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ORIGINAL ARTICLE

The Alpha Linolenic Acid Content of Flaxseed is Associated with an Induction of Adipose Leptin Expression

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Abstract Dietary flaxseed has cardioprotective effects that may be achieved through its rich content of the omega-3 fatty acid, alpha linolenic acid (ALA). Because ALA can be stored in adipose tissue, it is possible that some of its beneficial actions may be due to effects it has on the adipose tissue. We investigated the effects of dietary flaxseed both with and without an atherogenic cholesterol-enriched diet to determine the effects of dietary flaxseed on the expression of the adipose cytokines leptin and adiponectin. Rabbits were fed one of four diets: a regular (RG) diet, or a regular diet with added 0.5% cholesterol (CH), or 10% ground flaxseed (FX), or both (CF) for 8 weeks. Levels of leptin and adiponectin expression were assessed by RT-PCR in visceral adipose tissue. Consumption of flaxseed significantly increased plasma and adipose levels of ALA. Leptin protein and mRNA expression were lower in CH animals and were elevated in CF animals. Changes in leptin expression were strongly and positively correlated with adipose ALA levels and inversely correlated with levels of *en face* atherosclerosis. Adiponectin expression was not significantly affected by any of the dietary interventions. Our data demonstrate that the type of fat in the diet as well as its caloric content can specifically influence leptin expression. The findings support the hypothesis that the beneficial cardiovascular effects associated with flaxseed consumption may be related to a change in leptin expression.

Keywords Flaxseed · Alpha linolenic acid · Adipokine · Leptin · Adiponectin · Cholesterol · Atherosclerosis · Adipose tissue

Abbreviations

ALA	Alpha linolenic acid
LDL	Low density lipoprotein
eNOS	Endothelial nitric oxide synthase
RG	Regular rabbit diet
CH	Regular diet supplemented with 0.5% cholesterol
FX	Regular diet supplemented with 10% ground flaxseed
CF	Regular diet supplemented with 0.5% cholesterol and 10% ground flaxseed
EDTA	Ethylenediaminetetraacetic acid
ANOVA	Analysis of variance
LSD	Least significant difference
GC	Gas chromatography
SREBP	Sterol response element binding protein
C/EBP	CCAAT-enhancer-binding protein
PPAR	Peroxisome proliferator-activated receptor

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Introduction

Adipocytes, the cells that make up the bulk of adipose tissue in the body, have an important physiological role beyond their lipid storage capacity. They secrete a number

of important cellular signaling molecules, termed adipokines. These adipokines have consequences ranging from local autocrine and paracrine effects to systemic endocrine actions. Adipokines also vary widely in both their function and mechanisms of control. One such mechanism of control is the fatty acid composition of adipose tissue which can affect cellular signaling, fatty acid trafficking, gene expression and, consequently, metabolism [1]. Adipose tissue composition varies based on two main effectors: energy balance, which regulates the metabolism of free fatty acids within the adipose tissue, and diet, which will alter the fatty acid profile of the adipose tissue. Although the former has been examined extensively, particularly with regard to leptin expression and adipocyte differentiation, the effects of the latter on endocrine function have only recently begun to be investigated.

Adiponectin is the most highly expressed and secreted adipokine, with beneficial effects on metabolism, inflammation, and vascular function. Adiponectin has a paradoxical expression pattern. As adiposity increases, adiponectin expression and secretion decreases within the adipose tissue [2]. It is believed that this paradox is part of the pathology of obesity, and is symptomatic of dysfunctional adipose tissue. Adiponectin plays a role in insulin sensitivity, LDL oxidation, eNOS activation, inflammation suppression and fatty acid catabolism [3–5]. Thus, hypoadiponectinemia is of interest as a biomarker of both cardiovascular disease and metabolic syndrome.

Another important adipokine that could be stimulated by changes in fatty acid profile is leptin. It was first discovered as the protein encoded by the *obese* gene, named for the phenotype of the double knockout mouse. These mice experience no satiety, and thus eat continuously when fed ad libitum, leading to severe diet-induced obesity. In humans, leptin deficiency has been implicated in cases of morbid obesity, as either a genetic factor or a metabolic insufficiency [6, 7]. Leptin's role in hypothalamic-mediated appetite suppression in response to caloric intake is not its only function. Leptin may also be important in the modulation of T cell activity in the early stages of atherosclerotic development as well as other immune cells [8]. In obesity, leptin may be under-expressed by the adipose tissue in response to a consistently high caloric diet, or, leptin receptors may be down-regulated, thus leading to high plasma leptin levels and leptin resistance [9].

Flaxseed has recently gained popularity as a functional food. Alpha-linolenic acid (ALA) comprises approximately 55% of the total fatty acid content of flaxseed fatty acids [10]. ALA-rich diets, including diets enriched with ground flaxseed, have been shown in interventional and experimental trials to reduce both fatal and non-fatal myocardial infarction [11, 12], cardiac arrhythmias [12–15], and the incidence of atherosclerotic lesions [12, 14, 16, 17]. However, the mechanism whereby ALA and flaxseed induce this cardio-protective action is unclear. Previous data have indicated that ALA from a flaxseed enriched diet is deposited in adipose tissue [18]. It is possible, therefore, that this change in adipose tissue fatty acid content may influence adipose tissue function. We hypothesize that the change in lipid composition in adipose tissue in response to a flaxseed supplemented diet may affect the adipokine signaling from the adipocytes. It is possible, therefore, that the beneficial cardiovascular actions of flaxseed previously observed may be associated with changes in adipokine expression.

Materials and Methods

Diet and Feeding

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Sixteen male New Zealand White rabbits (2.8 ± 0.1 kg, Southern Rose Rabbitry) were randomly assigned to receive one of four diets. Diets were prepared as previously described [15, 17] by the addition of components to a regular (RG) rabbit diet (CO-OP Complete Rabbit Ration, Federated Co-operatives): 0.5% cholesterol (CH), or 10% ground flaxseed (FX), or both (CF) for 8 weeks ($n = 4$). The chow was stored at 4 °C and protected from light. The diets differed only in total fat content due to the inclusion of the naturally ALA-rich ground flaxseed (Tables 1, 2). The diet fatty acid composition is outlined in Table 2. Addition of flaxseed to the diet significantly increased the amount of C16:0, C18:0, C18:1 (oleic acid) and C18:3 (ALA) provided. The addition of cholesterol had no significant effect on dietary fatty acids provided in comparison to the RG diet. Rabbits were fed 125 g/day of the diet.

Table 1 Crude dietary composition

	RG	FX	CH	CF
Crude protein (%)	21.3	20.5	20.4	20.5
Carbohydrates (%)	51.4	51.7	52.5	50.6
Crude fat (%)	5.4	8.1	5.2	8.9
Crude fibre (%)	13.5	11.7	13.6	12.4
Ash (%)	8.4	8.1	8.1	7.7
ALA (mg/g of diet)	2.0	20.0	2.1	22.5
Digestible energy (kcal/g)	3.38	3.56	3.37	3.60

RG regular diet, *FX* 10% flaxseed supplemented diet, *CH* 0.5% cholesterol supplemented diet, *CF* 10% flaxseed and 0.5% cholesterol supplemented diet

Table 2 Fatty acid composition of rabbit diets

FAME (mg/g)	RG	FX	CH	CF
C14:0	0.293 ± 0.014	0.371 ± 0.013	0.267 ± 0.002	0.337 ± 0.016
C14:1	0.131 ± 0.001	ND	0.033 ± 0.033	ND
C16:0	6.502 ± 0.247	9.567 ± 0.349*	6.338 ± 0.044	9.364 ± 0.232*
C16:1	0.403 ± 0.024	0.540 ± 0.031	0.354 ± 0.001	0.442 ± 0.004
C18:0	2.200 ± 0.147	3.942 ± 0.157*	2.048 ± 0.006	3.643 ± 0.186*
C18:1 Ol	10.693 ± 0.325	17.055 ± 0.862*	10.292 ± 0.010	16.361 ± 0.840*
C18:1 Vac	1.719 ± 0.148	2.775 ± 0.002*	1.644 ± 0.064	2.611 ± 0.067
C18:2 LA	11.191 ± 0.343	11.781 ± 0.513	12.328 ± 0.170	13.713 ± 0.490
C20:0	0.113 ± 0.011	0.165 ± 0.009	0.117 ± 0.001	0.159 ± 0.025
C18:3n-6 GLA	ND	0.115 ± 0.002	ND	0.125 ± 0.004
C20:1	ND	ND	ND	ND
C18:3n-3 ALA	1.993 ± 0.120	20.077 ± 0.841*	2.119 ± 0.036	22.535 ± 0.679*^
C20:2	0.092 ± 0.012	0.125 ± 0.009	0.087 ± 0.002	0.106 ± 0.007
C22:0	0.160 ± 0.012	0.196 ± 0.002	0.166 ± 0.006	0.199 ± 0.036
C22:1	0.112 ± 0.032	0.292 ± 0.009	0.095 ± 0.009	0.094 ± 0.042
C20:3	ND	ND	ND	0.193 ± 0.003
C22:6 DHA	ND	ND	0.105 ± 0.105	0.115 ± 0.115

Values are means ± SE, as mg of lipid/g of diet. Fatty acids were extracted from rabbit chow

RG regular fed, *FX* 10% flaxseed fed, *CH* 0.5% cholesterol fed, *CF* 0.5% cholesterol plus 10% flaxseed fed, *ND* not detectable amounts present

* $p < 0.05$ versus RG

^ $p < 0.05$ versus FX

Blood Sampling and Analysis

Blood was drawn from the left marginal ear vein of rabbits that were fasted overnight before starting their experimental diets and at 8 weeks. It was collected in vacutainer tubes containing EDTA (Becton–Dickinson). Blood samples were centrifuged at 4,500×*g* at room temperature for 10 min, and plasma was then stored at −80 °C. Before analysis, plasma samples were thawed and centrifuged at 6,800×*g*. Plasma levels of cholesterol and triglycerides were analyzed using a VetTest 8008 blood chemistry analyzer (IDEXX Laboratories). Fatty acids were extracted from plasma and derivatized, as described previously [15, 18].

Tissue Collection

After 8 weeks of dietary treatment, animals were euthanized by 5% isoflurane gas delivered by face mask, followed by cardiac extraction. Retroperitoneal and epididymal adipose tissue were collected. To prevent RNase contamination, the animal and tools were sprayed with RNaseZap (Ambion) both before and during tissue collection. Adipose tissue was immediately placed in RNAlater, and kept overnight at 4 °C, as indicated in the manufacturer's instructions (Ambion). Preliminary testing indicated that there was successful stabilization of mRNA compared to flash freezing or maintenance overnight at 4 °C (as assessed by agarose gel electrophoresis and subsequent qRT-PCR) despite the high lipid content of this tissue. RNAlater was removed from the tissue by suction, and the samples were then flash frozen in liquid nitrogen and stored at −80 °C.

qRT-PCR

RNA was extracted from the adipose tissue in an RNase-free environment. Adipose tissue was homogenized in Trizol reagent (Invitrogen), and fat was removed. Phenol was separated from the solution by washing the solution twice with chloroform. The RNA was precipitated from solution with ethanol, and added to RNeasy columns for further purification (Qiagen). Extracted RNA was quantified and assessed for quality by spectrophotometer and agarose gel electrophoresis. It was then used for qRT-PCR (Quanta Biosystems) using a iQ5 Real-Time PCR Detection System (Bio-Rad). Primers designed using BLAST software (NCBI) and were as follows: Adiponectin: (Forward 5′ACCAGGACAAGAACGTGG AC3′, Reverse 5′TGGAGATGGAATCGTTGACA3′);

Leptin: (Forward 5′GTCGTCGGTTTGGACTTCATC3′, Reverse 5′CGGAGGTTCTCCAGGTCGTTG3′) [19];

GAPDH: (Forward 5′GATGGTGAAGGTCGGAG TGAA3′, Reverse 5′GGTGAAGACGCCAGTGGATT3′) [20].

Primers were validated using NCBI's BLAST software [21]. Unused samples were stored at −80 °C. cDNA was synthesized from 1 ug of RNA with qScript cDNA Supermix (Quanta) via the manufacturer's directions. qPCR proceeded for 2 min at 50 °C, 95 °C for 8.5 min, then 40 cycles of 95 °C for 15 s and 60 °C for 60 s, at which point the data were captured. A melt curve was obtained after cycling with 95 °C for 1 min followed by 55 °C for 1 min,

and 80 10-s capture cycles of 55 + 0.5 °C/cycle. Results were normalized by GAPDH expression and analyzed by the delta–delta-Ct method using iCycler Real-Time Detection Software.

Fatty Acid Extraction and Methylation

Plasma fatty acids were directly extracted and derivatized using a modification of the original method described by Lepage and Roy [22] and later modified by Garg et al. [23]. Briefly, 100 μL of plasma was combined with 2 mL of 4:1 (v/v) methanol:toluene in a borosilicate glass tube. The methanol:toluene solution contained 0.5 mg/ml of the internal standard, C19:0 (Nu-Chek Prep. Inc.). While vortexing, 200 μL of acetyl chloride was slowly added. Tubes were capped with a Teflon lined lid, weighed and then heated at 100 °C for 1 h. Once cooled to room temperature, tubes were re-weighed to ensure no sample loss had occurred. Five milliliters of an aqueous 6% K_2CO_3 solution was then added to terminate and neutralize the reaction. The sample tube was then centrifuged at 5,000 rpm for 5 min at room temperature after which the upper toluene layer was removed and subjected to gas chromatographic analysis using flame ionization detection (GC-FID). Methylation was verified by thin layer chromatography. Fatty acids from approximately 15 mg of adipose tissue were extracted and derivatized using the method outlined by Lepage and Roy [24].

Gas Chromatography

Fatty acid methyl esters (FAME) were injected onto a Varian CP 3800 gas chromatographic system using a Varian CP 8400 autosampler. Analytes were detected using flame ionization detection and analyzed on a Varian MS Workstation (vrs. 6.9.1). One microliter of sample was injected at 250 °C at a split ratio of 50:1 onto a Varian CP-Sil 88 capillary column (60 m × 0.25 mm × 0.20 μm). Helium gas (ultra pure) was used as the carrier gas at a constant flow rate of 1.5 mL/min. The oven temperature was maintained at 111 °C for 1 min then rapidly increased by 20 °C/min to 170 °C. It was then slowly increased at a rate of 5 °C/min to 190 °C and finally by 3 °C/min up to 225 °C where it was maintained for 10 min. FAME were quantified against an external standard, GLC 462 (Nu-Chek Prep, Inc.).

Quantification of Aortic Atherosclerosis

The aorta from the ascending arch to the iliac bifurcation was isolated from peripheral tissues and washed in cold PBS, then opened longitudinally and pinned flat. The aortic lumen was digitally photographed and luminal images were analyzed with Silicon Graphics Imaging software. Fatty streaks and complicated lesions were expressed as a percentile of total luminal surface area.

Statistics

Results were reported as means ± SE, and analyzed with Sigma-Stat software by one-way ANOVA, using Fisher's LSD test. A significant correlation was identified by a *t* test. $p \leq 0.05$ was considered statistically significant.

Results

Body Weight

After 8 weeks of dietary treatment, mean body weight significantly increased from 2.8 ± 0.06 to 3.7 ± 0.09 kg. However, there was no effect on weight with the experimental diets as compared to control diets (data not shown).

Plasma Lipids

There was no significant change in the plasma cholesterol of animals fed a regular or flaxseed supplemented diet after 8 weeks. Supplementation of the diet with dietary cholesterol for 8 weeks induced severe hypercholesterolemia (Fig. 1a). Addition of dietary flaxseed to a cholesterol-enriched diet did not lower the plasma cholesterol values from those observed in animals fed a diet supplemented with cholesterol alone.

Plasma triglycerides were not significantly affected by any of the diets (Fig. 1b). Addition of milled flaxseed to the diet, providing ALA, induced a 17-fold increase in the percentage composition of ALA in the plasma (Fig. 1c). Simultaneous consumption of flaxseed and cholesterol doubled the amount of ALA in the plasma as compared to the consumption of flaxseed alone, comprising 21% of all plasma fatty acids. an eightfold increase in plasma ALA, despite being provided with only 2 mg of ALA/gram, supporting the observation that cholesterol aids in absorption of ALA [15–18].

Atherosclerosis

Animals fed a regular or flaxseed-supplemented diet for 8 weeks did not develop any quantifiable atherosclerosis in the aortic arch (Fig. 2). Inclusion of 0.5% cholesterol in the diet induced atherosclerotic lesions in the aorta, covering 76.3 ± 8.5% of the aortic lumen ($p < 0.05$ vs. RG, $n = 3$). Addition of ground flaxseed to the cholesterol-supplemented diet ameliorated the atherogenic effects of cholesterol, significantly reducing lesions to 28 ± 4.3% of the aortic lumen ($p < 0.05$ vs. CH, $n = 3$).

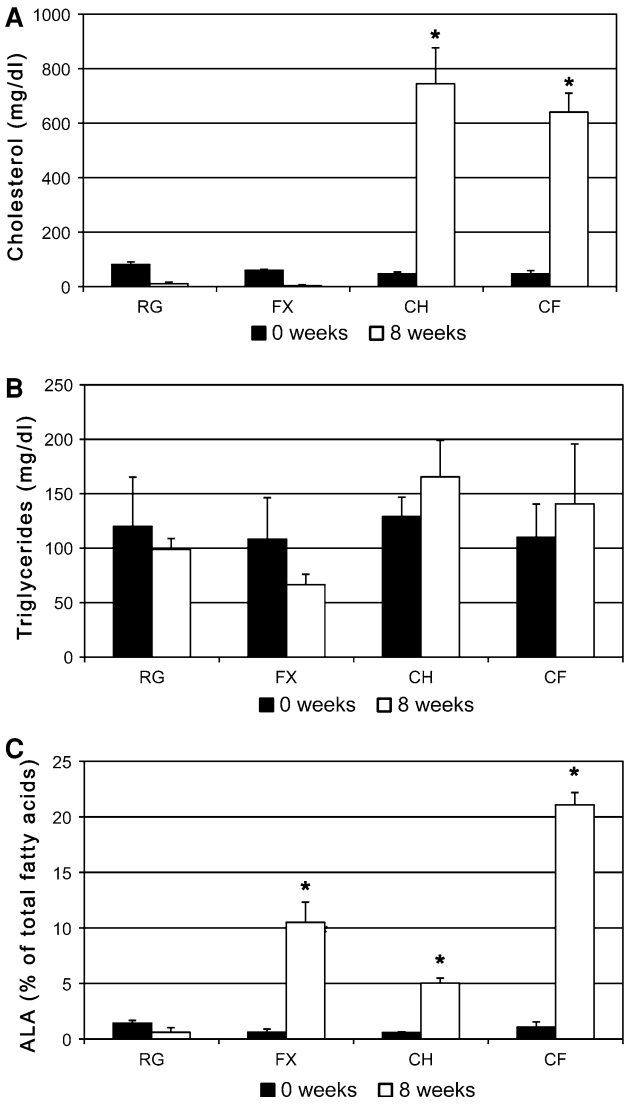

Fig. 1 Plasma cholesterol (**a**), triglycerides (**b**), and alpha-linolenic acid (**c**) levels in male New Zealand White rabbits at baseline and after 8 weeks of dietary treatment. *$p < 0.05$ versus RG; $n = 3$–4. *RG* regular chow, *FX* chow supplemented with 10% flaxseed, *CH* 0.5% cholesterol-supplemented diet, *CF* 10% flaxseed and 0.5% cholesterol-supplemented diet

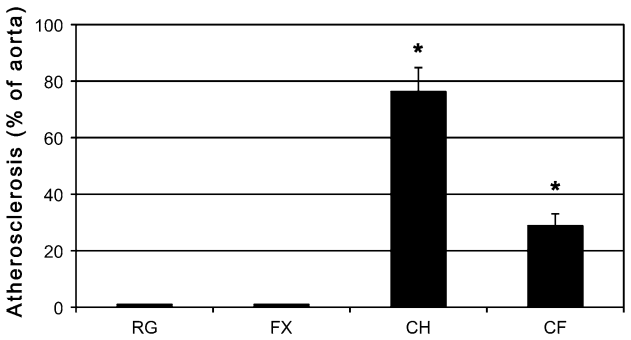

Fig. 2 Development of atherosclerotic lesions on the aorta of New Zealand White rabbits after 8 weeks of dietary treatment. Values are means ± SE; $n = 3$. *$p \leq 0.05$ versus RG; $p \leq 0.05$ versus CH. *RG* regular chow, *FX* chow supplemented with 10% flaxseed, *CH* 0.5% cholesterol-supplemented diet, *CF* 10% flaxseed and 0.5% cholesterol-supplemented diet

Fatty Acid Composition of Adipose Tissue

Total lipids were extracted from two primary sources of visceral fat. Epididymal and retroperitoneal fat consisted of 92.24% lipid by wet weight (range 83.3–99.5%), with no significant changes in total lipid between different dietary treatments, or either adipose source (Tables 3, 4).

The main component of adipose tissue was C18:1, oleic acid, which composed 32.3 ± 1.9% of total fatty acids in the retroperitoneal adipose of RG-fed animals (Table 3). C16:0, palmitic acid, and C18:2, linoleic acid, were also common, comprising 25.2 ± 1.4 and 26.7 ± 1.3% of the total retroperitoneal adipose tissue fatty acid content, respectively. Also stored in appreciable quantities in the retroperitoneal adipose tissue of animals fed a regular diet were ALA (6.34 ± 0.34%), steric acid (6.14 ± 0.57%), palmitoleic acid (2.12 ± 0.08%), vaccenic acid (1.98 ± 0.21%), and myristic acid (1.77 ± 0.12%) (Table 3).

The fatty acid proportions in the epididymal tissue of the animals fed a regular diet were not significantly different than the proportions of fatty acid in retroperitoneal tissue (Table 4). Dietary cholesterol did not significantly affect either total lipids or individual fatty acids as compared to RG. Addition of flaxseed to a regular diet significantly reduced levels of C16:0, C18:1-*cis*, C18:1-*trans*, and C18:2 in the adipose tissue, both in absolute concentrations and relative to total fatty acid content (Tables 3, 4). When consumed in conjunction with 0.5% cholesterol, dietary flaxseed induced a 3.1-fold increase in adipose tissue ALA, a significant increase as compared to RG, but significantly less than animals supplemented only with flaxseed. Dietary cholesterol did not affect any of the other fatty acids observed.

Adipokine Expression

Adiponectin and leptin are the two most highly expressed adipokines in adipose tissue. In epididymal adipose tissue, there was no significant change in either leptin or adiponectin mRNA expression after addition of either flaxseed or cholesterol to the diet (Fig. 3a). Adiponectin expression did not vary with dietary treatment in retroperitoneal adipose tissue (Fig. 3b). However, in retroperitoneal adipose tissue, dietary flaxseed induced a two-fold increase in leptin mRNA ($p < 0.05$ vs. RG). Conversely, dietary cholesterol reduced leptin mRNA expression by about one-half in comparison to control, and the addition of flaxseed to the cholesterol-supplemented diet induced a recovery of leptin expression, increasing expression beyond that of flaxseed alone.

Table 3 Fatty acids in retroperitoneal adipose tissue from rabbits fed diets for 8 weeks

FAME (μmol/g)	Retroperitoneal adipose tissue			
	RG	FX	CH	CF
10:0	ND	ND	ND	ND
12:0	ND	ND	1.54 ± 0.94	1.45 ± 0.87
12:1	ND	ND	ND	ND
14:0	74.40 ± 4.00	50.91 ± 6.76*	66.17 ± 2.12	60.90 ± 1.84
14:1	ND	ND	ND	ND
16:0	945.68 ± 37.82	699.88 ± 15.45*	905.45 ± 40.17	773.21 ± 25.07*
16:1	81.17 ± 7.50	46.95 ± 6.42	90.90 ± 10.74	83.39 ± 13.85
18:0	195.76 ± 12.98	169.31 ± 8.66	179.48 ± 6.93	169.35 ± 4.22
18:1 n-9	1,104.79 ± 75.25	821.93 ± 49.73*	981.88 ± 20.87	962.26 ± 21.53
18:1 *trans*	67.80 ± 7.42	42.39 ± 3.38*	55.96 ± 1.62	49.30 ± 1.26*
18:2 n-6	878.82 ± 26.06	705.14 ± 35.87*	938.47 ± 69.49	778.75 ± 19.54
20:0	0.51 ± 0.38	0.45 ± 0.24	0.88 ± 0.45	ND
18:3 n-6	ND	ND	ND	ND
18:3 n-3	218.96 ± 7.95	895.96 ± 63.33*	184.22 ± 6.98	672.52 ± 78.99*^
20:1	14.49 ± 1.69	11.83 ± 1.16	12.46 ± 1.29	11.14 ± 1.40
20:2	1.68 ± 0.57	0.27 ± 0.18	1.31 ± 0.51	0.60 ± 0.23
22:0	ND	ND	ND	ND
20:3 n-6	ND	ND	ND	ND
20:3 n-3	ND	5.76 ± 0.89	ND	3.32 ± 0.80
22:1	0.68 ± 0.28	0.22 ± 0.15	0.43 ± 0.26	0.83 ± 0.62
20:4 n-6	0.76 ± 0.44	0.38 ± 0.89	1.28 ± 0.75	0.83 ± 0.48
C22:2	ND	ND	ND	ND
C20:5 n-3	ND	ND	ND	ND
C24:0	ND	ND	ND	ND
C24:1	ND	ND	ND	ND
C22:4	ND	ND	ND	ND
C22:5	ND	0.58 ± 0.45	ND	0.48 ± 0.2
C22:6	ND	ND	ND	ND
TL (%)	90.5 ± 0.23	91.4 ± 3.70	95.7 ± 2.02	94.4 ± 1.35

Values are means ± SE. Fatty acids were extracted from retroperitoneal adipose tissue following 8 weeks of feeding ($n = 4$)

RG regular fed, *FX* 10% flaxseed fed, *CH* 0.5% cholesterol fed, *CF* 0.5% cholesterol plus 10% flaxseed fed, *ND* not detectable amounts present, *TL* total mg lipids extracted per mg of tissue

* $p < 0.05$ versus RG

^ $p < 0.05$ versus FX

These changes in leptin expression were positively correlated with plasma ALA and adipose ALA levels (Fig. 4). Plasma ALA correlated with leptin expression in the retroperitoneal adipose tissue ($p < 0.05$), but not in epididymal adipose (Fig. 4a). Adipose tissue ALA exhibited a stronger relationship than plasma ALA with leptin expression in retroperitoneal adipose tissue (Fig. 4b).

Relationship of Leptin Expression to Atherosclerosis

All animals that were not fed cholesterol did not exhibit any atherosclerosis. Consequently, in order to determine if there was a significant relationship between leptin expression and the development of atherosclerotic lesions, linear regression was performed only on data obtained from animals fed a cholesterol-supplemented diet. Increased leptin expression in the retroperitoneal adipose tissue significantly correlated ($p < 0.05$) with decreased atherosclerosis (Fig. 5).

Discussion

One of the primary purposes of this study was to determine if diets of very different lipid composition could influence the expression of leptin or adiponectin in adipose tissue when caloric balance was maintained. It is known that leptin expression varies with caloric balance and adiposity [25–28]. The present study was designed to avoid these potential variables by maintaining the diets in a manner which maintained a consistent level of caloric intake while at the same time insuring diversity in lipid composition. The differences in caloric values between the diets were so minor that they did not induce a significant change in body weight. The data obtained in the present study demonstrates that the lipid composition of the diet can have an important role to play in adipokine expression. The changes in adipokine expression were sensitive to the type of lipid in the diet, the adipose tissue examined and the

Table 4 Fatty acids in epididymal adipose tissue from rabbits fed diets for 8 weeks

FAME (μmol/g)	Epididymal adipose tissue			
	RG	FX	CH	CF
10:0	ND	ND	ND	ND
12:0	ND	1.13 ± 0.85	2.48 ± 1.70	ND
12:1	ND	ND	ND	ND
14:0	79.12 ± 3.28	59.37 ± 4.65	68.03 ± 4.33	57.85 ± 7.95
14:1	0.99 ± 0.52	ND	ND	ND
16:0	919.51 ± 32.27	676.35 ± 20.48*	862.90 ± 46.25	743.00 ± 44.11*
16:1	101.26 ± 11.89	64.76 ± 10.31	89.76 ± 14.79	92.60 ± 21.24
18:0	193.31 ± 7.60	162.54 ± 6.79	169.93 ± 11.23	158.20 ± 7.84
18:1 n-9	1,122.28 ± 38.65	889.86 ± 29.71*	925.11 ± 25.28	917.13 ± 73.87*
18:1 *trans*	69.78 ± 6.12	48.23 ± 1.50*	54.66 ± 3.68	49.07 ± 5.21*
18:2 n-6	893.65 ± 36.57	694.95 ± 22.56*	887.65 ± 17.06	789.54 ± 40.46
20:0	0.88 ± 0.45	ND	1.16 ± 0.61	1.27 ± 0.30
18:3 n-6	ND	ND	ND	ND
18:3 n-3	219.61 ± 7.33	916.35 ± 27.84*	176.76 ± 6.12	569.80 ± 96.29*^
20:1	15.06 ± 1.53	12.81 ± 0.92	11.97 ± 0.45	12.26 ± 0.26
20:2	1.13 ± 0.40	0.48 ± 0.28	0.98 ± 0.57	ND
22:0	ND	ND	ND	ND
20:3 n-6	ND	ND	ND	ND
20:3 n-3	ND	6.58 ± 2.16	ND	0.96 ± 0.81
22:1	1.67 ± 0.64	ND	1.83 ± 0.54	1.78 ± 0.34
20:4 n-6	ND	ND	ND	ND
C22:2	ND	ND	ND	ND
C20:5 n-3	ND	ND	ND	ND
C24:0	ND	ND	ND	ND
C24:1	ND	ND	ND	ND
C22:4	ND	ND	ND	ND
C22:5	ND	ND	ND	ND
C22:6	ND	ND	ND	ND
TL (%)	93.2 ± 2.87	90.5 ± 4.11	88.1 ± 3.36	94.1 ± 2.18

Values are means ± SE. Fatty acids were extracted from epididymal adipose tissue following 8 weeks of feeding ($n = 4$)

RG regular fed, *FX* 10% flaxseed fed, *CH* 0.5% cholesterol fed, *CF* 0.5% cholesterol plus 10% flaxseed fed, *ND* not detectable amounts present, *TL* total mg lipids extracted per mg of tissue

* $p < 0.05$ versus RG

^ $p < 0.05$ versus FX

adipokine studied as well. A high cholesterol diet suppressed leptin expression whereas a diet rich in the omega-3 fatty acid ALA increased leptin expression. Leptin expression was influenced by the diets whereas adiponectin was not. Retroperitoneal but not epididymal adipose tissue was affected by the diets. It is known that the location of the different adipose tissues will influence circulating adipokine levels [29].

The mechanism whereby leptin mRNA expression is regulated by dietary lipids is presently unclear. Although SREBP1c mRNA expression is directly related to polyunsaturated fatty acid content in adipose tissue [30] and an SREBP-like binding element is present in the leptin promoter, it is not responsive to SREBP itself [31]. However, polyunsaturated fatty acids may act as ligands for PPAR gamma to alter adipokine expression [32]. This may occur through an increase in translation [33]. Mason et al. [28] have demonstrated a novel binding site for an adipocyte-specific transcription factor in the -87 position of the leptin promoter which is conserved in both mice and humans, however, the consensus sequence does not match any known transcription factor. This region, termed LP1, presents an interesting possibility for a novel transcription factor which may regulate the response of leptin to dietary lipids in addition to PPAR-gamma.

The observation that the adipose tissue is responding to these diets in a very specific manner suggests that the changes are physiologically important. Increased leptin levels have been identified previously as a risk factor for atherosclerosis [34]. However, data correlating leptin and atherosclerosis have been derived from obese humans and animal models of obesity [35–39]. Our data indicates that in a non-obese population, leptin may have a previously unidentified role in cardioprotection. In support of this hypothesis, leptin expression was significantly correlated in a negative fashion with atherosclerosis. When leptin levels

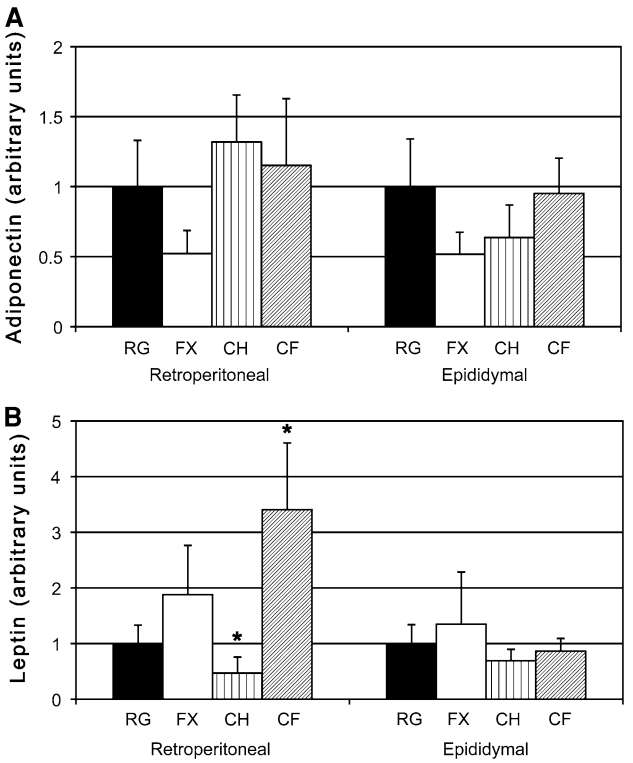

Fig. 3 mRNA expression of adiponectin (**a**) or leptin (**b**) in epididymal and retroperitoneal adipose tissue of New Zealand White rabbits after 8 weeks of dietary treatment, measured by quantitative real-time PCR. Values were normalized by GAPDH expression and expressed as means ± SE; $n = 3$–4. $^*p < 0.05$. *RG* regular chow, *FX* chow supplemented with 10% flaxseed, *CH* 0.5% cholesterol-supplemented diet, *CF* 10% flaxseed and 0.5% cholesterol-supplemented diet

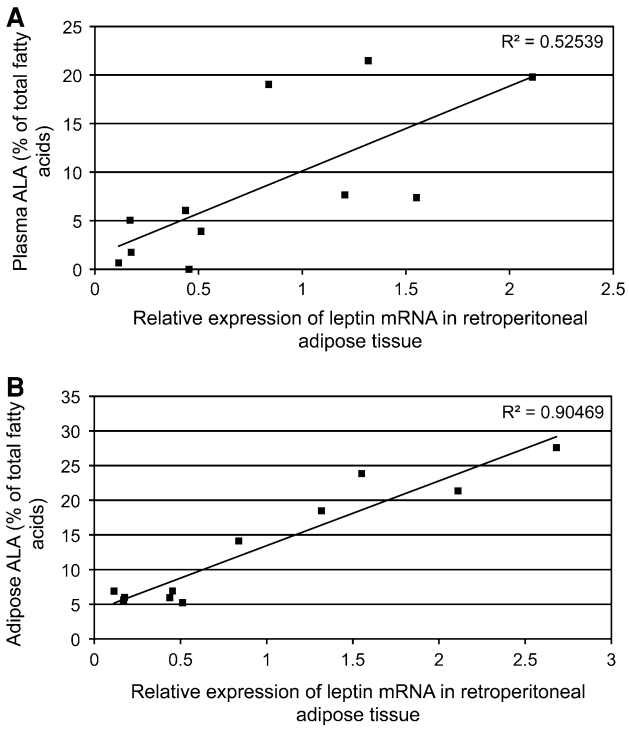

Fig. 4 Correlational analysis of expression of leptin mRNA from retroperitoneal adipose tissue with plasma alpha-linolenic acid (**a**), and adipose alpha-linolenic acid (**b**) of New Zealand White rabbits after 8 weeks of dietary treatment. **a** $^*p < 0.05$; **b** $p < 0.05$

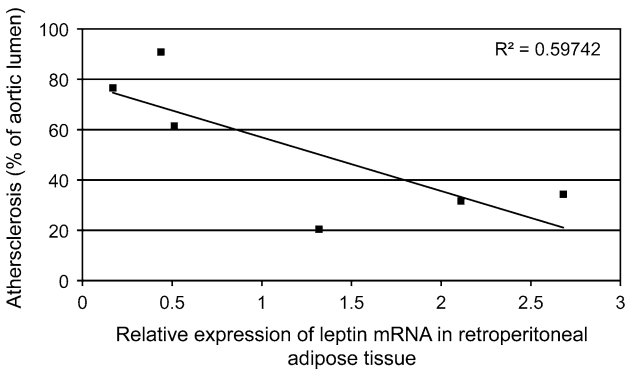

Fig. 5 Correlational analysis of the percentage of the aortic lumen covered with atherosclerotic plaque with leptin mRNA from retroperitoneal adipose tissue of hypercholesterolemic New Zealand White rabbits after 8 weeks of atherogenic dietary treatment. $^*p < 0.05$

were high, atherosclerosis was low and when leptin expression was depressed by circulating cholesterol, atherogenesis was stimulated.

Consumption of flaxseed has previously been shown to improve insulin resistance, hyperlipidemia, atherosclerosis and hypertension and decrease the incidence of cardiac arrhythmias [15–17, 40]. These effects of dietary flaxseed have been attributed, in part, to the rich ALA content of flaxseed [15–17, 40]. Dietary flaxseed provided a source of ALA in the present study which subsequently increased ALA both in the circulation and in the adipose tissue. With the addition of cholesterol to the flaxseed-supplemented diet, plasma ALA increased substantially but adipose tissue levels of ALA did not exhibit further increase beyond those observed when the diet was supplemented with flaxseed alone (Table 3). This could be due to one of several factors. ALA may be deposited in other organs preferentially, such as the heart and liver, as previously observed in the hypercholesterolemic rabbit [18]. Alternatively, a cholesterol-rich diet may direct the ALA to be used directly for beta-oxidation, thus reducing the amount of ALA available for storage [41]. Cholesterol may also induce an efflux of ALA from the adipose tissue, which is greater than the influx of ALA from the plasma, leading to a net decrease in ALA [42]. Further study is required to understand the relative lack of storage of ALA in the adipose tissue when it is presented with such high levels of circulating ALA.

Both beneficial and deleterious cytokines from the adipose tissue may be responsible for many of the links between diet, BMI and cardiovascular disease. The present data demonstrate that dietary cholesterol and flaxseed have

the capacity to alter leptin expression. The cardioprotective effects of flaxseed are thought to be provided in part by its delivery of ALA to the body [16, 40]. However, the mechanism to explain the induction of these effects by ALA remains elusive. In the present study, ALA in the adipose tissue was strongly associated with increased leptin expression and the subsequent reduction of atherosclerosis. Our data, therefore, suggests that flaxseed may induce its anti-atherogenic effects in part via an ALA-mediated modulation of the expression of leptin.

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