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INCREASED OXIDIZABILITY OF PLASMA LIPOPROTEINS IN DIABETIC PATIENTS CAN BE DECREASED BY PROBUCOL THERAPY AND IS NOT DUE TO GLYCATION

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Abstract—Atherosclerosis is considered to be the major complication of diabetes mellitus. Since diabetic patients have increased blood levels of lipid peroxidation products we investigated whether the susceptibility of blood components to oxidation is altered in this disease. We analysed the parameters characterizing the extent of oxidative change and the antioxidant status of low density lipoprotein (LDL) and high density lipoprotein (HDL) in a group of diabetic patients and in a control population. LDL oxidizability was significantly higher for patients ($P = 0.001$) than for individuals in the control group. There were no significant differences in the α-tocopherol content or levels of preformed peroxides in LDL samples isolated from diabetic patients and control individuals which could account for this effect. Similarly, LDL glycation, common in diabetes mellitus, was not responsible, since LDL glycated *in vitro* was more rather than less resistant to oxidation. Even the presence of unbound glucose at normal or elevated physiological concentrations had a delaying effect on the oxidation of LDL. The increased oxidizability of LDL isolated from diabetic patients could be reduced to control levels by a 6-week standard treatment with Probucol™, originally applied to reduce their blood cholesterol.

Atherosclerosis is considered to be the major complication of diabetes mellitus accounting for more than 70% of the total mortality in all forms of the disease [1].

The low density lipoprotein (LDL§) of human serum plays a central role in the mechanism of atherosclerotic damage to blood vessels. Modification of LDL by oxidative processes results in its unregulated uptake by the scavenger receptor of macrophages [2, 3] which is believed to lead to the eventual formation of cholesteryl ester-loaded cells, morphologically similar to tissue foam cells [4]. In addition, oxidatively modified LDL is likely to play a role in the recruitment and retention of monocytes/macrophages in the arterial walls [4] and has the potential to damage endothelial cells [5]. Many studies have found a correlation between increased levels of lipid peroxides and the atherosclerotic process [6–8], reinforcing the suggestion that LDL oxidation may be the cause of atherogenesis [9].

Diabetic patients have increased blood levels of lipid peroxidation products [10]. An attempt has been made to explain this by demonstrating that glucose autoxidation could be a potential source of oxidative stress [11], but a clear dependence of high peroxide levels on glucose is yet to be demonstrated.

We compared the properties of LDL and HDL isolated from normal individuals and from diabetic patients in an attempt to find factors which could be responsible for the positive correlation between diabetes and atherosclerosis. Parameters investigated were the relative oxidizabilities of the lipoproteins, their α-tocopherol and endogenous lipid peroxide contents and the effect of glycation on susceptibility to oxidation. We have also measured the effect of Probucol™ therapy on lipoprotein oxidizability because this drug lowers the total blood cholesterol and is a potent antioxidant [12].

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§ Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; TBARS, thiobarbituric acid reacting substances; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

All chemicals were of analytical grade and purchased from BDH Chemicals (Australia), AJAX Chemicals (Sydney, Australia) and Merck (Darmstadt, Germany). Fructosamine test kit was purchased from Roche (Switzerland). Solutions were made up in distilled water purified by passage through a Milli Q (Millipore-Waters, Sydney, Australia) apparatus.

Patients. Control blood samples were obtained from normal volunteers (10 males and 10 females, aged 20–59, mean age ± SD = 31.1 ± 8.1, mean wt = 68.4 ± 8.6 kg, total plasma cholesterol = 5.0 ± 1.1 mM) 2–3 hr after a light breakfast. The 12 Type II diabetic patients comprised five males and seven females aged between 51 and 69 years (mean age ± SD = 61.2 ± 3.4). They were not excessively obese (mean wt = 78.1 ± 11.8 kg) and the diabetic state was well controlled (Hbalc = 6.4 ± 1.6%; mean fasting plasma glucose = 6.8 ± 1.7 mM). The diabetic patients had Type IIA and Type IIB hyperlipidaemia (total cholesterol = 7.3 ± 0.9 mM; HDL cholesterol = 1.11 ± 0.19 mM; fasting plasma triglyceride = 2.8 ± 0.7 mM) but had not suffered significant renal impairment (creatinine = 73 ± 16 μM). LDL cholesterol cut-off value used to

select the patients for this study was 3.5 mM. Samples were collected before and 6 weeks after commencement of Probucol 500 mg BD.

Lipoprotein preparation. Whole blood was obtained by venipuncture and collected in tubes with EDTA (1 mg/mL) as anticoagulant. LDL and HDL were isolated from freshly drawn plasma using a Spinco-Beckman ultracentrifuge equipped with a SW-41 rotor, according to the method of Redgrave *et al.* [13]. After separation, the lipoprotein samples were dialysed for 24 hr at 4° against two changes of 200 volumes of PBS deoxygenated by bubbling with argon during the dialysis. EDTA (1 mg/mL) was present throughout the isolation procedure except for the last change of dialysis buffer. After dialysis, the lipoprotein samples were stored until use for not longer than 1 week under an argon atmosphere.

Measurement of LDL and HDL oxidizability. To test oxidizability of LDL and HDL, samples in PBS were exposed to γ-radiation from ^{60}Co source for 3 min at a dose rate of 54.4 Gy/min at room temperature (1 Gy = 1 J of energy absorbed/kg). Prior to irradiation, the concentration of LDL and HDL was adjusted to 1 mg protein/mL. Immediately following irradiation, aliquots of the samples were incubated with catalase (0.02 mg protein/mL) for 30 min to remove radiolytically generated hydrogen peroxide and then assayed for total hydroperoxide content. The amount of peroxide in the samples was determined by a modification of the tri-iodide assay [14], adapted for aerobic conditions as described elsewhere [15]. All measurements were performed in duplicate which differed by less than 5%.

LDL glycation. LDL glycation was carried out as described elsewhere [16]. Routinely 1–2 mg/mL solution of LDL, from control subjects, in PBS containing 100 mM glucose and 1 mM EDTA was sterilized by passing through 0.45 μm filter and then incubated for 7 days at 37°. The sample was then dialysed against PBS under anaerobic conditions. The extent of glycation was estimated by the fructosamine test.

Lipoprotein oxidation. The isolated LDL or HDL (1–3 mg/mL) was placed in a dialysis bag immersed in three to five volumes of PBS. Oxygen was bubbled through the dialysis buffer and the system was incubated at room temperature in the dark for various intervals of time. When the lipoproteins were oxidized in the presence of glucose, the concentration of glucose was kept the same inside and outside the dialysis bag.

Other assays. For the determination of TBARS, 50 μL of sample were mixed with 150 μL of 20% trichloracetic acid and 150 μL of 0.67% thiobarbituric acid. After incubation at 80° for 1 hr, the samples were centrifuged for 15 min and absorbance of the supernatant read at 532 nm. The result was expressed in units of TBARS. Freshly prepared malonaldehyde tetramethyl acetal solution was used as a standard.

α-Tocopherol was determined by the HPLC method [17]. Protein was determined by the Lowry method [18], with bovine serum albumin as standard.

Statistical evaluation. The results were analysed for statistical significance by Wilcoxon rank sum test using the corresponding MINITAB computer program—MANN-WHITNEY.

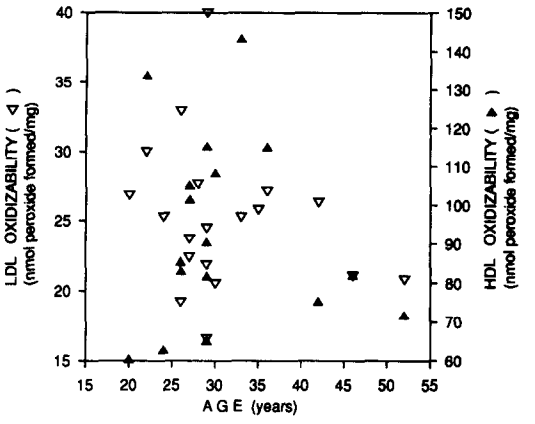

Fig. 1. Scatter plot of LDL and HDL oxidizabilities against age of normal subjects.

RESULTS

As a first step in this study we measured several parameters characterizing the antioxidant and lipid peroxidation status of blood lipoproteins in a group of normal subjects and in diabetic patients. To evaluate susceptibility of plasma lipoproteins to oxidation we used the oxidizability test which was developed by us recently [15]. In this, the lipoprotein sample is exposed to a standard quantity of free radicals generated by a cobalt-60 γ source. The amount of hydroperoxide groups formed is the measure of the oxidizability of the sample, inversely related to its antioxidant potential, which includes lipid composition and particle structure as well as the presence and amounts of antioxidant compounds. The other parameters measured were LDL TBARS and LDL α-tocopherol content. The control and patient groups have not been matched specifically for age but analysis of the data obtained for the control population did not show any correlation between LDL or HDL oxidizability and age of the normal subjects (Fig. 1). Similarly, we did not find any association between the lipoprotein oxidizabilities and the subjects' weights, sex or their plasma cholesterol levels.

The results of the measurements of LDL and HDL oxidizabilities in the group of patients and the control group demonstrated that there was a fairly wide range of variation in the lipoprotein oxidizabilities in both populations (Fig. 2). Since there was no evidence that the data obtained had normal Gaussian distributions we used non-parametric statistical test to analyse the results. LDL oxidizability was significantly higher for patients ($P = 0.0001$) than for individuals in the control group (Table 1). The other parameters analysed in diabetic and normal groups did not differ significantly.

Although there is no simple correlation between LDL oxidizability and its α-tocopherol content in normal individuals [15], any drastic lowering of α-tocopherol content in diabetes might account for the difference shown in Fig. 2. However, measurements of α-tocopherol in LDL samples obtained from

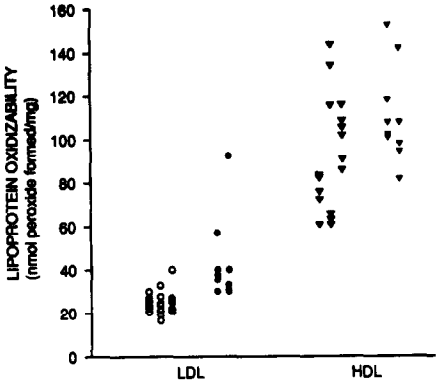

Fig. 2. Oxidizability of LDL and HDL isolated from diabetic patients. Oxidizability was defined as the amount of peroxide generated in 1 mg/mL solution after 3 min γ irradiation (dose rate = 54.5 Gy/min) at room temperature. Each point corresponds to duplicate measurements on a single patient, with a total of 10 results. Hollow symbols, control subjects; filled symbols, diabetic patients.

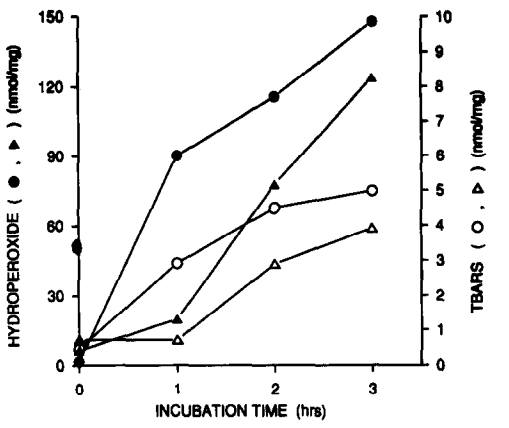

Fig. 3. Oxidation of glycated LDL. (○, ●) Control LDL; (▽, ▼) glycated LDL.

normal individuals and diabetic patients did not show any such difference (Table 1).

Individuals suffering from diabetes mellitus have periodically increased blood glucose levels and enhanced LDL glycation [19]. In an attempt to examine the effect of glycation on LDL oxidizability, lipoprotein samples glycated by prolonged exposure to glucose were compared with controls. The LDL preincubated with glucose contained typically 10–30 times more fructosamine than the control LDL. However, measurements of both hydroperoxides and TBARS showed that the glycated LDL resisted oxidation by prolonged contact with oxygenated buffer solution more effectively than control LDL (Fig. 3). The experiment was repeated twice with samples from different individuals. While there were wide variations in the extent to which glycation protected LDL from oxidation under these conditions, it always reduced the oxidative changes measured during 1–3 hr of oxidation.

The protective effect of glucose was measurable even when the oxidation resistance was determined immediately after addition of the sugar to the lipoprotein solution. In this case the appearance of hydroperoxides was slightly delayed in the presence of 15 mM and somewhat more so in that of 50 mM glucose (Fig. 4). HDL was protected much more effectively.

Probucol is an anticholesterolemic drug with considerable antioxidant properties [12]. The effect of Probucol on the oxidizability of blood lipoproteins was tested with a group of diabetic patients undergoing cholesterol lowering treatment. Administration of normal therapeutic doses of the drug in a 6-week treatment reduced the oxidizability of both LDL and HDL to control levels (Fig. 5). No such changes were found in control studies with placebo (results not shown).

DISCUSSION

Two factors are likely to be the cause of the high

Table 1. Comparison of blood lipoprotein oxidizabilities, LDL α-tocopherol and TBARS levels of normal and diabetic subjects

Parameter measured	Normal group	Diabetic group*
LDL oxidizability†	25.2 ± 5.3 (N = 20)	42.7 ± 19.2‡ (N = 10)
HDL oxidizability†	91.2 ± 24.9 (N = 18)	110.3 ± 21.6 (N = 10)
LDL TBARS (nmol/mg apoB)	0.5 ± 0.2 (N = 18)	0.6 ± 0.2 (N = 10)
LDL α-tocopherol (μg/mg apoB)	5.0 ± 1.7 (N = 20)	6.1 ± 1.6 (N = 10)

Values are means ± SD.
* Data obtained from 10 patients out of the 12 available.
† Measured as nmol peroxide/mL solution containing 1 mg protein.
‡ P = 0.0001.

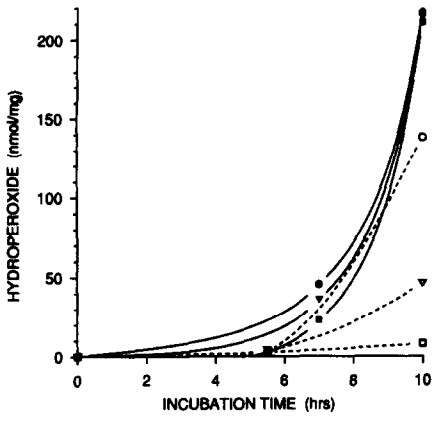

Fig. 4. Lipoprotein oxidation in the presence of glucose. Hollow symbols and dashed lines, HDL; filled symbols and solid lines, LDL. The oxidation was carried out in PBS at room temperature. Glucose concentration: (○, ●) 0 mM; (▽, ▼) 15 mM; (■, □) 50 mM.

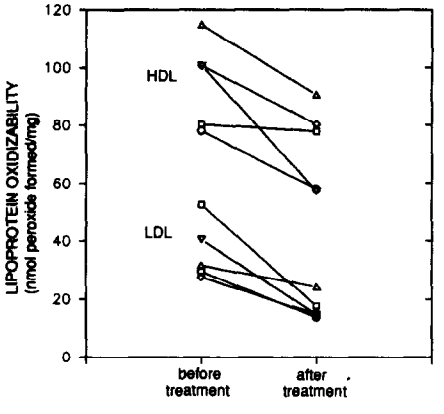

Fig. 5. Effect of Probucol treatment on LDL and HDL oxidizabilities in five diabetic patients. *Samples were collected 6 weeks after commencement of Probucol 500 mg BD.

blood level of lipid peroxidation products (assayed as TBARS) found in diabetic patients [10]: an increase in oxidative stress and/or lowering of the antioxidant potential of the normal defence systems. The aim of this study was to compare the antioxidant potentials of LDL and HDL fractions from diabetic patients and normal individuals. LDL oxidation particularly is thought to be an important factor in the development of atherosclerosis [9]. HDL has been reported to have antioxidant properties towards LDL which may be important in protecting LDL from oxidation *in vivo* [20, 21]. Therefore, we included in this study evaluation of the antioxidant potential of HDL.

It is known that LDL contains a number of lipid-soluble antioxidants [22]. Quantitatively the most abundant is α-tocopherol although recently retinol-stearate was reported in human LDL at concentrations similar to α-tocopherol [23]. We have shown that in normal subjects LDL oxidizability does not correlate with the α-tocopherol content [15] suggesting that some other factors must also be involved in determining the normal total antioxidant potential of human LDL.

According to the results of this study LDL oxidizability, which is inversely related to the LDL antioxidant status, was significantly higher in the diabetic than in the normal subjects (Table 1, Fig. 2). Analysis of the data obtained for HDL oxidizability and LDL TBARS by the non-parametric statistical test did not show a significant difference between the two populations studied. However, it must be mentioned that when Student's t-test was used for the same analysis both LDL and HDL oxidizabilities were found to be significantly higher ($P < 0.02$ and $P < 0.05$, respectively) for the diabetic than for the control group.

The observation of the higher oxidizability of HDL compared to LDL in both normal and diabetic subjects is consistent with our earlier results [15].

Surprisingly, the LDL α-tocopherol level in diabetic patients did not differ significantly from the control (Table 1). This suggests that either some other antioxidants are absent or are present in lower than normal concentrations in the diabetic LDL, or that some physico-chemical properties of LDL are perhaps altered in this disease, lowering the normal antioxidant potential of the lipoproteins. The second possibility is supported by a recent report that diabetic LDL differs structurally from normal LDL [24].

Since the diabetic and normal groups studied were not matched for age, weight and blood cholesterol level, differences in these parameters can not be excluded as possible contributing causes to the variations observed in the lipoprotein oxidizabilities (Table 1). However, as no correlation was found between the LDL or HDL oxidizabilities and the age, weight or blood cholesterol level within either the control or the diabetic groups tested, it seems unlikely that these variables can play any significant role in determining the blood lipoprotein oxidizability.

Our failure to find any increase in TBARS content of LDL in the diabetic patients shows that other components of plasma could perhaps be responsible for the higher total level of plasma TBARS reportedly found in diabetics [10, 25]. The observed low antioxidant potentials of diabetic LDL could result in elevated levels of plasma TBARS through a constant slow oxidation of the lipoproteins and the release of peroxide breakdown products into the bloodstream.

The group of patients studied was participating in a clinical test of the effects of Probucol on diabetes mellitus. The antioxidant properties of this drug are well known [12] and suggest that its administration might correct the abnormally high oxidizability of LDL in diabetic patients. The results obtained (Fig. 5) show that Probucol treatment did indeed increase substantially the resistance to oxidation of the patients' LDL and HDL. An inhibition of lipoprotein oxidation in diabetic rats [26] and in hyper-

cholesterolemic patients [27] after Probucol administration has also been reported recently by others.

In an attempt to explain the observed lowering of the antioxidant potential of LDL in diabetes, we have studied the effects of glucose and glycation on LDL oxidizability. It has been suggested in a number of studies that protein glycation products and glucose itself can induce free radical generation [11, 28–30] and LDL oxidative modification [31]. The process of glucose-induced peroxidation requires apparently rather long incubation times (many hours or days), higher than normal concentrations of glucose and the presence of copper ions, in the case of the LDL modification [31] to develop. The picture is complicated by the finding that, together with these pro-oxidant properties, glucose exhibits antioxidant characteristics when acting as a free radical scavenger [32].

Initially, we attempted to establish where the balance may lie between these two opposing effects of glucose in the case of short-term contact with the plasma lipoproteins. Additon of glucose to LDL solution inhibited the lipoprotein peroxidation induced either by γ-radiation (data not shown) or by oxygenation of the dialysis buffer (Fig. 4). Thus, at least in situations characterized by a localized rapid release of free radicals (such as at the sites of inflammation), a high concentration of glucose would be expected to protect the lipoproteins from free radical damage rather than promote their oxidation. It is reasonable therefore to suggest that glucose has a dual effect on the lipoproteins: it protects them from peroxidation during a short exposure to an oxidant stress, but it promotes oxidation in the presence of transition metals after long periods of contact. However, one should point out that in the *in vivo* situation transition metals are not likely to be available in free form in plasma especially for extended periods of time. The final balance between these two dual effects on LDL and HDL oxidation may also depend on the average concentration of glucose in the blood.

One of the characteristics of diabetes is glycation of LDL [19]. We prepared glycated LDL *in vitro* and tested whether this modification would alter its oxidizability. The results summarized in Fig. 3 demonstrate that glycated LDL was more resistant to oxidation than the control samples.

While the mechanism of this protection is unknown, it seems likely that under the conditions used in this study, glucose bound to the lipoprotein particles did not act as a free radical scavenger, or an effective antioxidant. Radicals reacting with glucose did not initiate lipid oxidation, thus reducing the yield of products detected by the peroxide or TBARS tests applied in this study.

It must be concluded that LDL glycation by itself is not the reason for the increased oxidizability of diabetic LDL.

We did notice that the glycated LDL samples obtained after a 7-day incubation with glucose consistently contained slightly higher amounts of peroxide and TBARS than control LDL samples (Fig. 3). This observation agrees with the results of earlier studies [29, 30] suggesting that protein glycation products can generate free radicals able to oxidize lipids present in the solution. It might also suggest that glucose can catalyse, at least to some extent, peroxidation of lipids after prolonged contact, even in the presence of transition metal chelators such as EDTA. Thus, LDL glycated in the course of diabetic disease may be slowly oxidized during its circulation life-time with the resulting depletion of antioxidant potential. Lipid peroxidation products formed during such an oxidation may dissolve in the blood and react with other plasma proteins present in abundance with the result that the LDL TBARS content will not differ significantly from that in normal subjects. Although we found no lowering of the α-tocopherol content in diabetic LDL, other more susceptible antioxidants may be absent or reduced in this disease. This would explain the increased oxidizability of diabetic LDL exposed to a sudden oxidant stress, as found in this study. Further tests are required to investigate the details of the lowered antioxidant potential of LDL in diabetes.

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