Effect of glycaemic control and age on low-density lipoprotein susceptibility to oxidation in diabetes mellitus type 1*

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Background Although individuals with diabetes mellitus frequently have dyslipidaemias and high blood pressure, much of the increased risk for coronary heart disease is not explained by these and other classical risk factors. Thus, other less widely recognized risk factors, including increased susceptibility of low-density lipoprotein (LDL) to oxidation, might enhance vascular dysfunction and atherogenesis in diabetes.

Aims We compared both the rate and extent of LDL oxidation ex vivo between 78 poorly controlled individuals with type 1 diabetes and 78 age- and sex-matched non-diabetic controls. We then initiated intensive insulin therapy for 3 months to determine the impact of improved glucose control on LDL composition and oxidation.

Results Diabetic and non-diabetic individuals did not have significantly different body weights, dietary intake, blood pressure, renal function or plasma lipid levels. LDL composition was also similar in both groups. In contrast, vitamin E content in LDL was significantly lower in diabetic patients. Measures of LDL lipid oxidation, including conjugated diene, lipid peroxide and thiobarbituric acid reactive substances formation, as well as measures of LDL protein modification, were significantly greater in diabetic patients. Levels of hyperglycaemia correlated strongly with each measure of LDL lipid oxidation (r ranges from 0.60–0.81, P<0.05 for each correlation). After improved glucose control (average reduction in % HbA1c of 5.5 units) all measures of LDL oxidation improved dramatically and approached values for non-diabetics. Absolute values of LDL oxidation increased among all categories of age in both diabetic and control individuals, and this relationship persisted even after adjustment for differences in glucose concentrations.

Conclusions We demonstrate that hyperglycaemia has a potent but reversible effect on LDL oxidation and that age may independently enhance LDL susceptibility to oxidation. These pathophysiological effects may play an important role in determining vascular complications and atherogenesis in poorly controlled type 1 diabetic patients.


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Key Words: LDL oxidation, diabetes, ageing, oxygen radicals, hyperglycaemia.

See page 2045 for the Editorial comment on this article.
Introduction

Diabetes mellitus type 1 is associated with increased morbidity and mortality, largely as a consequence of accelerated atherosclerotic disease. Unfortunately, our understanding of the mechanisms underlying this relationship is incomplete. Although diabetes is frequently associated with increased levels of coronary heart disease risk factors, these do not fully explain the accelerated development of atherosclerosis. This raises the possibility that other less widely recognized cardiovascular risk factors, such as increased LDL oxidation, may enhance the atherogenic process in individuals with diabetes. The mechanisms by which LDL oxidation may accelerate atherosclerosis have been extensively reviewed. There is increasing evidence that both LDL and plasma from individuals with diabetes may be more susceptible to oxidation. Moreover, hyperglycaemia and formation of advanced glycation end-products may contribute to these events. For example, addition of exogenous glucose in vitro and the process of glycosylation increase LDL susceptibility to oxidation. Hyperglycaemia also upregulates monocyte and endothelial 12/15-lipoxygenase activity that promotes LDL oxidation. Advanced glycation end-product can activate a wide variety of cells within the arterial wall leading to enhanced intracellular and extracellular production of free radicals and lipid oxidation. Thus, advanced glycation end-product formation and lipid peroxidation may be mutually reinforcing and their interaction may further enhance development of vascular dysfunction and atherogenesis.

Although the causal relationship of increased plasma LDL oxidation and coronary heart disease remains as yet theoretical in humans, several studies have evaluated LDL oxidizability in diabetes mellitus type 1 patients and the results have not been consistent. This may in part be related to differences in methodology of LDL isolation and oxidation as well as to the relatively small number of individuals that have been evaluated in each study to date. Perhaps a more important explanation may be the relatively large variation in glycaemic control between diabetic subjects in each study. Because the generation of radicals via glucose autoxidation and formation of redox-active glycated proteins, including glycated LDL and advanced glycation end-product proteins, is related to glycaemic control, study subjects with greater hyperglycaemia should presumably demonstrate greater LDL susceptibility to oxidation. Moreover, if this is indeed the case, it seems reasonable to expect that improving glycaemic control will reduce LDL susceptibility to oxidation.

Therefore, the present study was designed to compare the susceptibility of LDL to oxidation in a large and well-defined population of poorly controlled diabetes mellitus type 1 patients and healthy non-diabetic controls stratified by age. Furthermore, to determine whether hyperglycaemia has a direct influence on LDL oxidation, measures of LDL susceptibility to oxidation were repeated in the same diabetic population after returning glucose levels toward normal values with 3 months of intensive insulin therapy.

Patients, materials, and methods

Diabetes mellitus type 1 patients and healthy controls

Seventy-eight patients with diabetes mellitus type 1 and 78 healthy controls were studied. Subjects from each group were carefully matched for age, sex, body mass index, blood pressure and alcohol intake. Both groups underwent a thorough screening visit, including a medical and dietary history, physical examination, electrocardiograph (ECG), routine urine analysis (including microalbumin testing) and blood chemistry profile. Retinopathy was evaluated by retinal colour funds, photographs of two macular and optic disk fields and by fluorescein angiographs. Peripheral vascular disease was defined by the presence of symptoms of claudication or history of arterial occlusive diseases.

The medical history and ECG were used to provide information regarding the presence or absence of coronary heart disease and ischaemic stroke. Nutrient intake was assessed by means of a self-administered food-frequency questionnaire containing 277 of the most commonly eaten Italian foods. All meals consumed in 1 week were recorded. Mean daily energy intake and diet composition were estimated by using a computer program for nutrient analysis (Food Meter; Bayer Diagnostics, Milan, Italy). Dietary evaluation (assessed by the food questionnaire) was performed during the screening visit for both controls and diabetic subjects and monthly thereafter for the diabetic subjects during the period of intensive glucose control. Subjects were encouraged to maintain body weight during the study and dietary recommendations were provided as needed to ensure that this goal was achieved. Exclusion criteria included current smoking, hepatic, cardiac or renal disease, untreated hypothyroidism and pregnancy.

Diabetes mellitus type 1 was defined in accordance with the criteria of the American Diabetes Association. The average duration of diabetes was 9.2 ± 3 years (range 1 month–28 years). The diabetes mellitus type 1 group was composed of poorly controlled outpatients (HbA1c >8%) recruited at the Ospedale dei Pellegrini, Naples, Italy. Concentrations of the C-peptide were not measured. Four of the diabetes mellitus type 1 patients had non-proliferative retinopathy, one had proliferative retinopathy changes and none had macroalbuminuria. Healthy individuals were selected from hospital staff to serve as non-diabetic controls, with no family or personal history of hypertension or coronary heart disease. These subjects were excluded if they had elevated fasting or post-challenge glucose values. Non-diabetic and diabetic subjects were carefully matched for age, sex, body mass index, blood pressure and alcohol intake. Both groups underwent a thorough screening visit, including a medical and dietary history, physical examination, electrocardiograph (ECG), routine urine analysis (including microalbumin testing) and blood chemistry profile. Retinopathy was evaluated by retinal colour funds, photographs of two macular and optic disk fields and by fluorescein angiographs. Peripheral vascular disease was defined by the presence of symptoms of claudication or history of arterial occlusive diseases.

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were excluded if they were taking the following medications or supplements: oestrogens, glucocorticoids, \( \beta \)-adrenergic antagonists, calcium antagonists, diuretics, lipid lowering agents, antioxidant vitamins, fish oil or herbal compounds. In order to eliminate variability in results due to storage and handling of samples, clinic visits were scheduled such that individuals with diabetes mellitus and matched control subjects provided blood samples on the same days. LDL samples were prepared from freshly isolated plasma and were used immediately in the oxidation studies described below.

LDL was isolated from diabetes mellitus type 1 patients on two separate occasions; initially, when they were in poor glycaemic control, and again after approximately 3 months (range 2.8–3.3 months) of intensive insulin therapy. At the end of the intensive treatment period, 73 diabetes mellitus type 1 patients were receiving multiple-dose regimens of insulin (ultralente \( \pm \) regular insulin) and were on insulin pumps. The diet for both non-diabetic and diabetic groups had a composition that was on average 27–28% fat (>80% in the form of monounsaturated fatty acids), 12–14% protein and 52–58% carbohydrate (75% in the form of complex carbohydrate). Diabetic patients followed a similar diet. In both groups the cholesterol intake was less than 210 mg.day\(^{-1}\). The intake of dietary fibre was greater than 58 g.day\(^{-1}\). The present study was approved by the Human Study Ethical Committee of the Azienda Sanitaria Locale (ASL) NA1, Naples, Italy and it complied with the Declaration of Helsinki.

**Preparation of LDL**

LDL was isolated from plasma prepared from fasting blood drawn on the morning of each subject’s visit. LDL was prepared within 3.5 h by performing two rapid sequential KBr density gradient ultracentrifugations as described previously in detail\(^{[30]}\). This isolation procedure, to reduce the spontaneous oxidation of LDL, has been previously documented\(^{[30]}\). Each LDL preparation was examined for contamination with other lipoprotein particles by performing SDS-polyacrylamide and agarose gel electrophoresis. Protein content was measured by the Lowry assay\(^{[31]}\), using bovine serum albumin as the standard.

**Lipid analysis and vitamin E concentration of LDL**

Plasma total cholesterol and triglycerides were measured by immunoenzymatic kits (Boehringer Mannheim, Monza, Italy) and LDL was calculated. Plasma high density lipoprotein cholesterol (HDL) was measured in a similar fashion after precipitation of apolipoprotein B particles using phosphotungstic acid and MgCl\(_2\). LDL lipids of individual subjects were extracted by the methodology of Folch et al.\(^{[32]}\), modified as previously described in detail\(^{[33]}\). Separation of phospholipids, cholesteryl esters, triglycerides and free cholesterol was obtained by thin layer chromatography, and visualization was carried out according to the procedure of Bittman and Wood\(^{[34]}\). Lipids of each class were then measured by standard enzymatic procedures and are presented as a percentage of total LDL mass, as described\(^{[35]}\). Fatty acids were methylated with diazomethane and fatty acid methyl esters were injected into a gas chromatograph/mass spectrometer (GC 5890, MSD 5970, Hewlett Packard, Palo Alto, California, U.S.A.), as described in detail\(^{[35–37]}\). Content of vitamin E in LDL particle was determined by HPLC\(^{[33–35]}\). Briefly, LDL was precipitated with ethanol and vitamin E was extracted with hexane. The hexane phase was then evaporated and the residue dissolved in methanol and separated by HPLC using 3 mmol.l\(^{-1}\) particle size Supelcosil LC-8-DB guard (15 mm \( \times \) 4.6 mm) and analytical (150 mm \( \times \) 4.5 mm) columns (Supelco Inc., Milan, Italy).

**Oxygen radical generation**

LDL samples were oxidized by exposure to oxygen radicals generated by the reaction of xanthine (0.2 mm) with xanthine oxidase (100 mU.ml\(^{-1}\), salicylate-free, from bovine milk, specific activity 1 U.mg\(^{-1}\) of protein) in a 150 mm sodium chloride/100 mm sodium phosphate buffer, pH 7.4, as previously described\(^{[33,36]}\). The above reaction yields superoxide radicals, hydrogen peroxide and singlet oxygen, which in turn may give rise to hydroxyl radicals in the presence of trace amounts of iron or other transition metals\(^{[33,36]}\). At peak radical generation, the production of superoxide and hydrogen peroxide is approximately 20 nmoles min\(^{-1}\).ml\(^{-1}\) and 40 nmoles min\(^{-1}\).ml\(^{-1}\), respectively\(^{[33,36]}\).

**Oxidative modification of LDL**

To measure LDL susceptibility to oxidation in the presence of xanthine and xanthine oxidase, we determined the length of the lag-phase preceding the onset of rapid oxidation in LDL as previously described\(^{[33,35]}\). In additional experiments, LDL samples were also incubated with xanthine/xanthine oxidase for 18 h and aliquots were retained for measurement of lipid peroxides, malondialdehyde equivalents and modification of free lysine groups. Formation of lipid peroxides was measured using a commercial kit (Kamiya Biomedical Company, Thousand Oaks, CA, U.S.A.) and formation of malondialdehyde equivalents was determined by measurement of thiobarbituric acid (TBA) reactive substance\(^{[35,36]}\). Propagation of peroxidation during performance of the TBA assay was prevented by adding butylated hydroxytoluene (100 \( \mu \)mol final concentration) to all samples\(^{[35,36]}\). The percentage reduction in free lysine groups present in LDL after exposure to oxygen.

Table 1  Selected characteristics of the study population (healthy controls and diabetes mellitus type 1 patients before and after improved glycaemic control)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy controls (n=78)</th>
<th>Diabetes mellitus type 1 patients in poor glycaemic control (n=78)</th>
<th>Diabetes mellitus type 1 patients with improved glycaemic control (n=78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (F/M)</td>
<td>18/60</td>
<td>18/60</td>
<td>18/60</td>
</tr>
<tr>
<td>Body mass index (kg.m(^{-2}))</td>
<td>24.8 ± 2.9</td>
<td>24.3 ± 2.6</td>
<td>23.7 ± 4.0</td>
</tr>
<tr>
<td>Daily energy intake (kJ.kg(^{-1}))</td>
<td>125.6 ± 6.3</td>
<td>120.1 ± 8.4</td>
<td>123.6 ± 8.8</td>
</tr>
<tr>
<td>Daily protein intake (kJ.kg(^{-1}))</td>
<td>15.5 ± 0.7</td>
<td>14.9 ± 0.5</td>
<td>15.3 ± 0.6</td>
</tr>
<tr>
<td>Daily cholesterol intake (mg)</td>
<td>200 ± 15</td>
<td>199 ± 16</td>
<td>194 ± 13</td>
</tr>
<tr>
<td>Daily fat intake (%)</td>
<td>28.9 ± 1.0</td>
<td>27.7 ± 1.3</td>
<td>27.5 ± 1.2</td>
</tr>
<tr>
<td>Daily carbohydrate intake (%)</td>
<td>55.9 ± 6.5</td>
<td>53.2 ± 5.8</td>
<td>54.3 ± 6.2</td>
</tr>
<tr>
<td>Alcohol intake (ml.week(^{-1}))</td>
<td>78.5 ± 38.8</td>
<td>59.8 ± 13.2</td>
<td>61.2 ± 18.5</td>
</tr>
<tr>
<td>HbAlc (%)</td>
<td>3.94 ± 0.8</td>
<td>11.3 ± 2.5</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol.l(^{-1}))</td>
<td>4.2 ± 0.5</td>
<td>16.4 ± 2.4</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>Insulin dosage (IU.kg(^{-1}))</td>
<td>—</td>
<td>0.67 ± 0.06</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Arterial diastolic pressure (mmHg)</td>
<td>81 ± 9</td>
<td>83 ± 9</td>
<td>81 ± 10</td>
</tr>
<tr>
<td>Arterial systolic pressure (mmHg)</td>
<td>128 ± 13</td>
<td>131 ± 12</td>
<td>128 ± 15</td>
</tr>
<tr>
<td>Urinary albumin excretion rate (µg.min(^{-1}))</td>
<td>4 ± 1</td>
<td>7 ± 3</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Creatinine clearance (ml.min(^{-1}))</td>
<td>99 ± 24</td>
<td>89 ± 40</td>
<td>90 ± 48</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *P<0.001 vs healthy controls; †Insulin dosage is the mean dose at the time of improved glycaemic control. Note that five diabetic patients in stable glycaemic control were in on insulin pump.

radicals was measured using trinitrobenzenesulphonic acid[57], as previously described[14,35]. Briefly, native and oxidized LDL (50 µg of protein) were added to 1 ml of NaHCO\(_3\) (4% solution at pH 8.4) and 50 ml of trinitrobenzenesulphonic acid (0.1% solution) and heated for 70 min at 37°C. After this step, the absorbance at 340 nm was measured and compared with results obtained for native LDL. The enhanced electrophoretic mobility on agarose gels of oxidized LDL relative to native LDL was determined by measuring the distance (in centimetres) between the leading edge of each LDL band.

**Chemicals**

Agarose, acrylamide, SDS and other electrophoresis grade reagents were purchased from Bio-Rad (Richmond, CA, U.S.A.). Xanthine oxidase was obtained from Boehringer Mannheim (Monza, Italy). All other enzymes and chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). All solvents were of HLPC grade and purchased from Carlo Erba (Milan, Italy).

**Statistical analysis and calculations**

Results are expressed as means ± SD. Differences between subjects with diabetes and healthy subjects were evaluated by one-way ANOVA with post hoc tests performed by Student’s t-test, with significance defined at values of *P*<0.05. In order to assess the independent effects of age, gender, HbAlc level, fasting plasma glucose and vitamin E concentration on measures of LDL oxidation, multiple regression analyses was performed separately in each group. For multiple regression analyses, beta coefficients and *P* values are presented. Correlation between relevant variable were also evaluated by linear regression analysis. All data were analysed by SPSS statistical package (SPSS Inc., California, SPSS User’s Guide, 1992).

**Results**

**Diabetes mellitus type 1 subjects and healthy controls**

General characteristics and selected laboratory studies of the study population are presented in Table 1. Body mass index, dietary and alcohol intake were similar between the control and the diabetic groups (both before and after insulin therapy) as were measures of blood pressure and renal function. As expected, fasting blood glucose and HbAlc were higher in diabetes mellitus type 1 patients in poor glycaemic control than those in healthy individuals (Table 1). Intensive insulin therapy markedly reduced fasting plasma glucose and HbAlc levels; however HbAlc levels remained significantly higher than in controls.

**Plasma lipids and LDL composition**

No differences were observed in plasma lipid and lipoprotein levels between healthy controls and diabetic patients (Table 2). Furthermore, no significant changes in lipid levels occurred in diabetic subjects after improved glycaemic control. A reduction in plasma triglycerides might be expected after glycaemic control; however our study population had basal low levels of...
triglyceridaemia. In addition, no differences in total cholesterol, cholesterol ester, phospholipid or triglyceride mass in LDL were present between these groups. Fatty acid composition of LDL was also similar in these two groups. After improved glucose control, LDL lipid composition was not changed in subjects with type 1 diabetes. However, vitamin E content in LDL was significantly lower in patients with poorly controlled diabetes and subsequently returned to control levels after intensive insulin therapy.

**LDL lipid oxidation: effect of glycaemic control**

Measures of ex vivo LDL oxidation are shown in Table 3 for healthy controls and diabetic patients. LDL isolated from poorly controlled diabetic subjects was more susceptible to oxidation, as shown by a significantly reduced lag time compared with healthy controls. In addition, the extent of LDL oxidation after exposure to xanthine/xanthine oxidase, as measured by generation of thiobarbituric acid reactive substance, lipid peroxides and conjugated dienes, was also greater in poorly controlled diabetes subjects. In addition, lag time, thiobarbituric acid reactive substance and lipid peroxides concentrations were all significantly correlated with glucose levels (r=0·60, P<0·02 and r=0·74 and r=0·81, P<0·001; respectively).

As noted above (Table 1), after 3 months of intensive insulin therapy, fasting plasma glucose levels returned to near-normal values. This was mirrored by a significant rise in LDL vitamin E content in the diabetic subjects. LDL susceptibility to oxidation was reduced and was no

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy controls</th>
<th>Diabetes mellitus type 1 patients in poor glycaemic control</th>
<th>Diabetes mellitus type 1 patients after recovery of stable glycaemic control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma total cholesterol (mg.dl⁻¹)</td>
<td>185·6 ± 20·8</td>
<td>190·1 ± 50·3</td>
<td>188·2 ± 40·3</td>
</tr>
<tr>
<td>Plasma triglycerides (mg.dl⁻¹)</td>
<td>123·5 ± 29·6</td>
<td>129·2 ± 25·5</td>
<td>130·2 ± 26·1</td>
</tr>
<tr>
<td>Plasma LDL-cholesterol (mg.dl⁻¹)</td>
<td>104·3 ± 30·4</td>
<td>105·6 ± 23·9</td>
<td>104·6 ± 28·3</td>
</tr>
<tr>
<td>Plasma HDL-cholesterol (mg.dl⁻¹)</td>
<td>48·7 ± 9·3</td>
<td>45·2 ± 10·8</td>
<td>44·6 ± 9·1</td>
</tr>
</tbody>
</table>

**Table 2 Plasma lipid profile and chemical composition, fatty acid content and vitamin E concentrations of LDL from healthy individuals and diabetes mellitus type 1 patients**

Values are mean ± SD.

C/P ratio=ratio of cholesterol (free cholesterol+cholesterol moiety) of cholesteryl esters to proteins.

Values are expressed in percent distribution of fatty acids in triglycerides extracted from LDL, for more details see ref.[33]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy controls</th>
<th>Diabetes mellitus type 1 patients in poor glycaemic control</th>
<th>Diabetes mellitus type 1 patients after improved glycaemic control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-time (min)</td>
<td>120·3 ± 14·6</td>
<td>94·6 ± 9*</td>
<td>113·4 ± 12·8</td>
</tr>
<tr>
<td>TBARS (nmoles.mg protein⁻¹)</td>
<td>22·4 ± 4·2</td>
<td>38·3 ± 7·2*</td>
<td>24·4 ± 6·3</td>
</tr>
<tr>
<td>LPO (nmoles.mg protein⁻¹)</td>
<td>52·1 ± 28·9</td>
<td>153·2 ± 20·5*</td>
<td>84·1 ± 18·3</td>
</tr>
<tr>
<td>Dienes (nmoles.mg protein⁻¹)</td>
<td>355·8 ± 61·6</td>
<td>725·4 ± 54·8†</td>
<td>503·9 ± 41·6*</td>
</tr>
</tbody>
</table>

Values are mean ± SD. LPO=lipid peroxides; TBARS=thiobarbituric acid reactive substances; *P<0·05 vs healthy controls; †P<0·01 vs healthy controls.
longer different from that of LDL isolated from healthy non-diabetic controls (Table 3). Moreover, the extent of LDL oxidation (as measured by generation of thiobarbituric acid reactive substance and lipid peroxides) was also reduced and approached levels of those from LDL of non-diabetic controls. Formation of conjugated dienes upon LDL oxidation was also reduced but remained significantly greater than the level of conjugated dienes that developed in LDL from non-diabetic controls.

**LDL apolipoprotein B-100 modification: effect of glycaemic control**

To determine the differences in the extent of LDL protein modification between study groups, each LDL sample was analysed for relative electrophoretic mobility and reduction of free lysine groups (trinitrobenzenesulphonic acid assay) after oxidation with xanthine/xanthine oxidase. As shown in Table 4, LDL isolated from poorly controlled diabetes mellitus type 1 subjects demonstrated significantly greater relative electrophoretic mobility than did LDL from non-diabetic controls. Similarly, a significantly greater decrease in free lysine groups was also seen in LDL from individuals with poorly controlled diabetes. With improved glucose control the extent of apolipoprotein B-100 modification resulting from oxidation was reduced and similar to that present in LDL from non-diabetic controls that were exposed to similar conditions of oxidative stress. Relative electrophoretic mobility values correlated positively with glucose concentration in poorly controlled diabetic subjects (r=0.83; P<0.0001). Similarly, the percent decrease in free lysine groups remaining in LDL after oxidation correlated positively with glucose levels in the same subjects (r=0.66; P<0.02).

**Effects of age on parameters of oxidation**

The strong relationship, presented above (Table 3), between degree of hyperglycaemia and LDL oxidation raised the possibility that advanced glycation end-product formation (both on LDLs and in tissue) may contribute to this oxidation process. To explore this possibility further we evaluated the relationship of ageing, (another condition associated with increased tissue advanced glycation end-product formation), on LDL susceptibility to oxidation in both poorly controlled diabetes mellitus patients and non-diabetic healthy controls. We therefore compared parameters of LDL oxidation across three age groups (<25, 25–59, and >60 years of age). As shown in Table 4, measures of both LDL lipid oxidation and protein modification progressively increased with age in both controls and diabetics.

### Table 4  Plasma glucose, HbA1c, oxidative parameters and vitamin E levels on LDL obtained from healthy controls and diabetes mellitus type 1 patients in poor glycaemic control after age stratification. Age distribution in the healthy controls applies to the diabetic patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy controls (age in years (n))</th>
<th>Diabetes mellitus type 1 patients in poor metabolic control (age in years (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;25 (n=43)</td>
<td>25–59 (n=24)</td>
</tr>
<tr>
<td>Lag-time (min)</td>
<td>132.3 ± 11.2</td>
<td>121.8 ± 10.5</td>
</tr>
<tr>
<td>TBARS (nmole/mg protein⁻¹)</td>
<td>18.2 ± 2.6</td>
<td>22.3 ± 2.8</td>
</tr>
<tr>
<td>LPO (nmole/mg protein⁻¹)</td>
<td>33.2 ± 7.4</td>
<td>45.2 ± 13.7</td>
</tr>
<tr>
<td>Dienes (nmole/mg protein⁻¹)</td>
<td>299.4 ± 25.6</td>
<td>366.8 ± 46.2</td>
</tr>
<tr>
<td>REM (cm)</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>TNBS (%)</td>
<td>−26.3 ± 2.5</td>
<td>−29.9 ± 3.0</td>
</tr>
<tr>
<td>Vitamin E (nmole/mg protein⁻¹)</td>
<td>3.8 ± 0.3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Fasting plasma glucose (mm)</td>
<td>4.2 ± 0.4</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.6 ± 0.6</td>
<td>4.0 ± 0.6</td>
</tr>
</tbody>
</table>

TBARS, thiobarbituric acid reactive substances; LPO, lipid peroxides; REM=relative electrophoretic mobility; TNBS=trinitrobenzenesulphonic acid; values are mean ± SD; P for trend test after age stratification.

Age was particularly strongly correlated with generation of conjugated dienes (r=0.69; P<0.01), thiobarbituric acid reactive substance (r=0.75; P<0.001), and relative electrophoretic mobility (r=0.64; P<0.01) in the poorly controlled diabetes type 1 patients. Age was also strongly correlated with generation of conjugated dienes (r=0.61; P<0.01), thiobarbituric acid reactive substance (r=0.68; P<0.001), and relative electrophoretic mobility (r=0.49; P<0.005) in healthy controls. In each age group, all indices of LDL oxidation were greater in individuals with poorly controlled diabetes than in non-diabetic healthy controls.

Vitamin E content in LDL appeared to be reduced with age in both control and diabetic subjects; with the lowest values occurring in the oldest diabetes mellitus type 1 patients. Moreover, there was a strong inverse correlation between age and vitamin E content in LDL isolated from poorly controlled diabetic subjects (r=-0.72; P<0.04) and from controls (r=-0.63; P<0.05).

Although the extent of hyperglycaemia did not increase significantly with age in the non-diabetic healthy controls, the level of fasting plasma glucose and HbA1c increased with age in the patients with poorly controlled type 1 diabetes.

In order to evaluate the independent effects of age and the degree of hyperglycaemia on LDL oxidation, these variables, along with gender and LDL vitamin E content were analysed in multiple regression models (Table 5). The gender of the study population did not appear to influence any parameters of LDL oxidation. In contrast, age was independently related to length of the lag time of oxidation, generation of conjugated dienes and lipid peroxides, and modification of free lysine groups in healthy subjects. Age was also independently related to generation of thiobarbituric acid reactive substance and conjugated dienes in patients with diabetes mellitus, although this relationship appeared less consistent than the association of these parameters in healthy controls.

Fasting plasma glucose was independently related to the level of thiobarbituric acid reactive substance and conjugated dienes formed only in poorly controlled diabetics. However, after improved glucose control in these subjects, age but not fasting glucose was an independent predictor of formation of conjugated dienes (data not shown). Finally, levels of vitamin E in LDL were independently related to generation of thiobarbituric acid reactive substance in healthy controls, and lipid peroxides in LDL from poorly controlled diabetes mellitus type 1 patients.

**Discussion**

A major finding in this study was that LDL isolated from diabetes mellitus type 1 patients in poor glycaemic control was more rapidly oxidized and became more extensively oxidized under the experimental conditions utilized in this study. The differences in both susceptibility to oxidation and extent of oxidation between LDL from controls and diabetic subjects were substantial and reflected greater oxidative modification of both lipid and protein components of LDL. The lag time of LDL oxidation was nearly 20% shorter and extent of conjugated diene, lipid peroxide and thiobarbituric acid reactive substance formation was approximately 100%, 200% and 80% greater, respectively, in LDL from poorly controlled individuals with type 1 diabetes.

These data are consistent with several other studies examining LDL oxidation in diabetes[7,9,24]. Unlike previous studies, we assessed a large number of well matched subjects providing a more definitive comparison of LDL oxidation between healthy controls and type 1 diabetes mellitus patients. The clear demonstration of enhanced susceptibility to oxidation of LDL from diabetic type 1 patients in this study may be related to several issues. LDL was rapidly isolated and utilized immediately in oxidation experiments, thereby reducing...

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**Table 5** Multiple regression analysis in order to evaluate the effect of gender, age, recovery of metabolic control (FPG) and vitamin E levels on LDL on oxidative compounds generated after oxidation of lipid (thiobarbituric acid reactive substances, lag-time, dienes, lipid peroxides) and proteic (trinitrobenzenesulphonic acid) components

<table>
<thead>
<tr>
<th></th>
<th>TBARS</th>
<th>Lag-time</th>
<th>LPO</th>
<th>Dienes</th>
<th>TNBS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy controls</strong></td>
<td>β</td>
<td>P</td>
<td>β</td>
<td>P</td>
<td>β</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.26</td>
<td>ns</td>
<td>2.44</td>
<td>ns</td>
<td>0.72</td>
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<tr>
<td>Age</td>
<td>4.12</td>
<td>ns</td>
<td>-0.86</td>
<td>0.000</td>
<td>1.77</td>
</tr>
<tr>
<td>Vitamin E on LDL</td>
<td>0.04</td>
<td>0.029</td>
<td>0.77</td>
<td>ns</td>
<td>7.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.56</td>
</tr>
<tr>
<td><strong>Diabetes mellitus type 1 patients in poor glycemic control</strong></td>
<td>β</td>
<td>P</td>
<td>β</td>
<td>P</td>
<td>β</td>
</tr>
<tr>
<td>Sex</td>
<td>-6.12</td>
<td>ns</td>
<td>-0.41</td>
<td>ns</td>
<td>7.12</td>
</tr>
<tr>
<td>Age</td>
<td>0.16</td>
<td>0.018</td>
<td>-0.23</td>
<td>ns</td>
<td>1.86</td>
</tr>
<tr>
<td>Vitamin E on LDL</td>
<td>0.94</td>
<td>0.012</td>
<td>0.64</td>
<td>ns</td>
<td>5.62</td>
</tr>
</tbody>
</table>

TBARS = thiobarbituric acid reactive substances; LPO = lipid peroxides; TNBS = trinitrobenzenesulphonic acid.
autoxidation of samples during their preparation and storage. In addition, samples from diabetic and matching control subjects were isolated and run together to avoid a systematic bias resulting from inter-assay variation over time. It was important that many of the dietary or metabolic factors that may influence LDL oxidation and potentially obscure differences between diabetic and non-diabetic groups were eliminated or controlled in this study. The subjects consumed similar diets, were on few medications, and had similar lipid profiles. Perhaps most important, in comparison to prior studies was that the diabetic subjects were significantly more hyperglycaemic.

The present study strongly supports the notion that hyperglycaemia plays an important role in LDL susceptibility to oxidation. The major difference between non-diabetic and diabetic groups was the marked hyperglycaemia of the type 1 diabetes patients. Levels of hyperglycaemia also correlated strongly with measures of LDL oxidation and this relationship persisted after adjustment for other relevant factors such as gender, age and vitamin E content in LDL. Perhaps the strongest evidence supporting the importance of hyperglycaemia in enhancing LDL oxidation was the dramatic decrease in LDL oxidation that occurred after improving glucose control with intensive insulin therapy. Although several diabetes medications have been associated with modest ‘antioxidant properties’[38–40], this has not been demonstrated for insulin. Moreover, despite having comparable or elevated levels of endogenously produced insulin compared with non-diabetic controls, subjects with type 2 diabetes also appear to have LDL that is more susceptibility to oxidation[9,41].

As noted above, the control and diabetic groups in this study were similar in other ways. Their diets were closely monitored which resulted in similar lipid and fatty acid contents of their LDL. Because these variables, and plasma lipid profiles, were similar between the two groups there is little evidence to suggest that there were major differences in LDL particle size distribution in this study. In addition, it is unlikely that other metabolic abnormalities commonly associated with insulin resistance would contribute to oxidation of LDL isolated from individuals with type 1 diabetes. However, we observed a small difference in the percentage of protein toward LDL that was lower in the diabetic subjects prior to good glycaemic control. This could represent a pro-oxidant state of LDL in these patients.

Hyperglycaemia may enhance oxidative stress by several mechanisms. Glucose itself may directly accelerate LDL oxidation[13,14] by processes involving autoxidation-mediated production of free radicals and structural alteration of enzymes carried within LDLs, e.g. platelet activating factor acetylhydrolase[14,36,42]. Glucose has also been shown to upregulate enzymes such as 12/15 lipoxygenase, that are capable of oxidizing LDL in the arterial wall[15–17]. Hyperglycaemia also increases advanced glycation end-product formation and a consequence of the molecular rearrangements leading to advanced glycation end-product formation is the increased production of free radicals[21,43]. Advanced glycation end-product proteins may also contribute to cellular oxidative stress via stimulation of radical production by macrophages and other vascular cells[43]. An additional consequence of this enhanced oxidative stress, aside from greater oxidation of LDLs in vivo, would presumably be an accelerated loss of antioxidant molecules. This is consistent with the reduced level of vitamin E measured in LDL isolated from the diabetic subjects in poor glycaemic control and the rise in levels of this molecule with effective treatment of their hyperglycaemia. More importantly, by perturbing oxidative status in the arterial wall, several oxidation-sensitive signalling mechanisms can promote atherogenesis[44–46].

These alterations can be reversed by antioxidants in vitro and in vivo[44,45]. Stratification of LDL oxidation by categories of age demonstrated a strong relationship between age and LDL oxidation. LDL isolated from the oldest subjects were more susceptible to oxidation, developed greater levels of conjugated dienes and lipid peroxides and had lower levels of vitamin E. These age-related events were independent of glucose concentrations and were present in both non-diabetic and diabetic subjects. In each age range, LDL from the diabetic subjects was more readily and extensively oxidized when exposed to oxidative stress than was LDL from non-diabetics. Moreover, the effects of ageing and diabetes appear additive because LDL from the older diabetic individuals was the most susceptible to oxidation and had the lowest vitamin E content. These data indicate that LDL oxidation, which is already present in early atherosclerotic lesions of human fetuses[47] and children[48], increases with age; however, this effect is less potent than that of diabetes and hyperglycaemia. This latter conclusion is supported by the fact that the difference in LDL oxidation between control and diabetic subjects was substantially reduced after improved glucose control. Although several smaller studies have demonstrated trends between age and LDL oxidation[39,34], this study is the first to definitively demonstrate this effect in both normal and diabetic populations. The explanation for enhanced LDL oxidation in older subjects remains unclear. Vitamin E levels in LDL were decreased with age. Although this may have resulted from diminished vitamin E content in the diet, dietary records failed to reveal significant differences in vitamin E consumption between the groups. One may also speculate that oxidative stress was increased with age. Several, but not all, animal and human studies have demonstrated a decline in antioxidant content and/or antioxidant enzyme activity in plasma with age[55–58]. Moreover, it has been well demonstrated that tissue advanced glycation end-product content increases with age[59]. Age-related accumulation of advanced glycation end-product in artery wall collagen may increase oxidative stress[59], and thus enhance LDL oxidation. It is also interesting to note that the strength of the association between age and LDL oxidation appears to be less in the poorly controlled diabetics than in the normal subjects, suggesting
that both age and diabetes may be operating through common advanced glycation end-product mediated mechanisms. The pathophysiological importance of LDL oxidation and hypercholesterolaemia-induced effects in the vascular wall has recently been discussed elsewhere[40].

In summary, this study illustrates the important effect of diabetes and hyperglycaemia on LDL oxidation. These effects were exacerbated by age. Of potential clinical relevance are the findings that effective treatment of hyperglycaemia with intensive insulin therapy successfully reduced LDL susceptibility to oxidation and increased LDL vitamin E content towards levels of euglycaemic individuals. Although the exact roles of oxidative stress and LDL oxidation in the development of atherosclerosis and coronary heart disease are not fully elucidated, these data appear to provide additional support for treatment of hyperglycaemia in diabetes for the prevention of macrovascular disease. Although the UKPDS study[51] has demonstrated that intensive glucose lowering only modestly reduced the incidence of cardiovascular diseases, the difference in levels of HbA1c between treatment groups was a modest 11%. Prompt correction of marked hyperglycaemia, as demonstrated in this study, may induce a more profound inhibition of vascular dysfunction and atherogenesis.

We would like to dedicate this study to the memory of Prof. Gaetano Salvatore (Naples, Italy). We thank Ms Janan Skinner for her skillful technical help. The work was supported by grants from Consiglio Nazionale delle Ricerche, the Juvenile Diabetes Foundation and the Department of Veterans Affairs and from the American Federation of Aging Research.

References


