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Diet high in α-linolenic acid up-regulate PPAR-α gene expression in the liver of goats

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ABSTRACT

Background: There is little information on the effects of diets containing high α-linolenic acid (C18:3n-3) on liver lipid composition and lipogenic gene expressions. In this study fourteen goats (*Capra aegagrus hircus*) were fed either a flaxseed oil (FSO) supplemented diet containing high α-linolenic acid or a control diet without added flaxseed oil (CON) for 100-d to evaluate the effects on liver lipid composition and the gene expression of peroxisome proliferator-activated receptors (PPAR-α) and stearoyl-CoA-desaturase (SCD) in the liver.
Results: An increase in the levels of C18:3n-3 and C20:5n-3, C22:5n-3, C22:6n-3 was observed in the liver of FSO-treated goats. There was a significant ($P < 0.05$) up-regulation of PPAR-α gene expression and downregulation of SCD gene in the liver of goats fed the high α-linolenic acid diet.
Conclusions: In conclusion, genes associated with the control of fatty acid (FA) conversion (SCD and PPAR) were affected by the α-linolenic acid supplementation in the goat diet. It is suggested that PPAR-α is the key messenger responsible for the translation of nutritional stimuli into changes in hepatic gene expression.

1. Introduction

Altering omega-3 fatty acid (FA) profile of foods, specifically, decreasing the n-6:n-3 fatty acids ratio, acts as a beneficial factor in the prevention of disease in humans and animals. It is quite difficult to increase the n-3 polyunsaturated fatty acid (PUFA) content of ruminant meat using n-3 PUFA in animal feeds due to the microbial biohydrogenation of these PUFA in the rumen [1]. One alternative is to supplement the diet with oilseeds with high n-3 PUFA content such as flaxseed oil [2,3] which had been shown to increase the n-3 PUFA content in cattle [4] and lamb [5] and alters the fatty acid composition and cholesterol levels in some lamb tissues [6]. Bernard et al. [7] showed that dietary supplementation with flaxseed oil (FSO) led to positive results on the fatty acid composition of the goat milk and meat and downregulated the stearoyl-CoA-desaturase (SCD) genes in the mammary glands of the dairy goat.

Several researchers reported that dietary PUFA downregulated the expression of lipogenic enzymes [7,8] and enzyme synthesis with a decrease in hepatic fatty acid synthesis [9,10] in the rat liver. Compared to the bird, fish, and human in which the liver is the main organ in the body for fatty acid synthesis [11,12,13], the adipose tissue is a greater site of fatty acid synthesis [14]. Few experiments have investigated the lipogenic activities in the ruminant liver. Limited lipogenic activities of the ruminant liver and liver slices have been reported compared to the adipose tissue of the same animals and liver slices from the rat [14,15].

The regulation of gene expression plays a critical role in the adaptive response, in addition to altering the activity of enzymes in relevant metabolic pathways. Key transcription factors that regulate this adaptive response have been identified in the last decade. These include the peroxisome proliferator activated receptors (PPAR) [16], sterol regulatory element binding protein (SREBP) [17], and carbohydrate response element binding protein (ChREBP) [18]. Importantly, the activities of these transcription factors are regulated not only by hormones, but also by nutrients and metabolites [19,20]. The PPARs can induce the proliferation of peroxisomes in cells, a process that is accompanied by activation of the promoter of the acyl-CoA oxidase gene (ACOX1) encoding the key enzyme of β-oxidation for peroxisomal long-chain fatty acid [21]. The PPAR-α can also regulate the synthesis of highly unsaturated fatty acids, indicating the pleiotropic functions of PPAR-α in the regulation of lipid metabolic pathways [22].

Changes in dietary habits have modified the PUFA daily intake in humans. The reduction of n-6 and more importantly n-3 FA intake

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over the last decades is considered a contributing factor to the current obesity epidemic [23]. Different mechanisms by which dietary PUFAs influence obesity have been evidenced [24]. One of them relies on the ability of PUFAs to influence the expression of genes involved in metabolic processes. The PUFAs have been shown to regulate numerous transcription factors such as the PPARs [25], liver X receptors (LXRs) [26], SREBP [26] and ChREBP [27] that are involved in controlling metabolism.

The hypothesis of this study was that supplementing α-linolenic acid to the goat diet would result in changes on lipid deposition in the liver or gene expression in the liver. Manipulation to change the activity of PPAR and SCD may lead to increased production of omega-3 fatty acids and reduced SFA, thereby improving the tissue FA profiles. However, a clearer understanding of the expression of PPAR and SCD at gene levels in the goat liver may yield new information on the regulation of PPAR and SCD activity leading to improved goat products. In the current study, the hypotheses were that a positive correlation existed between changes in FA composition of the goat diet and gene expression of PPAR and SCD in the goat liver. Our specific objective was to determine any association between the gene expression of PPAR and SCD and deposition of omega-3 fatty acids in the liver of the goat.

2. Materials and methods

2.1. Animals and diets

Animal procedures described herein were approved by the Universiti Putra Malaysia Institutional Animal Care and Use Committee. Goats were individually housed and fed. Fourteen five month old Boer male goats (*Capra aegagrus hircus*) were randomly allocated into two groups of 7 animals each namely the control (CON) and FSO-fed goats. The goats were housed individually in slatted-floor pens in the animal farm of Universiti Putra Malaysia. A randomized complete design with 2 treatments was used. The concentrated feed of the experimental diet was obtained by adding FSO (1.3%) instead of palm kernel oil to the control diet. The chemical and nutrient compositions of the diets are presented in Table 1. The animals were fed for 100 d and slaughtered after an overnight fast (12 h). Immediately after slaughter, the liver tissue was quickly taken and snap-frozen in liquid nitrogen and stored at -80°C until fatty acid analysis and RNA extraction for gene expression analysis.

2.2. Procedures and analyses

For fatty acid analysis, total fat was extracted from liver samples using chloroform:methanol following the method of Folch et al. [28] modified by Rajion et al. [29] and Ebrahimi et al. [30]. The extracted fat was saponified with ethanolic KOH for 10 min at 90°C. Fatty acids were converted to fatty acid methyl esters (FAME) by transesterification with methanolic boron trifluoride (14% BF_3) at 90°C for 20 min. The FAME were then analyzed by gas–liquid chromatography (Agilent 7890A). One microliter of FAME was injected by an auto sampler into the chromatograph, equipped with a flame ionization detector (FID). The helium carrier gas was used at a flow rate of 1 mL × min^{-1}. The FAME were separated on a 100 m × 0.32 mm × 0.25 μm film thickness using Supelco SP-2560 capillary column (Supelco, Inc., Bellefonte, PA, USA). The injector and detector temperature was maintained at 250°C and 300°C respectively. The column was operated isothermally at 120°C for 5 min, then programmed to 250°C at 4°C/min, increased by 2°C/min up to 170°C, held at 170°C for 15 min, increased again by 5°C/min up to 200°C, and held at 200°C for 5 min and then increased again by 2°C/min to a final temperature of 235°C and held for 10 min. Peak identification was performed by using known standards (mix C4–C24 methyl esters; Sigma-Aldrich, Inc., St. Louis, Missouri, USA) and relative quantification was automatically carried out by peak integration.

Table 1
Ingredients and chemical composition of the experimental diets.

Ingredients (% of DM)	CON	FSO
OPF silage	30.00	30.00
Corn, grain	17.00	17.00
Soybean meal	13.30	13.30
Palm kernel cake	25.11	25.11
Rice bran	8.18	8.18
Flaxseed oil	0.00	1.30
Palm kernel oil	1.00	0.00
Sunflower oil	2.30	2.00
Mineral premix	0.50	0.50
Vitamin premix	0.50	0.50
Ammonium chloride	1.00	1.00
Limestone	1.00	1.00
Chemical composition		
ME (mcal/kg)[a]	2.51	2.51
CP%	13.00	13.00
EE%	7.00	7.00
NDF%	48.90	48.90
ADF%	33.00	33.00
CA%	0.68	0.68
P%	0.36	0.36
Fatty acid composition (% of total identified fatty acids)		
C10:0, capric	0.90	0.53
C12:0, lauric	7.04	3.53
C14:0, myristic	3.04	1.79
C16:0, palmitic	16.14	15.65
C16:1, palmitoleic	0.22	0.21
C17:0, margaric	0.29	0.29
C18:0, stearic	5.85	5.68
C18:1n-9, oleic	27.40	27.78
C18:2n-6, linoleic	35.68	30.92
C18:3n-3, α-linolenic	3.44	13.63

FSO: flaxseed oil group; CON: control group.
[a] Calculated values.

2.3. Liver gene expression

About 30 mg of the snap frozen liver samples from each animal was homogenized using a tissue homogenizer (IKA Analysentechnik GmBH, Germany). Total RNA was extracted from 30 mg of frozen liver tissue using the RNeasy® lipid tissue mini kit (Cat. No. 74804, Qiagen, Hilden, Germany) and DNase digestion was completed during RNA purification using the RNase-Free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described by Ebrahimi et al. [31]. Total RNA integrity was checked by agarose gel electrophoresis and total RNA was evaluated using a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) using 260/280 nm ratio of absorbance.

Single-stranded cDNA was reverse transcribed from total RNA by denaturing 1 μg of RNA in DNase/RNase-free water using a Quantitect® reverse transcription kit (Qiagen, Hilden, Germany).

Real-time PCR was performed with the Bio-Rad CFX96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) using optical grade plates

Table 2
Names and sequences of the primers used in this study.

Target group		Sequence 5′–3′	Length, nt	Reference
β-Actin	F	CGC CAT GGA TGA TGA TAT TGC3	123	[46]
	R	AAG CGG CCT TGC ACA T3		
PPAR-α	F	TGC CAA GAT CTG AAA AAG CA	101	[47]
	R	CCT CTT GGC CAG AGA CTT GA		
SCD	F	CCC AGC TGT CAG AGA AAA GG		
	R	GAT GAA GCA CAA CAG CAG GA	115	[47]

F: forward; R: reverse.

using Quantifast® SYBR Green PCR kit (Cat. No. 204054, Qiagen, Hilden, Germany). The sequences of primers are shown in Table 2.

Quantitative reverse transcription PCR (qPCR) reactions were carried out using a master mix consisting of 8.5 μL of RNase/Dnase-free water, 8.5 μL of Quantifast® SYBR green (Qiagen, Hilden, Germany), 1 μL cDNA, and 1 μL of each respective forward and reverse primer. Amplification was carried out in a Bio-Rad CFX96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) with the conditions set at 95°C for 10′, 40 PCR cycles at 95°C for 30″, 60°C for 20″ and 72°C for 20″. Fluorescence was measured at every 15″ to construct the melting curve. Negative assay controls included no template cDNA controls and no reverse transcriptase controls. Immediately after the qPCR experiment, a melt curve analysis was conducted to verify the purity of amplicons and absence of primer dimers. For this, the plate temperature was increased from 65 to 95°C in 1°C increment.

Each sample was run in triplicate, and averaged triplicates were used to assign cycle threshold (CT) values. The ΔCT values were generated by subtracting experimental CT values from the CT values for β-actin targets amplified with each sample. The group with the highest mean ΔCT value (lowest gene expression) per amplified gene target was set to zero and the mean ΔCT values of the other groups were set relative to this calibrator (ΔΔCT). The ΔΔCT values were calculated as powers of 2 (-2ΔΔCT), to account for the exponential doubling of the PCR as described by Ebrahimi et al. [32].

2.4. Data analysis

All data are presented as means ± SE. The assumption of the normal data distribution was checked using the UNIVARIATE procedure of SAS software. Statistical analysis was performed by analysis of variance (ANOVA) using SAS software package, version 9.1 (SAS Inst. Inc., Cary, NC) and the mean were compared for significance using T-test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Bodyweights

At slaughter, the bodyweight (kg) of the control (26.38) and FSO-fed goats (25.68) was not significantly different.

3.2. Dietary fatty acid composition

The control diet contained approximately 16.00% of C16:0 which was the main contributor for total saturated fatty acids, almost 27.40% of C18:1n-9 which was the main contributor for total monounsaturated fatty acids and 35.68% of C18:2n-6 which was the main contributor for PUFA n-6 and 3.44% of C18:3n-3 (Table 1). The inclusion of FSO increased the percentage of C18:3n-3 to 13.63%.

3.3. Liver fatty acid composition

The fatty acid composition of the liver in goats fed the CON and FSO diets is presented in Table 3. The major FA detected in the liver was C18:1n-9 (26.31%) with no significant differences among the treatment groups. Other major FA in the liver affected by the dietary treatment included C16:0 (14.95%) and C18:0 (23.72%). There were no significant effects ($P > 0.05$) on the percentage of C10:0, C12:0, C14:1 and C15:0. However, the dietary FSO lowered ($P < 0.05$) C16:0, C18:1 trans-11, CLA cis-9 trans-11 CLA and C20:4n-6 with no effects ($P > 0.05$) on the percentage of C18:1n-9 and trans-10 cis-12 CLA. Total saturated fatty acids showed no significant ($P > 0.05$) differences between the treatment groups. The percentage of C20:4n-6 was lowered with FSO supplementation ($P < 0.05$). At the end of the 100 day feed trial the liver total lipids of goats fed the FSO diet contained almost 41.72% SFA, 28.39% MUFA and 29.18% PUFA (Table 3). Increasing the dietary FSO content produced higher percentages of C18:3n-3 and PUFA n-3 accompanied by lower proportions of C20:4n-6 and C18:2n-6 (Table 3).

Table 3
Fatty composition of the liver in goats fed with different experimental diets.[a]

Fatty acids	Dietary treatments			
	CON	FSO	SEM	P-value
C10:0, capric	0.32	0.34	0.02	0.388
C12:0, lauric	0.26	0.35	0.04	0.898
C14:0, myristic	2.17	1.24	0.09	0.042
C14:1, myristoleic	0.06	0.09	0.005	0.066
C15:0, pentadecanoic	0.41	0.33	0.03	0.064
C15:1, pentadecenoic	0.34	0.59	0.04	0.124
C16:0, palmitic	16.12	13.77	0.49	0.019
C16:1, n-7 palmitoleic	1.13	0.65	0.08	0.010
C17:0, margaric	0.53	0.50	0.03	0.074
C17:1, margaroleic	0.57	0.35	0.05	0.319
C18:0, stearic	22.27	25.17	0.50	0.018
C18:1n-9, oleic	27.25	25.37	0.43	0.945
C18:1, trans-11 vaccenic	2.01	1.34	0.07	0.028
C18:2n-6, linoleic	13.65	9.73	0.23	0.039
CLA cis-9 trans-11	0.99	0.50	0.03	0.002
CLA cis-12 trans-10	0.25	0.21	0.02	0.010
C18:3n-3, linolenic	0.64	2.24	0.14	0.001
C20:4n-6, arachidonic	7.63	6.57	0.27	0.042
C20:5n-3, eicosapentaenoic	0.91	3.83	0.25	0.001
C22:5n-3, docosapentaenoic	1.64	4.68	0.27	0.001
C22:6n-3, docosahexaenoic	0.86	2.13	0.12	0.001
SFA[b]	42.06	41.72	0.78	0.054
MUFA[c]	31.36	28.39	0.45	0.899
PUFA n-3[d]	4.06	12.88	0.76	0.001
PUFA n-6[e]	21.28	16.30	0.41	0.422
Total trans FA[f]	2.01	1.34	0.07	0.028
Total CLA[g]	1.24	0.72	0.04	0.007
n-6:n-3 ratio[h]	5.24	1.27	0.32	0.001
UFA:SFA	1.38	1.4	0.04	0.001
PUFA:SFA ratio	0.60	0.70	0.03	0.061

CON: diet without FSO, FSO: diet with addition of flaxseed oil.
[a] The data are expressed as the percentage of total identified fatty acids.
[b] SFA: sum of C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0.
[c] MUFA: sum of C14:1 + C16:1 + C17:1 + C18:1n-9.
[d] PUFA n-3: sum of C18:3n-3 + C20:5n-3 + C22:5 n-3 + C22:6n-3.
[e] PUFAn-6: sum of 18:2n-6 + 20:4n-6.
[f] Total trans FA: C18:1trans.
[g] Total CLA: sum of cis-9 trans-11 CLA + cis-12 trans-10 CLA.
[h] n-6:n-3 fatty acid ratio: (C18:2n-6 + C20:4n-6) ÷ (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3).

There have been few reports on the fatty acid profile of the ruminant liver [33,34,35,36]. In agreement with these authors, the present results showed that C16:0 (16.12%) and C18:0 (22.27%) were the major SFA, with oleic acid (C18:1n-9; 27.25%) being the unsaturated fatty acid in the control goat liver.

The percentages of the main FA in the goat liver in this study were similar to those in the liver of sheep fed different n-6:n-3 fatty acid ratios (FAR) as reported by Kim et al. [37]. Palmitic acid can be converted to C16:1n-7 through palmitoyl-CoA Δ-9 desaturation [38]. As in the current study, Demirel et al. [36] showed that the amount of C16:1n-7 in the liver of lambs was elevated with greater consumption of dietary C16:0 from palm oil, although the amount of C16:1n-7 observed in this study was quite low (<1.13%; Table 3). Since brain and heart cannot synthesize enough C20:4n-6 from the circulating C18:2n-6, liver synthesis is necessary to maintain their homeostatic C20:4n-6 percentage in the absence of dietary C20:4n-6 [39]. The percentage of C20:4n-6 (7.63%) in the present study was lower compared with the 16.5% reported for the lambs fed different n-6:n-3 FAR [37] but greater compared with lambs (0.99%) fed different fat sources [36]. Dietary FSO supplementation produced a lower percentage of C20:4n-6 in the liver compared to the CON group. This decrease coincided with the lower dietary concentration of C18:2n-6. The increases in the percentage of n-3 FA (C18:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3) also coincided with the higher percentage of

C18:3n-3 in the FSO diet. These increases are in agreement with the reports of Demirel et al. [36] who supplemented the lamb diet with linseed oil, and with Kim et al. [37] who fed lambs with low n-6:n-3 FAR. The n-6:n-3 FAR ($P < 0.05$) in the liver of goats in the present study decreased, the lowest being 1.27:1. The elongation and desaturation of FA products synthesized from C18:2n-6 and C18:3n-3 in the liver play an important role in the n-6:n-3 FAR for the liver. In this study, the FSO diet contained large amounts of α-linolenic acid than the CON diet, which contained high linoleic acid. For this reason, it can be assumed that goats fed diets with greater amounts of α-linolenic acid would absorb more intact α-linolenic acid.

3.4. Liver gene expression

As key transcription factors of different genes participating in lipid homeostasis, three types of PPAR-α, β (δ) and γ are known to regulate gene expression, by sharing overlapping duties in a cell- and tissue-specific manner. Their actions are diverse, but those for PPAR-α are known for fatty acid catabolism and those for PPAR-γ are participating in adipogenesis [40]. Fatty acid synthesis and triglyceride (TG) assembly both occur in the liver. Fatty acid synthase is involved with *de novo* FA synthesis [41]. If fatty acids are not oxidized in the liver, they can work as a TG assembly. Based on the relative expression of testing genes, those associated with β-oxidation (SCD and PPAR-α) were more abundant in the goat liver.

Measurement of the gene expression by real time PCR showed that the expression of the PPAR-α genes was higher in the liver of FSO-fed goats. The expression of PPAR-α relative to CON was 1.23 fold in the liver of FSO-fed goats (Fig. 1). The fold changes for the SCD gene in FSO group were approximately 0.15 fold lower than that for the CON group (Fig. 1).

Several studies have shown that fatty acid desaturases are regulated by the PPAR gene in mammals, for instance stearoyl CoA Δ9 desaturase genes have been shown to be directly regulated by PPARs. In addition, rodent peroxisomal proliferators are known to up-regulate fatty acyl Δ-6, Δ-5 and Δ-9 desaturases [42,43].

The lower n-6:n-3 FAR in the liver of the FSO-fed goats was expected, due to the high content of n-3 in this oil. Omega-6 and omega-3 PUFA have been shown to be potent inhibitors/inducers of hepatic gene expression [44]. In line with this, the hepatic expression of SCD genes was low in FSO-fed goats (Fig. 1), thus paralleling the observed hepatic ratios between n-6 and n-3 PUFA. These findings suggest that the ratio between dietary n-6 and n-3 PUFA altered the hepatic expression of genes encoding enzymes involved in FA conversion. According to Jump et al. [44] and Zúñiga et al. [45], the n-6 and n-3 PUFA have shown to enhance lipid oxidation by inducing PPAR-α.

Large adaptations of transcription occurred in the liver when goats were supplemented with flaxseed oil, which can modulate energy metabolism and cell proliferation as well as the fatty acid synthesis pattern.

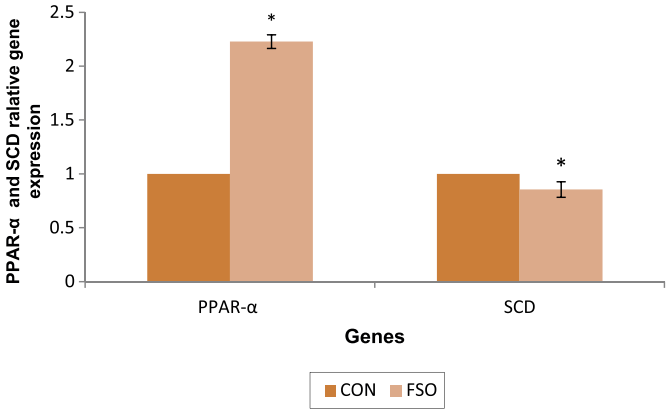

Fig. 1. Comparisons of PPAR-α and SCD gene expression in the goat's liver. Values indicated by the * show significant difference compared with the CON group ($P < 0.05$). CON: diet without FSO, FSO: diet with addition of flaxseed oil. Error bar: 1SE.

4. Conclusion

The results of this experiment showed that high α-linolenic acid supplemented in goats diet leads to alteration in liver fatty acid profile, upregulation of the PPAR-α, and downregulation of the SCD gene compared to feeding a diet supplemented with low α-linolenic acid. These findings may add to the knowledge of the mechanisms by which α-linolenic acid controls specific gene expression in the ruminant liver, and can provide the insight into the development of new nutritional strategies for a better management of the goats during the fattening period.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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