Is oxidative stress causally linked to unstable angina pectoris? A study in 100 CAD patients and matched controls


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Abstract

Objective: Unstable angina pectoris often leads to acute myocardial infarction. Since lipid peroxidation is thought to be causally related to chronic and acute events in atherosclerosis and coronary artery disease, we measured lipid peroxidation products and vitamin E in 100 patients with coronary artery disease and compared them to a matched control group.

Methods: 50 consecutive patients with stable angina pectoris SAP and 50 consecutive patients with unstable angina pectoris (UAP) were studied and compared to 100 clinically healthy individuals. In addition to conventional lipid and lipoprotein analysis, malondialdehydes were measured as thiobarbituric acid reactive substances (TBARS). Lipid hydroperoxides were assayed with the colorimetric methylene blue method. α-Tocopherol was quantitated by HPLC after extraction of serum with hexane-ethanol. In the patient group conjugated dienes were also measured.

Results: As expected, patients had significantly higher cholesterol, triglyceride LDL-C and Lp(a) values and lower HDL-C values than controls. When patients were divided into groups with SAP and UAP respectively, peroxides and TBARS were significantly higher in the latter group as compared to patients with SAP and to controls. Conjugated dienes were also significantly higher in patients with UAP as compared to patients with SAP. Total plasma α-tocopherol was comparable in all three groups, whereas the α-tocopherol content per LDL particle was lowest in patients with UAP, followed by patients with SAP and then controls.

Conclusion: It is concluded that lipid peroxidation parameters are increased in patients with UAP and discriminate SAP from UAP patients.

Keywords: Coronary artery disease; Humans; Lipid hydroperoxides; TBARS; α-Tocopherol

1. Introduction

Unstable angina pectoris (UAP) and acute myocardial infarction are hallmarks of acute coronary syndromes [1–3]. Underlying pathophysiological mechanisms of UAP are multifactorial, and include plaque rupture and consecutive thrombus formation as well as vasospasm, [4]. Depending on underlying mechanisms and onset of treatment, prognosis of UAP is rather poor. There is increasing evidence that oxidatively modified lipoproteins play a key role in the pathogenesis of atherosclerosis and that antioxidants may prevent atherosclerosis by inhibiting lipid peroxidation [5–9]. Most information on the role of oxidatively modified LDL in the pathogenesis of atherosclerosis is derived from in vitro studies, which have demonstrated that (1) oxidized LDL is taken up by macrophage scavenger receptors and that (2) oxidized LDL stimulates endothelial expression of macrophage chemotactants, adhesion molecules and cytokines [10–13]. There are several indications that oxidized LDL is also present in vivo, accumulating in atherosclerotic lesions [14,15]. It is generally thought that oxidized LDL present in the arterial intima is mostly derived from LDL particles that have been modified within the arterial wall [16], since oxidized LDL is removed rapidly from plasma immediately after intravenous injection [17]. Other studies suggest, however, that as much as 10% of...
LDL in plasma may be oxidized to a measurable extent [18,19]. Such oxidized LDL may be produced at sites of inflammation [20].

Unstable angina pectoris (UAP) is mainly the result of complex unstable atherosclerotic plaques which often predict poor in-hospital outcome [21]. The reason why UAP is so much more prone to complications than stable angina pectoris (SAP) is presently a subject of intensive research.

In this study we compared lipid peroxidation parameters in patients with SAP and UAP and in controls and demonstrate that some of these parameters discriminate UAP patients from SAP and controls.

2. Patients and controls

Fifty consecutive patients with SAP and 50 consecutive patients with UAP from regions of Vienna and Lower Austria who were admitted for diagnostic coronary arteriography to our department were included in this study. Patients referred for diagnostic coronary angiography or angioplasty for typical clinical indications, including evaluation of stable exertional angina or unstable angina (classified according to the CCS criteria) were considered eligible for the study. Exclusion criteria were: (1) previous angioplasty or bypass surgery; (2) progression to myocardial infarction during admission (enzyme elevation to twice normal levels or the presence of new Q waves, or both); (3) myocardial infarction within 12 weeks of study entry; (4) clinically significant valvular heart disease, serious conduction disturbances, inflammatory disease, heart failure or significant arrhythmia; and (5) age over 75 years.

Fifty patients had stable effort-related angina with no changes in symptoms in the preceding three months. Fifty patients had either unstable angina defined as recent onset angina or angina at rest (n = 31) or marked and rapid deterioration of preexisting chronic stable angina (n = 19). In every patient with unstable angina, medical stabilization of angina was achieved with a standard medical regimen.

From the 100 patients studied, 31 suffered from single-vessel disease, 33 from double-vessel and 36 from triple or multi-vessel coronary artery disease. Twenty-four patients were on lipid lowering therapy with lovastatin, 20–40 mg/day or bezafibrate 400 mg/day or gemfibrozil, 500 mg/day. Ninety-two patients received aspirin (100 mg/day), 32 calcium antagonists, 54 ACE inhibitors, 55 ß-blockers and 97 nitrates.

The control group consisted of 100 clinically healthy individuals participating in a health survey programme. Controls were matched for age, gender and socioeconomic status. Controls had no clinical signs of cardiovascular diseases and all controls had an exercise stress test performed. Only those controls with a negative exercise stress test were included in the study. Apart from occasional pain relievers, the controls did not take any medication. All patients and controls gave informed consent to this study and the study conforms with the principles outlined in the Declaration of Helsinki.

Collection of samples: Blood samples were collected before coronary angiography. Blood was drawn in the morning after a 12 h fasting period, allowed to clot at room temperature for 20 min and submitted immediately to the central laboratory for analysis of lipids, lipoproteins and routine parameters.

3. Methods

3.1. Determination of malondialdehyde with thiobarbituric acid

The measurement of TBARS is based on the reaction of malondialdehyde, a secondary breakdown product of lipid hydroperoxides, with thiobarbituric acid (TBA). The assay was performed as described previously [22]. The plasma sample was mixed with two volumes of cold 10% trichloroacetic acid for protein precipitation. Following centrifugation, the supernatant was mixed with an equal volume of 0.67% TBA in a boiling water bath for 10 min. After cooling, the absorbance was measured at 532 nm and the concentration of MDA was calculated from $e = 153\,000\,\text{M}^{-1}\,\text{cm}^{-1}$ [22]. Measurements were performed in duplicate.

3.2. α-Tocopherol measurement in human plasma samples

The neutral lipid fraction (200 µl) including α-tocopherol was extracted in a biphasic extraction system consisting of 200 µl ethanol, 200 µl H$_2$O and 200 µl hexane. The upper organic layer 200 µl including α-tocopherol was analyzed by high-pressure liquid chromatography using an ExSil 100 20 × 0.46 cm silica column (mobile phase: hexane/1% ethanol, 1 ml/min) and fluorescence detection (Hitachi, $E:\ 295\ nm/Em: 390\ nm$) [23]. Quantitation was performed by peak area comparison with external α-tocopherol standards of known concentrations.

3.3. Measurement of lipid hydroperoxides

Lipid hydroperoxides were determined by a method described previously [25], using a commercial kit from Kamya company. The assay is based on the reaction of lipid hydroperoxides with a derivative of methylene blue in the presence of hemoglobin, whereby free methylene blue is produced. The colorimetric assay was performed according to the manufacturer’s recommendations. Briefly, 20 µl of the serum sample was mixed with 200 µl of reagent 1 (containing ascorbate oxidase) and incubated at 30°C for 5 min under nitrogen. 400 µl of reagent 2 (containing a derivative of methylene blue and hemoglobin as a reaction catalyst) was then added to the samples,
mixed and incubated for at least 10 min at 30°C. The amount of free methylene blue generated from peroxides was measured photometrically at 675 nm. Cumene hydroperoxide (50 nmol/ml) was used as a standard. Measurements were performed in duplicate.

3.4. Measurement of conjugated dienes in plasma

Peroxidation of polyunsaturated fatty acids leads to the formation of a conjugated diene system, with a characteristic UV absorption maximum at 234 nm. The measurement of conjugated dienes was performed as described previously [24]. Plasma (100 µl) was mixed with 1 ml of water, followed by the addition of 3 ml chloroform:methanol (2:1, v/v) and vortexed vigorously for 2 min. Samples were then centrifuged for 5 min at 2000 rpm, the lower organic layer was removed and dried under nitrogen. The dry residue was solubilized in 1 ml of hexane and the absorbance was measured at 234 nm in a Hitachi double beam spectrophotometer using hexane as a blank. All measurements were performed in duplicate. Concentrations estimated by the 234 nm absorbance were calculated using the molar absorptivity of 2.8 × 10^4 M⁻¹ cm⁻¹.

3.5. Determination of lipids and lipoproteins

Cholesterol and triglycerides were measured enzymatically using the assay kits from Boehringer Mannheim. HDL-C was measured from the supernatant after precipitation with polyethylene glycol (Reagent A from Immuno A.G., Vienna). LDL-C was calculated according to the Friedewald equation. Lp(a) was measured by a sandwich assay on the DELFIA system (LKB-Pharmacia), as described in detail previously [26].

3.6. Statistical analyses

For data analysis we used the Statistical Package for Social Sciences (SPSS/MAc + ). For serum lipids, mean values ± SEM were calculated and analyzed by an one-way variance test (ANOVA). A Student’s t-test was applied to assess significant differences of continuous variables among groups.

4. Results

Table 1 lists demographic data of the studied CAD patients and controls. There was no difference in age, sex, smoking and drinking behaviour. As expected, CAD patients had higher total cholesterol, triglyceride, LDL-C and lower HDL-C values as compared to the control group (217 vs. 189 mg/dl; 192 vs. 106 mg/dl; 148 vs. 124 mg/dl and 35 vs. 47 mg/dl, respectively). All these differences were statistically highly significant (p < 0.001) (Table 2).

Lipoprotein (a) [Lp(a)] was also significantly higher in CAD patients (mean 32 mg/dl; median 18 mg/dl) as compared to controls (mean 16 mg/dl; median 12 mg/dl). Although UAP patients had a tendency toward higher triglyceride and cholesterol values, this did not reach statistical significance when compared to SAP patients.

Since there is no single parameter that reflects the oxidative status of plasma lipoproteins [24], and since it is not yet agreed which lipid peroxidation parameter is of greatest clinical relevance, we decided to measure TBARS (malondialdehyde), peroxides and α-tocopherol levels in CAD patients and in controls and additionally conjugated dienes in patients.

The mean concentration of lipid peroxides in all patients with CAD was 25.2 µg/l as compared to 21.9 µg/l in controls. This difference was not significant, as measured by ANOVA (p = 0.07). In patients with unstable angina by contrast, lipid peroxides were significantly higher as compared to stable angina (31.2 vs. 18.1 µg/l, p < 0.001) or controls (21.9 µg/l, p < 0.001).

We also measured the concentration of TBARS in CAD patients and controls. CAD patients showed significantly higher TBARS when compared to controls (2.0 vs. 1.6 µg/l, p < 0.02). Again when we divided CAD patients into two groups, we found that patients with unstable angina showed significantly higher TBARS than patients with stable angina (2.5 vs. 1.5 nmol/ml, p < 0.01), yet there was no significant difference between patients with stable angina and controls (1.5 vs. 1.6 nmol/ml).

Conjugated dienes were also measured in patients and controls. Again we found statistical significantly higher values in patients with unstable angina (13.4 vs. 8.9 ng/ml, p < 0.001) as compared to patients with stable angina.

Since vitamin E appears to be a very important antioxidant in plasma, α-tocopherol was subsequently measured in plasma of patients and controls. When we compared CAD patients and controls, no significant difference could be observed (9.5 vs. 9.7 µg/ml). Levels of α-tocopherol were also comparable in patients with unstable and stable angina (9.1 vs. 9.9 µg/ml). Since vitamin E is mainly transported by lipoproteins in plasma such as low density lipoproteins, it is more accurate to calculate the amount of vitamin E per LDL.

| Table 1 | Demographic data of the coronary artery disease patients and of controls |
| CAD patients | Controls | Significance |
| CAD patients | Controls |
| Number (m/f) | 100 (69/31) | 100 (74/26) | n.s. |
| Age | 63 ± 11 | 58 ± 16 | n.s. |
| BMI | 24.2 ± 3.3 | 23.1 ± 3.5 | n.s. |
| Hypertension | 35 | 5 | p < 0.001 |
| Family history | 52 | 11 | p < 0.001 |
| Diabetes mellitus | 11 | 0 | p < 0.001 |
| Smokers (n) | 31 | 37 | n.s. |
| Alcohol consumption | < 20 g/day | < 20 g/day | n.s. |

* BMI stands for Body Mass Index
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<tr>
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<th>Chol (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>Lp(a) (mg/dl)</th>
<th>Perox (µg/l)</th>
<th>TBARS (µmol/l)</th>
<th>α-Tocopherol (µg/ml)</th>
<th>α-Toc/LDL (µg/100 mg % LDL)</th>
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<tr>
<td>Controls</td>
<td>189±43</td>
<td>106±54</td>
<td>124±34</td>
<td>47±15</td>
<td>12</td>
<td>21.9±11.9</td>
<td>1.6±0.6</td>
<td>9.7±3.7</td>
<td>7.8±3.0</td>
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<tr>
<td>CAD</td>
<td>217±46</td>
<td>192±91</td>
<td>148±40</td>
<td>35±14</td>
<td>18</td>
<td>25.2±12.7</td>
<td>2.0±1.5</td>
<td>9.5±3.6</td>
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<td>SAP</td>
<td>214±42</td>
<td>190±95</td>
<td>144±39</td>
<td>34±12</td>
<td>18</td>
<td>18.1±11.4</td>
<td>1.5±1.1</td>
<td>9.9±4.1</td>
<td>6.7±2.8</td>
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<tr>
<td>UAP</td>
<td>219±51</td>
<td>201±87</td>
<td>150±42</td>
<td>32±14</td>
<td>17</td>
<td>31.2±14.0</td>
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<td>Con vs. SAP</td>
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<td>Con vs. UAP</td>
<td>p &lt; 0.001</td>
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<td>n.s.</td>
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<td>SAP vs. UAP</td>
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<td>n.s.</td>
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<td>n.s.</td>
<td>p = 0.02</td>
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<td>p &lt; 0.01</td>
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* Con: controls; CAD: total coronary artery diseases group (SAP + UAP); SAP: stable angina pectoris; UAP: unstable angina pectoris.
So when the α-tocopherol content of our samples was normalized to LDL-C, a significant difference between CAD patients and controls was noted (6.4 vs. 7.8 μg/100 mg% LDL-C, p < 0.01). Moreover, patients with unstable angina exhibited significantly lower α-tocopherol levels when normalized to LDL-C than patients with stable angina (6.1 vs. 6.7 μg α-T/100 mg% LDL-C, p < 0.01). There was also a significant difference in α-tocopherol per LDL particle between controls and stable patients (7.8 vs. 6.7 μg α-T/100 mg% LDL-C, p < 0.01) as can be seen in Fig. 1.

Even though there was no statistically significant difference in age between controls and CAD patients, the mean age of our patients was slightly higher. We therefore omitted the four oldest patients from the control group and the four youngest patients from the patient group, which resulted in a mean age of 60 ± 12 in the patient group and a mean age of 60 ± 11 in the control group. We then recalculated all our statistics and found virtually identical differences with the same statistical significance.

5. Discussion

Increased concentrations of C-reactive protein (CRP), a sensitive marker of inflammation, have been reported in patients with UAP in several studies [27–29]. Several experimental reports demonstrate that periods of ischemia as short as 15 min followed by reperfusion elicit a cascade of proinflammatory reactions that are known to lead to the production of oxygen-derived free radicals [30], activation of the complement system [31], adherence of neutrophils to the coronary endothelium [32], leukocyte mediated injury of myocardial cells [33] and production of cytokines [34,35] including IL-6 and IL-1, which are the major determinants of acute-phase protein production [36]. All these events are considered directly or indirectly pro-oxidative for plasma lipoproteins. Since unstable angina is a complex syndrome, the pathophysiological mechanisms of which are still incompletely understood, we were interested in measuring several parameters of lipoprotein oxidizability in a collective of CAD patients with SAP and UAP in comparison to a control group.

Interestingly, lipid hydroperoxides were not significantly different between CAD patients and controls. However, when the CAD group was divided, patients with UAP were found to have highly significant increased hydroperoxide levels as compared to patients with SAP or to controls. Unfortunately, there exists no single clinical parameter which reflects the oxidative stress in a given individual [24]. We therefore measured in addition to peroxides TBARS and conjugated dienes, as these have been reported to be an index of lipoprotein oxidation in vivo. TBARS are formed during the oxidation of polyunsaturated fatty acids (PUFAs), but may diminish during the later stages of lipid peroxidation. TBARS were found to be significantly higher in patients with UAP as compared to SAP patients and controls.

Conjugated dienes were also found to be significantly higher in patients with unstable angina.

Since α-tocopherol is an important lipid soluble antioxidant, we also analyzed plasma α-tocopherol levels in patients and controls. Total α-tocopherol levels were comparable in UAP, SAP and controls. However, when we calculated the amount of α-tocopherol per LDL-cholesterol, we found significantly lower levels in CAD patients as compared to controls. SAP patients also demonstrated a significantly lower α-tocopherol content in LDL, that was intermediate between controls and UAP patients. In that respect it is noteworthy, that Dieber–Rotheneder et al. [37] clearly demonstrated that the vitamin E content in LDL and not in whole plasma correlated with the susceptibility of LDL for oxidation. This probably relates to the fact that vitamin E distributes in plasma among all lipoproteins and the lipid free bottom fraction. Frei et al. also found that the susceptibility of LDL for oxidation correlates negatively with the vitamin E content in LDL [38].

Considering the inflammatory events which have been demonstrated in UAP patients, our data point towards an increased oxidative stress in this patient group which might be accompanied by an increased metabolic consumption of anti-oxidative vitamins. Since total plasma α-tocopherol levels were not different from controls, it seems unlikely that the intake of Vitamin E in this patient group was diminished.

On the basis of our results, we are unable to determine whether the elevated oxidative parameters are a cause or an effect of UAP. It is interesting to note that oxidized LDL rapidly impairs endothelium-dependent vessel dilatation [39]. This may relate to reduced biosynthesis or rapid inactivation of nitric oxide, formation of peroxinitrite, stimulation of endothelin production and others. If these
events are underlying mechanisms in UAP, the increased peroxides must be considered to be causally linked with UAP.

Patients suffering from UAP are amenable to drug treatment as demonstrated in the FRISC (Fragmin in Unstable Coronary Artery Disease) study where low molecular weight heparin and aspirin reduced the event rate during short-term and long-term treatment [38].

A randomised controlled trial of alpha tocopherol treatment in patients with coronary disease (CHAO) could recently demonstrate that alpha-tocopherol treatment substantially reduces the rate of non-fatal myocardial infarction [40].

Here we report for the first time that plasma lipid peroxidation products are increased in patients with UAP, and that these patients show a relative lack of alpha-tocopherol in their LDL. Therefore it might well be useful to add antioxidants such as alpha tocopherol to the drugs that are currently applied in the treatment of UAP. We are presently conducting such experiments in our department.

Acknowledgements

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