Changes in Dietary Fat Intake Alter Plasma Levels of Oxidized Low-Density Lipoprotein and Lipoprotein(a)

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Objective—To assess the effects of dietary modifications on oxidized low-density lipoprotein (LDL).

Methods and Results—Thirty-seven healthy women were fed two diets. Both diets contained a reduced amount of total and saturated fat. In addition, one diet was low in vegetables and the other was high in vegetables, berries, and fruit. The dietary intake of total fat was 70 g per day at baseline and decreased to 56 g (low-fat, low-vegetable diet) and to 59 g (low-fat, high-vegetable diet). The saturated fat intake decreased from 28 g to 20 g and to 19 g, and the amount of polyunsaturated fat intake increased from 11 g to 13 g and to 19 g (baseline; low-fat, low-vegetable; low-fat, high-vegetable; respectively). The amount of oxidized LDL in plasma was determined as the content of oxidized phospholipid per ApoB-100 using a monoclonal antibody EO6 (OxLDL-EO6). The median plasma OxLDL-EO6 increased by 27% ($P<0.01$) in response to the low-fat, low-vegetable diet and 19% ($P<0.01$) in response to the low-fat, high-vegetable diet. Also, the Lp(a) concentration was increased by 7% ($P<0.01$) and 9% ($P=0.01$), respectively.

Conclusion—Alterations in the dietary fat intake resulted in increased plasma concentrations of lipoprotein(a) and OxLDL-EO6. **(*Arterioscler Thromb Vasc Biol*. 2004;24:498-503.)**

Key Words: antioxidants ■ intervention ■ lipoprotein (a) ■ oxidized low-density lipoprotein ■ polyunsaturated fat

Oxidized low-density lipoprotein (OxLDL) has an important role in atherogenesis, in particular by attracting monocytes into the vascular intima and transforming them into foam cells.[1] Many studies have supplied evidence that OxLDL is present in vivo. OxLDL is found in atherosclerotic lesions of humans and animals,[2,3] autoantibodies to OxLDL are present in plasma and lesions of humans and animals,[4,5] and small amounts of minimal OxLDL can be demonstrated in plasma.[6]

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There has been a vast amount of interest in pharmacological agents and antioxidants that decrease LDL oxidation in vitro[7–9] and decrease atherosclerosis in animal models in vivo.[10] The data in humans, primarily with vitamin E, however, have been disappointing, although the reasons for this have been recently discussed.[11] Interest has also been focused on non-pharmacological agents and antioxidants, such as natural foods, and their ability to affect LDL oxidation.[12,13] Data on the effects of whole diets with a high intake of fruit and vegetables on LDL oxidation are limited and conflicting.[14–16]

In the present study, we investigated how alterations in the dietary intakes of fat, vegetables, berries, and fruit affected plasma levels of antioxidants and OxLDL. We performed a crossover dietary intervention with two diets, both low in total dietary fat but one was low in vegetables and the other was high in vegetables, berries, and fruit, to find out whether a high intake of natural antioxidants influences the plasma levels of OxLDL.

Methods

Subjects

We interviewed and examined 86 women, among whom 37 healthy volunteers were selected and completed the study. The inclusion criteria were: (1) body mass index (BMI) 20 to 29 kg/m^2; (2) blood glucose 3.7 to 6.2 mmol/L; (3) plasma cholesterol <7.0 mmol/L; (4) plasma triglycerides <3.0 mmol/L; (5) non-smoking; (6) no gastrointestinal, renal, or hepatic diseases or food allergies; (7) no use of vitamins and/or minerals for at least 6 month before the study; (8) not pregnant or lactating. Alcohol intake was assessed by an interview and from the food records. Most subjects were teetotalers and others reported occasional moderate alcohol intake. Six participants used oral contraceptives, and 3 were using postmenopausal estrogen/progestin supplementation. The average age, height, and weight of the subjects were 43±12 years, 163±5 cm, and 63.6±6.4 kg, respectively. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu and followed the Declaration of Helsinki.

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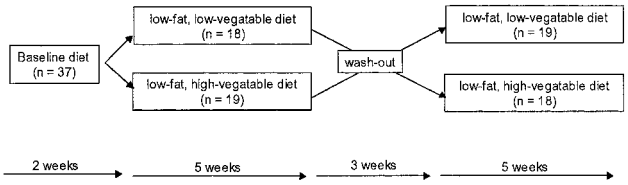

Figure 1. Design of the study.

Study Design

The study consisted of a baseline (2 weeks) and two 5-week study periods (a low-fat, low-vegetable diet and a low-fat, high-vegetable diet) with a washout period (3 weeks) in between (Figure 1). During the baseline and washout periods, the subjects followed their habitual diets.

Diets

All food, meals, and beverages during the intervention were provided by the hospital kitchen. The basic day's menu of both intervention diets included 8 portions of grain products, 3 to 4 portions of low-fat or fat-free dairy products, and 2 portions of lean meat, poultry, or fish. During the low-fat, low-vegetable diet, the subjects consumed 2 portions of both fruit and vegetables daily. On the low-fat, high-vegetable diet, they consumed 4 to 5 portions of fruit or berries and 5 to 6 portions of vegetables. The quantity and quality of dietary fat was controlled with low-fat or fat-free meat and dairy products, low-fat cooking methods, and vegetable oils and spreads. Rapeseed and sunflower oils were used during the low-fat, low-vegetable diet and the low-fat, high-vegetable diets, respectively. Soft vegetable oil spreads very low in trans-fatty acids were also used. To ensure the compliance, we provided the foods and meals and supervised the meals. Also, the subjects were weighed 5 times weekly and their energy intake was adjusted, when necessary, to maintain individual body weights. The subjects reported in writing any deviations in their diets. According to the surveyed meals and the subjects' reports, the subjects followed the diets without major exceptions. The changes in the plasma levels of carotenoids, vitamin C, and the changes in the blood folate concentrations[17] indicated that the compliance was good.

Plasma Lipids and Lipoprotein(a)

The total plasma triacylglycerol and cholesterol concentrations were determined enzymatically using Specific Clinical Chemistry Analyzer (kits by Boehringer Mannheim, GmbH, Germany). Heparin–manganese chloride precipitation of plasma was used to measure high-density lipoprotein (HDL) cholesterol.[18] LDL cholesterol concentration was calculated by the Friedewald formula.[19] Plasma Lp(a) concentrations were determined with a commercial enzyme-linked immunosorbent method (Biopool Ltd) according to the manufacture's instructions.

Autoantibodies to Oxidized LDL

The levels of IgM and IgG autoantibodies binding to copper OxLDL (CuOx-LDL) were determined in plasma samples by chemiluminescence-based ELISA.[20] CuOx-LDL was generated as described.[3] Antigens were added at 10 μg/mL in PBS-EDTA (PBS with 0.27 mmol/L EDTA, pH 7.5) and incubated overnight at 4°C. Plasma samples were diluted 1:1000 for IgM and 1:500 for IgG and the amount of immunoglobulin bound was detected with alkaline phosphatase-labeled goat anti-human IgM (Sigma) or goat anti-human IgG (Sigma) using Lumi-Phos 530 (Lumigen) as substrate. The luminescence was determined with a Victor^2 Luminometer (Wallac, PerkinElmer).

Sensitive CRP

Two different monoclonal mouse anti-human CRP antibodies (HyTest Ltd) and purified human CRP (HyTest Ltd) were used to measure sensitive CRP. Briefly, anti-human CRP antibody was coated at 2 μg/mL in PBS buffer overnight at 4°C. Plasma samples were diluted 1:2000 and the amount of CRP bound was detected with biotinylated anti-human CRP antibody. A commercial control CRP sample (Roche) was included in each assay.

Oxidized LDL

A sandwich chemiluminescent immunoassay using a well characterized murine monoclonal antibody EO6, which specifically binds to oxidized phospholipids, was used to measure plasma oxidized LDL.[20,21] First, a monoclonal anti-apolipoprotein B-100 antibody, MB47, was plated at 5 μg/mL overnight at 4°C. The plasma samples were diluted to 1:50 and the amount of oxidized phospholipid epitopes was measured with biotinylated antibody EO6. In parallel wells, the amount of LDL bound was detected with polyclonal biotinylated anti-apoB antibody. The results are expressed as amount of EO6 bound divided by the amount of anti-apoB bound into the wells, yielding the relative amount of oxidized phospholipid detected by EO6/apoB-100 (OxLDL-EO6). This measurement normalizes the oxidized phospholipid content per apoB-100 particle.

Analysis of Plasma Tocopherols, Carotenoids, and Vitamin C

Plasma concentrations of tocopherols[22] and carotenoids[23] were analyzed by HPLC. Total ascorbic acid was determined with an automated fluorometric method using orthophenylenediamine.[24] For this measurement, 4.5 mL of 5% metaphosphoric acid was added to 0.5 mL of plasma within 1 hour after venipuncture and stored at −70°C.

Dietary Analyses

The baseline dietary intakes were calculated from 4-day food records using the Nutrica software (Social Insurance Institution, Helsinki, Finland) based on the Finnish nutrient database. The nutrient contents of the intervention diets were analyzed at the Agricultural Research Center of Finland. The food portions of both diets were collected daily during the intervention at one energy level (7.5 MJ) and stored at −20°C until analysis. Before analysis, the samples were thawed and pooled for each diet period. The analysis included total energy, total fat, carbohydrate, fiber, fatty acids,[25] dietary cholesterol, potassium, sodium, calcium, iron, α-carotene,[26] β-carotene,[26] ascorbic acid (vitamin C),[27] and α-tocopherol.[28]

Statistical Analyses

The Saphiro-Wilk test was used to test the skewness of the distributions. Plasma carotenoids, triglycerides, OxLDL-EO6, sensitive CRP, and Lp(a) were not normally distributed; therefore, non-parametric tests were used. The differences were first tested by Friedman test for repeated measurements, and further by Wilcoxon signed rank test. The variables are expressed as medians with the lowest and highest quartiles. Total cholesterol, LDL cholesterol, HDL cholesterol, vitamin C, and vitamin E, which were normally distributed, were tested by Student t test and are expressed as mean±SD. Correlations were determined using Spearman and Pearson correlation coefficients. All the differences were considered significant at a 5% level. The SPSS computer program (9.0; SPSS Inc) was used in the statistical analyses.

Results

The Nutrient Contents of the Diets

The major differences between the baseline diet and the study diets were in the quantity and quality of dietary fat and in the amount of vegetables, berries, and fruit consumed. Both study diets were lower in total fat than the baseline diet (Table). In addition, there were slight changes in the quality of dietary fat between the diet periods. The amounts of saturated fatty acids (SAFA) and monounsaturated fatty acids were lower in both study diets compared with the baseline diets of the subjects.

Average Daily Nutrient Intake of the Women (n=37) at Baseline Period and the Average Nutrient Contents of the Study Diets

	Baseline	Low-Fat, Low-Vegetable Diet	Low-Fat, High-Vegetable Diet
Carbohydrate (E%)*	46±7	49	50
Protein (E%)	17±2	20	20
Total fat (E%)	36±6	31	31
Saturated fat (E%)	15±3	11	9.5
Monounsaturated fat (E%)	14±3	13	11
Polyunsaturated fat (E%)	6±1	7	9.5
Dietary fiber (g)	21±6	25	40
Calcium (mg)	1086±326	1210	1280
Potassium (mg)	3538±640	4200	5720
Iron (mg)	11±3	10.0	13.5
Carotenoids (mg)	3.4±2.3	4.6	18
Vitamin C (mg)	128±60	147	430
Vitamin E (mg)	10±4	8	17

The intakes at baseline are calculated from 4-day food records. The nutrient contents of the low-fat, low-vegetable and low-fat, high-vegetable diets are analyzed from the identical food portions. Values are mean±SD for calculated nutrients and the average daily intakes for analyzed nutrients.

*E% indicates percent of total energy intake.

The amount of polyunsaturated fatty acids (PUFA) increased, particularly in response to the low-fat, high-vegetable diet (Table).

Body Mass Index

The subjects' average BMI was 23.7±2.2 kg/m^2 at baseline. At the end of the low-fat, low-vegetable diet and the low-fat, high-vegetable diet, the average BMI of the subjects was 23.3±3.2 kg/m^2 and 23.3±3.3 kg/m^2, respectively.

Plasma Lipids

Plasma total cholesterol and triglyceride and the HDL cholesterol levels slightly decreased in response to the low-fat, high-vegetable diet (Figure 2). Interestingly, the low-fat, low-vegetable diet did not affect the total cholesterol levels but decreased the plasma total triglyceride and HDL cholesterol levels.

Plasma Antioxidant Levels

Plasma levels of several antioxidants increased in response to the low-fat, high-vegetable diet (Figure 3). The plasma levels of α-carotene, β-carotene, β-cryptoxanthin and lutein–zeaxanthin increased >2-fold during the low-fat, high-vegetable diet compared with the baseline diet (Figure 3). The low-fat, low-vegetable diet, however, contained quite similar amounts of antioxidants compared with the normal everyday diet of the subjects; therefore, less changes occurred in plasma antioxidant levels between the baseline and low-fat, low-vegetable diet.

At baseline, the plasma lutein–zeaxanthin concentration was correlated with the plasma OxLDL (r=0.44, P<0.05) and with the plasma Lp(a) (r=0.33, P<0.05). The basal plasma β-carotene was negatively correlated with the basal-sensitive CRP (r=−0.39, P<0.05). No other correlations were found between the basal plasma antioxidants and autoantibody titers to OxLDL, sensitive CRP level, OxLDL-EO6, or Lp(a) at the baseline.

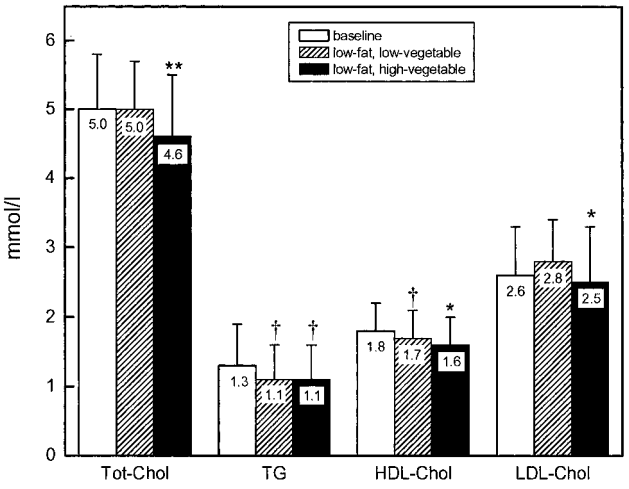

Figure 2. Plasma concentrations of total, HDL, and LDL cholesterol and triglycerides of all the study subjects (n=37) during the study diets. Tot-Chol indicates total cholesterol; TG, triglycerides; HDL-Chol, HDL cholesterol; LDL-Chol, LDL cholesterol. *P<0.05, **P<0.001; statistical significance of the difference between the low-fat, low-vegetable diet and the low-fat, high-vegetable diet (Student t test for paired samples and Wilcoxon signed ranks test). †P<0.05; statistical significance of the difference between the study diet and the baseline diet (Student t test for paired samples and Wilcoxon signed ranks test).

Autoantibody Titers to Oxidized LDL and Sensitive CRP

There were no significant changes in IgM or IgG autoantibody titers to OxLDL or sensitive CRP levels during the intervention (Table I, available online at http://atvb.ahajournals.org).

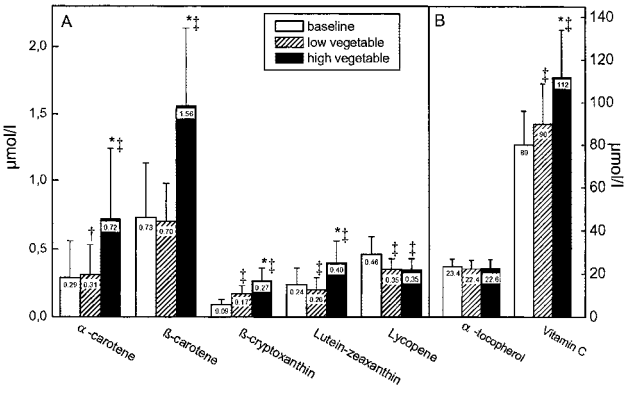

Figure 3. Plasma concentrations of carotenoids, vitamin C, and alpha-tocopherol of all the study subjects (n=37) during the study diets. Values are mean±SD. *P<0.001; statistical significance for the difference between the low-fat, low-vegetable diet and the low-fat, high-vegetable diet (Wilcoxon signed ranks test and Student t test for paired samples). †P<0.01; statistical significance of the difference between the baseline diet period and the study diet period (Wilcoxon signed ranks test). ‡P<0.001; statistical significance of the difference between the baseline diet period and the study diet period (Wilcoxon signed ranks test).

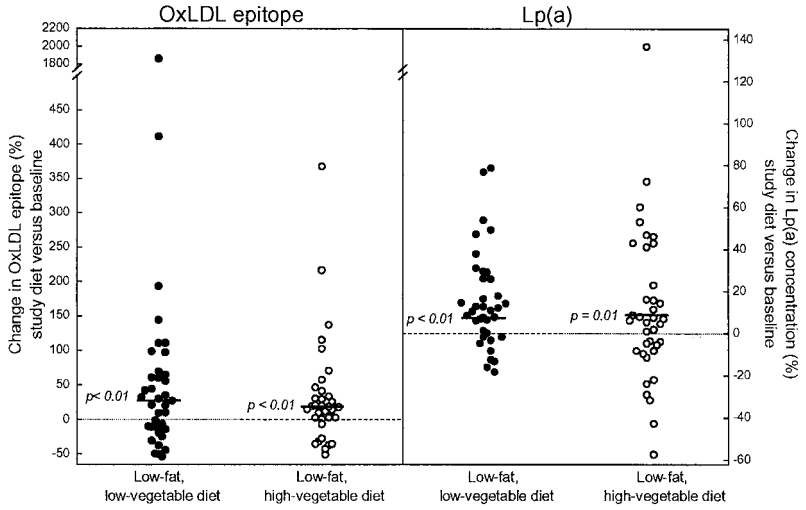

Figure 4. Relative median changes in the plasma concentrations of oxidized LDL (left) and lipoprotein(a) (right) of all the study subjects (n=37) from baseline to low-fat, low-vegetable diet and from baseline to the low-fat, high-vegetable diet.

Both Study Diets Increased Oxidized LDL and Lp(a) Levels

Oxidized epitopes in circulating LDL can be measured with a sensitive chemiluminescent capture immunoassay.[20,21] We used monoclonal antibody EO6-detecting oxidized phospholipid epitopes to assess if the study diets would influence the amount of oxidized epitopes in the apoB-100 particles of the subjects. Compared with the baseline levels, the median plasma OxLDL-EO6 increased by 27% (Q1, Q4: −14, 67) ($P<0.01$) in response to the low-fat, low-vegetable diet and by 19% (Q1, Q4: 1, 44) ($P<0.01$) in response to the low-fat, high-vegetable diet (Figure 4, left).

OxLDL-EO6 levels correlate strongly with the plasma Lp(a) level.[21] Although plasma levels of Lp(a) are considered to be regulated mainly by genetic factors, we explored the possibility of whether dietary factors influence the Lp(a) levels. Indeed, both study diets increased plasma Lp(a) concentration (Figure 4, right). The median plasma Lp(a) concentration of the subjects was 113 mg/L at baseline. The median plasma Lp(a) concentration increased by 7% (Q1, Q4: −4, 34) ($P<0.01$) when the subjects consumed the low-fat, low-vegetable diet compared with baseline. At the end of the low-fat, high-vegetable diet, the median plasma Lp(a) concentration was 9% (Q1, Q4: −4, 24) higher ($P=0.01$) compared with the baseline. There were no differences in the plasma Lp(a) concentrations between the intervention diets ($P=0.48$).

The baseline plasma OxLDL-EO6 correlated with the plasma Lp(a) concentration ($r=0.94$, $P<0.001$) (Figure I, available online at http://atvb.ahajournals.org). There were also strong correlations between the plasma OxLDL-EO6 and Lp(a) on the low-fat, low-vegetable diet ($r=0.97$, $P<0.001$) and on the low-fat, high-vegetable diet ($r=0.96$, $P<0.001$). The relative changes of plasma OxLDL-EO6 and plasma Lp(a) correlated as well ($r=0.65$, $P<0.001$; the change from the baseline to the low-fat, low-vegetable diet) ($r=0.38$; $P<0.05$; the change from the baseline to the low-fat, high-vegetable diet). The changes of plasma OxLDL-EO6 and plasma Lp(a) did not correlate with the changes of plasma antioxidants.

Discussion

The major, and unexpected, findings of the present study were that dietary changes altered the plasma concentrations of OxLDL-EO6 and lipoprotein(a). Our subjects consumed diets containing decreased amounts of total and saturated fat. The study protocol used a randomized crossover design for each of the study diets, which further adds confidence to the main findings that plasma levels of OxLDL-EO6 and Lp(a) were significantly increased on the low-fat diets, compared with the levels on their normal “everyday” diet. Although the amounts of vegetables, berries, and fruits in the diet significantly influenced the plasma antioxidant concentrations, it seemed that they did not affect the amount of OxLDL-EO6 and Lp(a) in the circulation.

The oxidative modification of LDL is one of the key events in early atherogenesis, both directly contributing to cholesterol accumulation in arterial wall macrophages and in promoting pro-inflammatory events that accelerate lesion development.[29] For this reason, factors influencing the oxidative susceptibility of LDL have been the subject of numerous investigations. One line of studies has looked into various antioxidant compounds. In fact, numerous animal studies have demonstrated that dietary supplementation with antioxidant compounds, such as probucol, vitamin E, coenzyme Q, diphenylphenylenediamine, or butylated hydroxytoluene, ameliorates progression of atherosclerosis.[11] In humans, however, the dietary antioxidant supplementation has not been proven to be protective. Large-scale placebo-controlled clinical trials in humans have shown that in general, supplementation with vitamin E, β-carotene, or various combinations of antioxidants has not reduced the risk of coronary artery disease.[30–33] Reasons for the negative results of these trials have been recently discussed.[11] One possibility is that the antioxidants used in these high-risk patients did not contain sufficient antioxidant activity. Another possibility is that because it is still not known with certainty how LDL oxidation occurs in vivo, the human trials may have used wrong antioxidants. Therefore, one of our goals in the present study was to assess the influence of a diet rich in berries, fruit, and vegetables that contains high amounts of several natu-

rally occurring antioxidants on plasma antioxidant levels and also on LDL oxidation.

Another line of studies searching for factors that influence the oxidative susceptibility of LDL have been those looking into dietary fat. Diets high in SAFA are known to increase total plasma cholesterol levels, whereas diets high in PUFA have a mostly favorable effect on plasma lipid profiles: a decrease in total and LDL cholesterol levels and also a decrease in HDL cholesterol levels.[34] For example, when linoleic acid replaces SAFA in the diet, it lowers serum cholesterol levels.[35] Despite this favorable effect of unsaturated fat on lipid profiles, concern exists that such diets could increase the susceptibility of LDL to oxidation, thus negating some of their cardioprotective effects. Previous studies show that diets high in linoleic acid lead to LDL particles enriched with linoleic acid, which are thought to be more susceptible to lipid peroxidation and also more atherogenic.[36] In our study, when the subjects consumed the study diets, the intake of total fat was reduced and SAFA was partly replaced by PUFA compared with the baseline diet. The amount of PUFA was particularly increased in response to the low-fat, high-vegetable diet.

Surprisingly, we found that in response to the low-fat, low-vegetable and the low-fat, high-vegetable diets, the average plasma levels of OxLDL-EO6 increased compared with the baseline diet. Because we expressed the oxidized phospholipids detected per apoB-100 particle, this measurement is independent of LDL values. However, because the absolute LDL levels were only minimally affected in response to the low-fat diets (Figure 2), the absolute number of apoB-100 particles did not change; thus, there was a net increase in the total content of oxidized phospholipid associated with apoB. In our experiments, we did not investigate the ability of the LDL particles to "resist" oxidation, such as by measurements of lag phase of conjugated diene formation when isolated LDL is exposed to copper ions. Instead, we measured the amount of oxidized phospholipid epitopes on the LDL particles assayed directly from the plasma samples with a sensitive immunoassay using a natural autoantibody EO6. This antibody was cloned from apo $E^{-/-}$ mice and it binds to oxidized phospholipid containing the phosphorylcholine head group, but not to native unoxidized phospholipids, even though they contain the PC head group.[20] Oxidized phospholipids are a prominent component of oxidized LDL and have been demonstrated to have many pro-inflammatory and pro-atherogenic properties.[37] Compared with the baseline diet, the plasma concentration of OxLDL-EO6 in the present study was equally elevated during the low-fat, low-vegetable diet and the low-fat, high-vegetable diet, yet plasma antioxidant levels were increased only during the low-fat, high-vegetable diet. Thus, the explanation for the increased OxLDL-EO6 may be found from dietary factors other than antioxidants of the intervention diets.

In addition to the increased concentration of OxLDL-EO6, the plasma Lp(a) concentration increased by 7% and 9% in response to the low-fat, low-vegetable and low-fat, high-vegetable diets, respectively, compared with the baseline. Lp(a) is believed to be an independent risk factor for CAD;[38] therefore, the factors affecting its concentrations are of major importance. The diets had no significant effect on levels of apoB-100 particles, VLDL, and LDL but surprisingly caused an increase in plasma levels of Lp(a). In a recent study, Tsimikas et al showed that a majority of the OxLDL-EO6 measured in human plasma is associated with Lp(a).[21] They also suggested that Lp(a) may act as a preferential acceptor that binds tightly oxidized phospholipids transferred from tissues or other lipoproteins.[21] In that report, the correlation between OxLDL-EO6 and Lp(a) was 0.91 ($P<0.0001$). This association was also very clear in our results. The correlation between OxLDL-EO6 and Lp(a) was as high as 0.97 ($P<0.001$). Therefore, we suggest that the most likely reason for the increase in OxLDL-EO6 in response to the diets was that the Lp(a) levels increased. If Lp(a) acts as a plasma transporter of oxidized phospholipids, it can be hypothesized that both low-fat diets resulted in favorable changes in the artery wall or other tissues that decreased oxidation. Increased Lp(a) would result in increased transport of oxidized phospholipid away from tissues, such as the artery wall. Our data suggest that Lp(a) levels are sensitive to these dietary changes and increase in plasma as a consequence of yet undefined mechanisms. In turn, Lp(a) binds the oxidized phospholipids that are being mobilized, which is reflected in the enhanced OxLDL-EO6 levels in plasma. Thus, it can be postulated that with respect to this parameter, both of these diets are beneficial. Support for this hypothesis can be found in our recent observation that in response to statin therapy in humans, a similar increase in OxLDL-EO6 occurs, possibly reflecting enhanced mobilization of oxidized phospholipids from the artery wall.[39]

The question remains as to why the Lp(a) levels increased in response to the dietary changes. The basal levels of Lp(a) are primarily genetically determined, but some data suggest that Lp(a) may act as an acute-phase reactant under some situations.[40] In a previous study, a diet high in SAFA was found to produce approximately 10% lower plasma Lp(a) concentration than diets high in oleic acid or trans-fatty acids.[41] This observation is consistent with our study in that both diets led to lower SAFA and consequently increased Lp(a).

In conclusion, we found that a diet traditionally considered to be anti-atherogenic (low in saturated fat and high in polyunsaturated fat and naturally occurring antioxidants) increased plasma levels of circulating oxidized LDL and Lp(a). The question of whether the changes observed in the present study are, in fact, pro-atherogenic or anti-atherogenic remains to be solved.

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