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Plasma levels of lipid and cholesterol oxidation products and cytokines in diabetes mellitus and cigarette smoking: effects of vitamin E treatment

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Abstract

To evaluate the role of both oxidation and inflammation in atherosclerosis, we compared LDL oxidizability, in vivo lipid and cholesterol oxidation, and basal and lipopolysaccharide (LPS)-stimulated production of various cytokines in normolipidemic patients with diabetes mellitus (DM; n = 11), cigarettes smokers (n = 12) and controls (n = 14). In addition, the effects of vitamin E (600 I.U./day for 4 weeks) on these parameters were evaluated. Initial LDL oxidation characteristics before and after vitamin E were identical in the 3 groups. Plasma thiobarbituric acid reactive substances were higher in DM and smokers versus controls (0.77 ± 0.22, 0.74 ± 0.14 versus 0.62 ± 0.10 μmol malondialdehyde equivalents/l, respectively; P versus controls <0.05) and normalized after vitamin E supplementation. Total plasma oxysterols were higher in smokers versus controls (354 ± 104 versus 265 ± 66 nmol/l, P < 0.05) and unaffected by vitamin E. The basal and LPS-stimulated levels of interleukin-1β and tumour necrosis factor α (TNFα) and the basal level of interleukin-1-receptor antagonist (IL-1RA) were identical for the 3 groups. LPS-stimulated IL-1RA was higher in DM versus controls (10.7 ± 2.0 versus 8.1 ± 1.7 pmol/l, P < 0.05). After vitamin E, TNFα dropped in controls and smokers, and IL-1RA in smokers only. Results suggest increased in vivo oxidative stress and inflammation in DM and smoking, which is partly overcome by vitamin E. © 1997 Elsevier Science Ireland Ltd.

Keywords: Atherosclerosis; Diabetes mellitus; Smoking; LDL oxidation; Antioxidants; Cytokines; Vitamin E

1. Introduction

The role of oxidative modification of LDL in the process of atherogenesis has been made likely by a number of observations in vitro models, in animal models and in humans [1,2]. Oxidized LDL present in atherosclerotic lesions is taken up avidly by monocytes rendering them into foam cells and induces chemotaxis of monocytes [1-5]. These observations together with the observation that antioxidants can inhibit the oxidative process hold promise for the development of new means of prevention of atherosclerosis [6-9]. Indeed, in animal models antioxidants have been shown to retard the atherosclerotic process [10-12], although these results have not always been confirmed [13,14]. Beneficial effects are also suggested by the results of observational studies in humans, but intervention studies are negative so far, or still on the way [15-21].

Atherosclerosis resembles a chronic inflammation in which immunocompetent cells are involved and cytokines are secreted [22,23]. In vitro, activation of mononuclear cells induces LDL oxidation [24,25]. Importantly, modified LDL itself is able to induce inflammation, as it induces adhesion and influx of monocytes, and influences cytokine release by monocytes [22,23,26-30]. A number of cytokines have been shown to modulate monocyte adhesion to endothelial cells and the oxidative potential of cells in the vascular wall, thus influencing early atherogenesis [22,23]. In addition, oxidized...
2. Methods

2.1. Study design

Volunteers were recruited from the outpatient clinic and from the healthy population. They were between 18 and 70 years of age, without hypertension or hyperlipidemia (serum cholesterol less than 6.5 mmol/l, fasting triglycerides less than 2.3 mmol/l, HDL-cholesterol more than 0.9 mmol/l in men, more than 1.0 mmol/l in women). They were not using any lipid lowering drugs, vitamin E or hormones apart from oral contraceptives. Diabetes mellitus patients all had non-insulin dependent diabetes mellitus, and were treated with oral drugs (glibenclamide: n = 2; glibenclamide and metformin: n = 2; metformin and acarbose: n = 1; subcutaneous insulin: n = 6. The duration of diabetes ranged from 3 to 24 years (mean 10 ± 7 years, median: 8 years). Smokers smoked at least 15 cigarettes per day, the average daily consumption amounted to 24 cigarettes. All other individuals were non-smokers. Three diabetes patients had established coronary artery disease (previous myocardial infarction, coronary angioplasty and stable angina pectoris, respectively). In the group with diabetes mellitus 3 out of 6 women were premenopausal, none were taking oral contraceptives; in the smokers group, 7 out of 8 women were premenopausal, 3 taking oral contraceptives and in the controls, 1 out of 6 women was premenopausal; she was not taking oral contraceptives.

Participants all underwent a brief physical examination, after which blood was taken after an overnight fast for the assays indicated below. Then they received DL-α-tocopherol acetate (vitamin E; Organon, Oss, The Netherlands) 600 mg (600 I.U.) per day in 3 equal doses for 4 weeks, after which blood sampling was repeated. Adherence to vitamin E was checked by tablet counting and amounted on the average to 98%. The protocol of the study was approved by the local medical ethical committee and written informed consent was obtained from all participants.

2.2. Isolation and storage of plasma

After an overnight fast, blood was collected in vacuum tubes in EDTA (final concentration: 1 mg/ml). Plasma was separated from cells by centrifugation. Plasma samples were stored after the addition of saccharose as cryopreservative (final concentration 6 g/l) at −80°C up to 12 months until further analysis of parameters indicated below. Storage conditions were identical for each study group. Plasma samples for the determination of cholesterol oxidation products were additionally supplemented with butylated hydroxytoluene (250 mg/l) before storage.

2.3. Measurement of LDL oxidation

LDL was isolated by a short-run ultracentrifugation procedure, essentially as described in [42]. We have shown earlier that storage under the conditions indicated does not influence isolation of LDL and the analyses indicated below [42]. The formation of conjugated dienes during copper-mediated oxidation of LDL was performed essentially as described [42,43]. From the resulting curve the following parameters were determined: (1) the lag time, which equals the time elapsed until the rapid increase of the absorbance; (2) the maximum rate of dienes formed, which is calculated from the maximum rate of change in absorbance; and (3) the maximum absorbance achieved [42,43].

2.4. Oxysterols in plasma

Cholesterol oxidation products were determined by a modification of a method described previously [44,45]. Plasma aliquots were thawed at room temperature and 19-OH-cholesterol was added as internal standard. Cholesteryl esters and oxysterol esters were hydrolyzed by saponification for 1 h at 37°C with 0.4 N ethanolic KOH. Lipids were extracted with hexane. Separation of cholesterol from oxysterols was achieved by solid phase extraction and HPLC. The oxysterols were derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide for 30 min at 60°C. After evaporation under nitrogen, the residue was dissolved in hexane and analysed by gas...
2.5. Basal and LPS induced levels of cytokines IL-1β, TNFα and IL-1 receptor antagonist in whole blood

Levels of cytokines IL-1β, TNFα, IL-1RA in whole blood both with and without stimulation with LPS were assayed as described [46,47]. Briefly, blood collected in EDTA (final concentration 1 mg/ml) was incubated with and without LPS in the presence of aprotinin at 37°C for 24 h, after which the cell supernatants were isolated for a further cytokine assay. Cytokines IL-1β, TNF-α and IL-1RA were measured with specific radio immuno assays, developed in our institute [46,47].

2.6. Other measurements

Thiobarbituric acid reactive substances (TBARS) in plasma were determined using a fluorescence method [48] modified as described [49]. Vitamin E levels in plasma and LDL were assayed on an HPLC (Spectra Physics Model 8800, Spectra Physics, Eindhoven, The Netherlands) with fluorescence as described [50].

Plasma cholesterol and triglycerides were measured by enzymatic methods (CHOD-PAP reagent no. 237574, Boehringer, Mannheim, Germany; and Sera-Pak, Miles, Milan, Italy, respectively). HDL-cholesterol was determined in plasma after precipitation of other lipoproteins with polyethylene glycol-6000 [51]. Lp(a) was measured with a specific radioimmunoassay (Apolipoprotein (a) RIA 100, Pharmacia Diagnostics, Uppsala, Sweden).

2.7. Statistical evaluation

Statistical evaluation was performed using the Student's t-test for paired and unpaired data. A 2-sided P < 0.05 was considered to be significant. Results are expressed as mean ± S.D.

3. Results

3.1. Characteristics of the study population

Characteristics of the study population are given in Table 1. Age, body mass index (BMI), total cholesterol, LDL cholesterol and HDL cholesterol were comparable, as were vitamin E levels in plasma and in LDL before and after supplementation. The basal triglyceride levels were slightly higher in the patients with diabetes mellitus and in smokers than in controls.

3.2. Effects of vitamin E on vitamin E content of plasma and LDL

Supplementation with vitamin E resulted in a 2- to 3-fold increase in the concentration of vitamin E in plasma, and a 2-fold-increase in that in LDL (Table 2). BMI, fasting blood glucose, HbA1c and lipid levels were unchanged after vitamin E as compared to the values before intervention.

3.3. Initial values of parameters of LDL oxidizability and lipid oxidation and the effects of vitamin E supplementation

Parameters of LDL oxidizability before and after supplementation with vitamin E are given in Table 2. In basal conditions, no differences between groups were found for lag time, maximal rate of diene formation and maximum dienes formed. As expected, after vita-
Table 2
Vitamin E concentrations, LDL oxidation characteristics and plasma TBARS before and after supplementation with vitamin E in the groups studied

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 14)</th>
<th>Diabetes (n = 11)</th>
<th>Smokers (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg/l plasma)</td>
<td>26 ± 6</td>
<td>29 ± 8</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Vitamin E (mg/mg LDL)</td>
<td>14 ± 4</td>
<td>11 ± 4</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>99 ± 23</td>
<td>97 ± 19</td>
<td>102 ± 16</td>
</tr>
<tr>
<td>Maximal rate*</td>
<td>14 ± 3</td>
<td>14 ± 2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Maximal dienes formed b</td>
<td>835 ± 119</td>
<td>765 ± 111</td>
<td>760 ± 115</td>
</tr>
<tr>
<td>TBARS c</td>
<td>0.62 ± 0.10</td>
<td>0.77 ± 0.22*</td>
<td>0.74 ± 0.14*</td>
</tr>
</tbody>
</table>

| After vitamin E       |                  |                  |                 |
| Vitamin E (mg/l plasma) | 52 ± 18          | 66 ± 21          | 57 ± 17         |
| Vitamin E (mg/mg LDL)  | 27 ± 9           | 25 ± 12          | 22 ± 7          |
| Lag time (min)        | 146 ± 14**       | 147 ± 29**       | 128 ± 28***     |
| Maximal rate*         | 11 ± 2***        | 11 ± 3***        | 11 ± 2          |
| Maximal dienes formed b | 787 ± 104        | 771 ± 123        | 750 ± 94        |
| TBARS c               | 0.57 ± 0.18      | 0.63 ± 0.21***   | 0.56 ± 0.15**** |

Results are mean ± S.D.

* In nmol dienes/mg LDL/min; b in nmol dienes/mg LDL; c in μmol MDA equivalents/l.

* P vs. controls < 0.05; ** P vs. initial value < 0.001; *** P vs. initial value < 0.01; **** P vs. initial value < 0.05.

Vitamin E supplementation increased the lag time in all 3 groups, the change being the smallest in smokers. The maximum rate of dienes formed decreased in controls and patients with diabetes, but was unchanged in smokers. Vitamin E did not influence the maximum amount of dienes formed.

TBARS concentrations in plasma of diabetes patients and smokers were significantly higher than in controls. After vitamin E treatment there was a significant drop in concentration of TBARS in both diabetes patients and smokers, to values comparable to those of the control subjects.

Oxysterol concentrations of 5 oxysterols in the various groups before and after vitamin E supplementation are given in Table 3. Before vitamin E supplementation the total amount of oxysterols was higher in smokers than in controls, due to higher concentrations of both 5,6α-epoxy-5α cholestan-3α-ol and cholest-5, 3β-ol, 7-one. After vitamin E supplementation, only 5,6α-epoxy-5α cholestan-3α-ol was found to be significantly decreased. In diabetes patients, initial concentrations were no different from those in controls, whereas after vitamin E supplementation levels of cholest-5 ene 3β,7β-diol, 5α-cholestan-3β,5,6-triol and cholest-5, 3β-ol, 7-one had decreased significantly.

3.4. Initial and LPS induced levels of IL-1β, TNFα and IL-1RA, and the effects of vitamin E

The basal and LPS induced levels of the various cytokines in the studied groups and the effects of vitamin E on these parameters are given in Table 4. Unstimulated levels of IL-1β, TNFα and IL-1RA before and after vitamin E supplementation were comparable within groups, except for the IL-1RA level in the smokers after vitamin E supplementation, which was higher than that in control subjects. After LPS stimulation, the level of each cytokine increased as expected. Before vitamin E supplementation, LPS-stimulated levels of IL-1RA were higher both in smokers and patients with diabetes mellitus than in controls. After vitamin E supplementation, the LPS-induced release of TNFα and IL-1RA dropped significantly in smokers and that of TNFα in controls, IL-1RA reaching levels in controls and smokers which were significantly lower than in diabetes patients.

4. Discussion

In the current study, parameters of in vitro oxidizability of LDL, in vivo fatty acid and cholesterol oxidation and basal and LPS-induced levels of cytokines were evaluated in patients with diabetes mellitus, cigarette smokers and in controls. In addition, the effects of vitamin E on these parameters were evaluated.

In each group, supplementation with 600 I.U. vitamin E per day during 4 weeks resulted in a comparable increase in the plasma vitamin E level by 2- to 3-fold and in LDL by 2-fold. Basal levels of vitamin E and the observed increase is grossly in accordance with other studies [6,7,15,52,53]. Vitamin E supplementation had no effect on lipid levels or glycemic control.

LDL oxidizability before vitamin E supplementation was comparable in the 3 groups studied. In studies on patients with diabetes mellitus, both increased and unchanged LDL oxidizability has been observed as compared to controls. An increased oxidizability has been postulated to be related to the presence of small dense
Table 3
Plasma concentrations of oxysterols before and after supplementation with vitamin E (600 I.U./day) for 4 weeks

<table>
<thead>
<tr>
<th>Oxysterol concentration (nmol/l)</th>
<th>Controls (n = 13)</th>
<th>Diabetes (n = 10)</th>
<th>Smokers (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholest-5 ene 3β, 7β-diol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial value</td>
<td>38.4 ± 12.3</td>
<td>47.1 ± 24.9</td>
<td>42.2 ± 12.8</td>
</tr>
<tr>
<td>After vitamin E</td>
<td>37.7 ± 12.9</td>
<td>35.8 ± 11.4*</td>
<td>40.8 ± 14.9</td>
</tr>
<tr>
<td>5,6α-epoxy-5α cholestan-3α-ol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial value</td>
<td>51.0 ± 18.6</td>
<td>49.1 ± 14.1</td>
<td>71.5 ± 23.3**</td>
</tr>
<tr>
<td>After vitamin E</td>
<td>56.4 ± 18.1</td>
<td>48.2 ± 15.8</td>
<td>55.6 ± 20.0***</td>
</tr>
<tr>
<td>5α-cholestan-3β,6,5,6-triol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial value</td>
<td>29.3 ± 7.3</td>
<td>36.6 ± 38.6</td>
<td>36.9 ± 16.4</td>
</tr>
<tr>
<td>After vitamin E</td>
<td>36.7 ± 17.7</td>
<td>26.0 ± 17.6*</td>
<td>31.7 ± 13.2</td>
</tr>
<tr>
<td>cholest-5 3β-ol, 7-one</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial value</td>
<td>121 ± 38</td>
<td>166 ± 120</td>
<td>181 ± 81**</td>
</tr>
<tr>
<td>After vitamin E</td>
<td>126 ± 41</td>
<td>120 ± 64*</td>
<td>135 ± 66</td>
</tr>
<tr>
<td>cholest-5 ene 3β, 25-diol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial value</td>
<td>25.6 ± 7.9</td>
<td>22.5 ± 5.7</td>
<td>22.5 ± 3.8</td>
</tr>
<tr>
<td>After vitamin E</td>
<td>22.8 ± 6.8</td>
<td>21.8 ± 5.1</td>
<td>22.1 ± 3.4</td>
</tr>
<tr>
<td>Total oxysterols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial value</td>
<td>265 ± 66</td>
<td>321 ± 191</td>
<td>354 ± 104**</td>
</tr>
<tr>
<td>After vitamin E</td>
<td>269 ± 55</td>
<td>252 ± 98</td>
<td>285 ± 102</td>
</tr>
</tbody>
</table>

* P<0.05 vs. initial value; **P<0.05 vs. control group; *** P<0.01 vs. initial value.

LDL, as found in diabetes mellitus patients with hypertriglyceridemia [40,54]. We studied normolipidemic patients only, possibly explaining the lack of difference. In smokers, parameters of LDL oxidizability were no different from controls, confirming the results of others [6].

After vitamin E supplementation the lag time increased by 50% as expected, and, although the response was smaller in the smokers group, there were no significant differences between the studied groups. Results of changes in lag time are well in accordance with the change in the vitamin E content in LDL, as reported by Prinzen et al. [6]. These findings indicate that neither smoking nor diabetes mellitus influences the effects of vitamin E supplementation on parameters of LDL oxidizability.

The concentration of products of fatty acid peroxidation, as measured by TBARS in plasma was higher in both diabetes patients and smokers than in controls. This may indicate the presence of increased oxidative stress in these two groups [9,37,55]. In diabetes patients increased levels of TBARS are probably related to an enhanced lipid peroxidation by the high glucose levels itself and to the generation of superoxide anion [36,37,56]. In accordance with studies on the effects of antioxidants in diabetes patients and diabetic rats, we observed a decrease in the concentration of TBARS after vitamin E treatment [40,41]. Increased concentrations of TBARS in plasma of smokers have been found by others [57]: normalization of the concentration after vitamin E is in accordance with its effect on acute smoking [9].

The elevated concentration of TBARS in diabetes mellitus and smokers contrasts with the observed unaltered susceptibility to in vitro oxidation of LDL. It suggests that the in vivo oxidation is not reflected by the in vitro oxidizability of LDL. It might point towards shortcomings of the method evaluating in vitro oxidizability of LDL, or to the presence of other oxidation processes apart from those related to circulating LDL occurring in vivo.

Analysis of TBARS in plasma is a widely used method for the evaluation of lipid peroxidation. In order to evaluate another parameter of oxidative stress, which aims at the evaluation of free radical-dependent cholesterol oxidation rather than that of fatty acids, we measured oxysterol levels in plasma. We postulated that cholesterol oxidation products, derived from both lipoproteins and cell membranes, can serve as an additional marker of in vivo oxidative stress, being complementary to the other parameters studied. Oxysterols identified in both human and rabbit atherosclerotic plaques [58,59] have been shown to exert biological activities, as reviewed by Smith et al. [60]. There have been reports on cytotoxicity of cholestanetriol, 7β,OH cholesterol, 5,6α-epoxy-5α cholestan-3α-ol and 25-OH cholesterol to smooth muscle cells and endothelial cells and the induction of cytokine release by macrophages [61–64]. Eventually, cell injury caused by incorporation of oxysterols into cell membranes can lead to the disruption of endothelial integrity and permeability, facilitating platelet aggregation, monocyte adhesion and penetration of LDL into the arterial wall [65]. Altogether, it is hypothesized that oxysterols are of importance in rela-
tion to oxidative stress and inflammation. Hitherto, in intervention studies with antioxidants, plasma concentrations of oxysterols have not been studied before. In the current study, we found higher initial oxysterol concentrations in the smokers compared to controls, but not in the diabetes patients. The sample size of the groups studied may have been too small to detect differences in the diabetes patients, since there was a trend towards higher oxysterol levels in this group.

The inflammatory status, as reflected by circulating and LPS-stimulated cytokine production showed no differences in levels of IL-1β and TNFα, whereas there was an increased LPS-stimulated level of IL-1RA in both patients with diabetes and smokers versus controls. Increased levels of the cytokine IL-1RA have been observed in the acute phase of meningococcal disease and in rheumatoid arthritis, and is supposed to reflect an anti-inflammatory reaction.[66,67] Thus, the results of IL-1RA levels in our study would indicate an increased inflammation in patients with diabetes mellitus and in smokers. This might be due to the increased oxidative stress, to hyperglycaemia and to toxic substances in cigarettes, but can also reflect an initiation of inflammation related to cardiovascular disease. Obviously, we were not able to correct for these variables. Supplementation with vitamin E partly corrected the increased IL-1RA response in smokers, but not in patients with diabetes mellitus. If the above hypothesis were true, this reflects a sustained stimulation of the inflammatory system, despite a decreased lipid peroxidation. The latter could be related to hyperglycaemia.

In summary, our data show elevated peroxidation of lipids and increased ex vivo LPS stimulated IL-1RA activity in patients with diabetes mellitus and in smokers and increased oxysterol levels in smokers, whereas the susceptibility of LDL is no different from that of normal controls. Vitamin E normalizes the differences with normal controls, except from the increased LPS stimulated IL-1RA activity in diabetes patients. Results suggest a role for in vivo oxidative stress in both conditions, but in diabetes patients stimulation of inflammation is possibly mediated by additional processes also, hyperglycaemia being a good candidate.

Acknowledgements

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