Oxidative stress and high-density lipoprotein function in Type I diabetes and end-stage renal disease

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ABSTRACT

In a cross-sectional study, oxidative stress in high vascular disease risk groups, ESRD (end-stage renal disease) and Type I diabetes, was assessed by measuring plasma protein carbonyls and comparing antioxidant capacity of HDL (high-density lipoprotein) as pertaining to PON1 (paraoxonase 1) activity and in vitro removal of LPO (lipid peroxides). ESRD subjects on haemodialysis (n = 22), Type I diabetes subjects (n = 20) without vascular complications and healthy subjects (n = 23) were compared. Plasma protein carbonyls were higher in ESRD patients [0.16 (0.050) nmol/mg of protein; P = 0.001; value is mean (SD)] relative to subjects with Type I diabetes [0.099 (0.014) nmol/mg of protein] and healthy subjects [0.093 (0.014) nmol/mg of protein]. Plasma PON1 activity, with and without correction for HDL-cholesterol, was lower in diabetes but did not differ in ESRD compared with healthy subjects. Plasma PON1 activity, without correction for HDL, did not differ between the three groups. In ESRD, plasma PON1 activity and plasma protein carbonyl concentrations were inversely related (r = −0.50, P < 0.05). In an in vitro assay, LPO removal by HDL in ESRD subjects was greater than HDL from healthy subjects (P < 0.01), whereas HDL from patients with Type I diabetes was less effective (P < 0.01). Efficacy of LPO removal was unrelated to plasma PON1 activity, in vitro glycation or mild oxidation, but was impaired by marked oxidation and glycoxidation. Protein carbonyl levels are increased in ESRD but not in complication-free Type I diabetes. HDL antioxidant function is increased in ESRD, perhaps a compensatory response to increased oxidative stress, but is lower in Type I diabetes. HDL dysfunction is related to glycoxidation rather than glycation or PON1 activity.

INTRODUCTION

Cardiovascular morbidity and mortality is increased in people with Type I diabetes [1,2] and also in patients with ESRD (end-stage renal disease) [3,4]. One proposed mechanism for the increased cardiovascular disease risk in these two conditions is increased oxidative stress. In diabetes, this may be mediated through carbonyl stress.

Key words: end-stage renal disease, high-density lipoprotein, lipid peroxide, oxidative stress, paraoxonase 1, protein carbonyl, Type I diabetes.

Abbreviations: AGE, advanced glycation end-product; BHT, butylated hydroxytoluene; CHD, coronary heart disease; CV, coefficient of variation; DNP, 2,4-dinitrophenylhydrazine; ESRD, end-stage renal disease; FOX2, ferrous oxidation of Xylenol Orange (version 2); HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPO, lipid peroxide; PAF, platelet-activating factor; PAF-AH, PAF-acetyl hydrolase; PON1, paraoxonase 1; RBC, red blood cell; TPP, triphenylphosphine.

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accelerating glycoxidation and lipid peroxidation, resulting in AGEs (advanced glycation end-products) [5]. Carbonyl groups (aldehydes or ketones) are produced on proteins as a result of oxidation of amino acid residues or by covalent attachment of aldehydes, such as 4-hydroxynonenal [5]. However, the presence of increased oxidative stress in human diabetes is controversial due to the lack of conclusive evidence from studies using well-validated markers. In ESRD, there is clearer evidence that oxidative stress, mediated by carbonyl formation, is a major contributor to the atherosclerotic process [6,7]. To establish whether plasma oxidative stress is increased in these two conditions, an assay of protein carbonyl levels was utilized.

One of the main defences against atherosclerosis is HDL (high-density lipoprotein). As well as its role in reverse cholesterol transport, HDL has an array of antioxidant mechanisms which may prevent the formation and promote removal of LPOs (lipid peroxides) from other lipoproteins (e.g. the pro-atherogenic LDL (low-density lipoprotein)) and from cell membranes [8–10]. The HDL-associated antioxidant enzyme PON1 (paraoxonase 1) is believed to be important in retarding atherosclerosis and has a wide range of activity dependent on genotype and phenotype factors [11–13]. To examine further the importance of oxidative stress in groups with a high risk of vascular disease, a bioassay was used in the present study to investigate whether the LPO-removing efficacy of HDL was altered in ESRD and Type I diabetes, and the relationships between HDL function, serum HDL-cholesterol levels and PON1 activity were evaluated.

METHODS

Subjects

For the protein carbonyl study, subjects with Type 1 diabetes (n = 20) were recruited from the Diabetes Outpatient Clinics, and subjects with ESRD (n = 22) were from the Haemodialysis Centre of St Vincent’s Hospital, Melbourne, Australia. Healthy control subjects (n = 23) were chosen from a group of volunteers and hospital staff completing a health screening survey questionnaire. For the HDL function study, subjects were recruited by the same method and consisted of control subjects (n = 14), subjects with Type 1 diabetes (n = 15) and subjects with ESRD (n = 14). Three additional healthy subjects with low HDL levels were also recruited for further analysis of observations made during the study.

This study was approved by the Human Research Ethics Committee of St Vincent’s Hospital, Melbourne, and each participant gave written informed consent.

Blood (50–75 ml) was taken after an overnight fast (during which water was permitted) and prior to pre-scribed medication. Biochemical characterization tests (performed by the St. Vincent’s Hospital Clinical Chemistry Department) included fasting lipids, renal and liver function tests, glucose and HbA1c (glycated haemoglobin). Plasma was prepared by centrifugation (2278 g at 4 °C for 20 min) and aliquots of plasma and serum were frozen at −86 °C until analysis of plasma protein carbonyls and serum PON1 activity. Lipoproteins were prepared from fresh plasma on the day of collection, and the LPO removal assay performed the next day (as described below).

Plasma protein carbonyl measurement

Plasma protein carbonyls were determined by ELISA, using a method described previously [14,15], involving derivatization with DNP (2,4-dinitrophenylhydrazine) and detection with an anti-DNP-biotinylate conjugate, followed by streptavidin/biotin–horseradish peroxidase. Intra- and inter-assay CV (coefficients of variation) were 8.0 and 4.5 % respectively, at a standard concentration in healthy control subjects. The limit of detection was 0.05 nmol/mg of protein.

RBC (red blood cell) membrane preparation

Human RBC ghosts were prepared by a modification of the method of Dodge and Phillips [16]. Briefly, centrifugation (1468 g at 4 °C for 20 min) was used to separate the RBC pellet from the blood of fasted healthy subjects. The RBC pellet was washed in buffer [10 mmol/l Na2HPO4 and 300 mmol/l NaCl, pH 8.0] three times (749 g at 4 °C for 25 min) and a final time in the same buffer at 15 849 g for 10 min. After washing, RBCs were lysed with the same buffer as above, but without NaCl. The lysed RBCs were washed three times (15 849 g for 25 min) with this buffer. Repeated spins (41 015 g for 25 min) of the lysed RBCs were performed until the RBC membrane pellet was washed free of haemoglobin and was visibly white. The RBC membranes were then washed twice (41 015 g for 15 min) in PBS. All centrifugations were carried out at 4 °C. The protein concentration of the RBC membranes was assessed by the modified method of Lowry et al. [17], and diluted to a final concentration of 1 mg/ml. This preparation was then oxidized in a UV-crosslinker (Fisher Biotek, Spectroline, Westbury, NY, U.S.A.) for 90 min at the unit’s standard level of intensity. Protein concentrations were assessed again, and both oxidized and unoxidized samples were aliquoted (250 µg) and stored at −86 °C. Preliminary studies (results not shown) demonstrated the stability of oxidized RBC membranes prepared and stored in this manner.

Lipoprotein preparation

Whole blood was collected into lithium-heparin tubes and HDL and LDL were isolated. Plasma density was adjusted to 1.3 g/ml with KBr and overlayed with...
d = 1.006 g/ml PBS buffer, followed by vertical spin ultracentrifugation using a Beckman ultracentrifuge (401 747 g at 4 °C for 90 min; VTi65.1 rotor). To preserve PON1 activity, all buffers used in lipoprotein preparation and the LPO removal assay were free of EDTA and contained 1 mmol/l CaCl₂. For the patient study, HDL and LDL samples were buffer-exchanged into PBS (1 mmol/l CaCl₂) by size-exclusion chromatography using PD-10 columns (Amersham Biosciences, Uppsala, Sweden). For the in vitro-modified lipoprotein studies, HDL and LDL samples were buffer-exchanged into PBS (1 mmol/l CaCl₂) using 100 000 M, Centricons (Millipore, Bedford, CA, U.S.A.). Samples from both protocols were then concentrated using Centricons. Protein concentrations were assessed by the modified method of Lowry et al. [17]. Lipoproteins were stored overnight under nitrogen in the dark at 4 °C.

**Lipoprotein modification and characterization**

Glycation of HDL was achieved by incubating HDL (4.35 mg/ml of protein) in a 50 mmol/l D-glucose solution with BHT (butylated hydroxytoluene; 80 mmol/l) under nitrogen for 4 days at 37 °C. Glycoxidation of HDL (glycation and oxidation) was achieved with the omission of BHT and nitrogen under the same conditions described above. Mildly oxidized HDL was produced by incubating 8 ml of HDL (1.0 mg of protein/ml) in a tissue culture plate (Corning, Acton, MA, U.S.A.) under air in a 37 °C incubator for 22 h. A UV crosslinker (Fisher Biotek) was used to generate UV-oxidized HDL by oxidizing 1.0 ml of 1.0 mg of protein/ml HDL for 45 min at the unit’s standard intensity level. Loss of coloration of HDL preparations was noted on UV oxidation, in keeping with loss of the lipid-soluble antioxidant carotenoids. Inactivation of PON1 on HDL was achieved by placing HDL under nitrogen for 20 min in a water bath preheated to 60 °C. Native HDL was kept under nitrogen in the dark at 4 °C. The extent of HDL oxidation for each condition was quantified by absorbance at 234 nm, FOX2 [ferrous oxidation of Xylenol Orange (version 2)] assay, fluorescence (excitation 360 nm/emission 430 nm) and Rf (ratio of electrophoretic mobility). Lipoprotein preparations were electrophoresed on native agarose gels (Helena Laboratories, Beaumont, TX, U.S.A.) to confirm lipoprotein purity and to assess the extent of oxidative modification, based on electrophoretic mobility relative to native lipoproteins.

**PON1 and arylesterase activities**

PON1 activity was determined by the rate of hydrolysis of paraoxon and of phenylacetate as described previously [18,19]. Consent for PON1 genotyping was not obtained. We demonstrated that PON1 activity does not differ between serum and plasma prepared from lithium-heparin tubes (results not shown). The method used is not a salt-stimulated assay. Before analysis of PON1 activity, samples were first incubated with eserine (final concentration, 5 µmol/l) for 10 min at room temperature to reduce butyrylcholinesterase activity, which may be increased in diabetes. PON1 activity was measured by adding 100 µl of assay buffer [100 mmol/l Tris/HCl (pH 8.0) and 2 mmol/l CaCl₂] to wells of a 96-well plate followed by 6 µl of each (serum or lipoprotein) sample. A portion (100 µl) of the working paraoxon solution (2 mmol/l) was added to each well, and the rate of change of absorbance (units/min), which represents p-nitrophenol production (molar absorption coefficient = 18 053 M⁻¹·cm⁻¹), was determined using a plate reader (Biotek Instruments, Winooski, VT, U.S.A.) at A₄₁₂, at 19 s intervals for 6 min at 25 °C. The units (units/l) represent the rate of p-nitrophenol production (nmol) per min per litre of plasma. Intra- and inter-assay CVs were 4.6 % and 6.8 % respectively. Arylesterase activity was assayed by incubating 2 ml of assay buffer [30 mmol/l Tris/HCl (pH 8.0) and 1.5 mmol/l CaCl₂] with 5 µl of sample at 25 °C for 5 min. A portion (1 ml) of phenylacetate (20 mmol/l) was then added. Reaction tubes were quickly vortexed, and 300 µl from each tube was transferred to a UV 96-well plate (Costar, New York, NY, U.S.A.), and the rate of hydrolysis of phenylacetate production (molar absorption coefficient = 1310 M⁻¹·cm⁻¹) was read on a plate reader at A₄₁₂, at 19 s intervals for 3 min at 25 °C. The units (units/ml) represent the rate of conversion of phenylacetate (µmol) per min per ml of plasma. Intra- and inter-assay CVs were 2.7 % and 3.7 % respectively. For the isolated HDL PON1, the units were calculated as above for each substrate, but instead of being unit/litre of plasma were units/µg of HDL protein.

**Lipoprotein incubation with oxidized RBC membranes**

Frozen samples (250 µg) of both oxidized and unoxidized RBC membranes were thawed to room temperature. Lipoprotein samples (500 µg) were then added to the tubes containing oxidized RBC membranes. Tubes containing oxidized RBC membranes with no added lipoproteins were used to establish a baseline value of the amount of lipid hydroperoxides present, with that in the oxidized preparation being taken as 100 %. Levels in unoxidized RBC membrane preparations were at low or undetectable levels. Samples were mixed by gentle inversion, and were placed in the dark in a 37 °C incubator for 2 h. Each condition was evaluated in triplicate. Four subjects were studied in each run, which always included a control subject. Intra- and inter-assay CVs were 5.7 % and 3.0 % respectively.

**Assessment of LPO removal by HDL from oxidized RBC membranes**

Samples were removed from the 37 °C incubator after 2 h, and lipid hydroperoxide content was assessed using
a modified version of the FOX2 assay as described by Nourooz-Zadeh et al. [20] and Deiana et al. [21]. Briefly, 180 µl from each sample was removed and divided equally between two microfuge tubes. A portion [10 µl of 100 mmol/l TPP (triphenylphosphine)] was added to one tube, and 10 µl of methanol added to the other to create a control for each sample. All tubes were vortexed and incubated in the dark (37 °C for 2 h). FOX2 reagent (900 µl) was added to each sample, tubes were vortexed and incubated for 30 min in the dark at room temperature. Samples were then centrifuged at 9940 g for 5 min. Supernatants were transferred to a 96-well plate (NUNC, Roskilde, Denmark), and A560 was read. The difference between the 'no TPP' sample and 'TPP' sample was calculated as the amount of authentic hydroperoxides present.

**Statistics**

All variables are expressed as means (S.D.), unless indicated otherwise. For comparisons between the three groups, one-way ANOVA was used. When appropriate, a post-hoc test comparing groups was performed by the Bonferroni method. All analyses included the Ryan Joiner normality test using standardized residuals. For any groups of data not normally distributed, a logarithmic transformation was performed and the normality test reapplied. Correlations between variables were calculated by the Pearson correlation coefficient (r). Prevalence of PON1 phenotype categories across patient groups was tested by $\chi^2$ analysis. $P < 0.05$ was taken as statistically significant.

**RESULTS**

**Clinical and biochemical parameters**

Clinical and biochemical parameters of the three study groups are shown in Table 1. Overall there was a significant difference in the age of the Type I diabetic and ESRD subjects compared with controls. There was no correlation between age and the key variables of the study, i.e. protein carbonyls, PON1 activity and HDL LPO removal. None of the Type I diabetic subjects had microvascular or macrovascular complications. Three of the ESRD subjects were smokers, six had Type II diabetes and eight had CHD (coronary heart disease). Exclusion of any or all of these subjects did not affect the comparative significance of any of the variables measured. All of the ESRD subjects, but none of the Type I diabetic or control subjects, were receiving vitamin E and vitamin C supplementation as part of their routine management.

As expected, Type I diabetic subjects had higher fasting glucose and Hba1C levels than controls. The overall glycaemic control in the Type I diabetic group was good [7.8 (1.2)%], with the highest Hba1C being 9.9% and the lowest 5.7%. Glucose and Hba1C were also higher in the ESRD group, which included six subjects with Type II diabetes. The only statistically significant differences in the lipid profiles of the three groups were lower HDL-cholesterol concentrations and higher triacylglycerol (triglyceride) concentrations in the ESRD subjects compared with the controls.

**Plasma protein carbonyls**

The plasma protein carbonyl concentrations (Table 2) were significantly higher in the ESRD group compared with the control and diabetes groups. When the ESRD subjects who also had diabetes and/or who smoked were taken out of the cohort, the remaining ESRD subjects still had significantly higher protein carbonyl concentrations [0.15 (0.028) nmol/mg of protein] compared with the control group [0.093 (0.014) nmol/mg of protein; $P < 0.01$]. ESRD subjects with CHD [0.17 (0.03) nmol/mg of protein] did not have higher protein carbonyls than those without CHD [0.16 (0.06) nmol/mg of protein; $P > 0.05$].

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**Table 1** Demographic and biochemical features of control subjects and subjects with Type I diabetes or ESRD in the plasma protein carbonyl study and the LPO removal by HDL study

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Type I diabetes</th>
<th>ESRD</th>
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<tbody>
<tr>
<td>Plasma protein carbonyl study</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>n (female/male)</td>
<td>11/12</td>
<td>11/9</td>
<td>8/14</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.5 (11.3)</td>
<td>32.6 (11.3)%</td>
<td>60.8 (17)%††</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.8 (4.4)</td>
<td>24.4 (3.5)</td>
<td>25.2 (4.2)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6 (0.80)</td>
<td>5.5 (1.00)</td>
<td>5.6 (1.9)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.6 (0.72)</td>
<td>3.2 (0.90)</td>
<td>3.0 (1.6)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.5 (0.40)</td>
<td>1.6 (0.53)</td>
<td>1.2 (0.50)‡</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>1.1 (0.40)</td>
<td>1.2 (0.83)</td>
<td>1.9 (1.0)‡‡</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>44 (4.0)</td>
<td>43 (4.5)</td>
<td>37 (6.8)‡‡</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>13 (7.3)</td>
<td>13 (7.5)%</td>
<td>77 (10)</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>0.09 (0.01)</td>
<td>0.09 (0.02)</td>
<td>0.85 (0.3)%‡</td>
</tr>
<tr>
<td>Creatinine (mmol/l)</td>
<td>5.6 (1.3)</td>
<td>5.2 (1.0)</td>
<td>24 (6.0)‡‡</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>5.0 (0.68)</td>
<td>14.6 (5.3)%</td>
<td>7.3 (3.7)%</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.9 (0.54)</td>
<td>7.8 (1.2)%</td>
<td>6.1 (1.8)%‡</td>
</tr>
</tbody>
</table>

| LPO removal by HDL study |         |                 |      |
| n (female/male)       | 7/7     | 6/9             | 7/7  |
| Age (years)           | 38 (12)  | 34 (12)         | 57 (15)†† |
| BMI (kg/m²)           | 24.3 (5.1) | 24.7 (3.9)     | 25.0 (4.1) |
| Total cholesterol (mmol/l) | 5.4 (0.78) | 5.4 (0.9)      | 4.9 (1.0) |
| LDL-cholesterol (mmol/l) | 3.2 (0.45) | 3.2 (0.68)     | 2.7 (0.87) |
| HDL-cholesterol (mmol/l) | 1.7 (0.41) | 1.5 (0.50)     | 1.3 (0.57) |
| Triacylglycerol (mmol/l) | 1.0 (0.41) | 1.2 (0.55)     | 1.9 (0.65)‡‡ |
| Creatinine (mmol/l)   | 0.09 (0.01) | 0.09 (0.02)    | 0.82 (0.22)‡‡ |
| Urea (mmol/l)         | 5.6 (1.5)   | 5.2 (1.1)      | 21 (6.2)‡‡ |
| Fasting glucose (mmol/l) | 5.0 (0.68) | 13 (5.3)%††    | 5.9 (1.1) |
| Hba1C (%I)           | 4.6 (0.36)  | 7.6 (1.1)%     | 5.2 (0.63) |

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Table 2  Plasma protein carbonyls and PON1 parameters in control subjects and subjects with Type I diabetes or ESRD

Values are means (S.D.). *P < 0.05, †P < 0.01 and ‡P = 0.09 compared with control; ¶P < 0.05 compared with Type I diabetes; ||P < 0.01 compared with ESRD.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Type I diabetes</th>
<th>ESRD</th>
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<tbody>
<tr>
<td>Protein carbonyl study</td>
<td></td>
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</tr>
<tr>
<td>Plasma protein carbonyls (nmol/mg of protein)</td>
<td>0.093 (0.014)</td>
<td>0.099 (0.014)</td>
<td>0.16 (0.050)</td>
</tr>
<tr>
<td>Plasma PON1 activity</td>
<td></td>
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</tr>
<tr>
<td>Paraoxon hydrolysis (units/l)</td>
<td>28.6 (14.9)</td>
<td>18.8 (10.3)*</td>
<td>27.3 (15.0)</td>
</tr>
<tr>
<td>Phenylacetate hydrolysis (units/ml)</td>
<td>125.2 (24.0)</td>
<td>129.5 (30.8)</td>
<td>87.8 (25.7)</td>
</tr>
<tr>
<td>PON1/HDL-cholesterol (units/mmol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraoxon hydrolysis</td>
<td>21.4 (13.5)</td>
<td>13.3 (8.4)*</td>
<td>24.9 (18.2)</td>
</tr>
<tr>
<td>Phenylacetate hydrolysis</td>
<td>84.6 (35.9)</td>
<td>88.4 (36.9)**</td>
<td>74.3 (30.1)</td>
</tr>
<tr>
<td>LPO removal by HDL study</td>
<td></td>
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<tr>
<td>HDL PON1 (units/µg of HDL protein)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Paraoxon hydrolysis</td>
<td>5.7 (3.2)</td>
<td>3.5 (1.7)**</td>
<td>5.6 (2.7)</td>
</tr>
<tr>
<td>Phenylacetate hydrolysis</td>
<td>27.7 (7.2)</td>
<td>26.9 (5.9)</td>
<td>21.8 (8.2)*</td>
</tr>
</tbody>
</table>

There was no significant correlation between protein carbonyl levels and the number of years on haemodialysis. There was no significant correlation between age and protein carbonyl levels in the control, Type I diabetic and ESRD groups, as separate groups or when combined. A subgroup analysis of age-matched controls (mean, 50 years of age; n = 14) and ESRD subjects (mean, 52 years of age; n = 14) still showed a highly significant (P < 0.001) elevation of protein carbonyl levels in ESRD [0.18 (0.06)] compared with controls [0.091 (0.01)].

Plasma protein carbonyl levels did not differ between subjects with complication-free Type I diabetes and healthy controls. In the diabetes group, there was a significant negative correlation between plasma protein carbonyl concentration and HbA1c (r = −0.49, P < 0.05). For the three groups, there was no statistically significant correlation between plasma protein carbonyls and age, serum albumin or total serum protein.

Plasma PON1 activity (specifically as assessed by paraoxon hydrolysis) was significantly lower in the diabetes group compared with healthy controls. Only the ESRD group, with high protein carbonyl levels, displayed a statistically significant negative correlation (r = −0.50, P < 0.05) between plasma PON1 activity (by paraoxon hydrolysis) and plasma protein carbonyl concentrations.

**HDL removal of LPO**

The efficacy of LPO removal by HDL from subjects with diabetes was significantly impaired relative to the control group (Figure 1). There was no significant difference in the LPO content of the isolated HDL from Type I diabetic subjects [0.54 (0.22) µmol/ml] compared with the control group [0.33 (0.29) µmol/ml; P > 0.05] nor did it correlate with removal of LPO or PON1 activity. The associated PON1 activity (specifically paraoxon hydrolysis) of the isolated HDL used in this bioassay tended to be lower in subjects with Type I diabetes compared with the control group (Table 2), but this did not reach statistical significance. HDL PON1 phenotype characterization was performed using the ratio of paraoxon and phenylacetate hydrolysis to categorize phenotype as 'low activity' or 'intermediate/high activity', which was not significantly different between the three groups. Specifically, further analysis of phenotype distributions between the three groups, in particular the proportion of high-activity phenotype (representing PON1<sup>-192 QR or RR</sup>) tended to be lower in Type I diabetes (20%) compared with controls (50%) and ESRD (57%), but this variation was not statistically significant (P = 0.097). Within the group with Type I diabetes, efficacy of HDL removal of LPO did not correlate with PON1 activity (paraoxon or phenylacetate hydrolysis) of plasma or of isolated HDL, nor was it related to fasting blood glucose or HbA1c (results not shown).
Correlation between the efficacy of LPO removal from oxidized RBC membranes by 250 μg of HDL protein for the three groups and the corresponding serum HDL-cholesterol concentrations

100 % represents removal of all LPOs. Control, $r = -0.49, P < 0.05 \ (n = 17)$; Type I diabetes, $r = -0.26, P > 0.05, \ (n = 15)$. ESRD, $r = -0.41, P = 0.08 \ (n = 14)$. IDDM, Type I diabetes.

The ability of HDL from subjects with ESRD to remove LPO was significantly greater [68 (14)%; $P < 0.01$] compared with the control and Type I diabetic groups. The LPO content of isolated ESRD HDL [0.67 (0.29) μmol/ml] was significantly higher than controls [0.33 (0.29) μmol/ml, $P < 0.05$], but did not correlate with in vitro LPO removal or PON1 activity. The higher efficacy of ESRD HDL could not be attributed to HDL-associated PON1 activity as it was not greater (Table 2) than that of the control group. As in the group with Type I diabetes, the efficacy of HDL removal of LPO from subjects with ESRD did not correlate with PON1 activity of plasma or of the isolated HDL.

The efficacy of HDL removal of LPO by HDL did not correlate with plasma protein carbonyls in the Type I diabetic and control groups. Evaluation was not possible in the ESRD group due to the smaller blood volume available.

Figure 2 Correlation between the efficacy of LPO removal from oxidized RBC membranes by 250 μg of HDL protein for the three groups and the corresponding serum HDL-cholesterol concentrations

<table>
<thead>
<tr>
<th>Serum HDL cholesterol (mmol/l)</th>
<th>% HDL LPO removal</th>
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<tbody>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Type I diabetes</td>
<td>Type I diabetes</td>
</tr>
<tr>
<td>ESRD</td>
<td>ESRD</td>
</tr>
</tbody>
</table>

In vitro modification of HDL and LPO removal

A negative relationship between plasma HDL-cholesterol levels and the efficacy of isolated HDL to remove LPO was observed (Figure 2). To verify these observations, three additional clinically healthy control subjects with serum HDL-cholesterol levels <1.0 mmol/l were recruited. There was a significant negative correlation between the extent of LPO removal by the isolated HDL and serum HDL-cholesterol concentrations ($r = -0.49, P < 0.05; n = 17$; Figure 2). This trend was also observed in ESRD, but it did not reach statistical significance ($r = -0.41, P = 0.08; n = 14$). This was not seen in the diabetes group ($r = -0.26, P = 0.21; n = 15$).

Inverse relationship between HDL function and HDL-cholesterol

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In vitro modification of HDL and LPO removal

Given the reduced efficacy of HDL function from subjects with Type I diabetes, we evaluated the effects of in vitro non-enzymatic glycation ($n = 2$) and/or oxidation ($n = 3$) on HDL from healthy non-diabetic subjects with normal renal function. In vitro glycation or mild oxidation (Figure 3) did not impair the ability of HDL to remove LPOs compared with native HDL. The combination of glycation and mild oxidation, glycoxidation, greatly diminished the ability of HDL to remove LPOs (Figure 3), despite the preservation of PON1 activity as assessed by artificial substrates (Table 3). The glycoxidized HDL showed increased protein oxidation, as indicated by fluorescence (excitation 360 nm/emission 430 nm), whereas markers of lipid oxidation ($\lambda_{234}$ and LPOs using FOX2) were not elevated. Extensive UV oxidation of HDL abolished its ability to remove LPOs in two of three subjects, and was associated with a total loss of PON1 activity. Heat inactivation of native HDL, which was associated with a loss of PON1 activity yet minimal oxidation (Table 3), decreased, but did not abolish, the LPO-reducing capacity of HDL (Figure 3).

Table 3 Characterization of in vitro-modified HDL in two series

| Values represent the average of the subjects’ results, which is indicative of individual results. The first series includes HDL in its native (N-HDL), in vitro glycated (Glyc-HDL) and glycoxidized (Glycox-HDL) states. The second series includes native (N-HDL), mildly oxidized (MO-HDL), UV-oxidized (UVox-HDL) and heat-inactivated (Ht-HDL) HDL. Fluorescence, excitation at 360 nm/emission at 430 nm. PON1 activity as measured by the hydrolysis of paraoxon.
<table>
<thead>
<tr>
<th>HDL FOX2</th>
<th>PON1 (unit/µg of HDL protein)</th>
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<tr>
<td>λ_{234}</td>
<td>Fluorescence</td>
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| N-HDL   | 0.07  | 0.28  | 1.0 | 19  | 8.7  |
| Glyc-HDL| 0.15  | 0.38  | 1.0 | 25  | 9.2  |
| Glycox-HDL| 0.15 | 0.35  | 1.0 | 35  | 3.0  |
| N-HDL   | 0.20  | 0.14  | 1.0 | 28  | 3.0  |
| MO-HDL  | 0.19  | 0.26  | 1.0 | 19  | 3.0  |
| Ht-HDL  | 0.19  | 0.35  | 1.0 | 23  | 0.0  |
| UVox-HDL| 0.26  | 3.0   | 1.1 | 550 | 0.0  |
DISCUSSION

Oxidative stress and HDL dysfunction may contribute to vascular damage in diabetes and ESRD. In a cross-sectional study, we have analysed plasma protein carbonyls and HDL function in healthy control subjects and two high vascular disease risk groups, one with Type I diabetes and the other with ESRD. Protein carbonyls were increased in ESRD, but not in well-controlled complication-free subjects with Type I diabetes. HDL from subjects with ESRD was more effective, and HDL from subjects with Type I diabetes less effective, in the removal of LPOs. In vitro studies showed that glycoxidation and extensive oxidation of HDL impaired its antioxidant function and this was unrelated to PON1 activity. HDL function was not impaired by glycation without oxidation.

Protein carbonyls are products of oxidative stress that are considered to be important pathogenic mediators of cardiovascular and other complications in ESRD [6]. The higher levels of plasma protein carbonyls in the subjects with ESRD in the present study corroborates the findings of another study, which show higher levels of carbonyl stress end products in the arterial tissues of dialysis patients [23]. Contributors to this pro-oxidant milieu include iron overload, accumulation of AGEs and the haemodialysis biomembrane, which is permeable to hydrosoluble antioxidants and activates neutrophils [6]. In addition, reactive carbonyl compounds have impaired renal excretion and enzymatic detoxification. The latter may be related to the decreased efficiency of the aldose reductases and aldehyde dehydrogenases that interplay with redox coenzymes [6]. An example of this is glutathione, which is inhibited by homocysteine, a pro-oxidant metabolite that is elevated in ESRD [7].

We did not assess PON1 genotype, a major influence on PON1 activity, in our relatively small groups. We did, however, use the ratio of paraoxonase/arylesterase activities as a surrogate marker for the presence of the high activity PON1*192R allele [24]. Use of this ratio showed concordance rates of 93% described by Vincent-Viry et al. [25] in healthy subjects. We have studied previously [24] a series of healthy subjects (n = 46) for whom PON1*192QR or RR, was not significantly different between the three groups. The complication-free Type I diabetic group showed no increase in their plasma protein carbonyl concentrations compared with controls. In contrast, Martin-Gallan et al. [32] showed increased plasma protein carbonyl levels in complication-free Type I diabetics, whereas Odetti et al. [33] observed no increase in Type II diabetic subjects despite poor glycaemic control. The controversy surrounding the hypothesis of increased oxidative stress in diabetes, and specifically in Type I diabetes, may be attributed to variations in study design and the paucity of reliable measures of oxidative stress. There is a tendency for published studies to be positive and to relate to subjects with relatively poor metabolic control and complications [34–37], or to not address complication status. As an example, two similar studies assessed LDL oxidizibility in Type I diabetes, with one showing no increased susceptibility in a metabolically well-controlled cohort [38] and the other, in a group with poor metabolic control, showing greater susceptibility [39].

The significantly lower mean plasma PON1 activity in Type I diabetic group in the Type I diabetic group is consistent with the literature [9]. In these diabetic subjects, there was no correlation between the activity of this HDL-associated antioxidant enzyme and carbonyl levels, but the lower PON1 activity in diabetes may still prove detrimental in less...
metabolically well-controlled individuals or in those with complications, such as renal disease.

Vascular disease is increased in spite of relatively normal or even high levels of HDL-cholesterol in well-controlled Type I diabetes [40,41]. Qualitative changes in lipoproteins, such as glycation and/or oxidation, may play a crucial role in the increased atherosclerotic risk of patients with Type I diabetes by altering lipoprotein function [42]. In light of this concept, we have utilized an assay to test HDL function in vitro. We found decreased efficacy of LPO removal by HDL from subjects with Type I diabetes. The tendency for PON1 activity to be lower in the isolated HDL of diabetic patients has been observed previously [43], but this does not entirely explain the antioxidant dysfunction of their HDL, as PON1 activity did not correlate with efficacy of LPO removal. In the ESRD group, PON1 activity did not mirror the increased efficacy of LPO removal by their HDL, nor was there a correlation between PON1 activity and LPO removal. There are several possible reasons why PON1 activity did not correlate with the LPO removal efficacy by HDL. Firstly, HDL has several other antioxidant factors [44], including LCAT (lecithin:cholesterol acyl transferase), PAF-AH (PAF platelet-activating factor)-acetyl hydrolase and apoA1 (apolipoprotein-A1). It is quite possible they interact and are interdependent in the overall ability of HDL to remove LPO. Thus, in this oxidized membrane assay system, PON1 may not be the rate-limiting enzyme in the overall process of LPO removal by HDL, but, instead, just one player in a series of reactions. Secondly, issues have been raised about the measurement of PON1 activity with the artificial substrates paraoxon and phenylacetate. These substrates do not bind and thus do not reflect physiological activity to the site responsible for the ability of the enzyme to prevent lipid oxidation [45] and, most probably, to remove LPOs. Bypassing this dilemma, Boemi et al. [46] used PON1 concentration rather than activity to analyse HDL antioxidant function and showed a protective effect of higher amounts of PON1. Greater knowledge of and the use of physiological substrates of PON1 would be preferable.

In a previous study, Hasselwander et al. [4] found the ability of HDL from subjects with ESRD on dialysis to inhibit copper-induced LDL lipid peroxidation was not significantly different compared with control subjects and, if anything, was better. However, they concluded that PON1 was not the enzyme preserving the antioxidant function of HDL in ESRD, as it tended to be lower in their patient group, consistent with other reports [47,48]. In contrast, PAF-AH was increased (although not statistically significantly) in the ESRD group compared with control subjects [4]. This enzyme, which can metabolize lipid peroxidation products alone and synergistically with PON1 [49,50], could be an important factor in the increased LPO removal by HDL seen in our present study. The intimacy of these two enzymes is highlighted by Rodrigo et al. [51], who showed that purified PON1 could hydrolyse PAF.

An interesting result from the present studies was an inverse relationship between serum HDL-cholesterol levels and the efficacy of equal amounts of HDL (protein) to remove LPO from oxidized membranes in vitro. This result suggests that there is a compensatory mechanism in vivo such that lower HDL-cholesterol concentrations (and therefore less quantitative antioxidant protection) may be offset by an increase in efficacy of HDL function. This ability to compensate for lower HDL levels may be impaired in diabetes, as suggested by the loss of this pattern in the Type I diabetic group. Importantly, Figure 2 shows that efficacy of LPO removal by HDL was higher in ESRD and lower in Type I diabetes throughout the whole range of serum HDL-cholesterol concentrations. Furthermore, the in vitro glycoxidation of HDL eradicates its ability to remove LPO compared with its native form with no change to the cholesterol content of the lipoproteins. This suggests there is a factor or factors independent of serum HDL-cholesterol that has led to the up-/down-regulation of HDL in this in vitro assay.

In exploring the potential mechanism for reduced antioxidant function of HDL in Type I diabetes, we have demonstrated adverse effects of in vitro glycoxidation and of extensive oxidation. Mild oxidation or glycation of HDL did not impair its ability to remove LPOs from oxidized membranes. Extensive oxidation, with loss of PON1 activity, measured by hydrolysis of paraoxon, impaired this HDL function. However, the combination of glycation and mild oxidation abolished the ability of HDL to remove LPO despite preservation of PON1 activity, as measured by paraoxon hydrolysis. This synergistic action between glycative and oxidative stress to impair HDL function is supported by other in vitro studies that show glycation may make proteins more susceptible to oxidation [52,53]. A study by Hedrick et al. [54] concluded that the glycation of HDL impaired its antioxidant function, but a confounding effect in this study is that their glycatcating method did not protect against oxidation, as demonstrated by an increase in carboxymethyl-lysine levels, a glycoxidation product. On the other hand, heat inactivation of HDL PON1, without significant oxidation, decreased but did not entirely eradicate the ability of HDL to remove LPOs, suggesting that PON1 plays a role in LPO removal. These findings add more evidence to the argument that PON1, as currently measured by the hydrolysis of paraoxon and phenylacetate, is not the sole crucial factor in ability of HDL to remove LPO. These in vitro findings of a detrimental effect of glycoxidation on HDL function may explain why the HDL of patients with Type I diabetes was less effective at removing LPOs. Further evidence of this is a study by Ferretti et al. [55], which also showed impaired HDL LPO removal in
Type I diabetes using a similar assay system. Our present study results in Type I diabetes are in keeping with these observations. In contrast, their diabetic cohort was 10 years older, had poorer glycemic control (HbA1C, 9.6%) and significantly higher HDL LPO content. This suggests higher in vivo glycoxidative stress which may explain the negative correlations between HDL LPO content and the HDL LPO removal efficacy and HDL PON1 activity. Subsequently, this potential for in vivo HDL damage may explain the positive correlation between LPO removal and HDL PON1 activity that they described [55]. This hypothesis should not be negated on the grounds that our Type I diabetic group showed no increase in plasma oxidative stress, as measured by the protein carbonyl ELISA, and no significant elevation in HDL LPO content, because any major oxidative modification of HDL in vivo will most probably occur in the subendothelial space. In this microenvironment, there is a concentration of oxidative processes resulting in the formation of AGEs and free radicals that can damage HDL and propagate a pro-atherogenic vicious cycle [56].

In conclusion, the level of protein carbonyls, a measure of oxidative stress in vivo, is increased in ESRD, but not in well-controlled complication-free Type I diabetes. HDL dysfunction, evident in Type I diabetes but not in ESRD, cannot be attributed to decreased PON1 activity alone. Other HDL-related antioxidant factors and damage to HDL due to glycoxidation may contribute. Assays to measure oxidative and carbonyl stress and HDL dysfunction may facilitate an understanding of the mechanisms for vascular disease in high-risk groups and aid in the testing of interventions.

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