The effects of N-6 polyunsaturated fatty acid supplementation on the lipid composition and atherogenesis in mouse models of atherosclerosis

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Abstract

Despite numerous studies, the precise role of dietary n-6 polyunsaturated fatty acids in the pathogenesis of atherosclerosis remains controversial. It has been shown that feeding an n-6-enriched diet resulted in decreased atherosclerosis in African green monkeys and was associated with a reduction in LDL levels. However, other authors reported that n-6 supplementation increased the oxidative stress and the susceptibility of LDL to undergo in vitro oxidation, thus potentially enhancing atherosclerosis. The present study was designed to investigate the effect of dietary supplementation of n-6 polyunsaturated fats (safflower oil), as compared with a saturated fat-rich diet (Paigen), on the blood lipid profile and atherosclerosis in two mouse models. In the first experiment, female C57BL/6 mice (n = 23–30 per group) were fed a cholate containing Paigen diet, a safflower oil-rich diet (with cholate), or normal chow for 15 weeks. No significant differences between the high fat diet groups were evident with respect to total cholesterol, LDL, HDL or triglyceride levels. The extent of aortic sinus fatty streaks did not differ significantly between the two groups. In the second experiment, LDL-receptor-deficient (LDL-RD) mice (n = 20–30 per group) were randomized into similar dietary regimens. Mice consuming a safflower oil-enriched diet developed significantly less atherosclerosis, in comparison with Paigen diet-fed mice. A reduction in LDL levels, although not of a similar magnitude as the reduction in atherosclerosis, was evident in the safflower oil-fed mice when compared to the Paigen diet-fed littermates. In both mouse models of atherosclerosis, LDL isolated from the plasma of mice on the n-6 polyunsaturated diet was rendered slightly more susceptible to oxidation in vitro, as indicated by a shorter lag period for diene formation. Thus, the effects of n-6 fatty acids on the lipoprotein composition and other potential influences may have contributed to the anti-atherogenic effect in the LDL-RD mouse model. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atherosclerosis; Mouse; Polyunsaturated; LDL; Safflower oil; Oxidation

1. Introduction

It has been recently suggested that enrichment of diet with linoleic acid (n-6; 18:2) could play a dominant role in the pathogenesis of atherosclerosis [1]. Accordingly, the extent of coronary atherosclerosis has been shown to correlate with levels of 18:2 in adipose tissue, which mirror dietary intake [2]. Additionally, phospholipid fractions obtained from coronary arteries of humans dying of ischemic heart disease were shown to contain increased concentrations of n-6 [3]. Consumption of diets enriched with linoleate or corn oil (in comparison with saturated-fat diets) were found to increase the susceptibility of LDL from the human subjects to oxidative modification, and promoted LDL-induced disruption of endothelial functions [4]. Similar findings were subsequently reported in rabbits [5]. The mechanisms proposed to account for 18:2-mediated endothelial cell (EC) disruption include: decreased intracellular
2. Materials and methods

2.1. Animals

Female LDL receptor-deficient (LDL-RD) mice (hybrids between the C57BL/6J and 129Sv strains) were created by homologous recombination as described by Ishibashi et al. [23] and were from an in-house colony. Female C57BL/6J mice were obtained from Charles River Laboratories.

2.2. Diets and experimental design

Three diets were tested in the two mouse strains:

1. Teklad Premier Laboratory Diet, No. TD 90221 (Paigen high-fat diet) 1.25% cholesterol, 7.5% cocoa butter, ~17% total fat and 0.5% cholic-acid.

2. Teklad Premier Laboratory Diet, No. TD 94272 (safflower oil high-fat diet) 1.25% cholesterol, 7.5% safflower oil, ~17% total fat and 0.5% cholic acid.

3. Normal fat diet, Purina Rodent Laboratory Chow No. 5001, 0.027% cholesterol, ~4.5% total fat.

The precise composition of each diet is outlined in Table 1.

In the first experiment, the C57BL/6 mice (9–10 weeks old) were randomized to groups and fed one of the three diets for 15 weeks. Body weights and serial bleedings were carried out at baseline, 4, 8, 12 and 15 weeks after initiation of the diets. Hearts were removed after the 15-week bleed and embedded for histological assessment of atherosclerosis as described below. In the second experiment, LDL-RD mice (4–5 weeks old) were randomly divided into three groups and fed one of the three diets for 6 weeks. Body weights and serial bleedings were carried out at baseline, 2 and 6 weeks after initiation of the diets. Hearts were removed after the 6-week bleed and embedded for estimation of the extent of atherosclerosis. In both experiments, diets were fed ad libitum.

2.3. Preparation and oxidation of LDL

Mice were fasted for 12 h before blood samples were collected in EDTA (1 mg/ml) and pooled within each diet group for lipoprotein isolation. LDL (density = 1.019–1.063 g/l) was isolated from plasma as previously described [24], by density adjustment with KBr and preparative ultracentrifugation at 50 000 rpm (Bekman) for 22 h, using type 50 rotor. LDL preparations were washed by ultracentrifugation, dialyzed against 0.15 mol/l EDTA (for 24 h; four baths; pH 7.4), passed though an acrodisc filter (0.22 μm pore size, Gelman) to remove aggregates, and stored under nitrogen at 4°C in the dark. The total protein content of the LDL preparation was determined by the Lowry technique. LDL protein (50 μg/ml) in PBS was incubated with 15 μM CuCl₂ and conjugated diene formation was measured by the increase in absorbance at 234 nm [25].

2.4. Lipid profile

During each experiment, blood was drawn from the retroorbital sinus (prior to which the animals were mildly anesthetized with ether) for serial lipid assessments. At the end of the experiment, 1–1.5 ml of blood was obtained by cardiac puncture into EDTA containing tubes. Total plasma cholesterol and triglyceride levels were determined by using an automated enzymatic technique (Sigma). Because the densities of both mouse and human lipoproteins are the same, it was possible to determine serum HDL cholesterol concentrations after selective precipitation of VLDL and LDL using polyethylene-glycol 6000. Results are expressed as means ± SD.
2.5. Assessment of atherosclerosis

Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described [26]. Briefly, the heart and the upper section of the aorta were removed and imbedded in OCT compound for 48 h. Each heart was frozen on a cryostat, and 10 μm horizontal sections were made beginning with the distal (cut surface) of the embedded portion with the plane of sectioning parallel to a line between the tips of the atria. Sections were discarded until the first section that showed the three valve cusps determined by examining sections by microscopy without staining. Once the appropriate section was located, the next 20 consecutive 10 μm thick sections caudal to the area were placed on microscope slides and stained with oil red O. The men lesion size was calculated by averaging the 20 sections obtained per animal. The total lesions per group was determined by averaging the means from all animals in the group (means of means). The lesions in the aortic sinus were counted using a grid [27] by an observer unfamiliar with the tested specimen. Lesions were traced as either accumulating in the base of the aortic cusps or in the free wall of the same section (not in contact with the valve leaflet). Total aortic lesion size represents the sum of the two measurements (cusp + free wall). Samples from both strains of mice and from the various experimental groups were treated the same with respect to their placement, sectioning and orientation. Results are expressed as means ± SD.

2.6. Statistical analysis

All three groups (body weights, lipid levels and lesion size) in the two experiments were compared using a one way ANOVA test. \( P < 0.05 \) was considered as statistically significant.

3. Results

3.1. Body weights

In the first experiment, no statistically significant differences were noted between the final body weight in

Table 1
Fat composition (%) in the three diets

<table>
<thead>
<tr>
<th></th>
<th>Paigen high-fat diet (1.25% cholesterol)</th>
<th>Safflower oil high-fat diet (1.25% cholesterol)</th>
<th>Normal chow diet (0.027% cholesterol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Purina mouse chow}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 (Polyunsaturated, linoleic acid)</td>
<td>1.8</td>
<td>18:2 (Polyunsaturated, linoleic acid)</td>
<td>1.8</td>
</tr>
<tr>
<td>18:1 (Monounsaturated, oleic acid)</td>
<td>3.25</td>
<td>18:1 (Monounsaturated, oleic acid)</td>
<td>3.25</td>
</tr>
<tr>
<td>16:0; 18:0 (Saturated fats)</td>
<td>2.8</td>
<td>16:0; 18:0 (Saturated fats)</td>
<td>2.8</td>
</tr>
<tr>
<td>Others</td>
<td>0.4</td>
<td>Others</td>
<td>0.4</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>0.5</td>
<td>Cholic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>8.25</td>
<td>Total</td>
<td>8.25</td>
</tr>
<tr>
<td>\textit{Cocoa butter}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 (Polyunsaturated, linoleic acid)</td>
<td>0.25</td>
<td>18:2 (Polyunsaturated, linoleic acid)</td>
<td>5.8</td>
</tr>
<tr>
<td>18:1 (Monounsaturated, oleic acid)</td>
<td>2.7</td>
<td>18:1 (Monounsaturated, oleic acid)</td>
<td>0.9</td>
</tr>
<tr>
<td>16:0; 18:0 (Saturated fats)</td>
<td>4.45</td>
<td>16:0; 18:0 (Saturated fats)</td>
<td>0.8</td>
</tr>
<tr>
<td>Others</td>
<td>0.1</td>
<td>Others</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>7.5</td>
<td>Total</td>
<td>7.5</td>
</tr>
</tbody>
</table>

|                  | 17% total fat |                                               |                                     |
|------------------|---------------|                                               |                                     |
| 18:2 (Polyunsaturated, linoleic acid) | 12            | 18:2 (Polyunsaturated, linoleic acid)          | 45                                  |
| 18:1 (Monounsaturated, oleic acid)   | 35            | 18:1 (Monounsaturated, oleic acid)             | 24                                  |
| 16:0; 18:0 (Saturated fats)          | 43            | 16:0; 18:0 (Saturated fats)                    | 21                                  |
| Cholesterol                    | 7             | Cholesterol                                   | 7                                   |
| Other                          | 3             | Other                                         | 3                                   |
| Total                           | 100           | Total                                          | 100                                 |
Fig. 1. Weights of C57BL/6 and LDL-RD mice throughout the study. C57BL/6 (A) and LDL-RD (B) mice were fed the three experimental diets and weighed immediately before sacrifice. Data represent mean ± SD (n = 28, 30 and 23 for the Paigen, Safflower and chow fed C57BL/6 mice, respectively; n = 30, 26 and 20 for the Paigen, Safflower and chow fed LDL-RD mice, respectively).

Fig. 2. Lipid profile in C57BL/6 mice. Female C57BL/6 mice were fed a Paigen diet (n = 28), a safflower oil-enriched diet (n = 30) or a normal chow diet (n = 23). Blood (in EDTA containing tubes) was collected from the retroorbital plexus, and levels of total cholesterol (A), LDL + VLDL (B), HDL (C) and triglycerides (D) were determined. Data are presented as mean ± SD of all mice per group.

the three groups: the weights were 25.3 ± 1.8 g for Paigen diet-fed C57BL/6 mice, 24 ± 2.4 g (P = ns) for safflower oil-fed mice and 24.2 ± 1.5 g (P = ns as compared to Paigen fed mice) for the chow-fed mice (Fig. 1A). Likewise, in the second experiment the final body weights did not differ significantly: 19.6 ± 1.8 g in the Paigen diet-fed LDL-RD mice, 18.3 ± 1.3 g (P = ns) in the safflower oil-fed mouse and 18.9 ± 2.6 g (P = ns as compared to Paigen fed mice) in the chow-fed mice (Fig. 1B). No significant differences in body weights among the groups were noted throughout the course of either study.

3.2. Lipid analysis

3.2.1. Experiment 1

At sacrifice, total cholesterol levels in the Paigen diet-and safflower oil-fed mice did not differ significantly (mean ± SD; 130.3 ± 27.7 and 129.9 ± 38.3 mg/dl, respectively) and were higher than in the chow-fed mice (55.8 ± 11 mg/dl; P < 0.001) (Fig. 2A). Similarly, LDL + VLDL levels were higher in the Paigen diet-and safflower oil-fed mice (101.3 ± 25.3 and 96 ± 38 mg/dl, respectively) in comparison with the chow-fed mice (49 ± 7.1, P < 0.0001) (Fig. 2B).
HDL-cholesterol levels were reduced by the high fat diets: 29.1 ± 7.6 mg/dl for the Paigen diet-fed and 34 ± 12.6 mg/dl in the safflower oil-fed mice, as compared with 52.8 ± 8.7 mg/dl in the chow-fed mice at sacrifice (Fig. 2C).

Triglyceride levels were similarly reduced by the two high fat diets. At sacrifice, the levels measured were: 30.4 ± 6.7 mg/dl for the Paigen diet-fed mice, 37.2 ± 14.2 mg/dl for the safflower oil-fed and 53.6 ± 13.2 mg/dl for the chow-fed mice (Fig. 2D).

3.2.2. Experiment 2

The total cholesterol levels in safflower oil-fed mice (1994 ± 215 mg/dl) were significantly reduced in comparison with the Paigen diet-fed mice (2177 ± 257 mg/dl; P < 0.05) at sacrifice (Fig. 3A). A similar trend was observed in the values of LDL + VLDL: a reduction in the safflower oil group (1974 ± 217 mg/dl) as compared with the Paigen diet group (2177 ± 270 mg/dl; P < 0.05). The differences were also significant at the 2 week bleed (Fig. 3B).

No statistically significant differences were evident between the two high fat diet-fed groups with respect to HDL or triglyceride levels (Fig. 3C,D) and both were reduced in comparison with values obtained for the chow-fed mice.

3.3. LDL oxidation in vitro

The lag period for conjugated diene formation was found to be significantly reduced in LDL obtained from safflower oil-fed C57BL/6 mice, in comparison with that from Paigen diet-fed mice (mean ± SD; 68 ± 14 versus 97 ± 18 min; P < 0.002). Similarly, the lag period was shortened for the LDL from safflower oil-fed LDL-RD mice, as compared with those fed the Paigen diet (90 ± 22 versus 64 ± 17 min; P < 0.002) (Table 2). No statistically significant differences were evident between the $V_{\max}$ or the extent of total oxidation between the LDL obtained from the three experimental groups in the C57BL/6 or in the LDL-RD mice.

3.4. Atherosclerotic plaque formation

3.4.1. Experiment 1

There were overall significant differences among the three C57BL/6 groups in all three parameters of plaque formation measured (all: $P < 0.0001$). Pairwise com-

Fig. 3. Lipid profile in LDL-RD mice. Female LDL-RD mice were bled into tubes in the presence of EDTA at baseline, 2 and 6 weeks after consumption of one of the three diets for determination of (A) total cholesterol, (B) LDL + VLDL, (C) HDL, and (D) triglycerides. Data are presented as mean ± SD of all mice per group ($n =$ 30 for the Paigen-fed, $n =$ 26 for the Safflower-oil rich diet fed and $n =$ 20 for the chow-fed mice).
Table 2

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>110 ± 26</td>
<td>97 ± 18*</td>
<td>68 ± 14</td>
</tr>
<tr>
<td>LDL-RD</td>
<td>98 ± 31</td>
<td>90 ± 22*</td>
<td>64 ± 17</td>
</tr>
</tbody>
</table>

* Plasma was pooled from every three C57BL/6 mice (achieving a volume of 0.5 ml) and from every single LDL-RD mouse (a volume of 0.5 ml). Oxidability of the LDL (in a concentration of 50 μg/ml sample of LDL protein) from the experimental groups was expressed as the lag time in minutes for diene formation. The lag time was determined by monitoring the changes measured at 234 nm in absorbance, recorded every 10 min for 3 h [25]. The results are expressed as mean ± SD. The number in the parenthesis represents the number (pools of 3 C57BL/6 mice and single in the LDL-RD mice) of samples evaluated.

* P < 0.001 when compared to safflower oil-fed group.

Comparisons revealed that mice fed the Paigen diet or the safflower oil diet had significantly larger plaque area than those on the chow diet: at the cusp (mean ± SD; 13 500 ± 7400 and 12 200 ± 5300 μm² versus 600 ± 1000 μm²), at the wall (10 200 ± 1250 and 6600 ± 5700 μm² versus 200 ± 500 μm²) and with respect to total plaque (23 400 ± 17 500 and 18 600 ± 9000 μm² versus 800 ± 1300 μm²) (all P < 0.01) (Fig. 4). The lesions in safflower and Paigen-diet fed mice were principally fatty streaks.

### 3.4.2. Experiment 2

Pairwise comparison revealed that safflower oil-fed group was significantly lower than the Paigen diet-fed group as to the total plaque area (37 300 ± 19 4000 μm² versus 64 700 ± 27 700 μm²; P < 0.05). A reduction in plaque area was also evident in the safflower oil group, in comparison with the Paigen diet group, in the cusp (35% reduction) and the wall (52% reduction). Plaques in the safflower and Paigen-diet fed LDL-RD mice were larger than the ones observed in the respective groups in the C57BL/6 mice and contained more lipid filled cells (Figs. 5 and 6). Furthermore, some lesions in the LDL-RD mice appeared more complex and contained cholesterol clefts evidenced by the H&E staining.

### 4. Discussion

In the current study, we have shown that dietary consumption of n-6 polyunsaturated fat was associated with decreased atherosclerotic lesion formation in LDL-RD mice. A similar trend, although not reaching statistical significance, was obtained in the inbred C57BL/6 strain. This study is the first to analyze the effect of 18:2 supplementation on the blood lipid profile and atherosclerosis progression in mice.

![Fig. 4](image_url)

Aortic fatty streak accumulation in C57BL/6 mice. Upon sacrifice, hearts of all female C57BL/6 mice were embedded in OCT, frozen, and subsequently sectioned by a cryostat and stained by Oil red O for determination of fatty streaks. The extent of aortic atherosclerosis was determined at the level of the cusp (A), wall (B) and total (C), which included the sum of both (A + B). Data are presented as mean ± SD; n = 28 for the paigen-fed, n = 30 for the Safflower-oil rich diet fed and n = 23 for the chow-fed mice.

![Fig. 5](image_url)

The extent of aortic sinus atherosclerosis in LDL-RD mice. Six weeks after initiation of feeding with one of the three diets, LDL-RD mice were sacrificed and hearts removed for quantitation of atherosclerosis by staining of frozen sections with Oil red O. Data are presented as mean ± SD; n = 30 for the Paigen-fed, n = 26 for the Safflower-oil rich diet fed and n = 20 for the chow-fed mice.
Fig. 6. A representative lesion from a LDL-RD and a C57BL/6 mouse fed a Safflower diet. Representative Oil-red O staining of a characteristic frozen section of lesion from a Safflower diet-fed LDL-RD (A) or C57BL/6 (B) mouse, original magnification × 25.

The influence of dietary n-6 fatty acid was investigated in the African green monkey in a 5 year study [20], and it was found that animals fed polyunsaturated fatty acids had significantly less coronary atherosclerosis. This protective effect was observed despite a reduction in HDL levels, and was associated with a reduction in LDL levels and particle size. These observations were subsequently reinforced in the same animal strain, by the authors showing that dietary intervention early in life (from birth until young adulthood) decreased the development of atherosclerosis [28]. In this study, a positive correlation was also found between LDL concentration and particle size and the extent of atherosclerosis.

The mechanisms by which n-6 polyunsaturated fatty acids influence atherogenesis are still under investigation and conflicting results have been reported. It has been
shown that an increased percentage of 18:2 in LDL correlated strongly with the rate of conjugated diene formation in vitro and with macrophage uptake and degradation [4]. This effect, combined with the peroxidant properties of 18:2 and its endothelial-disrupting characteristics [5–16], may antagonize the beneficial effects of polyunsaturated fatty acids on the lipoprotein profiles.

Several characteristics of n-6 have been demonstrated which may indicate anti-atherogenic effects. For example, the proliferation of cultured human smooth muscle cells, incubated with human sera from subjects fed a n-6-rich, polyunsaturated fatty acid diet, was significantly reduced when compared with cells incubated with sera from subjects fed a diet rich in saturated fats [29]. Moreover, Manning et al. [30] have shown in cynomolgus monkeys that a safflower oil-enriched diet led to a modification in LDL properties resulting in decreased interaction between LDL and arterial proteoglycans, thus potentially reducing atherosclerosis progression.

In a recent study, Rudel et al. [31] have shown that increased dietary content of n-6 polyunsaturated fatty acids (primarily as linoleic acid) decreased atherosclerosis in the cynomolgus monkey, which is more atherosclerosis-susceptible than the African green monkey. Due to the more pronounced correlation between oleate enrichment of LDL and coronary atherosclerosis, these authors attributed a more important role for LDL composition in the pathogenesis of atherosclerosis.

In our study, which is the first to involve a small animal model, we have supported Rudel’s conclusion. The study was designed to include two mouse strains. The C57BL/6 mouse is the more susceptible to develop early atherosclerosis among the inbred mouse strains. Even so, the lesions induced after feeding a high-fat diet are early fatty streaks containing principally cholesterol-rich macrophages. The LDL-RD mice however, can be induced to develop larger and more mature lesions which may shed light on the pathogenesis of more complicated atherosclerotic plaques.

The C57BL/6 mice did not develop significantly less atherosclerosis with dietary n-6 fat enrichment, and LDL levels were similarly elevated in comparison with the saturated fat diet-fed mice. However, the safflower oil-fed LDL-RD mice, which developed significantly less aortic sinus atherosclerosis, also had lower levels of total cholesterol and LDL (although not to the same extent). The effect on atherogenesis in this mouse model of atherosclerosis occurred despite the slightly greater susceptibility of the LDL isolated from mice in this group to oxidation, as indicated by the shorter lag period for diene formation in vitro.

Several conclusions can be drawn from these results: (1) The extent of LDL-cholesterol reduction in the safflower-fed LDL-RD mice was not as significant as the extent of lesion size reduction in these mice. These observations clearly highlight the importance of additional effects of n-6 fatty acids on atherogenesis. (2) The effect of n-6 supplementation on the lipid composition may be genetically determined since the lipoprotein profile in both strains of mice in our study was differentially effected by the diet. (3) The lack of effect of n-6 on lipid composition in the C57BL/6 mouse in the face of enhanced susceptibility to oxidation suggests that properties related to oxidizability of LDL does not necessarily predispose to a more ‘efficient’ foam cell formation. In this context, other effects of n-6 on the cellular constituents of the atherosclerotic plaque may produce inhibitory influence on atherogenesis.

An important issue in this study is the cholic acid supplementation. Cholic acid has been shown to possess proinflammatory properties which may have an accelerating influence on atherosclerosis progression. To overcome this limitation, the safflower diet was made with the addition of cholic acid thus allowing for more accurate comparison between both high fat diet.

In conclusion, we have shown that dietary enrichment of polyunsaturated n-6 fatty acid decreases the serum concentration of LDL and is associated with a reduction in the extent of atherosclerotic plaque development in the atherosclerosis-susceptible LDL-RD but not in the C57BL/6 mouse strain.

References