

Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats

Masao Sato, Kenichi Shibata, Run Nomura, Daisuke Kawamoto, Rika Nagamine and Katsumi Imaizumi*

Laboratory of Nutrition Chemistry, Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University, Fukuoka 812-8581, Japan

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The relative benefit of replacing saturated fatty acid with linoleic acids is still being debated because a linoleic acid-enriched diet increases oxidative and inflammatory stresses, although it is associated with a reduction in serum cholesterol levels. The present study was conducted to evaluate the effect of dietary supplementation of linoleic acid-rich (HL) fat, compared with a saturated fatty acid-rich (SF) fat on atherosclerotic lesion areas, serum and liver cholesterol levels, oxidative stress (urinary isoprostanes and serum malondialdehyde) and inflammatory stress (expression of aortic monocyte chemoattractant protein-1; MCP-1) in apo E-deficient mice. Male and female apo E-deficient mice (8 weeks old; seven to eight per group) were fed an AIN-76-based diet containing SF fat (50 g palm oil and 50 g lard/kg) or HL fat (100 g high-linoleic safflower-seed oil/kg) for 9 weeks. Compared with the SF diet, the HL diet lowered atherosclerosis ($P < 0.05$). It reduced serum total cholesterol levels ($P < 0.05$), increased HDL-cholesterol levels ($P < 0.05$) and lowered liver esterified cholesterol levels ($P < 0.01$). The HL diet-fed mice showed increased expression of MCP-1 mRNA ($P < 0.05$), serum levels of malondialdehyde ($P < 0.05$) and urinary excretion of 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F_{2α}; ($P < 0.05$). These results suggest that having biomarkers *in vivo* for oxidative stress and inflammatory status of endothelial cells does not necessarily indicate predisposition to an increased lesion area in the aortic root in apo E-deficient mice fed an HL or SF diet.

Apolipoprotein E-deficient mice: Linoleic acid: Monocyte chemoattractant protein-1: Saturated fatty acids

Endothelial dysfunction is a key variable of atherosclerosis where elevated serum cholesterol levels are associated with endothelial dysfunction (Vogel, 1999). Endothelial dysfunction is also associated with increased oxidative stress, an important promoter of inflammatory processes (Napoli *et al.* 2001). In man, saturated fatty acids increase the levels of LDL- and VLDL-cholesterol, and current recommendations include decreasing their intake as part of a heart-healthy diet (Krause *et al.* 2000). Non-human primate studies have shown that consuming a saturated fatty acid-rich (SF) diet compared with a linoleic acid-rich (HL) diet results in increased serum levels of LDL-cholesterol (Rudel *et al.* 1995). In contrast, observations from *in vitro* studies show that increased intake of linoleic acid leads to increased oxidative stress, which may be associated with endothelial dysfunction (Mertens & Holvoet, 2001; Moreno & Mitjavila, 2003). However, compelling evidence that this occurs *in vivo* is still lacking, although Turpeinen *et al.* (1998) showed that urinary excretion of 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}), which increases in situations associated with oxidative stress (Morrow *et al.* 1995), is increased after consumption of an HL diet by healthy subjects. Thus, the status of endothelial function after consumption of these fatty acids can be regarded as an integrated index of all atherogenic and atheroprotective factors in a given individual, because

cardiovascular risk factors such as hyperlipidaemia, hypertension, diabetes and smoking, local factors including shear stress, genetic factors, and unknown factors determine the status of endothelial function (for a review, see Bonetti *et al.* 2003).

The advantage of using animal models to examine the effect of dietary fats is that the level of serum cholesterol, the *in vivo* status of oxidative or inflammatory stresses and atherosclerosis can simultaneously be determined at the end of the study. Thus, we conducted the present study to investigate the effects of dietary enrichment of linoleic acid on serum and liver lipid levels, urinary excretion of isoprostanes as a biomarker of oxidative stress, expression of aortic monocyte chemoattractant protein-1 (MCP-1) as a biomarker of inflammatory status of endothelial cells (Bursill *et al.* 2004) and the extent of atherosclerosis in apo E-deficient mice.

Materials and methods

Animals and diets

ApoE-deficient mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) in 1994 were used (Ni *et al.* 1998). Male and female apo E-deficient mice (8 weeks old) with an initial weight of 26.4 (SE 0.6) g for male mice and 22.0 (SE 0.6) g for female mice were divided into two groups, and were fed an AIN-76 diet

Abbreviations: 15-F_{2t}-IsoP-M, 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F_{2α}; HL, linoleic acid-rich; 8-iso-PGF_{2α}, 8-iso-prostaglandin F_{2α}; MCP-1, monocyte chemoattractant protein-1; SF, saturated fatty acid-rich; TBARS, thiobarbituric acid reactive substances.

*Corresponding author: Dr Katsumi Imaizumi, fax +81 92 642 3003, email imaizumi@agr.kyushu-u.ac.jp

(Anonymous, 1977) containing saturated and monounsaturated fatty acid-rich fat (SF diet; 50 g palm oil and 50 g lard/kg) or HL fat (HL diet; 100 g high-linoleic safflower-seed oil/kg). The fatty acid composition of the dietary fats is shown in Table 1. The cholesterol diets were supplemented at the level of 0.4 g/kg diet. Safflower-seed oil contained approximately a 10-fold higher amount of α -tocopherol compared with the mixed fat composed of palm oil and lard (236 and 24.9 mg/kg oil for safflower-seed oil and the mixed fat, respectively). Simultaneously, the vitamin mixture of the AIN-76 diet supplies large amounts of α -tocopherol, so that the α -tocopherol level in the HL diet (69.1 mg/kg diet) was approximately 1.5-fold higher than that in the SF diet (48.0 mg/kg diet). The animals were individually housed in a temperature-controlled room at 22–25°C with a 12 h light–dark cycle (lights on, 08.00–20.00 hours). Experimental diets were packaged in a pouch containing an O₂ absorbent (Ageless S-200; Mitsubishi Gas Chemical Co., Tokyo, Japan), flushed with N₂ and stored at 4°C. The diet was freshly prepared every week and changed every 2 d and any remaining diet was discarded. At the end of the 9-week feeding period, the mice were deprived of food for 4 h before killing. During the week before killing, they were placed in a metabolism cage (Shinano Seisakusho, Tokyo, Japan) where they were freely given the diet and water, and their urine was collected in a container containing butylated hydroxytoluene (final concentration of 453 nmol/l) for 24 h (Tomoyori *et al.* 2004). The urine was frozen with liquid N₂ after blowing Ar through it and then kept at –85°C. At the end of the experiment, the mice were anaesthetised by intraperitoneal injection of 50 mg/kg body weight sodium pentobarbital and killed by withdrawing blood from the left ventricle. The blood was transferred into 1 ml microcentrifuge tubes containing 50 μ g butylated hydroxytoluene; the serum was then separated, bubbled with Ar and finally stored at –85°C after being frozen with liquid N₂. Livers and aortas were immediately removed from the carcasses, frozen in liquid N₂ and stored at –85°C. Before freezing, adipose tissue around the aorta was removed, rinsed in fresh PBS and blotted dry between filter paper.

The experiments were carried out following the Guidelines for Animal Experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and law no. 105 and notification no. 6 of the Government of Japan.

Determination of 8-iso-prostaglandin F₂ α

Purification and measurement of urinary 8-iso-PGF₂ α or 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F₂ α (15-F₂t-IsoP-M) was carried out by combining four methods (Morrow & Roberts, 1994; Wübert *et al.* 1997; Schwedhelm *et al.* 2000; Zhao *et al.* 2001). 8-Iso-PGF₂ α and 15-F₂t-IsoP-M were obtained

from Cayman Chemicals (Ann Arbor, MI, USA). The urine isoprostanes were analysed by GC negative ion chemical ionisation MS; GC negative ion chemical ionisation MS was performed using a Shimadzu QP5050A GC/MS (Shimadzu, Kyoto, Japan). GC was carried out using a 30 m, 0.25 mm diameter, 0.25 μ m film thick SPB-20 fused silica capillary column (Supelco, Bellefonte, PA, USA). The column temperature was initially maintained at 100°C for 2 min, was next heated to 250°C in 7 min and then to 290°C at 2°C/min and maintained at this temperature. Isobutane was used as a reactant gas for negative chemical ionisation and the carrier gas used was He at 1.1 ml/min. The ion source temperature was 290°C and the electron energy was 70 eV. The ion monitor for endogenous 8-iso-PGF₂ α or 15-F₂t-IsoP-M was set at *m/z* 569 (M-181) and *m/z* 547, respectively. 8-Iso-PGF₂ α -d₄ (Cayman Chemicals) or 15-F₂t-IsoP-M [¹⁸O₂] (kindly donated by Dr J. D. Morrow, Department of Medicine and Pharmacology, Vanderbilt University, Nashville, TN 37232, USA) was used as an internal standard and ions at *m/z* 573 or *m/z* 547 were monitored. The quantification of endogenous 8-iso-PGF₂ α or 15-F₂t-IsoP-M was accomplished by SIM analysis of the ratio of *m/z* 569 to *m/z* 573 or *m/z* 543 to *m/z* 547, where the lower limit of detection (signal:noise ratio of 4:1) of 8-iso-PGF₂ α and 15-F₂t-IsoP-M were 28 and 109 pg, respectively. A standard curve was constructed by adding varying amounts of unlabelled 8-iso-PGF₂ α to 1 ng 8-iso-PGF₂ α -d₄ or 15-F₂t-IsoP-M to 1 ng of 15-F₂t-IsoP-M [¹⁸O₂]. The concentration of urine isoprostanes was expressed as a function of urinary creatinine (Wako Pure Chemicals, Osaka, Japan).

Determination of aortic monocyte chemoattractant protein-1 mRNA level by reverse transcription polymerase chain reaction Southern hybridisation analysis

Total RNA from the mouse aorta was extracted following the method of Chomczynski & Sacchi (1987). The reverse transcription-PCR was carried out according to the method of Sokolov & Prockop (1994). Total RNA (5 μ g) was transcribed into first-strand cDNA using a You-primed cDNA single step kit (Pharmacia Japan, Tokyo, Japan). The primers of mouse MCP-1 were designed according to the data of Kawahara *et al.* (1991). The sequences were 5'- CACCAGCAAGATGATCCCAATG for the 5'-primer and 5'-AAGGCATCACAGTCCGAGTCACAC for the 3'-primer. Also, the measurement of aortic β -actin mRNA levels was carried out as a housekeeping gene to confirm stable extraction from these aortic samples. The primer sequences of mouse β -actin (Shimano *et al.* 1996) were 5'-GGATCCCAGATCATGTTTGAGACCTTCAA for the 5'-primer and 5'-GAATTCGGAGAGCATAGCCCTCGTAGATGG for the 3'-primer.

PCR amplification of MCP-1 as well as β -actin was carried out in 10 μ l reaction mixtures composed of 1 μ l cDNA solution, 2.5 units of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), 1 μ l 10 \times PCR buffer containing 2.5 mM-MgCl₂ (attached to the enzyme), 1.6 mM of each dNTP and 5 pmol each of the two oligonucleotide primers. The amplification conditions were as follows: 95°C, 2 min; 95°C, 1 min, 54°C, 30 s, 72°C, 1 min, for forty-two cycles; and finally 72°C, 5 min. The amplification products obtained using the two primer pairs had 357 bp of MCP-1 or 254 bp of β -actin. We cloned the PCR products into pGEM-T Easy (Promega, Madison, WI, USA) using a sequence vector to check the sequence with

Table 1. Fatty acid composition of saturated fatty acid-rich fat (SF) and linoleic acid rich-fat (HL) (g/100 g total fatty acids)

(Mean values with triplicate determination for each group)

Fatty acid	SF	HL
14:0	1.4	–
16:0	34.4	7.0
16:1	2.2	–
18:0	9.2	2.5
18:1	43.4	15.2
18:2	9.8	75.4

a DNA sequencer; Gene Rapid (Amersham Pharmacia Biosciences, Tokyo, Japan).

The aorta total RNA samples from these mice were subjected to RT-PCR as described earlier, but the cycle number was changed from forty-two to twenty cycles for MCP-1 and to sixteen cycles for β -actin. Southern-blotting hybridisation of the PCR products was carried out as described elsewhere (Wahl *et al.* 1979). The products on the nitrocellulose filter, Hybond NX (Amersham Pharmacia Biosciences, Tokyo, Japan) were hybridised to 32 P-labelled probe of the cloned mouse MCP-1 or β -actin. The exposition intensities of these bands were then quantified by a Bio-imaging analyser FLA-5000 (Fuji Photo Film, Tokyo, Japan). We have confirmed that the concentrations (0.5, 1, 2 and 3 μ l per 10 μ l reaction solution) of the cDNA from the aorta total RNA were linearly correlated with the radiation activities for MCP-1 (r 0.991; P <0.01).

Analyses of serum and liver lipids

Serum lipid levels were determined using commercially available kits (Cholesterol C test, Triglyceride G Test and Phospholipid B Test from Wako Pure Chemicals, Osaka, Japan and HDL-C:2 from Daiichi Chemicals, Tokyo, Japan) and liver lipids were chemically determined as previously described (Tomoyori *et al.* 2004). The fatty acid composition of the serum and liver phosphatidylcholine was determined according to methods described previously (Carvajal *et al.* 2000).

Serum lipid peroxidation

Lipid peroxidation was quantified by measuring the serum concentration of thiobarbituric acid reactive substances (TBARS) determined to be malonyldialdehyde (MDA), a product of lipid peroxidation as previously described by Yagi (1976).

Serum nitrite plus nitrate

Serum nitrite plus nitrate (NO_2 plus NO_3) was determined as the final metabolites of NO as described previously (Ni *et al.* 2003).

Morphometric determination of atherosclerosis

Apo E-deficient mice were perfused with 50 ml PBS (pH 7.4) via a cannula inserted into the left ventricle, which allowed unrestricted efflux from an incision in the vena cava. After the aorta and its main branches were dissected from the aortic valve to the iliac bifurcation, perfusion of the heart was immediately continued with 50 ml of 10% (v/v) neutral formalin buffer solution (pH 7.4). The heart was removed and fixed in 10% (v/v) neutral formalin-buffered solution (Ni *et al.* 1998). To determine the cross-sectional lesion area, hearts containing aortic roots were processed for quantitative atherosclerosis assay as previously described (Tomoyori *et al.* 2004).

Statistics

The data were expressed as the means with their standard errors and were analysed by two-way ANOVA followed by a *post hoc* test (Bonferroni–Dunn method). Statistical analysis was carried out with Statcel (OMS, Saitama, Japan) and Excel 2000

(Microsoft, Redmond, WA, USA) and differences were considered to be statistically significant for P <0.05.

Results

There was a difference in the initial mean body weight between females (21.9 (SE 0.6) and 22.1 (SE 0.5) g for the SF and HL group, respectively) and males (26.4 (SE 0.7) and 26.4 (SE 0.5) g for the SF and HL group, respectively) (P <0.01). The final mean body weight for the females (32.0 (SE 1.4) and 33.1 (SE 1.5) g for SF and HL group, respectively) was smaller than that for males (41.9 (SE 1.0) and 43.1 (SE 0.8) g for SF and HL group, respectively) (P <0.01), but there was no significant dietary fat effect observed. The sex and type of dietary fat had no significant effect on the mean daily food intake: 4.2 (SE 0.1) and 4.2 (SE 0.1) g for SF- and LH-fed females, respectively; and 4.8 (SE 0.1) and 4.5 (SE 0.1) g for SF- and HL-fed males, respectively. There was also no significant difference due to sex and type of dietary fat on the relative liver weight (data not shown).

Table 2 shows the degree of atherosclerotic lesions as well as concentrations of the serum and liver lipids, serum NO_2 plus NO_3 , serum TBARS and urinary isoprostanes in the apo E-deficient female and male mice fed a diet containing different fats. Two-way ANOVA revealed main effect of fats on the lesion size in the aortic root (P <0.01) and the serum HDL-cholesterol concentrations (P <0.01): the HL diet resulted in a more decreased lesion size and increased HDL-cholesterol concentrations than did the SF diet. There was no main effect of sex on these characteristics. A main effect of the fats was observed on the levels of serum total cholesterol (P <0.01), liver total cholesterol (P <0.01) and esterified cholesterol (P <0.01) and the serum TBARS (P <0.01). The mice fed the HL diet had a decreased concentration of serum total cholesterol and liver total or esterified cholesterol and increased concentration of serum TBARS compared with the SF-diet mice and these values were higher for males than for females. The atherosclerotic lesion area was significantly negatively correlated to the serum HDL-cholesterol level (r -0.59; P <0.05), but was not significantly related to the serum total cholesterol level (r 0.13; P >0.1). There was no main effect of fats on the concentrations of serum triacylglycerols and NO_2 plus NO_3 . Table 2 also shows the urinary excretion of 8-iso-PGF 2α or the β -oxidation product of 15-F $2t$ -IsoP-M. Two-way ANOVA revealed a main effect of fats on the urinary levels of 15-F $2t$ -IsoP-M (P <0.01), but not of 8-iso-PGF 2α . Here, HL diet-fed mice showed increased excretion of the former isoprostane than compared with the SF diet-fed mice and females tended to excrete greater amounts of both isoprostanes. The atherosclerotic lesion area was not significantly related to 8-iso-PGF 2α (r -0.11; P >0.1) or 15-F $2t$ -IsoP-M (r -0.18; P >0.1) in the urine.

Fig. 1 shows MCP-1 mRNA expression over the whole aorta, where aortas that were unbroken from the aortic valve to the iliac bifurcation were used for RNA extraction. Main effects were found for fats on this expression: the HL diet-fed males exhibited increased expression of the mRNA compared with the SF diet-fed males; MCP-1 mRNA expression in females also tended to be lower.

Table 3 shows the fatty acid composition of the serum phosphatidylcholine. There was a prominent difference in the proportion of oleic, linoleic and arachidonic acids between the HL diet-fed and SF diet-fed mice, where a significant sex effect on their

Table 2. Effect of dietary saturated fatty acid-rich fat (SF) and linoleic acid-rich fat (HL) on atherosclerotic lesions, serum and liver lipids, serum nitric oxides and peroxidation parameters for female and male apolipoprotein E-deficient mice (Mean values with their standard errors for seven to eight mice per sex for each group)

	Female				Male				ANOVA		
	SF		HL		SF		HL		Fat	Sex	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Lesion size at aortic root (mm ²)	0.251 ^a	0.016	0.146 ^b	0.015	0.271 ^a	0.025	0.130 ^b	0.011	0.01	NS	NS
Serum											
TG (mmol/l)	1.24 ^{a,b}	0.15	1.05 ^b	0.10	1.47 ^{a,b}	0.27	1.81 ^a	0.23	NS	0.01	NS
Total chol (mmol/l)	22.2 ^b	1.5	14.9 ^c	0.8	31.0 ^a	3.2	25.6 ^{a,b}	1.9	0.01	0.01	NS
HDL-chol (mmol/l)	0.68 ^b	0.08	1.19 ^a	0.25	0.69 ^b	0.13	1.19 ^a	0.25	0.01	NS	NS
NO ₂ plus NO ₃ (μmol/l)	13.9 ^b	2.3	16.6 ^b	1.9	26.1 ^{a,b}	6.2	31.8 ^a	4.4	NS	0.01	NS
MDA (μmol/l)	10.6 ^b	0.9	16.3 ^b	2.2	13.2 ^b	1.4	45.2 ^a	11.8	0.01	0.01	0.05
Liver											
Total chol (μmol/g liver)	25.8 ^a	1.8	14.4 ^b	0.7	20.8 ^a	2.2	14.6 ^b	0.9	0.01	NS	NS
Esterified chol (μmol/g liver)	19.6 ^a	1.7	9.4 ^b	0.5	15.2 ^a	2.2	9.7 ^b	0.9	0.01	NS	NS
Urine											
8-Iso-PGF2α (ng/μmol creatinine)	0.114	0.036	0.139	0.053	0.058	0.018	0.078	0.011	NS	0.08	NS
15-F2t-IsoP-M (ng/μmol creatinine)	2.12 ^a	0.29	2.42 ^a	0.28	0.53 ^b	0.05	1.55 ^a	0.24	0.01	0.01	NS

Chol, cholesterol; TG, triacylglycerol; MDA, malondialdehyde; 8-iso-PGF2α, 8-iso-prostaglandin F2α; 15-F2t-IsoP-M, 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2α.
^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$) (Bonferroni–Dunn test).

proportions was seen, but only to a small extent. ANOVA showed a significant diet effect on the proportion of saturated fatty acids (16:0 and 18:0), but the extent was smaller compared with the differences for the unsaturated fatty acids (18:1, 18:2 and 20:4n-6). For liver phosphatidylcholine, HL diet-fed mice showed an increased proportion of PUFA and a decreased proportion of oleic acid than did the SF group (data not shown).

Discussion

The novel findings of the present study were the following: (1) atherosclerotic lesion area was greater in the apo E-deficient

mice fed the SF diet than in those fed the HL diet, whereas the extent of biomarkers for oxidative stress (15-F2t-IsoP-M) and inflammatory status (MCP-1 mRNA) in the arterial wall was greater in the latter group than in the former group; (2) the serum total cholesterol level was lower in the HL group than in the SF group, whereas the HDL-cholesterol level was higher in the former group than in the latter group. These results suggest that in apo E-deficient mice, the cholesterol traffic between the serum and arterial wall is the primary determinant for the atherosclerosis process.

In the present study, the HL diet-fed apo E-deficient mice had an increased proportion of linoleic and arachidonic acids in their serum phosphatidylcholine than did the SF diet-fed mice. It is probable that the elevation of these PUFA was associated with increases in serum TBARS and urinary isoprostanes, which are derived from arachidonic acid (Morrow *et al.* 1999). The results that the HL diet makes serum lipoproteins more susceptible to oxidation are in agreement with those of a previous *in vitro* study which found an increased rate in conjugate diene formation in LDL enriched with linoleic acid (Reaven *et al.* 1993), and an *in vivo* study that found increased urinary excretion of 8-iso-PGF2α in human subjects after consumption of a diet rich in linoleic acid compared with an oleic acid-rich diet (Turpeinen *et al.* 1998). Furthermore, the HL diet compared with the SF diet resulted in increased inflammatory stress as reflected in the increased expression of aortic MCP-1. It is probable that the elevation of MCP-1 mRNA was in part related to increased oxidative stress in mice of the HL group than in those of the SF group, because linoleic and arachidonic acids induce MCP-1 gene expression in cultured cells by activating the oxidative stress-responsive transcription factor NF-κB (Hennig *et al.* 1996; Lee *et al.* 2001).

Despite the above unfavourable effects of the HL diet, the present study found that compared with the apo E-deficient mice fed the SF diet, the HL diet-fed mice had decreased lesion sizes in the aortic root compared with those of the SF group, followed by a decreased serum cholesterol level and an increased level of the serum HDL compared with the SF

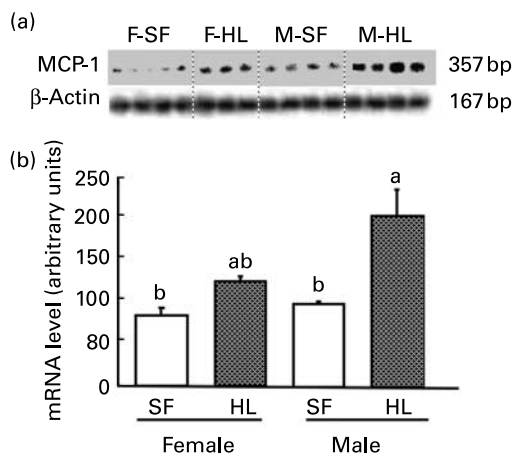


Fig. 1. The effect of dietary saturated fatty acid-rich fat (SF) and linoleic acid-rich fat (HL) on aortic mRNA levels of monocyte chemoattractant protein-1 (MCP-1) in apo E-deficient mice. (a) RT-PCR Southern hybridisation analyses for MCP-1 and β-actin mRNA for each sex and dietary treatment (F-SF, female fed SF diet; F-HL, female fed HL diet; M-SF, male fed SF diet; M-HL, male fed HL diet). (b) Mean values for three to five mice per group, with the corresponding standard errors represented by vertical bars. The relative levels of MCP-1 mRNA were normalised to the mean for the aorta from male mice fed an SF diet. ^{a,b}Mean values with unlike letters were significantly different ($P < 0.05$). There were significant effects for fat ($P < 0.001$) and for sex ($P < 0.005$), but not for their interaction (ANOVA).

Table 3. Effect of dietary saturated fatty acid-rich fat (SF) and linoleic acid-rich fat (HL) on the fatty acid composition of serum phosphatidylcholine in female and male apolipoprotein E-deficient mice (g/100 g total fatty acids)

(Mean values with their standard errors for seven to eight mice per sex for each group)

Fatty acid	Female				Male				ANOVA		
	SF		HL		SF		HL		Fat	Sex	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
16:0	27.8 ^b	0.4	26.0 ^c	0.7	31.3 ^a	0.5	28.5 ^b	0.4	0.01	0.01	NS
16:1	0.9 ^b	0.2	0.7 ^b	0.2	1.9 ^a	0.2	0.8 ^b	0.1	0.01	0.01	0.01
18:0	18.9 ^b	0.4	20.1 ^d	0.6	13.6 ^a	0.2	16.0 ^c	0.3	0.01	0.01	NS
18:1	18.8 ^b	0.4	7.0 ^c	0.3	21.3 ^a	0.5	7.9 ^c	0.3	0.01	0.01	0.05
18:2	14.7 ^a	0.5	24.3 ^c	1.0	15.5 ^a	0.8	26.9 ^b	1.0	0.01	NS	NS
20:4n-6	10.5 ^a	0.5	15.1 ^b	0.7	9.0 ^a	0.8	13.4 ^b	0.6	0.01	0.05	NS
22:6n-3	4.1 ^b	0.3	3.6 ^{a,b}	0.4	3.3 ^a	0.2	3.0 ^a	0.2	NS	0.05	NS

^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$) (Bonferroni–Dunn test).

diet group. These favourable effects of the HL diet have also been observed in studies on the African green monkey (Rudel *et al.* 1995), LDL receptor-deficient human apo B-transgenic mice (Rudel *et al.* 1998), and LDL receptor-deficient mice (George *et al.* 2000). In these experiments, the SF diet contained palm oil (49% palmitic acid and 37% oleic acid; Rudel *et al.* 1995, 1998) or cocoa butter (43% palmitic plus stearic acid and 35% oleic acid; George *et al.* 2000) as the primary source of dietary fat. Therefore, the diets designated SF diets in the present and previous studies (Rudel *et al.* 1995, 1998; George *et al.* 2000) contained almost equal proportions of saturated fatty acids and MUFA. Hence, it may not be appropriate to term them SF diets. In addition, Calleja *et al.* (1999) reported that male apo E-deficient mice fed a chow-based diet containing 10% sunflower-seed oil (56% linoleic acid) had fewer lesions than did those fed 10% palm oil. In contrast, Merkel *et al.* (2001) reported that there were no significant differences in the atherosclerotic lesion areas between HL diet-fed LDL receptor-deficient mice and SF diet-fed LDL receptor-deficient mice, and between HL diet-fed apo E-deficient mice and SF diet-fed apo E-deficient mice. In their experiments they (Merkel *et al.* 2001) used coconut oil (71% saturated fatty acids and 19% MUFA) as a primary source of dietary fats for the SF diet. For the same experiments with the LDL receptor-deficient mice, compared with the SF and HL diets, the MUFA-rich diet (oleic acid-enriched safflower-seed oil as primary fat; 71% MUFA) significantly increased atherosclerosis in both sexes (Merkel *et al.* 2001). As described earlier, Rudel and colleagues (Rudel *et al.* 1995, 1998) used two animal models to compare the effect of various dietary fats on atherosclerosis. Animals fed an SF diet (palm oil as the primary fat source; 49% palmitic acid and 37% oleic acid) and a MUFA-rich diet (oleic acid-enriched safflower-seed oil as the primary fat source; 71% MUFA) developed equivalent numbers of atherosclerotic lesions and those fed the HL diet developed fewer. From these results, it is probable that dietary enrichment of linoleic acid is associated with a reduction in the extent of atherosclerotic lesion development in the atherosclerosis-susceptible mice model. Furthermore, dietary fats rich in saturated fatty acids and MUFA rather than dietary fats enriched in saturated fatty acid alone appear to be more atherogenic than did linoleic acid-rich diets.

Although the present study was not designed to explore the mechanism whereby an HL diet positively prevents

atherosclerosis susceptibility, the opposing results for the atherosclerotic lesion area and the MCP-1 mRNA expression between apo E-deficient mice fed the HL diet and SF diet suggests that dietary factors could be involved in influencing monocyte chemoattraction to arterial intima. Several animal studies found a role for the CC-chemokine receptor 2 in atherosclerosis (Boring *et al.* 1998; Guo *et al.* 2003). CC-chemokine receptor 2 is expressed on circulating monocytes, and this expression is up regulated in hypercholesterolaemia and suppressed by elevated levels of HDL (Han *et al.* 1999). In the present study, the HL diet resulted in lower serum cholesterol and elevated HDL-cholesterol levels. Therefore, it remains to be determined if an HL diet is involved in lowering the recruitment of circulating monocytes into the arterial intima by reducing the expression of CC-chemokine receptor 2.

In summary, the present study found that an HL diet *in vivo* induces oxidative and inflammatory stresses as an adverse effect but improves serum lipoprotein cholesterol levels as a beneficial effect, and their net effect is an anti-atherogenic state in apo E-deficient mice. Therefore, having biomarkers *in vivo* for oxidative stress and inflammatory status of endothelial cells does not necessarily show a predisposition toward atherosclerosis initiation or development in this animal model when fed different dietary fats.

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