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A high linoleic acid diet increases oxidative stress in vivo and affects nitric oxide metabolism in humans

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Summary Evidence from in vitro studies shows that increased intake of polyunsaturated fatty acids leads to increased oxidative stress, which may be associated with endothelial damage. We measured the urinary levels of 8-iso-$PGF_{2\alpha}$ and nitric oxide metabolites as well as plasma sICAM-1 levels from healthy subjects after strictly controlled diets rich in either linoleic acid (LA, C18:2 n-6) or oleic acid (OA, C18:1 n-9).

Thirty-eight volunteers (20 women and 18 men, mean age 27 years) consumed a baseline diet rich in saturated fatty acids (SFA) for 4 weeks and were then switched to either a high LA diet (11.5 en%) or a high OA diet (18.0 en%) also for 4 weeks. During the LA and OA diets, nearly all food was provided for the whole day. A control group of 13 subjects consumed their habitual diet throughout the study.

Urinary excretion of 8-iso-$PGF_{2\alpha}$ was significantly increased after the LA diet (170 vs 241 ng/mmol creatinine, $P=0.04$), whereas the urinary concentration of nitric oxide metabolites decreased (4.2 vs 2.6 mg/mmol creatinine, $P=0.03$). No significant changes were seen in the OA group. Significant differences between the LA and control group were found for both 8-oxo-$PGF_{2\alpha}$ ($P=0.03$) and NO ($P=0.02$), whereas the OA and LA groups did not differ with respect to any parameter. Also plasma sICAM-1 remained unchanged in both groups throughout the study. In conclusion, the high-LA diet increased oxidative stress and affected endothelial function in a way which may in the long-term predispose to endothelial dysfunction.

INTRODUCTION

Oxidative stress is considered to be involved in the initial stages of atherosclerosis. Generation of oxidized LDL (ox-LDL), a proatherogenic particle, is generally accepted to have a role in early lesion formation and adversely affect the arterial wall. A hypothesis based on in vitro findings suggests that polyunsaturated fatty acids are a potential source of oxidative stress in humans. Diets enriched with linoleic acid (LA) lead to LA-enriched LDL particles, which in turn have been shown to be more susceptible to oxidation.[1,2] In another study, the formation of DNA adducts was significantly increased after a high-LA diet in women, indicating oxidative damage.[3] Results from in vivo studies on oxidative injury are in general controversial, mainly due to the shortcomings with methods used to measure in vivo lipid peroxidation.

Endothelial-derived nitric oxide (NO) can potentially inhibit several components of the atherogenic process, including the oxidative modification of LDL.[4] Oxidized LDL, on the other hand, has been shown to have an inhibitory effect on NO production.[5–7] To date, few studies have addressed the issue of dietary factors and NO. Yet, there is evidence that dietary n-3 fatty acids enhance NO production in humans.[8–10]

Atherogenesis requires the adhesion of circulating leucocytes to the endothelium and their subsequent migration into the subendothelium. The process is regulated by the endothelial cell adhesion molecules, e.g. ICAM-1, VCAM-1 and E-selectin.[11] Circulating levels of these molecules are raised during various disease processes.[12] Endothelial nuclear factor-κB (NF-κB) is an inducible transcription factor which specifically activates transcription of cell adhesion molecules in endothelial

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cells. Activation of NF-κB in vitro is significantly enhanced by LA.[13]

To study whether high-LA diets cause oxidative stress in a way that affects endothelial function, we measured the urinary excretion of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), plasma soluble ICAM levels and urinary nitric oxide metabolites from healthy subjects participating a controlled dietary intervention with either high-LA or high-oleic acid (OA) diets.

PATIENTS AND METHODS

Subjects and study design

Thirty-eight healthy volunteers, 20 women and 18 men, aged 20–48 years (mean 26.6 years) were selected from university students and employees. Exclusion criteria were serum cholesterol >7.0 mmol/L, hypertension, anemia, glycosuria and proteinuria. Only subjects who agreed to refrain from ingestion of aspirin or aspirin-like drugs during the study period were considered eligible for the study. The subjects were allocated into two groups pairwise according to the concentration of an indicator of platelet activation (urinary 2,3-dinor-thromboxane B_2) known to have a high interindividual variation, menstrual cycle and sex. The baseline serum cholesterol and triglyceride concentrations were similar in both groups. All subjects were asked to maintain their smoking habits, alcohol consumption and physical activity. Apart from free food, they received no payment. Furthermore, 13 control subjects were selected to the study. They had their habitual diets throughout the study but gave the same samples as the participating subjects.

The experiment lasted 8 weeks. During the first 4 weeks, the subjects were advised to use butter and other foods rich in dairy fat. The purpose of this period was to stabilize the baseline plasma values. For the second 4 weeks, 19 subjects were given a high-LA diet and 19 subjects a high-OA diet in a controlled manner.[14] The two diets were designed to contain similar proportions of energy from fats, carbohydrates and proteins, similar proportions of saturated fatty acids (SFA) and similar amounts of cholesterol. They differed only in their proportions of LA and OA.

The edible oils mainly used as margarines and on bread, in bakery products, desserts and in cooking were high-LA sunflower oil and high-OA Trisu® sunflower oil.

On weekdays, the subjects lunched at the Division of Nutrition, where they also received take-away food for the evening and the following morning. Food for the whole weekend was provided on Fridays. All food was weighed for each participant. In addition, the subjects chose 10% of their energy intake from foods free of fat and cholesterol. They recorded in diaries the freely chosen foods (including alcohol) and all leftover food. The subjects were weighed twice a week and energy intake was adjusted to avoid weight changes. Duplicate portions of each diet were collected daily, pooled for each period and analyzed. The analyzed values and those calculated from free-choice and leftover diaries were combined giving the final composition of the diets (Table 1).

Blood and urine sampling

Venous blood samples were collected during the last week of the baseline and the experimental diet periods with minimal stasis from fasting (>12 h) subjects in the morning (between 07:30 and 09:30) after 10 min rest. The subjects had abstained from alcohol and heavy physical activity (for 24 h) before blood sampling. Siliconized, evacuated EDTA tubes and 20-gauge needles were used. Plasma was separated and stored at −70°C.

At the end of each diet period, the subjects and controls collected five 24 h urine samples using a Japanese aliquot cup (KK Izumi Seisakusyo Co. Ltd., Japan). The urine was carefully mixed and frozen in aliquots at −20°C.

Analytical methods

Percentual distribution of methylated plasma fatty acids was determined by gas chromatography as described previously.[15] Plasma samples for the analysis of α-tocopherol (α-T) were extracted as described previously[16] and thereafter analyzed by HPLC.

The urinary excretion of 8-iso-$PGF_{2\alpha}$ was measured from three 24 h urine samples per diet period using a newly developed radioimmunoassay.[17] The samples from

Table 1 Mean daily intake of energy and nutrients according to duplicate-portion analysis plus calculated contribution of freely selected items

Nutrient	OA-diet	LA-diet
Energy (MJ)	10.8	10.9
Protein (en%)*	14.2	13.8
Fat (en%)	33.4	34.4
SFA (en%)†	10.0	10.6
MUFA (en%)†	18.7	11.4
Oleic acid (C18:1)	18.0	10.7
PUFA (en%)†	4.6	12.3
Linoleic acid (C18:2)	3.8	11.5
Carbohydrates (en%)‡	52.4	51.8
Cholesterol (mg/day)§	303	305
Vitamin C (mg/day)§	129	132
Vitamin E (mg/day)§	12.5	16.1

*en% = percent of total energy; †SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids; ‡calculated as the difference between total energy intake and energy intake from protein, fat and alcohol; §calculated

each period were pooled and unextracted urine of 0.1 ml was used in the assay. In brief, an antibody was raised in rabbits by immunization with 8-iso-$PGF_{2\alpha}$ coupled to BSA at the carboxylic acid. The cross-reactivity of the 8-iso-$PGF_{2\alpha}$ antibody with 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, TXB_2, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$ was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%, respectively. The detection limit of the assay was about 8 pg/ml.

Plasma concentrations of soluble ICAM-1 were measured with an ELISA technique using a human soluble ICAM-1 immunoassay (R & D systems, Minneapolis, USA). A nitrate/nitrite immunoassay (Cayman Co, USA) based on the Griess reaction was used for the measurement of urinary NO metabolite concentrations from pooled urine samples of two days. In this assay, nitrate is converted to nitrite utilizing nitrate reductase and with the addition of the Griess reagent[18] further into an azo compound. The results for 8-iso-$PGF_{2\alpha}$ and NO metabolites were corrected for creatinine, analyzed with a commercial kit (Merck), and presented as ng/μmol creatinine. All analyses were performed in duplicate (α-T in triplicate) in one series for each subject.

Statistics

Differences from baseline to experimental diets were analyzed with paired *t*-tests. Differences between experimental diets were analyzed with the General Linear Models procedure of the Systat program[19] using values at the end of the experimental periods as dependent variables and treatment and baseline values as covariates.

Ethics

The protocol and the aims of the study were explained to the subjects, who gave their written consent. The research protocol was approved by the Ethic Committee of the University of Helsinki. The participants and laboratory staff were blinded to the subject grouping.

RESULTS

The body weight of the subjects was monitored twice a week and it remained unchanged during both diets. Dietary intake was checked by analyzing the energy and nutrient content of the pooled diets. Intake of energy, total fat, SFA, carbohydrates and protein was comparable in the experimental diets. The high-LA diet contained 11.5 and 10.7 en% LA and OA, and the high-OA diet 3.8 and 18.0 en% LA and OA, respectively.

Dietary compliance was also monitored by the plasma fatty acid profile. During the high-LA diet the proportion of OA decreased from 22 to 18% ($P=0.002$) and that of LA increased from 29 to 39% ($P<0.001$) of total fatty acids. The change from baseline diet to the high-OA diet increased the proportion of OA from 23 to 25% ($P=0.003$) of total fatty acids, while the proportion of LA did not change (31% of total fatty acids).

The LA diet significantly increased the excretion of 8-iso-$PGF_{2\alpha}$ (from 170 to 241 ng/mmol creatinine, $P=0.04$) and decreased levels of urinary NO metabolites (4.2 vs 2.6 mg/mmol creatinine, $P=0.03$). No significant changes were found after the OA diet. In the control group the excretion of nitrate slightly increased ($P=0.05$) during the study (Table 2) and a significant difference between the controls and the LA diet group was found both for 8-oxo-$PGF_{2\alpha}$ ($P=0.03$) and NO metabolites ($P=0.02$). The OA and LA groups did not, however, differ with respect to any parameter.

The plasma concentration of sICAM-1 and α-T stayed unaltered both during the dietary treatments and in the control group (Table 2).

DISCUSSION

The possibility arose by in vitro experiments that a high intake of LA would increase oxidative stress in the body is supported by the results of our strictly controlled human experiment: urinary excretion of 8-iso-$PGF_{2\alpha}$ was significantly increased after the high-LA diet, whereas excretion

Table 2 Plasma α-tocopherol, plasma soluble ICAM-1, urinary 8-iso-$PGF_{2\alpha}$ and urinary nitrate metabolites of 38 subjects and 13 controls who consumed high-linoleic acid (LA) or high-oleic acid (OA) diets after a saturated fat baseline diet

	LA-group (n = 19)		OA-group (n = 19)		Controls (n = 13)	
	Baseline	Experimental	Baseline	Experimental	Baseline	Experimental
α-tocopherol (mg/L)	10.1 (2.2)	10.6 (2.8)	10.0 (0.9)	10.3 (2.2)	10.5 (3.4)	10.2 (2.6)
8-iso-$PGF_{2\alpha}$ (mg/mmol creatinine)	170 (100)	241 (117)*	147 (73)	184 (86)	160 (90)	127 (62)
NO metabolites (mg/mmol creatinine)	4.2 (4.1)	2.6 (2.3)*	4.7 (3.2)	3.9 (3.8)	3.1(2.5)	5.8 (4.6)†
sICAM-1 (ng/mL)	288 (102)	277 (91)	297 (83)	294 (92)	305 (89)	298 (67)

Values are mean (SD); *$P<0.05$, †$P=0.05$ denote significance of difference from the preceding baseline diet.

of urinary NO metabolites was decreased, although the intake of antioxidants and plasma levels of α-tocopherol of our subjects were well above recommendations.[20] The LA diet also differed from the control group with respect to both 8-oxo-$PGF_{2\alpha}$ and excretion of NO metabolites. We could not, however, show a difference between the OA and LA diets.

The measurement of prostaglandin F_2-like compounds (F_2 isoprostanes), which are produced in vivo by a non-cyclooxygenase mediated pathway involving free radical-catalyzed peroxidation of arachidonic acid, are considered to be an important advancement in assessing in vivo lipid peroxidation.[21] Isoprostanes are stable molecules that can be detected in most biological fluids of healthy subjects.[21,22] Evidence from both animal and human studies shows that the production of these compounds increases markedly in situations associated with oxidative stress.[23–25]

Furthermore, elevated levels have been found in patients with hypercholesterolemia[26] and in situ in atherosclerotic tissue and circulating LDL.[27] In vitro oxidation of human LDL was also associated with parallel formation of lipid hydroperoxides and F_2-isoprostanes after depletion of α-tocopherol and ubiquinol-10.[28,29] Although increased intake of LA has been associated with increased susceptibility of LDL to oxidation in vitro, compelling evidence that this occurs in vivo is lacking. The present results are strong indications of increased in vivo lipid peroxidation caused by a high-LA diet.

Oxidation products may have detrimental effects on the vascular endothelium. Endothelium-dependent vasodilation, mediated mainly by NO, is impaired in atherosclerosis and hypercholesterolemia. Although normal or elevated NO synthesis has been found in atherosclerotic and hypercholesterolemic arteries,[6] its biological activity seems to be attenuated.[30] A role for ox-LDL as scavenging NO before it reaches its target has been suggested.[7] The possible mechanisms by which ox-LDL could exert its action include a direct reaction with nitric oxide or a stimulatory effect on NO scavengers.[21] Whether the decrease in urinary NO metabolites in the present study was due to an increase in reactive oxygen metabolites capable of inhibiting NO cannot be answered. Dysfunction of the endothelium is unlikely due to the short duration of the study and the health status of our subjects. This is supported by the unchanged sICAM levels. Increased levels of circulating ICAM-1 have been found in several inflammatory diseases involving leucocyte activation, e.g. in diabetes, impaired renal function and septic shock.[12]

Previous data on the effects of n-6 PUFA on cell adhesion molecule expression and NO production is scarce, whereas n-3 PUFA have been shown to affect both. In vitro studies have demonstrated attenuated induction of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin by n-3 PUFA in human vein endothelial cell cultures.[32,33] Recent results with rabbits suggest that OA might also inhibit VCAM-1 expression in vivo.[34] The possibility of a protective effect of monounsaturated OA was not supported by our findings: ICAM-1 remained unchanged.

Several studies have shown enhanced production of NO in humans after supplementation with n-3 PUFA,[8–10] although suppressed synthesis has also been found.[35] In diabetic subjects, fish oil supplementation enhanced NO production compared with matching olive oil capsules or baseline values,[9] indicating different effects of n-3 PUFA and MUFA. However, most of these studies have analyzed NO production of stimulated cells, whereas our results reflect changes in basal synthesis. Also control of diet is necessary as food is a significant contributor to body nitrate levels. Our strict study design allows us to conclude that NO synthesis may be modulated by dietary means to a significant degree in healthy subjects, devoid of any signs of vascular disease.

In conclusion, our results indicate that both the synthesis of F_2-isoprostanes and NO may be modulated by dietary fatty acid composition. Significant evidence of oxidative stress in vivo, caused by a high-LA diet, was observed in these healthy subjects although their intake and plasma levels of antioxidants were within recommendations. The oxidative stress was accompanied by decreased excretion of NO, probably as a result of scavenging of the oxidation products. Although endothelial dysfuction after this short period is unlikely, even a slight decrease in NO for a longer period may be of consequence to human health.

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