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## Enhanced Susceptibility to in Vitro Oxidation of Apolipoprotein B-Containing Lipoproteins and Antioxidant Status in Patients with Coronary Artery Disease

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**Abstract:** Lipoprotein oxidation may play a pivotal role in the pathogenesis of atherosclerosis, and vitamin E and  $\beta$ -carotene have been proposed to prevent the development and progression of atherosclerosis.

The aim of this study was to investigate the oxidative susceptibility of apolipoprotein B (apo B)-containing lipoproteins and their relation with vitamin E and total carotene levels.

The susceptibility of apo B-containing lipoproteins to oxidation, plasma malondialdehyde (MDA), serum vitamin E, total carotene, lipid and lipoprotein levels were studied in 177 patients with coronary artery disease (CAD) and 75 non-CAD subjects. All subjects (n = 252) had undergone coronary catheterization. Student's unpaired t-test, chi-square test and

Pearson's correlation test were used for the statistical analyses.

CAD patients demonstrated significant decreases in serum vitamin E and total carotene levels and significant increases in low density lipoprotein-cholesterol (LDL-C) and MDA levels.  $\Delta$  MDA (oxidizability of apo B-containing lipoproteins) was negatively correlated with vitamin E and inversely correlated with LDL-C, apo B and MDA levels in CAD patients.

In conclusion, oxidized forms of apo B-containing lipoproteins may play an important role in the pathogenesis of atherosclerosis, and antioxidant vitamin supplementation may be useful in CAD by improving the oxidative balance.

**Key Words:** Lipoprotein oxidation, Vitamin E, Total carotene, Coronary artery disease

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### Introduction

There is increasing evidence that oxidation of low density lipoprotein (LDL) plays a major role in the pathogenesis of coronary artery disease (CAD). However, results from some clinical studies on LDL oxidation and CAD are not consistent and the pathway linking LDL to CAD remains unclear. In addition to LDL, all apolipoprotein B (apo B)-containing lipoproteins have been considered atherogenic (1). The atherogenic characteristics of oxidized apo B-containing lipoproteins include accelerated uptake by macrophages, facilitation of foam cell formation, chemoattraction for monocytes and T lymphocytes and cytotoxicity to endothelial cells (2).

Antioxidants such as vitamin E and beta carotene can prevent or delay atherogenesis by inhibiting the oxidation of lipoproteins (3,4). Furthermore, antioxidants may represent new preventive and therapeutic agents for CAD (5). Some of the epidemiologic data show an inverse correlation between CAD and vitamin E and total carotene

levels (6). On the other hand, several other studies found no direct link between CAD and decreased levels of antioxidant vitamins (7,8). Although many investigators have worked on LDL oxidation, studies examining the oxidation of apo B-containing lipoproteins are limited and data from these studies are contradictory (9,10).

We, therefore, were interested in investigating the susceptibility of apo B-containing lipoproteins to in vitro oxidation in CAD patients.

### Materials and Methods

One hundred and seventy-seven symptomatic CAD patients (32 females and 145 males, age 36-73 years) and 75 non-CAD subjects (45 females and 30 males, age 38-72 years), who had undergone coronary catheterization (total 252) were included in this study. Consent for coronary angiography and associated blood tests were obtained from all patients. The disease group included 21 patients with single-vessel, 41 patients with

two-vessel, 115 patients with three-vessel CAD. By the use of a questionnaire, histories of hypertension (treated hypertension or untreated subjects with diastolic blood pressure > 90 mmHg, systolic blood pressure > 140 mmHg in two measurements), diabetes mellitus, smoking and family history of cardiovascular disease were recorded. Family history was defined as positive if a parent or sibling aged < 55 years had angina, had a myocardial infarction or died unexpectedly. Height and weight were measured and the body mass index (BMI) was calculated. None of the subjects had been treated with lipid lowering drugs.

Venous blood was drawn from the subjects after overnight fasting and 4 mL of blood was transferred into tubes containing EDTA (final concentration 4.08 mM), and plasma was separated immediately. Lipoprotein oxidation studies were performed within 24 hours. Blood for measuring vitamin E and carotenoid was covered with aluminium foil to protect it from light. Serum aliquots separated for vitamin E, carotenoid and plasma aliquots for malondialdehyde (MDA) were kept at -20 °C and assayed within two months, as suggested previously (11,12).

Total cholesterol (TC) and triglyceride (TG) were determined according to standard laboratory methods using a Technicon-Dax 72 analyser (Technicon Instruments Corporation, Tarrytown, NY, USA). High density lipoprotein cholesterol (HDL-C) was quantified after precipitation of apo B-containing lipoproteins with dextran-sulphate and magnesium chloride (13). Apo A and apo B were determined nephelometrically (Sanofi Pasteur, Kallestad QM 300, France). LDL-C was calculated according to the Friedewald equation (14). Vitamin E was measured according to the principle that tocopherols reduce ferric ions to ferrous ions and then the latter form a red complex with  $\alpha$   $\alpha$ -dipyridyl (15). Serum total carotene was measured by using spectrophotometric method described by Neeld and Pearson (16). Vitamin E and total carotene were assayed in a dark room. Plasma malondialdehyde concentration was determined using a colorimetric assay (17). In order to determine the susceptibility of apo B-containing lipoproteins (non-HDL fraction) to oxidation, this fraction was separated by precipitation, and then the cholesterol content of this fraction was adjusted to 200  $\mu$ g/mL with phosphate buffered saline (PBS). The non-HDL fraction (0.5 mL) was oxidized in the presence of copper (final

concentration 0.5 mM) in PBS at 37 °C. The lipid peroxide content of the oxidized non-HDL fraction was determined by the thiobarbituric acid reacting substances (TBARS) assay (18). TBARS was expressed as MDA equivalents content using 1,1,3,3 tetraethoxypropane as standard. MDA levels of the apo B-containing lipoproteins were measured after 0, 30, 60, 90, 120, 150 and 180 minutes of incubation.  $\Delta$  MDA (oxidizability of apo B-containing lipoproteins) was recovered by taking the difference between MDA levels of apo B-containing lipoproteins at 180 and 0 minutes (19).

Data are expressed as mean  $\pm$  SD. Student's unpaired t-test was used to determine the significance of difference between means. Differences in prevalence were evaluated by the chi-square test and differences were considered significant when the probability was  $p < 0.05$ . Pearson's linear correlation was adopted for correlation analysis.

## Results

Demographic characteristics of the groups are given in Table 1. Table 2 shows the lipid profile (TC, TG, HDL-C, LDL-C, apo AI and apo B), vitamin E, total carotene and MDA levels. TC, TG, LDL-C, MDA and apo B levels were significantly higher ( $p < 0.001$  for each) while HDL-C, apo AI, vitamin E and total carotene levels were significantly lower ( $p < 0.001$  for each) in the CAD patients than those in non-CAD subjects (Table 2). MDA levels of apo B-containing lipoproteins measured at time intervals (except 0 minutes) and  $\Delta$  MDA were significantly higher in the CAD group than in the non-CAD group (Table 3).

Table 1. Demographic Data of All the Subjects.

	Non-CAD (n = 75)	CAD (n = 177)
Age (year)	51 $\pm$ 11	57 $\pm$ 10
Sex (Male/Female)	30 / 45	145 / 32
BMI (kg/m <sup>2</sup> )	25 $\pm$ 0.6	27 $\pm$ 0.4
Hypertension (%)	29	32
Smoking (%)	32	41
Diabetes (%)	11	12
Family history (%)	32	35

CAD = Coronary artery disease

BMI = Body mass index

Table 2. Lipid Profile, Antioxidants and Malondialdehyde Levels on Subjects.

Parameters	Non-CAD (n = 75)	CAD (n = 177)	p values*
Total cholesterol (mmol/L)	5.05 ± 0.85	5.83 ± 1.09	< 0.001
Triglycerides (mmol/L)	1.66 ± 0.52	2.23 ± 0.78	< 0.001
HDL- cholesterol (mmol/L)	1.06 ± 0.18	0.85 ± 0.16	< 0.001
LDL-cholesterol (mmol/L)	3.21 ± 0.73	3.94 ± 0.85	< 0.001
Apo AI (g/L)	1.46 ± 0.24	1.31 ± 0.21	< 0.001
Apo B (g/L)	1.29 ± 0.33	1.42 ± 0.35	< 0.001
Malondialdehyde (nmol/mL)	5.9 ± 1.8	8.2 ± 2.0	< 0.001
Total carotene (µmol/L)	3.11 ± 0.76	2.36 ± 0.65	< 0.001
Vitamin E (µmol/L)	35.0 ± 7.2	27.8 ± 8.1	< 0.001

Data are expressed as mean ± SD. HDL = High density lipoprotein  
 \* = Student's unpaired t-test LDL = Low density lipoprotein  
 CAD = Coronary artery disease Apo = Apolipoprotein

Table 3. Malondialdehyde Levels\* of Apolipoprotein B-Containing Lipoproteins Measured at Time Intervals.

Time intervals (min)	Non-CAD	CAD	p value
0	6.75 ± 0.84	6.84 ± 0.75	NS
30	7.61 ± 0.88	8.58 ± 1.16	< 0.01
60	12.01 ± 2.60	14.80 ± 4.56	< 0.001
90	31.36 ± 9.21	34.23 ± 7.85	< 0.02
120	43.97 ± 9.95	49.81 ± 11.05	< 0.02
150	55.30 ± 12.45	66.77 ± 13.47	< 0.0001
180	82.04 ± 18.10	98.30 ± 21.73	< 0.0001
Δ MDA	75.29 ± 18.39	91.46 ± 20.41	< 0.0001

\* nmol/mg cholesterol

NS: Non-Significant

Correlations among Δ MDA, vitamin E, total carotene, MDA and serum lipid parameters were evaluated with Pearson's correlation coefficient. In CAD patients, Δ MDA was significantly positively correlated with LDL-C, apo B and MDA levels and significantly inversely correlated with vitamin E levels. MDA was significantly correlated with LDL-C and apo B levels and significantly inversely correlated with HDL-C, apo AI, vitamin E and total carotene levels (Table 4). In non-CAD subjects, there was a significant positive correlation between MDA and TC levels. There was a significant negative correlation between MDA and HDL-C levels (Table 4).

## Discussion

The biologic modification of lipoproteins seems to play an important role in the development of atherosclerosis (20). Low, very-low density lipoproteins and lipoprotein (a) are apo B-containing lipoproteins, and are susceptible to oxidative modification. In vivo, the most likely process responsible for this modification is lipid peroxidation, in which the polyunsaturated fatty acids are rapidly converted to lipid hydroperoxides and then to some reactive aldehydes such as MDA, one of the most frequently used indicators of lipid peroxidation (21,22).

Oxidized forms of apo B-containing lipoproteins are similar in structure and play a role in atherogenesis (23). In vitro, lipoproteins are subjected to oxidative stress by adding micromolar amounts of copper and their susceptibility to oxidation can be evaluated (24). The oxidative susceptibility of lipoproteins has been shown to correlate with the severity of coronary atherosclerosis (25). In the present study, we found MDA levels of apo B-containing lipoproteins at all time intervals and Δ MDA was significantly higher in patients with CAD than those of subjects without CAD. Smoking is a well-established risk factor for atherosclerosis and the proatherogenic effects of smoking may be related to oxidative modification of apo B-containing lipoproteins and decrements in the levels of some antioxidants, such as β-carotene and vitamin E (26). The higher number of smokers in the patient group may contribute to higher levels of MDA in the plasma and apo B-containing lipoprotein fraction of this group. Lipoproteins are protected from oxidation by lipid-soluble antioxidants such as α-tocopherol and β-carotene (27). α-tocopherol is the most prevalent and biologically active form of vitamin E and it traps peroxy free radicals and thus acts as a chain-breaking antioxidant (28). When vitamin E and carotenoids are depleted from the lipoprotein particles, they become more susceptible to oxidation (29,30). Numerous human studies have shown that α-tocopherol supplementation reduced LDL oxidizability and progression of atherosclerosis in CAD patients (28,31). In the present study we found that vitamin E levels were significantly lower ( $p < 0.001$ ) in patients with CAD and there was a significantly negative correlation ( $p < 0.01$ ) between vitamin E and Δ MDA. β-carotene is a member of the carotenoid family of plant pigments that is also carried in the blood within the LDL particle. Carotenoids may protect low-density lipoprotein from oxidation, a

Table 4. Correlation of Plasma Malondialdehyde (MDA) and  $\Delta$  MDA with Lipid Parameters, Apolipoprotein AI, apolipoprotein B and Vitamin E and Total Carotene.

Correlated parameters	CAD				non-CAD			
	Plasma MDA		$\Delta$ MDA		Plasma MDA		$\Delta$ MDA	
Total Cholesterol	r = 0.07	p = NS	r = 0.09	p = NS	r = 0.27	p < 0.01	r = 0.16	p = NS
Triglycerides	r = 0.01	p = NS	r = 0.15	p = NS	r = 0.06	p = NS	r = 0.10	p = NS
HDL-Cholesterol	r = -0.20	p < 0.01	r = -0.03	p = NS	r = -0.22	p < 0.05	r = -0.08	p = NS
LDL-Cholesterol	r = 0.20	p < 0.01	r = 0.29	p < 0.001	r = 0.18	p = NS	r = 0.19	p = NS
Apolipoprotein AI	r = -0.18	p < 0.05	r = -0.06	p = NS	r = -0.15	p = NS	r = -0.19	p = NS
Apolipoprotein B	r = 0.17	p < 0.05	r = 0.28	p < 0.001	r = 0.19	p = NS	r = 0.05	p = NS
Vitamin E	r = -0.19	p < 0.01	r = -0.21	p < 0.01	r = -0.11	p = NS	r = -0.19	p = NS
Total Carotene	r = -0.25	p < 0.001	r = -0.14	p = NS	r = -0.07	p = NS	r = -0.10	p = NS

CAD: Coronary Artery Disease, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, NS: Non-Significant

process implicated in the development of atherosclerosis (32). We found that total carotene levels were significantly lower ( $p < 0.001$ ) in patients with CAD. Prospective epidemiologic studies have shown a strong inverse correlation between HDL-C and CAD. In vitro, HDL can partially protect LDL from oxidation mediated by cells or by copper. Therefore, HDL could play an important role in preventing or reducing the atherosclerotic process by inhibiting LDL oxidation (33). In this study, we observed a negative correlation between HDL-C and plasma MDA in CAD patients.

In conclusion, we have shown that in CAD patients plasma apo B-containing lipoproteins were more susceptible to  $\text{Cu}^{++}$ -induced oxidation and also serum

vitamin E and total carotene levels were lower in patients with CAD than those in non-CAD subjects. Therefore, oxidized forms of apo B-containing lipoproteins may play an important role in the pathogenesis of atherosclerosis, and antioxidant vitamin supplementation may be useful in CAD by improving the oxidative balance.

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