrate ratios reported in Table 4 are considered a proxy of SCD activity (Malau-Aduli et al., 1997). Palmquist et al. (2004) proposed that the DI14 can be used to quantify endogenous RA synthesis in lamb tissues. Results from the present study, indicated that DI14 was not related to RA concentration in intramuscular fat (Figure 3). Keeping into consideration also the other product:substrate ratios, the pattern of SCD activity did not seem related to the pattern of SCD mRNA expression (Table 4). The only FA ratio significantly, but marginally, affected by the replacement of SO with LO was the RA/VA ratio (Table 4). In particular, intramuscular fat from lambs fed 6L diet shown significant lower RA/VA ratio (-11.4%) than that from lamb fed the other diets (Table 4). In a previous study, Bas et al. (2007) reported that feeding lamb diet supplemented with linseed oil did not affect Δ9-desaturase indexes in the intramuscular fat.

The lack of association between desatura-

### Table 3. Descriptive statistics of desaturase index and expression level (expressed as mRNA amount). mRNA amount was expressed as mRNA copy number of SCD gene relative to cyclophilin gene.

| Trait      | Mean   | SD    | CV, % | Minimum | Maximum |
|------------|--------|-------|-------|---------|---------|
| DI14       | 0.041  | 0.012 | 29    | 0.020   | 0.068   |
| DI16       | 0.042  | 0.006 | 14    | 0.032   | 0.057   |
| DI18       | 1.926  | 0.215 | 11    | 1.385   | 2.415   |
| RA/VA      | 0.342  | 0.039 | 11    | 0.262   | 0.427   |
| mRNA       | 2.520  | 2.143 | 85    | 1.00    | 6.60    |

### Table 4. Diet effect on SCD activity (expressed as product to substrate ratio) and expression level (expressed as mRNA amount). mRNA amount was expressed as mRNA copy number of SCD gene relative to cyclophilin.

| Trait      | SO replaced with LO, %° | SE   | P values |
|------------|-------------------------|------|----------|
| SO         | (6S)                    | (4S2L) | (2S4L) | (6L)     |
| DI14       | 0.038                   | 0.038 | 0.037   | 0.048    | 0.013    | 0.228   | 0.685   |
| DI16       | 0.039                   | 0.048 | 0.039   | 0.039    | 0.009    | 0.839   | 0.060   |
| DI18       | 1.837                   | 1.958 | 1.917   | 1.989    | 0.070    | 0.230   | 0.279   |
| RA/VA      | 0.350 a                 | 0.359 a| 0.337 a| 0.308 b  | 0.010    | 0.049   | 0.048   |
| mRNA       | 5.840 A                 | 2.720 B| 0.875 C| 0.676 C  | 0.260    | <0.001  | <0.001  |

°Zero percentage of sunflower oil (SO) by linseed oil (LO) replacement – diet 6S; 33.3% of SO with LO replacement – diet 4S2L; 66.6% of SO with LO replacement – diet 2S4L; 100% of SO with LO replacement – diet 6L. Desaturation index for C14:0 (c9-14:1 to c9-14:1 + 14:0); DI16, desaturation index for C16:0 (c9-16:1 to c9-16:1 + 16:0); DI18, desaturation index for C18:0 (c9-18:1 to c9-18:1 + 18:0); RA/VA, desaturation index for vaccenic acid (c9,t11-18:2 to c9,t11-18:2 + t11-18:1). A,B,CMeans within rows with different superscripts differ (P<0.01). a,bMeans within rows with different superscripts differ (P<0.05).

Figure 2. Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on abundance of SCD mRNA (y=5.86–0.12x + 0.0007x^2, R^2=0.99) and n-3 LC-PUFA (y=0.78 0.01x–0.00007x^2, R^2=0.94). Concentration of n-3 LC-PUFA is expressed as g/100 g of total FA (data from Jerónimo et al., 2009), while SCD mRNA abundance is expressed as relative to cyclophilin gene expression level x10.

Figure 3. Effect of dietary replacement of sunflower oil (SO) with linseed oil (LO) on DI14 (c9-14:1 to c9-14:1 + 14:0) (y=0.0405 – 0.0001x + 2e^{-6}x^2, R^2=0.94) and Rumenic Acid (RA) (y=-2.1346 - 0.0011x + 5e^{-5}x^2, R^2=0.99).
tion indexes and SCD mRNA expression was reported also in previous studies on beef cattle (Archibeque et al., 2005), on dairy cow (Bionaz and Loo, 2008) and goat (Bernard et al., 2009a). On the contrary, Herdman et al. (2010) revealed that the SCD protein expression was significantly affected by dietary PUFA n-3 in bovine adipocytes. According to Archibeque et al. (2005), these contrasting results may be due to individual variation of enzyme activity not related with indices of desaturation, probably because ingestion of SCD substrates and products may vary between diets (Renaville et al., 2006).

Therefore, data of the present study seemed to suggest that in the intramuscular fat of lambs, dietary PUFA n-3 affected mainly SCD gene transcription rather than the activity of the SCD enzyme, as reported also in mouse and cow by Sessler et al. (1996) and Hiller et al. (2011) respectively. Herdman and co-workers (2010) observed a reduction of SCD enzyme content in adipose tissue of bulls fed flaxseed油, without significant decreasing of MUFA. The authors concluded that the lack of significant differences might be related to large between-individual variations within the groups and relatively small number of animals per group. However, further investigation based on direct measurements of SCD enzyme activity in vivo are needed, in order to obtain additional insights about the regulation of SCD enzyme by PUFA n-3 in ruminant tissues.

Some hypothesis have been proposed in order to explain the regulation of SCD gene transcription by PUFA n-3. Some authors suggested that PUFA may regulate the expression of the adipocyte SCD gene by acting on the stability of mRNA transcripts (Sessler et al., 1996; Bernard et al., 2009a). Other authors proposed that PUFA n-3 may affect SCD gene transcription by regulating the nuclear abundance of the mature sterol response element binding protein-1 (i.e. nSREBP-1) (Ntambi, 1999; Hiller et al., 2011). However, at present it is still not completely clear the mechanism of the SCD gene regulation by PUFA n-3 in ruminants. Despite the relatively small number of animals per group, results of this work suggested an active role of these FA in lipid metabolism and gave more information about their dietary effect on quality of ruminant product. Since PUFA n-3 are implicated in the prevention of various diseases, including obesity and diabetes, by several different molecular mechanisms, including signal transduction and regulation of gene expression in liver, adipose tissue and brain (Al-Hasani and Joost, 2005), further researches with a larger number of animals are required to completely understand the role of PUFA n-3 in the gene expression.

Conclusions

Replacing SO with LO in the diet of lambs resulted in a reduction of SCD mRNA level, and a marginally decrease of the SCD enzyme activity (expressed as RAVA ratio). This results may be due either to a lack of effect of PUFA n-3 on the enzyme activity or to a lack of correlation between SCD product:substrate ratios and actual enzyme activity. Finally, results seemed to indicate that PUFA n-3 are more effective in affecting SCD expression in lamb muscle than PUFA n-6.

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