

Feeding a thermally oxidised fat inhibits atherosclerotic plaque formation in the aortic root of LDL receptor-deficient mice

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Abstract

Activators of PPAR α have been demonstrated to inhibit atherosclerosis development due to lipid lowering in plasma and direct protective effects on the vasculature. Because dietary oxidised fats (OF) have strong PPAR α -activating and lipid-lowering properties, we hypothesised that dietary OF has also an inhibitory influence on atherosclerosis development. To verify our hypothesis, we investigated the effect of feeding diets containing an OF (a 92:8 mixture of heated (170°C, 48 h) hydrogenated palm fat and fresh sunflower oil) compared with a fresh fat (fresh hydrogenated palm fat) on the development of atherosclerotic lesions in LDL receptor-deficient (LDLR $^{-/-}$) mice. We observed that a dietary OF caused a strong up-regulation of PPAR α -regulated genes in the liver and a marked reduction in plasma concentrations of cholesterol and TAG ($P < 0.05$). Cross-sectional lesion area and the lipids and collagen levels in the aortic root were approximately 40–50% lower in mice fed diets containing OF than in those fed diets containing fresh fat ($P < 0.05$). Immunohistochemical analysis of aortic root sections revealed an about 8-fold increased expression of PPAR α and a markedly reduced expression of the pro-inflammatory vascular cell adhesion molecule-1 and smooth muscle cell (SMC)-specific marker α -actin in LDLR $^{-/-}$ mice fed OF ($P < 0.05$). We postulate that OF exert anti-atherogenic effects by activation of PPAR α both in the liver, which contributes to lipid lowering in plasma, and in the vasculature, which inhibits pro-atherogenic events such as monocyte recruitment and SMC proliferation and migration.

Key words: Oxidised fat: Atherosclerosis: LDL receptor-deficient mice: PPAR α

In recent years, the contribution of oxidised fats (OF) to total energy intake has markedly increased in industrialised countries⁽¹⁾ due to the rising consumption of deep-fried products. During deep-frying, several chemical reactions occur within the frying oil resulting in the formation of a mixture of chemically distinct lipid peroxidation products. Large quantities of the frying oil are absorbed into the fried foods during deep-frying and are therefore ingested during their consumption.

Although OF are widely considered to have detrimental effects on human health^(2–4), feeding experiments in rats have consistently demonstrated an improvement in the blood lipid profile, i.e. a reduction in TAG and cholesterol levels in plasma and VLDL, by OF^(5–7). This effect of OF has been attributed to the ability of OF to activate hepatic PPAR α ^(8–10), a ligand-activated transcription factor that controls a comprehensive set of genes regulating most aspects of lipid catabolism, glucose homeostasis and inflammation^(11,12). Thus, activation of PPAR α results in decreased lipid concentrations in plasma and VLDL,

improved glucose tolerance and reduced inflammatory processes. The components of OF supposed to be responsible for PPAR α activation are hydroxy and hydroperoxy fatty acids⁽¹³⁾ and cyclic fatty acid monomers⁽¹⁴⁾. Indeed, feeding a diet supplemented with 13-hydroperoxy octadecadienoic acid strongly reduced TAG concentrations in plasma via PPAR α -dependent effects⁽¹⁵⁾.

PPAR α is also expressed in all the major cells of the vessel wall which are implicated in atherosclerotic lesion development⁽¹¹⁾. Activation of PPAR α in these cells modulates the expression of several genes implicated in the atherosclerotic process, resulting in decreased monocyte recruitment to endothelial cells⁽¹⁶⁾, enhanced cholesterol removal from macrophages⁽¹⁷⁾ and reduced smooth muscle cell (SMC) proliferation and migration⁽¹⁸⁾. These direct atheroprotective effects together with the lipid-lowering effects are largely responsible for the observation that pharmacological PPAR α activators cause an inhibition of atherosclerosis development^(19–22). Because dietary OF have strong PPAR α -activating and lipid-lowering

Abbreviations: CYP4A10, cytochrome P450 isoform 4A10; FF, fresh fat; LDLR $^{-/-}$, LDL receptor deficient; OF, oxidised fat; SMC, smooth muscle cells; VCAM-1, vascular cell adhesion molecule-1.

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properties, it would be expected that dietary OF have also an inhibitory influence on atherosclerosis development. Nevertheless, several earlier reports^(2,23–25) demonstrated that feeding OF has pro-atherogenic effects. However, this may be due to the fact that these studies used fats which were strongly oxidised and which contained lipid oxidation products, which are clearly above the limit allowed for 'used frying fats'. Thus, feeding such strongly OF does not reflect the physiological situation in human nutrition. Moreover, feeding such strongly OF causes intense oxidative stress due to the depletion of antioxidants such as tocopherols in serum and tissues^(26,27), which is considered to promote the development of atherosclerosis⁽²⁸⁾. Hence, a possible atheroprotective effect of OF due to activation of PPAR α is probably compromised by the simultaneous induction of intense oxidative stress. It could be demonstrated, however, that oxidative stress and depletion of antioxidants induced by feeding OF is alleviated by supplementation of the diet with a high vitamin E level⁽²⁷⁾. The aim of the present study was to investigate the effect of a thermally OF prepared under deep-frying conditions on the development of atherosclerotic lesions. In order to find out whether the effects of the OF in this respect are influenced by oxidative stress, we used diets with moderate or high vitamin E concentrations. As an experimental model of atherosclerosis, we used LDL receptor-deficient (LDLR^{-/-}) mice. These mice mimic human lipoprotein disorders that are associated with an increased risk of CHD and develop extensive aortic atherosclerosis which resembles human lesions⁽²⁹⁾.

Materials and methods

Animals and diets

A total of thirty-six male, adult, 15-week-old LDLR^{-/-} mice (B6.129S7-Ldlr^{tm1Her}/J mice; Charles River, Germany) with an initial body weight of 27 (SD 1) g were randomly assigned to three groups of twelve mice each. All mice were kept individually in Macrolon cages in a room maintained at 22 \pm 1°C and 50–60% relative humidity with lighting from 06.00 to 18.00 hours. All the experimental procedures described followed established guidelines for the care and handling of laboratory animals⁽³⁰⁾ and were approved by the local Animal Care and Use Committee. The mice were fed a semi-purified Western-type diet which consisted of (g/kg diet) maize starch, 285.5; casein, 200; saccharose, 200; experimental fat, 200; vitamin and mineral mixture, 60; cellulose, 50; linseed oil as a source of α -linolenic acid, 3; cholesterol, 1.5. Vitamins and minerals were supplemented according to the recommendations of the American Institute of Nutrition-93M⁽³¹⁾.

The experimental fat was varied as follows. The first group (fresh fat group, 'FF25') received 200 g/kg diet of fresh hydrogenated palm fat (Enco, Hamburg, Germany),

which is a typical fat used for deep-frying in restaurants. Both the second (OF group 25, 'OF25') and the third groups (OF group 250, 'OF250') received 200 g/kg of a mixture of heated hydrogenated palm fat (Enco) and fresh sunflower oil (92:8, w/w) (AOP, Riesa, Germany). This ratio was chosen to equalise the concentrations of the major fatty acids of the OF diets to that of the FF diet, since the heating process caused a partial loss of PUFA. The OF was prepared by heating the hydrogenated palm fat at a temperature of 170 \pm 3°C for 48 h in a domestic fryer (Fryer Model PROFRI 4; Saro Gastro Products, Emmerich, Germany). During the 48 h heating process, a portion of 70 g French fries obtained from a local cafeteria was deep-fried for 6 min every 30 min. The extent of lipid peroxidation in the fats was estimated by assaying the peroxide value⁽³²⁾ and the percentages of polar and unpolar compounds⁽³³⁾ before and after inclusion into the diets.

Because the frying process caused a dramatic loss of tocopherols in the heated hydrogenated palm fat, the native concentrations of tocopherols of all the experimental fats were analysed. Based on the native concentrations of the fats, the vitamin E concentration of the diets was adjusted to 25 mg α -tocopherol equivalents/kg diet in the FF25 diet and the OF25 diet and 250 mg α -tocopherol equivalents/kg diet in the OF250 diet by individually supplementing with all-*rac*- α -tocopheryl acetate (the biopotency of all-*rac*- α -tocopheryl acetate is considered to be 67% of that of α -tocopherol). Diets were prepared by mixing the dry components with the fat and water and subsequent freeze-drying. The residual water content of the diet was below 5 g/100 g diet. Food was administered daily at 12.00 hours in controlled amounts to standardise the intake.

Experimental diets were fed for 14 weeks. To standardise the food intake, diets were fed in a controlled feeding regimen, whereby each mouse received 2.5 g diet/d during the whole experiment. Energy supplied by this amount of diet was close to the energy requirement of the mice for maintenance⁽³⁴⁾. Water was available *ad libitum* for nipple drinkers during the whole experiment.

Sample collection

The mice were killed by decapitation under light anaesthesia with diethyl ether in the non-fasted state. Whole blood was collected into EDTA polyethylene tubes (Sarstedt, Nürnberg, Germany). Plasma was separated from the whole blood by centrifugation (1100 g; 10 min) at 4°C. Liver, skeletal muscle (*Musculus gastrocnemius*) and visceral adipose tissue were excised immediately and shock frozen with liquid N₂. All the samples were stored at -80°C for pending analysis.

Lipoproteins (VLDL, LDL and HDL) were separated by step-wise ultracentrifugation (900 000 g, 1.5 h, 4°C; Mikro-Ultrazentrifuge, Sorvall Products, Bad Homburg, Germany) as described elsewhere⁽³⁵⁾.

Preparation of aortic tissue and morphometric determination of atherosclerosis

To quantify atherosclerosis, aortic root sections (10 μm thick slices; beginning at the aortic valve area) were prepared and sections were stained with haematoxylin–eosin, oil red O for vascular lipids, Goldner's trichrome for collagen structures and von Kossa for vascular calcification, as described recently in detail⁽³⁵⁾. Histomorphological characterisation and computerised morphometric quantification of the atherosclerotic lesions were performed and blinded to the protocol. The cross-sectional surface area of the total vessel, the cross-sectional surface area of the lesion, the calcification area, the collagen area and the lipid area were assessed. The relative lesion area, the relative collagen area, the relative lipid area and the relative calcification area (expressed relative to the total surface area) were used to show individual atherosclerosis development in the aortic root.

Immunohistochemistry

For immunohistochemistry, aortic root sections were immediately fixed in acetone at -20°C for 10 min, and endogenous peroxidase was blocked in 0.3% H_2O_2 in methanol. Three sections were incubated each with 5% blocking serum (either goat, rabbit or sheep depending on the secondary antibody used) in PBS at room temperature for 20 min. Following a washing step, the sections were incubated with primary antibodies against SMC α -actin (Sigma, Taufkirchen, Germany; sections from 190 to 220 μm), vascular cell adhesion molecule (VCAM)-1 (Abcam, Cambridge, UK; sections from 250 to 280 μm), PPAR α (Abcam; sections from 280 to 310 μm) and PPAR γ (Axxora, Lörrach, Germany; sections from 310 to 340 μm) in a humidifying chamber for various periods (2–14 h depending on the antibody used) at 4°C . After washing in PBS, the sections were incubated with horseradish peroxidase-labelled secondary antibodies (goat anti-rat IgG and sheep anti-rabbit IgG (Serotec, Oxford, UK), and rabbit anti-mouse IgG (Dako, Hamburg, Germany)) at room temperature for 1 h. The immunocomplex was visualised using either diaminobenzidine chromogen (Dako) or Nova Red (Axxora). Subsequently, sections were counterstained with Harris haematoxylin solution. Intensity of staining was measured using LuciaG 3.2 software.

Lipid analysis

TAG and cholesterol concentrations in plasma and lipoproteins were determined using enzymatic reagent kits (DiaSys Diagnostic Systems, Holzheim, Germany, ref. 1.13009990314 and 1.57609990314). The fatty acid composition of the dietary fats was determined by GC. Fats were methylated with trimethylsulphonium hydroxide⁽³⁶⁾. Fatty acid methyl esters were separated by GC, using a system

(HP 5890; Hewlett-Packard GmbH, Böblingen, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm internal diameter, Macherey and Nagel, Düren, Germany) and a flame ionisation detector. Helium was used as the carrier gas with a flow rate of 5.4 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards.

Determination of vitamin E concentrations

Concentrations of α -tocopherol in liver, skeletal muscle and epididymal adipose tissue were determined, as described recently, in more detail⁽³⁷⁾.

RNA isolation and real-time detection PCR

For the determination of hepatic mRNA expression levels of cytochrome P450 isoform 4A10 (CYP4A10), acyl-CoA oxidase and lipoprotein lipase, total RNA was isolated, mRNA reverse transcribed, and target gene mRNA concentrations were determined by real-time detection PCR, as described previously⁽³⁸⁾. Sequences of gene-specific primers were as follows (forward, reverse; NCBI GenBank): glyceraldehyde 3-phosphate dehydrogenase (5'-AACG-ACCCCTTCATTGAC-3', 5'-TCCACGACATACTCAGCAC-3'; NM_008084), CYP4A10 (5'-TGAGGGAGAGCTGGAAAA-GA-3', 5'-CTGTTGGTGATCAGGGTGTG-3'; NM_010011), acyl-CoA oxidase (5'-CAGGAAGAGCAAGGAAG TGG-3', 5'-CCTTTCTGGCTGATCCCATATA-3'; NM_015729), lipoprotein lipase (5'-GGGCTCTGCCTGAGTTGTAG-3', 5'-AGAA-ATTTCTGAAGGCTTGGT-3'; BC_158040).

Statistical analysis

Values presented in the text are means and standard deviations. Treatment effects were analysed using one-way ANOVA. For significant *F* values, means were compared by Fisher's multiple range test. Differences with $P < 0.05$ were considered significant.

Results

Characterisation of the dietary fat of the experimental diets

In the OF diets, the dietary fat represented a mixture (92:8, w/w) of heated hydrogenated palm fat and fresh sunflower oil in order to equalise the dietary fat of the experimental diets for their fatty acid composition. This was necessary to avoid the confounding effects resulting from differences in the concentrations of major fatty acids between the experimental diets. As revealed by GC-flame ionisation detector analysis, the concentrations of the major fatty acids and of the essential fatty acids, linoleic acid (18:2 *n*-6) and α -linolenic acid (18:3 *n*-3) were similar between

Table 1. Fatty acid composition and concentrations of peroxidation products in the dietary fats after inclusion into the diets

	FF25	OF25	OF250
Major fatty acids (% of total FAME)			
8:0	0.5	0.4	0.5
10:0	0.7	0.8	0.7
12:0	2.5	1.2	1.4
14:0	2.5	1.8	1.9
16:0	42.7	49.0	48.7
18:0	4.3	5.0	4.8
18:1 <i>n</i> -9	35.5	31.4	32.0
18:2 <i>n</i> -6	9.9	9.5	9.3
18:3 <i>n</i> -3	1.1	0.9	0.7
20:0	0.2	0.1	0.1
Peroxidation products			
POV (mEq O ₂ /kg diet)	5.0	7.5	9.5
Total polar compounds (%)	8.8	26.9	29.4
Total unpolar compounds (%)	91.2	73.1	70.6

FF25, fresh fat group; OF25 and OF250, oxidised fat groups; FAME, fatty acid methyl esters; POV, peroxide value.

all the three experimental diets (Table 1). The concentrations of *trans*-fatty acids such as 18:1 *n*-9, 18:2 *c*9*t*11 and 18:2 *t*10*c*12 were below 0.1% of total fatty acids in all the three experimental diets. In contrast, the peroxide value and the percentage of polar compounds in the dietary fat were about 2- and 3-fold, respectively, higher in the OF diets than in the FF25 diet. The percentage of unpolar compounds was lower in the dietary fat of the OF diets than in the FF25 diet (Table 1).

Food intake, body weight changes and relative liver weights

To exclude secondary food intake effects, a controlled feeding system was applied in which each mouse was given an identical amount of diet. Nevertheless, mice of the FF25 group had a slightly higher final body weight at the end of the 14-week feeding period than those of the OF groups (FF25, 35.4 (SD 1.5) g; OF25, 29.5 (SD 1.6) g; OF250, 28.9 (SD 1.9) g; *n* 12, *P*<0.05). No difference in final body weights was observed between the mice of the OF25 group and the mice of the OF250 group. Daily body weight gain during the 14-week feeding period was also slightly higher in the FF group than in the OF groups (FF25, 0.08 (SD 0.02) g; OF25, 0.02 (SD 0.02) g; OF250, 0.02 (SD 0.03) g; *n* 12, *P*<0.05). No difference in daily body weight gain was observed between mice of the two OF groups. Relative liver weights were higher in mice fed the OF diets than in those fed the FF diet (FF25, 4.8 (SD 0.2) g/100 g body weight; OF25, 6.1 (SD 0.3) g/100 g body weight; OF250, 6.1 (SD 0.4) g/100 g body weight; *n* 12, *P*<0.05).

Atherosclerosis in the aortic root

To examine the effect of treatment on atherosclerotic lesion development, serial sections through the aortic

root beginning at the level of the aortic valves were taken. Subsequent analysis of the aortic root sections showed that all mice developed severe atherosclerotic lesions covering approximately 20–30% of total vessel area. Atherosclerotic lesion size (cross-sectional lesion area) and the lipids and collagen levels in the aortic root were approximately 40–50% lower in mice of the OF groups than in those of the FF25 group (Figs. 1(A)–(C) and 2(a) and (b); *P*<0.05). The levels of calcifications in the aortic root did not differ between the three groups of mice (Figs. 1(D) and 2(c)).

Lipid concentrations in plasma and lipoproteins

To evaluate whether the dietary OF also exerts a lipid-lowering action in LDLR^{-/-} mice, the lipid concentrations in plasma and lipoproteins were determined. Concentrations of TAG in plasma and VLDL + chylomicrons were markedly lower in the OF groups than in the FF25 group (Table 2; *P*<0.05). TAG concentrations in plasma and VLDL + chylomicrons did not differ between both OF groups.

Concentrations of cholesterol in plasma, LDL and HDL were lower in mice fed the OF250 diet than in those fed the FF25 diet (Table 2; *P*<0.05). In mice fed the OF25 diet, only the concentrations of cholesterol in HDL, but not in plasma and LDL, were lower than in those fed the FF25 diet (Table 2). Cholesterol concentrations in VLDL + chylomicrons did not differ among the three groups of mice.

Expression of PPAR α and PPAR γ in the aortic root

PPAR agonists have been shown to exert antiatherogenic effects through the activation of PPAR in the vasculature. To examine the effect of OF on expression of PPAR, sections of the aortic root were stained for PPAR α and PPAR γ by immunohistochemistry. Both PPAR α and PPAR γ were well detectable in the aortic root of LDLR^{-/-} mice, with staining localised largely to the atherosclerotic lesion. Expression of PPAR α in the aortic root was about 6- to 8-fold higher in mice fed the OF diets than in those fed the FF25 diet (Fig. 3(A); *P*<0.05). In contrast to PPAR α , expression of PPAR γ in the aortic root was not different among the three groups of mice (Fig. 3(B)).

Expression of smooth muscle cell α -actin in the aortic root

SMC are the major collagen-producing cell types in the atherosclerotic plaque. To investigate whether changes in SMC content of plaques might be responsible for the reduction of collagen content by OF, sections of the aortic root were stained for the SMC-specific marker α -actin. Immunostaining for SMC α -actin showed a strong expression in the aortic root of mice fed the FF25 diet, with staining localised to atherosclerotic lesions. In the aortic root of mice fed the OF diets, expression of SMC α -actin was strongly reduced (Fig. 4; *P*<0.05).

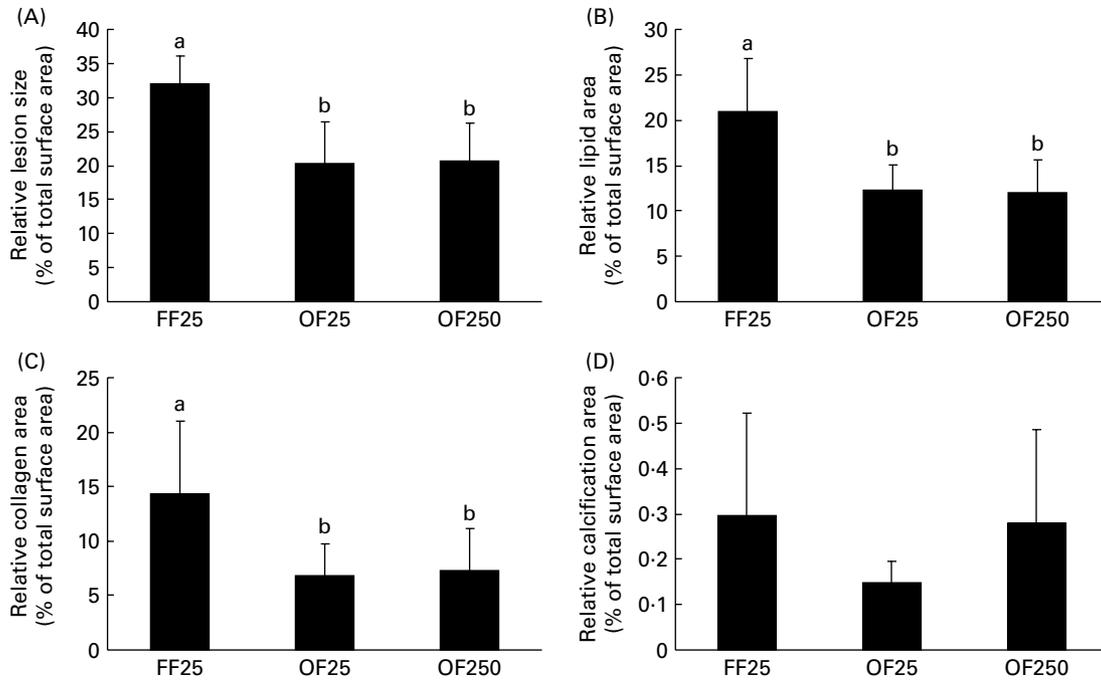


Fig. 1. Effect of treatment on cross-sectional lesion size and lesion composition in the aortic root of LDL receptor-deficient $^{-/-}$ mice fed experimental diets for 14 weeks. (A) Lesion size, (B) lipid area, (C) collagen area and (D) calcified area relative to total surface area. Bars represent means and standard deviations (n 9). ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

Expression of vascular cell adhesion molecule-1 in the aortic root

To evaluate the effect of dietary OF on inflammation, expression of the inflammatory adhesion molecule VCAM-1 in the aortic root sections was determined by immunohistochemistry. Expression of VCAM-1 in the aortic root was approximately 70% lower in mice fed the OF diets than in those fed the FF25 diet (Fig. 5; $P < 0.05$). Staining for VCAM-1 was localised mainly to the core region of the atherosclerotic lesions.

Vitamin E status

To evaluate the induction of oxidative stress by the OF, vitamin E concentrations in various tissues were determined in the LDLR $^{-/-}$ mice. Concentrations of total tocopherols in liver, skeletal muscle and epididymal adipose tissue were markedly lower in mice fed the OF25 diet than in those fed the FF25 diet (Table 3; $P < 0.05$). In mice fed the OF250 diet, concentrations of total tocopherols in liver and epididymal adipose tissue were higher than in mice fed the FF25 diet (Table 3; $P < 0.05$). Concentrations of total tocopherols in skeletal muscle did not differ between the mice fed the OF250 diet and those fed the FF25 diet (Table 3).

Transcript levels of PPAR α target genes in the liver

To investigate whether dietary OF also activates hepatic PPAR α in LDLR $^{-/-}$ mice, transcript levels of classical

PPAR α target genes were determined in the liver. Relative mRNA levels of the PPAR α target genes CYP4A10, acyl-CoA oxidase and lipoprotein lipase in the liver were about 4-fold, 2-fold and 1.5-fold higher, respectively, in mice fed the OF diets than in those fed the FF25 diet (Fig. 6; $P < 0.05$).

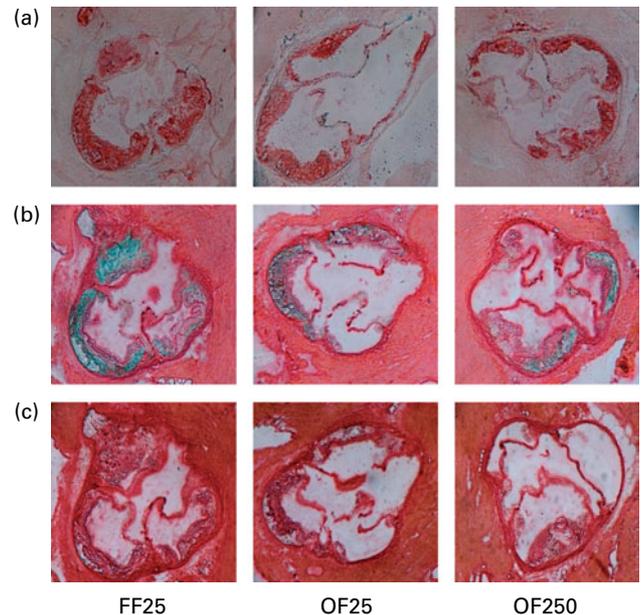


Fig. 2. Stained aortic root sections of LDL receptor-deficient $^{-/-}$ mice fed experimental diets for 14 weeks. (a) Oil red O staining for lipids, (b) Golder's trichrome staining of collagen structures, (c) von Kossa staining of calcifications (3 \times magnification). The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root. FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

Table 2. Concentrations of lipids in plasma and lipoproteins of LDL receptor-deficient mice fed the experimental diets for 14 weeks(Mean values and standard deviations, *n* 12)

	FF25		OF25		OF250	
	Mean	SD	Mean	SD	Mean	SD
TAG (mmol/l)						
Plasma	7.45 ^a	1.98	3.54 ^b	1.21	3.67 ^b	1.11
VLDL + chylomicrons	2.03 ^a	0.27	1.03 ^b	0.33	1.19 ^b	0.43
Cholesterol (mmol/l)						
Plasma	39.4 ^a	10.1	33.7 ^{a,b}	6.5	31.6 ^b	5.5
VLDL + chylomicrons	20.9	7.5	17.7	4.6	16.7	2.1
LDL	12.6 ^a	1.1	11.4 ^{a,b}	1.4	10.6 ^b	1.3
HDL	5.9 ^a	1.5	4.6 ^b	0.6	4.4 ^b	0.5

FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$).

Transcript levels of lipogenic and cholesterologenic genes in the liver

In order to evaluate whether the reduction of lipid concentrations in plasma and lipoproteins by dietary OF is due to decreased lipogenesis and cholesterologenesis in the liver, transcript levels of hepatic lipogenic and cholesterologenic genes were determined. Transcript levels of genes encoding lipogenic enzymes such as fatty acid synthase and acyl-CoA carboxylase and of the rate-limiting enzyme of cholesterol synthesis, hydroxymethylglutaryl-CoA reductase, did not differ among the three groups of mice (data not shown). In addition, transcript levels of the key transcription factors controlling lipogenic and cholesterologenic genes, sterol regulatory element-binding protein-1 and -2, were not different among the three groups (data not shown).

Discussion

In feeding studies dealing with OF, a markedly reduced food intake and growth of the experimental animals has been frequently observed^(27,39,40). This has been attributed to the use of strongly OF containing less PUFA and antioxidants than the equivalent FF and high levels of polymerisation products, thereby causing toxic effects, pronounced oxidative stress and reduction of nutrient digestibility. To avoid these confounding effects, we used a moderately OF (as shown by the comparatively low amount of peroxidation products), which was prepared under deep-frying conditions using hydrogenated palm fat, a typical fat used for such purposes in German restaurants. In addition, dietary fats were equalised for their fatty acid composition by using fat mixtures, and vitamin E concentrations in the diets were adjusted. Moreover, a controlled feeding regimen in which mice of all groups were fed identical amounts of fat was applied. Because we used non-growing mice and the food administered was close to the energy requirement for maintenance, there was only a slight change of body weight during the 14-week feeding

period in the three groups of mice. Despite the controlled feeding regimen, weight gain was slightly higher in the FF group than in the OF groups, which might be due to the fact that OF show a slightly lower digestibility than FF^(27,39,40). Nevertheless, the observation that differences in daily weight gains were small between mice fed the FF and those fed the OF indicates that intake of digestible energy did not considerably differ between these groups of mice. We are therefore confident that the metabolic effects of OF reported in this study are not confounded by the slightly reduced weight gain of the OF-fed mice.

The main finding of the present study is that a moderately OF containing levels of lipid oxidation products that are below the limit allowed for 'used frying fats' when fed together with a hyperlipidaemic diet inhibits atherosclerosis development in LDLR^{-/-} mice, as evidenced by

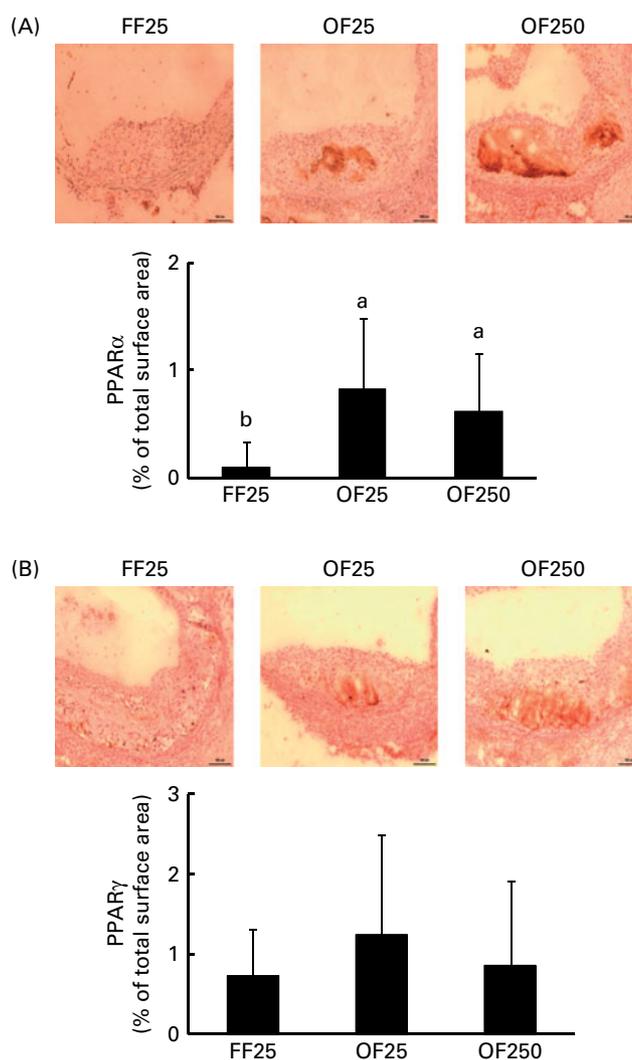


Fig. 3. Quantification of immunohistochemical staining for (A) PPAR α and (B) PPAR γ in aortic root sections of LDL receptor-deficient^{-/-} mice fed experimental diets for 14 weeks. The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root (10 \times magnification). Bars represent means and standard deviations (*n* 9). ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

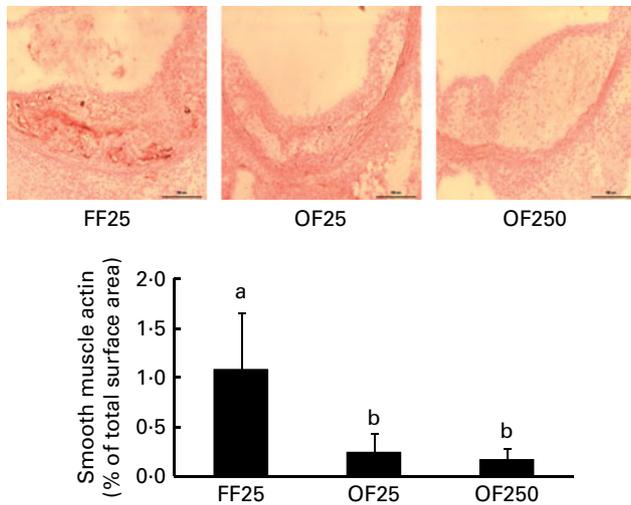


Fig. 4. Quantification of immunohistochemical staining for smooth muscle α -actin in aortic root sections of LDL receptor-deficient $^{-/-}$ mice fed experimental diets for 14 weeks. The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root (10 \times magnification). Bars represent means and standard deviations (n 9). ^{a,b}Mean values with unlike letters were significantly different (P <0.05). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

a markedly lower lesion size (cross-sectional lesion area) and strongly reduced lipid and collagen contents in the aortic root. Moreover the present study shows that the inhibitory effect of the moderately OF on atherosclerosis development could even be observed when the vitamin E concentration in the diet was moderate. It is likely that this concentration of dietary vitamin E was sufficient to prevent the induction of oxidative stress by OF. In agreement with the recent findings^(26,27), we observed that the

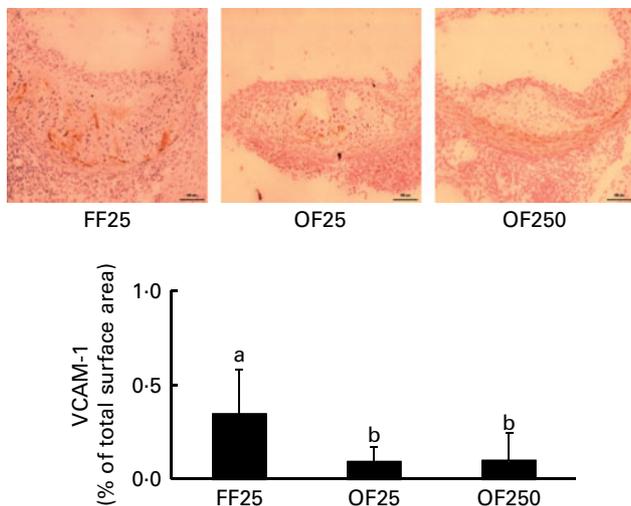


Fig. 5. Quantification of immunohistochemical staining for vascular cell adhesion molecule (VCAM)-1 in aortic root sections of LDL receptor-deficient $^{-/-}$ mice fed experimental diets for 14 weeks. The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root (10 \times magnification). Bars represent means and standard deviations (n 9). ^{a,b}Mean values with unlike letters were significantly different (P <0.05). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

Table 3. Concentrations of total tocopherols in tissues of LDL receptor-deficient mice fed the experimental diets for 14 weeks (Mean values and standard deviations, n 12)

	FF25		OF25		OF250	
	Mean	SD	Mean	SD	Mean	SD
α -Tocopherol equivalents (nmol/g)						
Liver	93 ^b	10	31 ^c	5	221 ^a	79
Skeletal muscle	15.1 ^a	3.3	8.5 ^b	1.4	16.1 ^a	4.4
White adipose tissue	47.5 ^b	8.5	26.6 ^c	5.9	85.3 ^a	14.9

^{a,b,c}Mean values with unlike superscript letters were significantly different (P <0.05).

vitamin E status of the OF-fed mice was compromised by the OF, which suggests induction of oxidative stress.

As a mechanism of action, we suggest that inhibition of atherosclerotic lesion development in LDLR $^{-/-}$ mice fed diets containing OF is, at least partially, due to reduction in plasma cholesterol and TAG concentrations, because elevated blood lipid concentrations are known risk factors for the development of atherosclerosis. It has been shown that the lipid-lowering action of OF is mediated in part by activation of PPAR α in the liver, leading to an enhanced fatty acid catabolism and an increased lipolysis of VLDL particles⁽⁵⁻⁷⁾. Due to the activation of hepatic PPAR α , dietary OF prevent the excessive accumulation of TAG induced by steatosis-inducing agents such as ethanol⁽⁴¹⁾. Herein, activation of hepatic PPAR α could also be observed in LDLR $^{-/-}$ mice as evidenced by the up-regulation of PPAR α -dependent genes such as acyl-CoA oxidase, CYP4A10 and lipoprotein lipase in the liver and elevated relative liver weights, which is a typical response to PPAR α agonists. A recent study with mice also revealed a strong up-regulation of PPAR α and a marked reduction in plasma lipid concentrations in response to feeding a diet supplemented with 13-hydroperoxy octadecadienoic acid, which is derived from peroxidation of linoleic acid⁽¹⁵⁾. It is therefore likely that, through the activation of hepatic PPAR α , components of OF are capable of favourably influencing the blood lipid profile. Thus, we suggest that PPAR α activation in the liver contributes to lipid lowering in plasma of LDLR $^{-/-}$ mice, which might in part be responsible for the inhibition of atherosclerotic lesion development. In contrast, transcription of sterol regulatory element-binding protein-regulated lipogenic and cholesterologenic genes, such as fatty acid synthase, LDLR and hydroxymethylglutaryl-CoA reductase, was not influenced by OF in the liver of the mice, suggesting that reduced lipid concentrations in plasma are not due to a decreased synthesis of fatty acids and cholesterol in the liver and/or uptake of cholesterol into the liver^(42,43).

We propose that direct activation of PPAR α in the vasculature also contributed to the inhibitory effect of OF on atherosclerosis development, because we could observe a markedly increased PPAR α expression in the aortic root

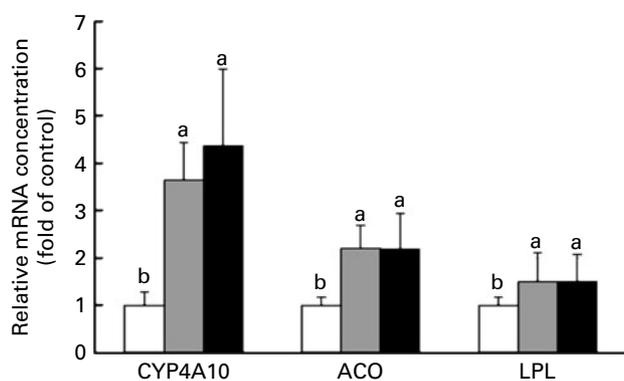


Fig. 6. Effect of treatment on relative mRNA concentrations of PPAR α -responsive genes in livers of LDL receptor-deficient^{-/-} mice fed experimental diets for 14 weeks. Bars represent means and standard deviations (n 12). ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). FF25 (□), fresh fat groups; OF25 (■) and OF250 (■), oxidised fat groups. ACO, acyl-CoA oxidase; LPL, lipoprotein lipase.

lesions of mice that fed the OF. This is probably indicative of an increased expression of PPAR α by the plaque cells because lesion size was markedly reduced by the OF. The increased expression of PPAR α protein in atherosclerotic lesions by OF has to be considered beneficial because inhibition of atherosclerosis development by anti-atherogenic dietary agents was accompanied by an increased PPAR α expression in the atherosclerotic plaque and the aorta, respectively⁽⁴⁴⁾. In line with the increased expression of PPAR α by dietary OF is the observation that the expression of the inflammatory protein VCAM-1 and the SMC-specific marker α -actin as well as the lipid and collagen content in the aortic root was also significantly reduced by the OF. VCAM-1 and other adhesion molecules, the expression of which is negatively regulated by PPAR α ⁽¹⁶⁾, are responsible for monocyte attachment to the luminal surface of the blood vessels and are required for subsequent infiltration of the subendothelial space by monocyte-derived macrophages. Consequently, inhibition of endothelial adhesion molecule expression by PPAR α activators inhibits atherosclerotic plaque formation^(19,20). The decreased expression of SMC α -actin suggests that the content of SMC in the aortic root of LDLR^{-/-} mice was reduced by the OF. This might be indicative of an inhibitory effect of OF on the proliferation and/or migration of SMC into the intima, which was shown to be inhibited by PPAR α activation⁽¹⁸⁾. Because SMC are the major collagen-producing cells in the atherosclerotic plaque and collagens substantially contribute to lesion volume⁽⁴⁵⁾, it is likely that the decreased aortic SMC content is responsible for the reduced collagen content and lesion size in mice fed OF. In contrast to lipid and collagen content of atherosclerotic lesions, no effect of OF could be observed on the levels of calcification in the aortic root of LDLR^{-/-} mice, suggesting that dietary OF has no major influence on the calcification process and on the complex mechanisms regulating vascular calcification.

Expression of PPAR γ , another PPAR isotype with athero-protective effects that can also be activated by hydroxylated fatty acids present in OF, was not influenced by the OF in the aortic root of LDLR^{-/-} mice. Although this finding does not definitely exclude the possibility that OF caused some of its effects by activation of PPAR γ , it is less likely because a recent study revealed only a weak activation of this receptor by OF⁽³⁷⁾.

Heated oils are a complex mixture of a great number of oxidation products formed during heat treatment. Therefore, it remains unclear which of the components of the OF were responsible for the effects observed in this study. Hydroxy and hydroperoxy fatty acids as well as cyclic fatty acid monomers have been identified as strong PPAR α agonists^(13–15). Therefore, these oxidation products are potential candidates which could be responsible for the anti-atherogenic effects induced by the OF. However, Khan-Merchant *et al.*⁽⁴⁾ observed that feeding 13-hydroxy octadecadienoic acid, an oxidation product of linoleic acid, did not inhibit but even enhanced the development of atherosclerosis in LDLR^{-/-} mice. Recently, Litvinov *et al.*⁽⁴⁶⁾ observed that administration of azelaic acid, an end product of linoleic acid peroxidation, inhibits the development of atherosclerosis in LDLR^{-/-} mice, probably by preventing macrophage accumulation in the arterial wall. Thus, this substance could also account for the anti-atherogenic effect of OF observed in the present study.

In the present study, we used LDLR^{-/-} mice as a well-established experimental model of atherosclerosis. When trying to transfer these findings to human subjects, it must be considered that mice, in contrast to human subjects, have a much higher tissue expression level of PPAR α and that the response of many genes to PPAR α activation is much stronger^(47,48). As the beneficial effects of the OF observed in the present study might be primarily caused by activation of PPAR α , it is expected that the same effects are much weaker in human subjects. Moreover, the results of the present study must not be interpreted in the way that OF could be regarded as a health-promoting component of the diet, as components of OF might have several adverse effects in human subjects. The results of the present study rather suggest that OF are a mixture of chemically distinct substances, some of which exhibit a significant biological activity.

In conclusion, the present study demonstrates that feeding an OF prepared under deep-frying conditions containing levels of lipid oxidation products which are below the limit allowed for 'used frying fats' causes anti-atherogenic effects in LDLR^{-/-} mice – effects that are probably due to activation of PPAR α in the liver and the vasculature.

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analysed data; I. K., R. R. and K. E. wrote the paper. K. E. had primary responsibility for the final content. All the authors read and approved the final manuscript. The authors have no conflicts of interest.

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