Diabetologia (1995) 38: 1300–1306

Diabetologia

Oxidation of low-density lipoprotein in NIDDM: its relationship to fatty acid composition

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Summary The increased risk of atherosclerotic disease in diabetic subjects may be due to enhanced foam cell formation following an increased susceptibility of low density lipoprotein to oxidative modification. This study has compared fatty acid content and lipoprotein oxidisability in 10 non-insulin-dependent diabetic subjects with that in 10 control subjects. Both groups were normocholesterolaemic and the diabetic subjects had higher triglyceride levels (2.2 ± 0.4 vs 1.2 ± 0.2 mmol/l, $p < 0.05$). The fatty acid composition was compared in low density lipoprotein following Folch extraction, separation by thin layer chromatography (for the lipid classes) and analysis by gas liquid chromatography. Low density lipoprotein oxidisability was assessed by conjugated diene and thiobarbituric acid reacting substance formation in the presence of copper ions. The esterified/free cholesterol ratio was higher in the low density lipoprotein from patients compared to control subjects (2.9 ± 0.1 vs 1.9 ± 0.3, $p < 0.05$). Linoleic acid in the cholesteryl ester fraction of the lipoprotein was higher in the patients than in the control subjects (48.2 ± 2.2 % vs 42.4 ± 3.4 %, $p < 0.05$) as was the total quantity of linoleic acid in the cholesteryl ester fraction (317.8 ± 68.0 vs 213.2 ± 28.0 μg/mg protein, $p < 0.05$) and in the low-density lipoprotein as a whole (443.2 ± 70.0 vs 340.2 ± 28.2 μg/mg protein, $p < 0.05$). Lipoprotein oxidisability was also increased in the diabetic group with increased formation of thiobarbituric acid reacting substances (35.6 ± 7.2 vs 22.3 ± 3.5 nmol/mg protein, $p < 0.05$, increased total diene formation (502 ± 60 vs 400 ± 30 nmol/mg protein, $p < 0.05$) and increased rate of diene formation (7.2 ± 0.6 vs 5.1 ± 0.9 nmol diene · mg protein^{-1} · min^{-1}, $p < 0.05$). This study indicates that low-density lipoprotein from diabetic subjects is more susceptible to oxidation. This could, in vivo, accelerate foam-cell formation thereby increasing atherosclerotic risk in diabetic subjects. [Diabetologia (1995) 38: 1300–1306]

Key words Non-insulin-dependent diabetes mellitus, low-density lipoprotein oxidation, dietary fatty acids, low-density lipoprotein composition, glycated low-density lipoprotein.

Although hypercholesterolaemia is an important risk factor in both diabetic and non-diabetic subjects, low-density lipoprotein (LDL) cholesterol levels are often normal in patients with atherosclerosis. In this study attention has been focused on abnormalities in the composition of LDL the major cholesterol-carrying particle, rather than the quantity. These investigations have been prompted by the finding that oxidised rather than native LDL delivers cholesterol to the macrophage [1], the precursor of the foam cell and the major cholesterol-containing cell in the atherosclerotic plaque. Therefore, the potential for LDL to be oxidised in the vessel wall may be of importance in atherogenesis. Babiy et al. [2] have shown that LDL from non-insulin-dependent diabetic (NIDDM)

Received: 5 January 1995 and in revised form: 19 April 1995

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Abbreviations: BHT, Butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; TBARS thiobarbituric reacting substances; HPLC, high performance liquid chromatography; MDA, malondialdehyde; HbA_{1c}, glycated haemoglobin.

patients with hypercholesterolaemia is more easily oxidised and we have demonstrated similar findings in normocholesterolaemic NIDDM patients [3]. Several studies have shown an increase in lipid peroxidation products in diabetic subjects [4–6,], although some studies have shown no difference [7], or an increase only in diabetic subjects with complications [8, 9] or with poor control [10] compared with control subjects. The oxidation of LDL involves the peroxidation of its polyunsaturated fatty acids [11–13,] and LDL oxidation by copper can be divided into three phases; lag phase, propogation phase and decomposition phase. During the lag phase LDL antioxidants are consumed and only minimal lipid peroxidation takes place. When antioxidants have been consumed the polyunsaturated fatty acids then become oxidised. Chisolm et al. [14] have shown that lipoprotein fractions isolated from diabetic patients had elevated levels of lipid peroxides. Several studies have investigated the fatty acid composition of plasma lipids and LDL lipids in diabetes with conflicting results. Some studies have shown lower levels of linoleic acid and higher levels of the more highly unsaturated linoleic acid metabolites, one being arachidonic acid [15, 16]. Other studies have shown the plasma linoleic acid levels from diabetic patients to be similar to control subjects but with different levels of linoleic acid metabolites [17–19]. Peterson et al. [20] have recently shown higher proportions of linoleic acid and lower proportions of arachidonic acid and oleic acid in erythrocytes and plasma triglycerides isolated from Asian diabetic subjects compared with European diabetic subjects. However, so far few studies have examined the fatty acid composition of LDL isolated from diabetic subjects. The increased susceptibility of LDL from normocholesterolaemic diabetic patients to oxidise when compared to control non-diabetic subjects suggests that the fatty acid composition of LDL might contribute to the increase in LDL oxidisability. The purpose of this study was to examine the fatty acid composition of LDL in NIDDM patients and to explore the relationship between the composition of LDL and its susceptibility to oxidation.

Subjects and methods

Subjects. NIDDM subjects with normal serum cholesterol levels were randomly selected from the hospital diabetic clinic. Since plasma lipid peroxidation has been shown to be influenced by smoking [21], smokers were excluded from this study. Patients with hepatic or thyroid disease and patients with nephropathy, retinopathy and neuropathy were excluded from the study. Nine of the patients were treated with sulphonylureas and one with diet alone. Non-diabetic subjects were recruited from a pool of healthy hospital and laboratory personnel with normal serum lipid levels. All subjects gave informed consent and the study was approved by the Adelaide Hospital Ethics Committee. More patient details are given in Table 1.

LDL isolation. Blood was collected following a 12-h fast and centrifuged at 4 °C. The plasma was removed and ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) were added at a concentration of 1 g/l plasma and 4.4 mg/l, respectively [22]. Plasma LDL was isolated by sequential ultracentrifugation at a density range of 1.025–1.063 g/ml [23]. LDL was stored at 4 °C in a nitrogen atmosphere in the dark for the measurement of conjugated diene and TBARS formation and LDL composition. The effect on conjugated diene lag time (min) of storing LDL for up to 7 days was examined. Lag time did not vary when LDL was stored for up to 3 days (95 ± 10, 102 ± 7 and 98 ± 5 min) but by day 5 they were reduced (days 5 and 7 lag times were 72 ± 8 and 40 ± 15 min, respectively $n = 4$). For the determination of vitamin E and fatty acids the samples were stored for up to 8 weeks at –20 °C. Plasma cholesterol and LDL total and free cholesterol levels were measured by enzymatic colorimetric methods (Boehringer Mannheim GmbH, Mannheim, Germany) and plasma triglycerides were measured with kits from Biomerieux (Marcy-l'Etoile, France). HbA_{1c} levels were determined as a percentage of total haemoglobin by an enzyme linked immunoassay where the monoclonal antibody was specific for HbA_{1c} (normal values < 4.9 %). Protein levels were determined by a modification of the method of Markwell et al. [24].

LDL fatty acids. Heptadecanoic acid (100 µg) was added as an internal standard to LDL (1 mg/ml of protein) and the lipids were extracted by a modification of the method of Folch et al [25]. The organic fraction was dried with anhydrous sodium sulphate. The samples were dried under nitrogen and either transmethylated [26] for total LDL fatty acid analysis or subjected to thin layer chromatography for the analysis of the fatty acids in the LDL cholesteryl ester, triglyceride and phospholipid lipid classes prior to transmethylation.

Fatty acids in LDL lipid classes. Dried extracts were redissolved in chloroform and applied to activated LK6DF S60 TLC plates (Whatman, Maidstone, U.K.) using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:0.7, v:v:v) for the separation of cholesteryl ester, triglyceride and phospholipid classes. Following transmethylation of the total LDL fatty acids and the fatty acids in the LDL lipid classes, the fatty acid methyl esters were extracted after the addition of 1.3 ml of H_20 and 3 volumes of 2.5 ml of hexane. The hexane phase was dried under nitrogen and reconstituted in iso-octane immediately prior to determination by gas liquid chromatography. The fatty acids were analysed in a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) equipped with a capillary fused silica Permabond FFAP-DF-0.1 (25 m × 0.25 mm internal diameter) column (Duren, Germany). Calculations of fatty acid amounts were determined from peak area ratios of sample to internal standard with a Shimadzu CR6A Chromapac (Kyoto, Japan). The intra and inter-assay variations were 1.8 and 2.6 %, respectively.

LDL oxidation. LDL oxidation was measured by two methods – the formation of conjugated dienes and measurement of thiobarbituric acid-reactive substances (TBARS). The measurement of conjugated dienes was performed using a modification of the procedure described by Esterbauer et al. [27]. Oxidation of freshly prepared LDL samples (100 µg/ml) was initiated using $CuCl_2$ (1.66 µM). The kinetics of lipoprotein oxidation were determined by continually monitoring the change

in absorbance at 234 nm at room temperature with a SP6–500 Pye Unicam UV spectrophotometer (Cambridge, UK) attached to a Perkin Elmer (023) recorder (Cambridge, UK). Three different phases were determined [27]; the lag phase, the propagation phase and the decomposition phase. The lag phase was determined manually by drawing a tangent to the slope of the linear portion of the propagation phase and extrapolating it to the horizontal axis at time zero. The rate of LDL oxidation was determined from the propagation phase of the time course. The maximum diene concentration was determined from the A_{234} value represented by the points of intersection of the propagation phase line with that of the lag and decomposition phases. The concentration of dienes represented by this absorbance value was calculated from the known extinction coefficient for conjugated dienes (29500 litre · mol · $^{-1}$ · cm^{-1})[28]. The inter assay variation was 4.2 %. For the TBARS method LDL was diluted to a concentration of 300 μg protein/ml and extensively dialysed in 0.01 mol/l phosphate-buffered saline (pH 7.4) and assayed as described by Buege and Aust [29]. LDL was oxidised in the presence of 10 μmol/l copper at 37 °C in phosphate buffered saline for up to 4 h. Oxidation was stopped at 0, 1, 2, 3 and 4 h by refrigeration to 4 °C and the addition of 200 μmol/l EDTA and 40 μmol/l BHT. Lipid peroxidation was determined by measuring TBARS and results are expressed as malondialdehyde (MDA) equivalents using freshly prepared MDA standard. The intra-assay variation for the TBARS method ($n = 6$) was 5.9 % and each assay included LDL from both the diabetic and control groups.

LDL vitamin E. Vitamin E was measured by high performance liquid chromatography (HPLC) according to the method of Kaplan et al., [30]. To determine the vitamin E content of LDL a-200 μl aliquot of LDL was taken and 5-mg α-tocopherol acetate was added as an internal standard. The samples were extracted with ethanol and dried under nitrogen. The extract was reconstituted in a mobile phase which consisted of acetonitrile: methanol (75:25, v:v) and run at a flow rate of 3.5 ml/min. The chromatography analysis was performed with a 4.6-mm particle Ultracarb 5 column (, Cheshire, UK) fitted to a Shimadzu LC-6A HPLC (Kyoto, Japan) with a Shimadzu SPD-6A ultraviolet detector (Kyoto, Japan). Calculations were determined from the standard curve of peak area ratios of sample to internal standard.

LDL glycation. LDL glycation was measured by affinity chromatography using aminophenyl borate gel [31]. Microcolumns containing 1 ml of Glycogel B (Pierce Ltd, Cambridge, UK) were prepared and equilibrated with ammonium acetate buffer (pH 8.5). LDL (400 μl) was applied to the columns and non-glycated LDL was washed off with 10 ml of the same buffer. Glycated LDL, which adheres to the gel, was subsequently eluted with 4 ml of sorbitol buffer (pH 8.5). Glycated LDL was expressed as a percentage of total LDL.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA). Correlation coefficients are determined by linear regression analysis. Results were expressed as mean ± SEM and values of less than 0.05 were considered to be statistically significant.

Table 1. Patient characteristics, plasma lipoproteins and LDL composition

	Diabetic patients	Control subjects
Number	10	10
Age	69 ± 10	63 ± 10
Sex ratio (female/male)	5/5	6/4
BMI (kg/m^2)	27.4 ± 2.9[a]	22.5 ± 0.2
Serum triglyceride (mmol/l)	2.2 ± 0.4[a]	1.2 ± 0.2
Serum cholesterol (mmol/l)	5.3 ± 0.5	5.1 ± 0.4
LDL glycation (%)	4.6 ± 0.33[b]	2.2 ± 0.11
LDL esterified/free cholesterol (ratio)	2.9 ± 0.12[b]	1.9 ± 0.26
LDL vitamin E (nmol/mg LDL protein)	268 ± 15	255 ± 23

Mean ± SEM
[a] $p < 0.05$, [b] $p < 0.01$ with respect to control subjects

Results

The subjects were of a similar age but the diabetic patients were significantly more obese with a body mass index (BMI of 27.4 ± 2.9 kg/m^2 as shown in Table 1. Serum triglycerides were higher in the diabetic group (2.2 ± 0.4 mmol/l) compared to the control group (1.2 ± 0.2 mmol/l) ($p < 0.05$). The diabetic patients were moderately well controlled (HbA_{1c} = 7.3 ± 0.8 %) and the esterified to free cholesterol ratio in LDL was significantly higher in the diabetic group (2.9 ± 0.12 vs 1.9 ± 0.26, $p < 0.01$, Table 1). The vitamin E content was not significantly different between the groups (Table 1). A significant increase was seen in the percentage of glycated LDL in the diabetic patients compared to the control group (4.6 ± 0.33 vs 2.2 ± 0.11 %, $p < 0.01$). Total fatty acids in the LDL particle did not differ significantly between the groups, although there was a slight increase in the percentage of both linoleic acid and arachidonic acid in the diabetic subjects; however, when expressed as μg/mg LDL protein there was a significantly higher linoleic acid/LDL protein in the diabetic patients (443.2 ± 70 vs 340.2 ± 28.2, $p < 0.05$) (Table 2). The levels of the main unsaturated fatty acids in the three lipid classes – cholesteryl esters, triglycerides and phospholipids are shown in Table 3. There was a significantly greater percentage of linoleic acid in the cholesteryl ester class in the diabetic patients compared to the control group (48.2 ± 2.2 vs 42.8 ± 3.4 %, $p < 0.05$) and there was also more arachidonic acid in their cholesteryl esters although this did not reach statistical significance (Table 3). Cholesterol linoleate, expressed as μg cholesteryl linoleate/mg LDL protein was also significantly higher in the diabetic patients 317.8 ± 68.0 vs 213.2 ± 28.0 μg/mg LDL protein ($p < 0.05$).

LDL oxidisability was measured by two different methods and a significant increase was found in both the formation of TBARS and conjugated dienes in

Table 2. Percent of total fatty acids in LDL

Fatty acid	Diabetic patients		Control subjects	
	% total LDL fatty acids	μg/mg LDL protein	% total LDL fatty acids	μg/mg LDL protein
16:0	22.3 ± 1.1	243.5 ± 26.5	22.8 ± 1.2	190.5 ± 26.2
16:1	2.9 ± 0.4	28.8 ± 8.2	2.7 ± 1.2	24.8 ± 3.2
18:0	9.9 ± 1.7	96.2 ± 8.5	9.0 ± 1.3	84.6 ± 6.1
18:1	19.1 ± 1.8	183.7 ± 20.1	17.2 ± 1.6	163.3 ± 11.1
18:2	39.8 ± 1.2	443.2 ± 70.7[a]	41.5 ± 2.2	340.2 ± 28.2
20:4	6.0 ± 1.6	72.6 ± 11.9	6.8 ± 0.8	51.3 ± 10.3

Mean ± SEM
[a] $p < 0.05$ with respect to control subjects

Table 3. Fatty acid composition in LDL lipid fractions (%)

Fatty acid	Cholesteryl ester		Triglyceride		Phospholipid	
	Control subjects	Diabetic patients	Control subjects	Diabetic patients	Control subjects	Diabetic patients
16:0	14.7 ± 3.2	10.0 ± 1.3	26.3 ± 2.1	27.2 ± 3.1	36.0 ± 4.5	36.8 ± 2.9
16:1	4.9 ± 0.8	4.5 ± 0.6	1.2 ± 0.4	2.6 ± 0.9	0.8 ± 0.2	1.0 ± 0.3
18:0	3.8 ± 0.6	4.0 ± 1.3	8.1 ± 2.2	6.5 ± 1.9	16.5 ± 2.8	17.5 ± 2.0
18:1	29.1 ± 4.2	24.0 ± 3.1	46.4 ± 4.8	42.7 ± 3.8	17.4 ± 1.8	17.8 ± 1.4
18:2	42.8 ± 3.4	48.2 ± 2.2[a]	15.5 ± 1.9	15.0 ± 2.0	22.8 ± 3.1	19.7 ± 3.6
20:4	4.7 ± 1.9	5.9 ± 2.2	2.5 ± 1.5	6.0 ± 3.0	4.0 ± 1.3	7.2 ± 3.1

Mean ± SEM
[a] $p < 0.05$ with respect to control subjects

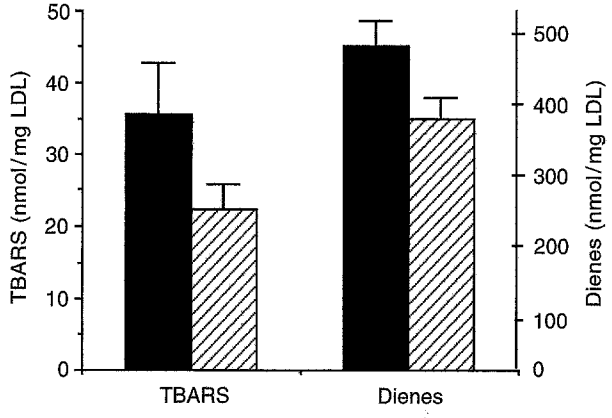

Fig. 1. LDL oxidation in non-diabetic ■ ($n = 10$) and non-diabetic subjects ▨ ($n = 10$). LDL oxidisability was determined by two methods under copper-induced stress. The formation of thiobarbituricacid reacting substances (TBARS) was significantly higher in the diabetic group and the total amount of conjugated dienes formed over a 5-h time period was significantly higher in the diabetic group compared to the control group (both $p < 0.05$)

the diabetic patients (Fig. 1). Both the TBARS produced (35.2 ± 7.2 vs 22.3 ± 3.5 nmol/mg LDL protein, $p < 0.05$) and the total amount of conjugated dienes produced (502 ± 60 vs 400 ± 30 nmol/mg LDL protein, $p < 0.05$) were significantly greater in the diabetic group compared to the control group as shown in Figure 1. The conjugated diene lag time was shorter in the diabetic patients compared to the control group (84 ± 19 vs 125 ± 15 min, $p < 0.05$) and the rate of increase in diene formation was greater in the diabetic patients (7.22 ± 0.6 vs 5.05 ± 0.9 nmol dienes · mg LDL^{-1} · min^{-1}, $p < 0.05$) (Fig. 2). The relationship between LDL fatty acid composition and oxidisability was examined (Fig. 3). There was a significant positive correlation between the percentage of linoleic acid in the LDL cholesteryl ester fraction and the maximum amount of dienes produced ($r = 0.87$, $p < 0.01$). The LDL esterified-to-free cholesterol ratio also significantly correlated with the percentage of linoleic acid in the cholesteryl ester fraction of LDL ($r = 0.69$, $p < 0.01$) (Fig. 4) but no significant relationship was found with LDL glycation and linoleic acid.

Discussion

There is evidence to suggest that the oxidation of LDL is important in the atherosclerotic process. Free radical production is increased in diabetes suggesting an increased potential for oxidation of LDL in diabetic patients [4]. We have previously demonstrated an increased esterified/free cholesterol ratio in LDL from NIDDM patients[3] which was related to an increased LDL oxidisability. The present study demonstrates that an increased LDL oxidisability, measured as the formation of conjugated dienes, in the diabetic patient is related to an increase in the percentage of linoleic acid in the LDL cholesteryl ester in these patients. The reason why diabetic patients

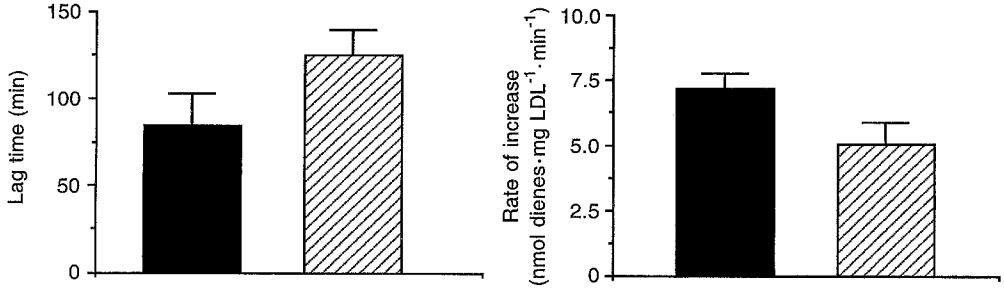

Fig. 2. Susceptibility of LDL from diabetic patients ■ and control subjects ▨ to copper-induced oxidation measured by conjugated diene formation. Conjugated diene lag time was significantly shorter and rate of increase in diene formation significantly greater (both $p < 0.05$) using LDL from the diabetic patients

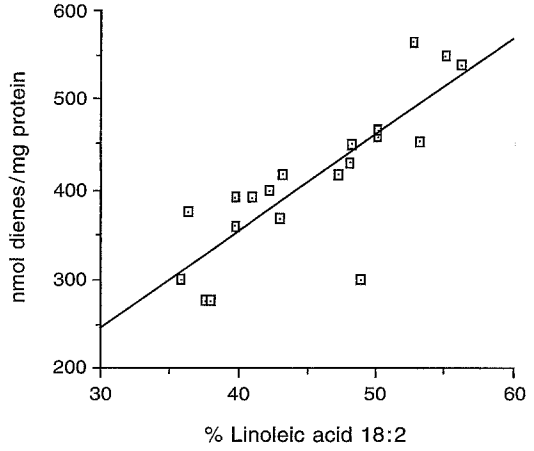

Fig. 3. Correlation was found between the maximum amount of conjugated dienes produced and the percentage of linoleic acid in the LDL of the subjects studied ($n = 20$), $r = 0.87$, $p < 0.05$

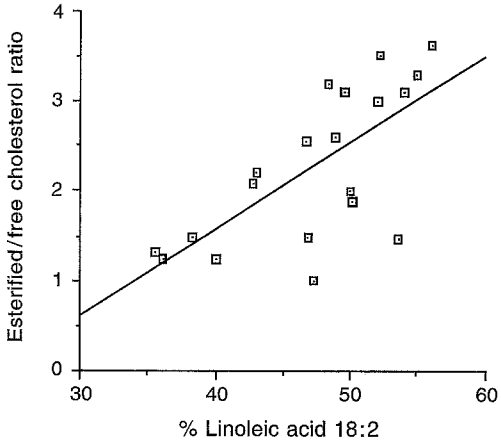

Fig. 4. A significant positive correlation was found between the esterified/free cholesterol ratio and the percentage of linoleic acid in the LDL cholesteryl ester fraction, ($n = 20$), $r = 0.69$, $p < 0.05$

have an increased linoleic acid in the LDL cholesteryl ester is uncertain but it may be due to diet. Dietary advice for the diabetic patient has usually been to decrease saturated fat, and to increase polyunsaturated fat and this has been shown to increase serum linoleic acid [32]. Furthermore, a recent study by Laitinen et al. [33] showed that the serum fatty acid composition of recently-diagnosed diabetic patients could be changed by diet therapy. The increase may also be related to an impairment in the activity of the insulin sensitive Δ-6 desaturase in diabetes [18]. This enzyme is responsible for the initial step in the metabolism of linoleic acid via γ-linolenic acid to arachidonic acid. Reaven et al. [34] showed that the extent of LDL oxidation is strongly influenced by the LDL linoleic acid content. More recently, dietary studies have shown that the enrichment of LDL linoleic acid by dietary manipulation resulted in an increase in oxidisability [35, 36]; our results confirm those findings. The diabetic subjects in this study had a raised percentage of linoleic acid in their cholesteryl ester and this positively correlated with oxidisability.

Lipid peroxidation is a complex non-linear process [37] and methods of in vitro oxidation measure different stages of lipid peroxidation. In the present study, two methods were used to measure the oxidisability of LDL under copper-induced stress. In our study the lag time for LDL oxidation was shorter than in LDL from control subjects in spite of similar vitamin E levels suggesting that the fatty acid composition of the LDL does have an effect on the initiation phase. The rate of increase in diene formation was also significantly greater in LDL from the diabetic patients which would be expected because of the greater amount of available substrate present. In LDL from non-diabetic subjects the rate of increase during the propogation phase has been shown previously to be related to fatty acid composition of LDL [34]. The formation of conjugated dienes is an early step in lipid peroxidation and is dependent on the fatty acid composition and the endogenous antioxidants [38]. The TBARS assay measures MDA which is an end-stage product of lipid peroxidation, and can be used as an indicator of oxidation. The MDA is formed from fatty acids with more than two double bonds [37]; thus, in LDL linoleic acid having two double bonds is not a potential source of MDA as is arachidonic acid. Therefore, the TBARS assay can be used as an indicator of oxidation but, to get a more complete picture, other methods for measuring oxidation should also be used. Öhrvall [39] recently showed a negative correlation with MDA and linoleic acid; however, they showed a positive correlation with arachidonic acid and γ-linolenic acid in serum lipo-

protein lipids. This result would be expected according to Esterbauer et al. [37] since MDA is formed from fatty acids with more than two double bonds. Glycation of LDL has previously been shown to be related to the oxidisability of LDL [40]. In this study we failed to find such a correlation possibly because there was little variation in the range of HBA_{1c} and LDL glycation values in the diabetic patients studied and this may also be a reason for the lack of correlation with LDL linoleic acid.

Oxidative susceptibility of LDL has been shown to be increased in the most dense of three LDL subfractions [22, 41, 42] demonstrating an increased susceptibility to oxidative modification of small dense LDL in patients with atherogenic lipoprotein phenotype pattern B. Analysis of the composition of small dense LDL was not shown in these papers. The present study suggests that the small dense LDL, a cholesterol-rich particle, is more easily oxidisable due to the increased polyunsaturated fatty acids. This is supported by a significant increase in linoleic acid in LDL isolated from diabetic subjects as shown in Table 2. It is likely that the increased oxidisability of LDL occurred in the small dense particles which are known to be increased in diabetes [43]

This study suggests that even though diabetic subjects may be under fairly reasonable control important factors such as LDL core fatty acid composition should be examined because of the possibility of enhanced oxidisability of this lipoprotein with its ensuing implications for foam-cell formation.

Acknowledgements. The authors wish to thank the European Association for the Study of Diabetes for their financial support of the project.

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