Enhanced Susceptibility to Oxidation and Diminished Vitamin E Content of LDL from Patients with Stable Coronary Artery Disease

Mehran Haidari,* Ebrahim Javadi, Mehry Kadkhodaee, and Arashmidos Sanati

Background: Convincing evidence points to oxidative modification of LDL as an important trigger in a complex chain of events leading to atherosclerosis. We investigated the occurrence of enhanced susceptibility of LDL to oxidation and decreased vitamin E concentration in LDL as additional risk factors promoting atherosclerosis among patients with established coronary artery disease (CAD).

Methods: We examined 132 patients with angiographically confirmed CAD and compared them with 111 healthy control individuals. We measured conjugated diene production to assess susceptibility of LDL to copper-mediated oxidation. Vitamin E content of LDL was measured by HPLC.

Results: The mean lag time of LDL oxidation and LDL α-tocopherol/LDL-cholesterol ratio were lower in the patients with CAD (55 ± 14 min and 2.4 ± 1.0 mmol/mol) than in the controls (63 ± 13 min and 2.9 ± 1.1 mmol/mmol; P <0.0001 and <0.001, respectively). Multiple stepwise regression analysis demonstrated the lag time (odds ratio, 1.96; 95% confidence interval, 1.34–2.87; P <0.0001) and concentration of vitamin E in LDL (odds ratio, 1.65; 95% confidence interval, 1.16–2.33; P <0.005) as independent determinants of CAD. Significant inverse Spearman rank correlations were found between lag time (r = −0.285; P <0.001) or concentration of vitamin E in LDL (r = −0.197; P <0.002) and severity of CAD. Lag times were not significantly correlated with serum C-reactive protein or ferritin.

Conclusions: Our data suggest that a short LDL oxidation lag time and a low concentration of vitamin E in LDL might be independent coronary risk factors for stable CAD in Iranian people.

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In vitro and animal model studies provide an ever-increasing body of evidence that oxidation of LDL or related oxidative mechanisms play a major role in atherogenesis (1). Oxidative modification of LDL confers several properties to LDL that render the particle atherogenic. Oxidized LDL induces the transformation of macrophages into foam cells; in addition, it is cytotoxic and immunogenic and may have vasoconstrictor and prothrombotic effects (2, 3). The evaluation of LDL oxidation in vivo is difficult. One of the main problems is that LDL oxidation is likely to occur in the intima of the artery wall rather than in the general circulation (4). Even if some lipoproteins are oxidized in the circulation, the concentrations of these modified lipoproteins may be difficult to detect and may not reflect the extent of oxidation occurring in the arterial wall. Thus, susceptibility of LDL to in vitro oxidation has been proposed as an indicator of the response of LDL to in vivo oxidative stress. Much information relating to oxidized LDL has been acquired from studies focusing on various animals. However, the role of LDL susceptibility to oxidation as a risk factor for coronary artery disease (CAD) in humans has not been fully explored. Divergent information is available in this field, which may be explained in part by the use by investigators of small sample sizes (5–8) or nonspecific methods for evaluation of susceptibility of LDL to oxidation (9).

Quantitatively, vitamin E is the major antioxidant in LDL and is generally considered to protect LDL against oxidation (10). However, conflicting results were also reported concerning the role of vitamin E in atherosclerosis (11). Recently, one interventional study showed a null
effect of vitamin E on atherosclerosis (12). Moreover, a recent in vitro study has shown that vitamin E can be prooxidative rather than protective for lipids in isolated LDL (13).

We undertook the present study to investigate the occurrence of enhanced susceptibility of LDL to oxidation and decreased vitamin E concentration in LDL as additional risk factors promoting atherosclerosis among patients with established CAD. Recently, a relationship between inflammation and LDL susceptibility to oxidation has been reported (14). To address this question, we also studied the association of ultrasensitive C-reactive protein (CRP) with LDL susceptibility to oxidation.

**Materials and Methods**

**Patients and controls**

This study included 132 angiographically confirmed CAD patients (41 females and 91 males; mean age 57 ± 10 years) and 111 controls (66 females and 45 males; mean age, 52 ± 8 years). All had given written consent. The CAD patients were recruited from the Cardiology Department of Shariaty Hospital of Tehran University of Medical Sciences from July 1999 to February 2000. The indications for angiography were suspicion of CAD or preoperative screening for CAD in subjects with valvular disease.

Individuals with a history of acute coronary syndrome during the previous 4 weeks or percutaneous transluminal coronary angioplasty were not included. In addition, individuals with concomitant inflammatory diseases, cancer, or other diseases possibly associated with an acute-phase reaction and those who took lipid-lowering drugs, antioxidants, or supplements with vitamin E, vitamin A, or iron were excluded. Diabetic and hypertensive subjects adhering to the necessary diets and medications; the other subjects consumed a typical Iranian diet [rich in carbohydrates, vegetables, and fruits with moderate fat intake (15)]. Coronary angiographies were performed according to the standard Judkins technique (16). The patients were classified as positive for CAD if one or more coronary arteries had a stenosis ≥50%. A panel of three cardiologists read all coronary arteriographies blindly.

The control group was recruited randomly from the city census, and potential participants were identified from the desired residential area. Of subjects contacted and interviewed, 111 met requirement for inclusion in this study. Although control individuals were not subjected to coronary angiography, they underwent a comprehensive examination by a physician and completed a rose angina questionnaire. None of controls had angina or a prior history of CAD, and all controls had a normal resting electrocardiogram. Interviews were conducted and clinical data were collected using a common protocol. Hypertension was defined as resting systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg. Cigarette smoking was defined as ever vs never smoked. Current smoking was defined as smoking cigarettes within the past month. Diabetes was defined as fasting blood glucose >7.8 mmol/L or a diagnosis of diabetes requiring diet or drug therapy. Blood samples were obtained the day before angiographic procedures after a 12-h fast. LDL susceptibility to oxidation, the LDL vitamin E concentration, total cholesterol, triglycerides, HDL-cholesterol (HDL-C), ultrasensitive CRP, and ferritin were determined for all individuals. Serum samples were separated immediately after collection by centrifugation at 2000g for 15 min and stored at −80 °C until analysis.

**Preparation of LDL**

Blood samples were collected in polypropylene tubes containing EDTA (final concentration, 1 mg/mL of blood). Aliquots (1 mL) of plasma were collected in polypropylene vials and supplemented with 10 µL of sucrose solution (60 g/L) to prevent LDL aggregation (17). The vials were stored at −80 °C until use. LDL (d = 1.019–1.063 kg/L) was isolated by sequential ultracentrifugation (18). We used an Optima TLX tabletop ultracentrifuge with a TLA-120.2 rotor and thick-walled polycarbonate tubes without caps (Beckman Instruments) with the following run conditions: 625 000g; temperature, 10 °C; run time of 120 min for VLDL and 150 min for LDL. EDTA and salts were removed from LDL by gel filtration on Sephadex G-25 (Pharmacia) just before analysis. The protein concentrations were determined by the Bradford method (19).

**LDL Susceptibility to Oxidation**

The production of conjugated diene in LDL (final protein concentration, 0.05 g/L) mixed with 3.2 µmol/L CuSO_4 was monitored at 234 nm and 30 °C for 240 min (20) in a DU 640 spectrophotometer with six cuvettes (Beckman Instruments). The oxidative resistance of LDL was estimated in terms of the period when no oxidation occurred (lag phase), calculated as the intercept of extrapolations of the parts of the curve representing the lag and propagation phases.

The rate of propagation (µmol dienes·min⁻¹·g⁻¹) was obtained from the slope of the absorbance curve during the propagation phase, using the molar absorptivity of conjugated dienes (ε_234 nm = 29 500 mol·L⁻¹·cm⁻¹). The intraassay CV of the lag time measurement was 3.2% (n = 20), and the interassay CV was 3.8% (n = 20).

**LDL Vitamin E (α-Tocopherol) Content**

For α-tocopherol measurements, LDL samples were extracted with n-hexane, and the evaporated extracts were analyzed by HPLC using α-tocopherol acetate as the internal standard. The HPLC system was equipped with a Waters pump and a Nova-Pack C₁₈ 60A column [3.9 (i.d.) × 150 mm; 4-µm bead size; Waters]; the effluent was monitored at 292 nm with a diode array detector (Beckman Instruments). The mobile phase consisted of absolute methanol (gradient grade; Merck) at a flow rate of 1 mL/min (21). The within-day (n = 20) and between-day
(n = 25) CVs at a concentration of 10 μmol/L were 3.2% and 4.1%, respectively, and the recovery for α-tocopherol was 90%.

**OTHER LABORATORY DETERMINATIONS**

Cholesterol and triglycerides were measured enzymatically (Kone Diagnosis) on a Kone specific automated analyzer. HDL-C was determined after precipitation of apolipoprotein B-containing particles by phosphotungstic acid–MgCl₂. LDL-cholesterol (LDL-C) was estimated using the Friedewald equation (22). Serum CRP concentrations were measured by an ultrasensitive latex-enhanced immunoturbidimetric method (Randox Laboratory LTD), which has a detection range of 0.0–15 mg/L. The method was standardized against the international reference preparation CRM 470 (23). The run-to-run CVs (n = 20) for CRP at concentrations of 1 and 7 mg/L were 4.6% and 3.4%, respectively. Ferritin was measured using a radioimmunoassay (Amersham International). The between- and within-batch CVs (n = 20) were 7% and 3.5%, respectively. All biochemical measurements were carried out without knowledge of the angiographic findings.

**STATISTICAL ANALYSIS**

All data are presented as the mean ± SD. Statistical analyses were performed with SPSS for Windows, Ver. 10 (SPSS Inc). The Kolmogorov–Smirnov test of normality was used to test whether the distribution of variables was gaussian. Because the CRP and ferritin frequency distributions were skewed rightward, a natural logarithmic transformation was applied to normalize the data for analysis. The discrete variables were compared by the Pearson χ² test, and the Student t-test was used to compare the continuous variables. We used the Pearson correlation test to assess correlation between the continuous variables. To assess correlation between ordinal variables, we used the nonparametric Spearman rank correlation test. To determine factors independently correlated with CAD, multivariate analyses were carried out by multiple logistic regression analysis using the forward stepwise likelihood ratio method. Adjusted odds ratios (ORs) for the exposure variables and CAD were obtained. All P values were two-tailed, and values <0.05 were considered statistically significant. All confidence intervals (CIs) were calculated at the 95% level.

**Results**

The baseline characteristics of the study participants are shown in Table 1. The prevalence of hypertension, diabetes, and cigarette smoking were higher in the individuals with angiographically confirmed CAD. Total cholesterol, LDL-C, and CRP concentrations were higher in the patients with CAD relative to the controls. The controls demonstrated higher concentrations of HDL-C. Furthermore, the CAD patients were older and had a larger body mass index than the control group. Table 2 shows the data obtained from monitoring conjugated diene formation after CuSO₄-induced LDL oxidation as well as the α-tocopherol content of LDL. The mean lag phase for LDL particles obtained from the CAD patients was significantly shorter than the lag phase for the LDL particles from the healthy subjects, whereas the difference in oxidation rate was not statistically significant. The mean α-tocopherol concentration in LDL was significantly lower in the coronary patients than in the controls. After controlling for hypertension, diabetes, smoking, body mass index, and the other established coronary risk factors, the multiple logistic regression model demonstrated lag time, LDL α-tocopherol concentration, HDL-C, CRP, and age as the only independent discriminating factors, which remained significantly associated with CAD.

The quality of the prediction model, calculated as the percentage of individuals correctly classified as coronary patients and controls, was 80% for the model. The age-adjusted ORs for the highest compared with the lowest quartiles of lag time, LDL α-tocopherol concentration, and CRP are shown in Table 3. The association between lag time or LDL α-tocopherol concentration and the severity of CAD was evaluated. Individuals with angiographically confirmed CAD were divided into three groups: those with ≥50% stenosis in one vessel, two vessels, or three vessels. Comparisons of the lag time and LDL α-tocopherol concentration in these three groups are presented in Table 4. There were reverse age-adjusted Spearman rank

**Table 1. Baseline characteristics of the study participants.**

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 132)</th>
<th>Controls (n = 111)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>91/41</td>
<td>45/66</td>
<td></td>
</tr>
<tr>
<td>Age, a years</td>
<td>57 ± 10</td>
<td>52 ± 8</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI, a kg/m²</td>
<td>27.11 ± 4.1</td>
<td>26.2 ± 3.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>28 (21)</td>
<td>9 (8)</td>
<td>0.003</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>55 (41)</td>
<td>24 (21)</td>
<td>0.001</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>50 (37)</td>
<td>33 (29)</td>
<td>0.031</td>
</tr>
<tr>
<td>Triglycerides, a mmol/L</td>
<td>2.45 ± 1.22</td>
<td>2.19 ± 1.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Total cholesterol, a mmol/L</td>
<td>6.49 ± 1.33</td>
<td>5.77 ± 1.20</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDL-C, a mmol/L</td>
<td>4.40 ± 1.17</td>
<td>3.67 ± 1.14</td>
<td>0.0001</td>
</tr>
<tr>
<td>HDL-C, a mmol/L</td>
<td>0.96 ± 0.23</td>
<td>1.09 ± 0.27</td>
<td>0.0002</td>
</tr>
<tr>
<td>CRP, a mg/L</td>
<td>2.17 ± 1.30</td>
<td>1.65 ± 0.96</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

a Value given in mean ± SD.

b BMI, body mass index.

**Table 2. Parameters of conjugated diene formation and vitamin E content of LDL in the CAD patients and the controls.**

<table>
<thead>
<tr>
<th></th>
<th>CAD patients</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time, min</td>
<td>55 ± 14</td>
<td>63 ± 13</td>
<td>0.0001</td>
</tr>
<tr>
<td>Rate of oxidation, μmol dienes · min⁻¹ · g⁻¹</td>
<td>2.76 ± 0.93</td>
<td>2.53 ± 0.82</td>
<td>0.65</td>
</tr>
<tr>
<td>LDL-α-tocopherol/LDL-C, mmol/mmol</td>
<td>2.4 ± 1.0</td>
<td>2.9 ± 1.1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values given as mean ± SD.
correlations between the extent of CAD (indicated by the number of vessels with obstruction) and the lag time ($r = -0.285; P < 0.001$) and the LDL $\alpha$-tocopherol concentration ($r = -0.197; P < 0.002$).

To evaluate the susceptibility of LDL to oxidation among individuals with LDL concentrations within the reference interval, we performed a subgroup analysis of the study participants with LDL-C concentrations $<3.36$ mmol/L (130 mg/dL), the target value for the primary prevention of CAD according to the National Cholesterol Education Program (24). When we used this cutoff point, 26% of the individuals ($n = 43$ and $n = 19$ in the control and patient groups, respectively) had LDL-C concentrations below the cutoff (mean LDL-C, 2.54 $\pm$ 0.52 mmol/L). Among this normolipidemic subgroup, the patients with CAD showed shorter lag times relative to the controls, and the difference was more pronounced than in the total population ($50 \pm 12$ vs $63 \pm 14$ min; $P < 0.002$). The logistic regression model demonstrated that lag time remained an independent determinant of CAD (after controlling for age and other confounding risk factors) among the individuals with LDL-C $<3.36$ mmol/L ($P < 0.005$). The OR for the highest as compared with the lowest quartile of lag time was 2.29 (95% CI, 1.23–4.41; $P < 0.01$). The correlation between the lag time and the severity of CAD was also stronger in the population with LDL below the National Cholesterol Education Program cutoff relative to the total population ($r = -0.343; P < 0.006$).

There was no correlation between the LDL $\alpha$-tocopherol concentration and duration of the lag phase, nor was there a significant correlation between CRP, ferritin, or the measured lipid values and lag time or the LDL $\alpha$-tocopherol concentration. No association was found between lag time or LDL $\alpha$-tocopherol content and sex, hypertension, diabetes, and cigarette smoking among the study participants.

### Table 3. Age-adjusted ORs for highest compared with lowest quartiles of lag time, $\alpha$-tocopherol content of LDL, and CRP in the total population.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time</td>
<td>1.96</td>
<td>1.34–2.87</td>
<td>0.0001</td>
</tr>
<tr>
<td>$\alpha$-Tocopherol content of LDL</td>
<td>1.65</td>
<td>1.16–2.33</td>
<td>0.005</td>
</tr>
<tr>
<td>Ultrasensitive CRP</td>
<td>1.50</td>
<td>1.03–2.1</td>
<td>0.026</td>
</tr>
</tbody>
</table>

### Table 4. Lag time and $\alpha$-tocopherol content of LDL and the severity of CAD.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>1-Vessel disease</th>
<th>2-Vessel disease</th>
<th>3-Vessel disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>111</td>
<td>36</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Lag time, $^a$ min</td>
<td>63 $\pm$ 13</td>
<td>57 $\pm$ 17</td>
<td>55 $\pm$ 13</td>
<td>54 $\pm$ 14</td>
</tr>
<tr>
<td>LDL-$\alpha$-tocopherol, $^a$ mmol/mmol</td>
<td>2.9 $\pm$ 1.0</td>
<td>2.6 $\pm$ 0.9</td>
<td>2.5 $\pm$ 1.2</td>
<td>2.4 $\pm$ 1.0</td>
</tr>
</tbody>
</table>

$^a$ Values given in mean $\pm$ SD.

**Discussion**

Although reliable population-based data on CAD prevalence and mortality in Iran are lacking in the literature, two recent reports revealed relatively high prevalences of both CAD and the established coronary risk factors among the Iranian population (25, 26). An important role of oxidatively modified LDL in the development of CAD is supported by data from in vitro and animal studies (2). Antibodies against epitopes of oxidized LDL recognize materials in atherosclerotic lesions of rabbits and humans but not in healthy arteries (27, 28). LDL extracted from human atherosclerotic lesions contains nearly all of the physicochemical and immunologic properties of in vitro oxidized LDL (29). An increase in the in vitro oxidation of lipoproteins is considered one way to assess predisposition to in vivo development of oxidized LDL inside the arterial wall. The oxidative susceptibility of LDL appears to be increased with established coronary risk factors such as diabetes, smoking, hypertension, and hyperlipidemia (30–33). Several authors (7, 29, 34–36), but not others (37–41), have reported that LDL from individuals with increased risk of CAD shows enhanced susceptibility to oxidation. Our study showed an increased susceptibility to copper-induced formation of conjugated dienes in LDL particles from the coronary patients, as evaluated by the length of the lag phase. Because the methods for measuring the lag phase of LDL oxidation are not well standardized, it is more likely that results differ between studies. Our finding of a reverse correlation between lag time and severity of CAD is in agreement with the results obtained by Regnstrom et al. (7). The separate analysis for the individuals with LDL-C below the National Cholesterol Education Program cutoff revealed a greater difference between the lag times of the coronary patients and the controls in addition to a stronger correlation between the lag times and the severity of CAD. This may explain, in part, why CAD is clinically manifested in patients with a desirable LDL-C concentration.

Endogenous antioxidants, mainly $\alpha$-tocopherol, contained in LDL particles are rapidly consumed after induced oxidation, and propagation of the oxidative process does not begin until antioxidant molecules are largely exhausted (42). The oxidative resistance of LDL increases when the $\alpha$-tocopherol content is increased either in vitro or through dietary vitamin E supplementation (43). There are many studies demonstrating the effectiveness of vitamin E against atherosclerosis in small animals (44, 45). In humans, prospective cohort studies support a beneficial effect of vitamin E in CAD (46, 47). In the five major prospective clinical trials that have tested the effect of vitamin E supplementation on cardiovascular events, four have shown a beneficial effect on cardiovascular endpoints (48). The Heart Outcomes Prevention Evaluation Study demonstrated that treatment with vitamin E for a mean of 4.5 years had no apparent effect on cardiovascular outcomes (12). However, the study suffers from certain deficiencies (49). Plasma vitamin E concentrations
were not provided to confirm the supplementation. Furthermore, because the animal intervention evidence on which this trial was based deals primarily with very early lesions (fatty streaks), that evidence does not necessarily provide a basis for predicting what antioxidant intervention will do in patients with advanced lesions, particularly when the end-points used relate to unstable plaques and fatal thrombosis events, for which no adequate animal models are currently available (11). Therefore, although the published data from the prospective clinical trials are not overwhelming, the majority of studies appear to suggest a benefit of vitamin E supplementation.

In the current study, we found a significant difference in the LDL \( \alpha \)-tocopherol content between the coronary patients and the healthy controls, which is consistent with some other studies (5). Furthermore, Regnstrom et al. (50) and Miwa et al. (51), as in our study, found an inverse correlation between the severity of CAD and the LDL vitamin E concentration. However, some investigators have found no association between the LDL vitamin E concentration and CAD (35, 52). The lack of association in these studies may be attributable to methodologic issues, such as differences in vitamin E status determination (e.g., dietary intake, vitamin supplementation, and lipid-standardized or absolute plasma vitamin E concentration).

During the lag phase, LDL-associated antioxidants such as \( \alpha \)-tocopherol, carotenoids, and ubiquinol are consumed, illustrating that the complement of antioxidants should predict the susceptibility to oxidation. However, several studies (including ours) have failed to demonstrate a relationship between vitamin E in LDL and the diene lag time (10, 53), which indeed suggests that still other factors govern LDL oxidation.

Inflammation of the vessel wall is now considered to play an essential role in the initiation and progression of atherosclerosis (54). Our results demonstrated significantly increased CRP in coronary patients with enhanced susceptibility of LDL to oxidation. Increased LDL oxidation during inflammation may promote atherogenesis and could be a mechanism for the increased incidence of CAD in patients with inflammatory and chronic infectious disorders. However, no significant correlation existed between CRP concentrations and lag times in the study participants; therefore, further investigations are required to confirm this observation.

Iron has been implicated in atherogenesis mainly because of its capacity to initiate severe oxidative stress, which is supported by some epidemiologic (55) and experimental (56) studies. Our findings of no correlation between ferritin concentrations and the susceptibility of LDL to oxidation or the LDL \( \alpha \)-tocopherol concentration do not support a role for body iron in promoting oxidation of LDL.

The potential limitations of this study need further consideration. We used the number of coronary vessels with >50% obstruction as an indicator of the severity of CAD. A linear relationship may not reflect the hemodynamic problems of the vessels, whereby the severity of CAD may be over- or underestimated. Although it is accepted that the oxidizability of LDL may play a key role in the development of atherosclerosis, one must question the validity of this, or indeed any assay performed in vitro to predict the in vivo existence, development, or progression of cardiovascular disease. The presence of occult ischemic coronary disease in our controls could not be excluded and may have attenuated the differences seen among the two groups.

In conclusion, our observation of an association between the predisposition to oxidative modification of LDL and the development of atherosclerosis supports the hypothesis that lipid oxidation is an important factor in atherogenesis.

We gratefully acknowledge the excellent editorial assistance of Taryne M. Chong.

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