Increased Glycation and Oxidative Damage to Apolipoprotein B100 of LDL Cholesterol in Patients With Type 2 Diabetes and Effect of Metformin

Naila Rabbani, Madhu Varma Chittari, Charles W. Bodmer, Daniel Zehnder, Antonio Ceriello, and Paul J. Thornalley

OBJECTIVE—The aim of this study was to investigate whether apolipoprotein B100 of LDL suffers increased damage by glycation, oxidation, and nitrination in patients with type 2 diabetes, including patients receiving metformin therapy.

RESEARCH DESIGN AND METHODS—For this study, 32 type 2 diabetic patients and 21 healthy control subjects were recruited; 15 diabetic patients were receiving metformin therapy (median dose: 1.50 g/day). LDL was isolated from venous plasma by ultracentrifugation, delipidated, digested, and analyzed for protein glycation, oxidation, and nitrination adducts by stable isotopic dilution analysis tandem mass spectrometry.

RESULTS—Advanced glycation end product (AGE) content of apolipoprotein B100 of LDL from type 2 diabetic patients was higher than from healthy subjects: arginine-derived AGE, 15.8 vs. 5.3 mol% (P < 0.001); and lysine-derived AGE, 2.5 vs. 1.5 mol% (P < 0.05). Oxidative damage, mainly methionine sulfoxide residues, was also increased: 2.5 vs. 1.1 molar equivalents (P < 0.01). Nitrotyrosine content was decreased: 0.04 vs. 0.12 mol% (P < 0.001); and lysine-derived AGE, 2.5 vs. 1.1 mol% (P < 0.05). In diabetic patients receiving metformin therapy, arginine-derived AGE and methionine sulfoxide were lower than in patients not receiving metformin: 19.3 vs. 8.9 mol% (P < 0.01) and 2.9 vs. 1.9 mol% (P < 0.05), respectively; 3-nitrotyrosine content was higher: 0.10 vs. 0.03 mol% (P < 0.05). Fructosyl-lysine residue content correlated positively with fasting plasma glucose. Arginine-derived AGE residue contents were intercorrelated and also correlated positively with methionine sulfoxide.

CONCLUSIONS—Patients with type 2 diabetes had increased arginine-derived AGEs and oxidative damage in apolipoprotein B100 of LDL. This was lower in patients receiving metformin therapy, which may contribute to decreased oxidative damage, atherogenicity, and cardiovascular disease. Diabetes 59:1038–1045, 2010

Cardiovascular disease (CVD) is the major cause of premature death in diabetes. Type 2 diabetes is associated with a twofold to threefold increased risk of coronary heart disease in men and a threefold to fivefold increased risk in women, relative to the nondiabetic population (1). Dyslipidemia is a key feature of diabetic CVD where small dense LDL particles pose a major atherogenic threat. The underlying mechanism producing small, dense LDL is related to hepatic oversecretion of apolipoprotein B100 (apoB100) and impaired clearance of LDL by the high-affinity LDL receptor in which both hepatic and peripheral tissues participate (2). The normal residence time of LDL in plasma is 3 days but this is increased to 5 days for small, dense, highly atherogenic LDL (3). Atherogenicity and plasma residence time of LDL may be influenced by damage to apoB100 by glycation, oxidation, and nitrination but the quantitative amounts of damage in healthy human subjects and diabetic patients remain unclear.

Glycation of proteins is a complex series of parallel and sequential reactions collectively called the Maillard reaction. Early stage reactions are directed to lysine and NH₂-terminal amino acid residues leading to the formation of the early glycation adduct, fructosyl-lysine (FL), and other fructosamine derivatives. Later stage reactions form advanced glycation end products (AGEs). FL degrades slowly to form AGEs. Glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG) are physiological dicarbonyl metabolites and potent glycating agents formed by the degradation of glycolytic intermediates, glycated proteins, and lipid peroxidation. They react with proteins to form AGEs directed mainly to arginine residues—often functionally important arginine residues. The most important AGEs quantitatively are hydromidazolones derived from arginine residues modified by glyoxal, methylglyoxal, and 3-DG: G-H₁, MG-H₁, and 3DG-H, respectively. Nε-carboxymethyl-arginine (CMA) is a further arginine-derived adduct formed by glyoxal. Other important and widely studied AGEs are Nε-carboxymethyl-lysine (CML), Nε-carboxyl-ethyl-lysine (CEL), and pentosidine. Markers of oxidative damage to proteins are methionine sulfoxide (MetSO), formed by the oxidation of methionine, and dityrosine, formed by oxidative cross-linking of tyrosine. A widely studied marker of nitrination damage to proteins is 3-nitrotyrosine (3-NT) (rev. in 4) (Fig. 1).

Metformin is the most widely prescribed oral glucose-lowering agent for the treatment of type 2 diabetes. It improves glycemic control and decreases the risk of CVD (5). Metformin therapy of type 2 diabetic patients increased LDL particle size (6) and decreased plasma con-
centrations of remnant lipoprotein cholesterol, a predictor of myocardial infarction and thought to reflect increased residence time and atherogenicity of cholesterol ester–rich chylomicrons and VLDL (7). Metformin also decreased the plasma concentrations of methylglyoxal in diabetic patients (8) and may decrease oxidative stress and related oxidation of LDL (9).

In this study, we used the gold standard method of stable isotopic dilution analysis liquid chromatography–tandem mass spectrometry (LC-MS/MS) to measure protein glycation, oxidation, and nitration adducts in apoB100 of LDL to assess whether there is increased lipoprotein damage in patients with type 2 diabetes with respect to normal healthy control subjects and to investigate the effect of metformin therapy.

**RESEARCH DESIGN AND METHODS**

**Patients and normal healthy volunteers.** Diabetic patients were recruited from patients attending the Diabetes Clinics at Colchester General Hospital (Colchester, U.K.) and University Hospital of Coventry and Warwickshire (Coventry, U.K.). Healthy control volunteers were recruited from partners and friends of the patients and investigators. Ethical approval for the study was given by the local ethics committees (North and Mid-Essex Local Research Ethics Committee, Chelmsford, U.K. and Coventry Research Ethics Committee, Coventry, U.K.). Inclusion criteria were type 2 diabetes with normoalbuminuria (albumin excretion rate <30 mg/24 h), age 40–80 years, diabetes duration of ≥1 year, and A1C <13%. Exclusion criteria were individuals with significant comorbidities who participated in an intervention study within 30 days, recipients of renal and/or pancreatic transplants, and women who were pregnant or breastfeeding or of child-bearing potential not using adequate contraceptive potential not using adequate contraceptive precautions. Metformin therapy was given in the dose range 0.85–3 g/day; median 1.50. The duration of metformin therapy was in the range 1–20 years; median 4 years. Other therapy (number of patients without/with metformin therapy) was insulin (17/5), gliclazide (2/3), glimepiride (1/2), and antihypertensive therapy (0/4). Participant characteristics are shown in Table 1. Venous blood samples (fasting) were taken after informed consent. Plasma was separated immediately and stored at −80°C until analysis.

**Isolation of LDL.** For rapid, same-day preparation of LDL, a self-generating gradient of iodixanol in a vertical rotor (S120VT) was used in a Sorvall MTX 150 microcentrifuge (Hitachi). The density of plasma was increased to 12% using 60% iodixanol solution (OptiPrep; Axis-Shield). Plasma (0.9 ml) was layered under 0.9 ml of 9% iodixanol in a 2-ml ultracentrifuge tube (polyal-

**FIG. 1. Molecular structures of protein glycation, oxidation, and nitration residues.**
lomer, no. S302807A; Hitachi) and further void filled with 0.2 ml nitrogen-purged PBS. The sample was centrifuged (50,000g, 16°C, 2.5 h) with low acceleration and deceleration. LDL was washed with nitrogen-purged water (4 ml x 3) over 100-kDa microspin filters (Amicon) to remove iodixanol. The LDL was stored at -20°C until further analysis. Sample handling was performed under subdued light. Protein concentration was measured by Bradford assay. Lipid peroxidation of LDL was assessed by measuring thiobarbituric acid reactive substances (TBARS). TBARS were quantified by reference of the chromophoric response to a standard curve constructed from malondialdehyde tetramethyl acetal and expressed as malondialdehyde equivalents (10). The purity of LDL was assessed by SDS-PAGE denaturing and agarose nondenaturing electrophoresis.

In vitro modification of LDL. LDL was glycated minimally by methylglyoxal and glucose in vitro to assess the major glycation adducts formed. LDL glycated minimally by methylglyoxal (MG$_{min}$-LDL) was prepared by incubation of methylglyoxal (200 μmol/l) with LDL (4.2 mg/ml) in PBS (0.4 mmol/l diethylenetriaminepentaacetic acid [DETAPAC], pH 7.4) at 37°C for 6 h. The glycated and control LDL was washed extensively with argon-purged ice-cold water using ultra-spin filters (Amicon 100-kDa cutoff membrane from Millipore) at 4°C, and stored at 4°C under argon and used within 2 weeks. LDL glycated minimally by glucose (AGE$_{min}$-LDL) was prepared by incubation of glucose (25 mmol/l) with LDL (3 mg/ml) in PBS (0.4 mmol/l DETAPAC, pH 7.4) at 37°C for 7 days under sterile conditions. Control LDL was incubated without glucose. The glycated and control LDL was washed with argon-purged ice-cold water ultrafiltration at 4°C, and stored at 4°C under argon until further analysis. Electrophoretic mobility of native and modified LDL on agarose gel electrophoresis was performed using a gel lipoprotein electrophoresis kit using barbital buffer, pH 8.6 (Helena).

Delipidation of LDL. An aliquot of LDL solution (20 μl, 100 μg) was transferred into a glass tube (50 x 7.5 mm) containing butylated hydroxytoluene in methanol (5 μl, 2 mg/ml), 20% trichloroacetic acid (100 μl), and water (75 μl), mixed well, left on ice for 10 min, and then centrifuged (10,000g, 15 min, 4°C). The supernatant was removed and the pellet washed with acetone (200 μl) and diethyl ether (200 μl) and dried under argon.

Enzymatic digestion of apoB100. Delipidated protein was hydrolyzed exhaustively by modification of our published procedure (11). Protein was suspended in 100 μmol/l potassium phosphate buffer, pH 7.4 (50 μl). Pronase E (20 μl, 2 mg/ml in 10 mmol/l KH$_2$PO$_4$ buffer, pH 7.4) and 10 μl penicillin (50 units/ml) and streptomycin (50 μg/ml) were added and the samples incubated at 37°C for 24 h. Thereafter, 10 μl each of proelidase and aminopeptidase solutions (2 mg/ml in 10 mmol/l KH$_2$PO$_4$ buffer, pH 7.4) was added, and samples were incubated for a further 48 h. All steps were performed under argon. A similar method was used previously to quantify the oxidative marker 5-hydroxy-2-aminovinlaldehyde in apoB100 (12).

Protein biomarker determination by LC-MS/MS. Fructosyl-lysine, advanced glycation end products, and oxidation and nitration markers were determined in enzymatic hydrolysates of delipidated lipoprotein by stable isotopic dilution analysis LC-MS/MS (13).

Statistics. Data are mean ± SD for parametric data and median (minimum – maximum) or (lower – upper quartile) for nonparametric data. Significance of difference between means was assessed by Student t test and significance of difference between medians, by the Mann-Whitney U test. Difference of proportions was assessed using Fishere contingency tables.

### RESULTS

**Isolation of LDL and glycation by methylglyoxal and glucose in vitro.** A new rapid method for isolation of LDL was developed and used in this study. This method used a single ultracentrifugation step for 2.5 h only with samples at 16°C (14). High purity was confirmed by a single protein band in denaturing SDS-PAGE and agarose nondenaturing electrophoresis (data not shown). Lipid peroxidation of isolated LDL from normal healthy control subjects, as judged by TBARS content, was low: 0.81 ± 0.45 nmol/mg protein (n = 12).

LDL glycated minimally by methylglyoxal and glucose showed increased levels of AGE residues. For glycation by methylglyoxal, MG$_{min}$-LDL showed increased content of MG-H1, CEL, and MOLD residues. The major AGE formed by glycation with methylglyoxal was MG-H1 (98.4%) with minor formation of CEL (1.4%) and MOLD (0.2%). For glycation of LDL by glucose, the major increase in glycation adducts was of FL residues with a minor increase in CML residues (Table 2).

**Protein damage markers in apolipoprotein B100 of LDL of healthy human subjects and patients with type 2 diabetes.** In apoB100 of LDL of healthy human subjects, the mean FL residue content was 2.90 pmol/mg apoB100, equivalent to 1.49 mol/mol apoB100 or 4.17 mmol/mol Lys. Major AGE residues quantitatively were MG-H1, median content 46.8 pmol/mg apoB100, equivalent to 0.204 mol/mol apoB100 or 0.16 mmol/mol Arg; and CML, median content 24.0 pmol/mg apoB100, equivalent to 0.012 mol/mol apoB100 or 0.035 mmol/mol Lys. Median total arginine-derived AGE residue content (G-H1 + MG-H1 + 3DG-H + CMA + pentosidine) was 103 pmol/mg apoB100, equivalent to 0.062 mol/mol or 0.42 mmol/mol Arg. Median total lysine-derived AGE residue content (CML + CEL + MOLD + pentosidine) was 33 pmol/mg apoB100, equivalent to 0.017 mol/mol or 0.047 mmol/mol Lys. The major oxidative marker was MetSO residues with mean content of 2084 pmol/mg apoB100, equivalent to 1.07 mol/mol apoB100 or

### Table 1

Characteristics of type 2 diabetic patients and normal healthy control subjects

<table>
<thead>
<tr>
<th>Study group</th>
<th>Control subjects</th>
<th>Type 2 diabetic subjects not receiving metformin therapy</th>
<th>Type 2 diabetic subjects receiving metformin therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.5 ± 9.7</td>
<td>60.5 ± 12.2</td>
<td>64.1 ± 12.8*</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10/11</td>
<td>14/18</td>
<td>11/8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.6 ± 3.6</td>
<td>33.7 ± 6.3‡</td>
<td>31.5 ± 6.3</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>—</td>
<td>11 (1–35)</td>
<td>13 (1–35)</td>
</tr>
<tr>
<td>FPG (mM)</td>
<td>5.14 ± 0.74</td>
<td>8.75 ± 2.51‡</td>
<td>9.15 ± 2.41‡</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.48 ± 0.57</td>
<td>8.20 ± 1.94</td>
<td>8.25 ± 2.04‡</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>5.11 ± 1.43</td>
<td>4.90 ± 1.02</td>
<td>4.71 ± 1.00</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>3.18 ± 1.41</td>
<td>3.01 ± 1.01</td>
<td>2.89 ± 1.04</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.55 ± 0.81</td>
<td>1.60 ± 0.30</td>
<td>1.18 ± 0.23</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.16 ± 0.31</td>
<td>2.23 ± 1.02‡</td>
<td>2.13 ± 1.09§</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 ± 20</td>
<td>141 ± 22</td>
<td>140 ± 24</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>77 ± 8</td>
<td>78 ± 8</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>GFR (ml/min)</td>
<td>99 ± 20</td>
<td>100 ± 42</td>
<td>80 ± 30</td>
</tr>
</tbody>
</table>

Data are mean ± SD or median (minimum – maximum). Significance: *P < 0.05, §P < 0.01, and ‡P < 0.001, with respect to normal healthy control subjects; and †P < 0.05, with respect to type 2 diabetic patients receiving conventional therapy. GFR, glomerular filtration rate.
Correlation analysis for markers of glycemic control

diabetic patients. There was no correlation of protein damage marker content of apoB100 with patient age, suggesting that the significant age difference of diabetic patients with and without metformin therapy did not compromise protein damage marker of these study groups. For markers of glycemic control, FPG concentration correlated positively with A1C and also with FL residue content of apoB100. FL residue content correlated positively with CEL, MG-H1, and 3DG-H residue contents. There was a cluster of correlations of dicarbonyl-derived AGE residue contents: G-H1 correlated positively with MG-H1 and CMA; MG-H1 also correlated positively with CMA and pentosidine; and CMA and MOLD correlated positively with dityrosine (Table 4). There were negative correlations of 3-NT with MG-H1 and 3DG-H.

**Protein damage markers in apolipoprotein B100 of LDL of patients with type 2 diabetes receiving metformin.**Patients receiving metformin therapy were slightly younger and more obese than those not receiving metformin therapy, although all other conventional clinical variables were not significantly different (Table 1). ApoB100 of LDL from patients receiving metformin therapy had lower contents of AGEs (G-H1, MG-H1, 3DG-H, and CMA) and MetSO but higher 3-NT content.

### TABLE 2

Changes in protein glycation adduct residues in human LDL minimally modified by methylglyoxal and glucose in vitro

<table>
<thead>
<tr>
<th>Glycation adduct</th>
<th>Control 1</th>
<th>MG&lt;sub&gt;min&lt;/sub&gt;-LDL</th>
<th>Control 2</th>
<th>AGE&lt;sub&gt;min&lt;/sub&gt;-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>2.21 ± 0.21</td>
<td>1.99 ± 0.06</td>
<td>3.10 ± 0.67</td>
<td>6.08 ± 0.37*</td>
</tr>
<tr>
<td>CML</td>
<td>0.031 ± 0.004</td>
<td>0.032 ± 0.007</td>
<td>0.056 ± 0.009</td>
<td>0.070 ± 0.003†</td>
</tr>
<tr>
<td>CEL</td>
<td>0.004 ± 0.001</td>
<td>0.024 ± 0.003*</td>
<td>0.011 ± 0.003</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>MG-H1</td>
<td>0.15 ± 0.02</td>
<td>1.57 ± 0.37*</td>
<td>0.23 ± 0.03</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>MOLD</td>
<td>0.0002 ± 0.0001</td>
<td>0.0025 ± 0.0009*</td>
<td>0.0057 ± 0.001</td>
<td>0.0092 ± 0.002</td>
</tr>
</tbody>
</table>

Data are mol adduct/mol apoB100; mean ± SD (n = 3). Control 1 and control 2 are incubations of LDL for 6 h and 7 days without methylglyoxal and glucose, respectively. Significance: *P < 0.001, †P < 0.01. Other adduct residues, G-H1, 3DG-H, CMA, pentosidine, MetSO, dityrosine, and 3-NT, were not changed significantly during the incubation with glyciating agents.

### TABLE 3

Markers of protein damage in apolipoprotein B100 of LDL

<table>
<thead>
<tr>
<th>Type of modification/Analyte</th>
<th>Control subjects</th>
<th>All type 2 diabetic subjects</th>
<th>Type 2 diabetic subjects not receiving metformin therapy</th>
<th>Type 2 diabetic subjects receiving metformin therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>32</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Fructosamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>2,900 ± 1,402</td>
<td>3,347 ± 1,914</td>
<td>3,789 ± 1,971</td>
<td>2,682 ± 1,688</td>
</tr>
<tr>
<td>AGE</td>
<td>24.0 (0.7–143.7)</td>
<td>20.6 (1.7–58.9)</td>
<td>20.5 (3.9–58.9)</td>
<td>24.1 (1.7–56.4)</td>
</tr>
<tr>
<td>CML</td>
<td>3.5 (0.2–38.9)</td>
<td>17.3 (3.5–96.9)*</td>
<td>21.9 (3.5–96.9)*</td>
<td>14.6 (4.6–33.4)*</td>
</tr>
<tr>
<td>CEL</td>
<td>3.6 (0.1–50.4)</td>
<td>31.5 (12.2–188.3)</td>
<td>44.0 (18.1–188.3)*</td>
<td>25.0 (12.5–59.0)*</td>
</tr>
<tr>
<td>G-H1</td>
<td>46.8 (15.9–219.4)</td>
<td>197.0 (30.4–474.4)*</td>
<td>235.8 (45.5–474.4)*</td>
<td>91.3 (30.3–309.4)*</td>
</tr>
<tr>
<td>MG-H1</td>
<td>19.4 (2.2–138.9)</td>
<td>60.0 (4.8–163.8)*</td>
<td>82.3 (4.8–163.8)*</td>
<td>39.4 (6.3–86.2)*</td>
</tr>
<tr>
<td>3DG-H</td>
<td>20.3 (0.4–47.9)</td>
<td>26.8 (0.7–112.8)</td>
<td>38.3 (0.7–112.8)</td>
<td>8.9 (1.6–74.6)*</td>
</tr>
<tr>
<td>CMA</td>
<td>1.8 (0.3–51.5)</td>
<td>9.0 (0.2–31.8)</td>
<td>12.2 (0.2–31.8)</td>
<td>7.6 (1.5–27.3)*</td>
</tr>
<tr>
<td>MOLD</td>
<td>0.26 (0.03–0.84)</td>
<td>0.76 (0.08–2.13)*</td>
<td>0.75 (0.18–2.13)*</td>
<td>0.76 (0.08–1.61)*</td>
</tr>
<tr>
<td>Pentosidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MetSO</td>
<td>2.084 ± 1,360</td>
<td>4.738 ± 3,367*</td>
<td>5.633 ± 3,837*</td>
<td>3.857 ± 2,641†</td>
</tr>
<tr>
<td>Dityrosine</td>
<td>0.26 (0.05–6.86)</td>
<td>16.7 (0.2–6.84)*</td>
<td>16.8 (5.8–34.8)*</td>
<td>11.0 (0.2–47.1)*</td>
</tr>
<tr>
<td>Nitration</td>
<td>2.3 (0.3–49.1)</td>
<td>0.9 (0.1–24.1)†</td>
<td>0.7 (0.1–15.4)‡</td>
<td>2.0 (0.1–24.1)‡</td>
</tr>
</tbody>
</table>

Data are pmol/mg apoB100; mean ± SD or median (minimum – maximum). Significance: †P < 0.05, ‡P < 0.01, and *P < 0.001, with respect to normal healthy control subjects; and $P < 0.05 and $P < 0.01, with respect to type 2 diabetic patients not receiving metformin therapy.
that the rate of glycation was first order with respect to approximately constant during the incubation time) and
Assuming initial rate conditions (the rate of glycation was adduct formed in apoB100 was MG-H1 residues with
the rate of LDL glycation by glucose is
rates of glycation of LDL rLDL, Glycating agent by glucose reactive with LDL than is glucose. The predicted in situ
due to the markedly lower concentration of methylglyoxal than glucose in plasma: 100 nmol/l vs. 5 mmol/l.
this rapid method has potential to decrease the risk of apoB100 damage in preanalytic processing and could facilitate clinical studies of LDL.
Glycation of LDL by glucose in vitro to form AGEmin-LDL showed that the major glycation adduct formed in apoB100 was FL residues with related minor increase of CML residue content. CML is formed by the oxidation degradation of FL. Glycation of LDL by methylglyoxal in vitro to form MGmin-LDL showed that the major glycation adduct formed in apoB100 was MG-H1 residues with concurrent minor formation of CEL and MOLD residues. Assuming initial rate conditions (the rate of glycation was approximately constant during the incubation time) and that the rate of glycation was first order with respect to LDL and glycatine agent, the rate constants kL, Glycatine agent for glycation of LDL by glucose and methylglyoxal are kL, Glucose = 11.2 (mol/l)^-1\cdot day^-1 and kL, MG = 28,800 (mol/l)^-1\cdot day^-1, respectively, at pH 7.4 and 37°C. This suggests that methylglyoxal is ~2,600-fold more reactive with LDL than is glucose. The predicted in situ rates of glycation of LDL rL, Glycatine agent by glucose and methylglyoxal in plasma, assuming concentrations of LDL, glucose, and methylglyoxal of 1.3 μmol/l, 5 mmol/l, and 100 nmol/l, respectively (8,15), are rL, Glucose = 73 mmol/l\cdot day^-1 and rL, MG = 4 nmol/l\cdot day^-1, suggesting that the rate of LDL glycation by glucose is ~18-fold faster than by methylglyoxal in plasma. The apparent switch of relative reactivity of glucose and methylglyoxal with LDL in situ is due to the markedly lower concentration of methylglyoxal than glucose in plasma: 100 nmol/l vs. 5 mmol/l.

### DISCUSSION

A new method for rapid isolation of LDL is described and used here with a single ultracentrifugation step of only 2.5 h at 16°C, whereas the conventional method of LDL isolation involves ultracentrifugation for 20–22 h at 15°C (14). This rapid method has potential to decrease the risk of apoB100 damage in preanalytic processing and could facilitate clinical studies of LDL.

Glycation of LDL by glucose in vitro to form AGEmin-LDL showed that the major glycation adduct formed in apoB100 was FL residues with related minor increase of CML residue content. CML is formed by the oxidation degradation of FL. Glycation of LDL by methylglyoxal in vitro to form MGmin-LDL showed that the major glycation adduct formed in apoB100 was MG-H1 residues with concurrent minor formation of CEL and MOLD residues. Assuming initial rate conditions (the rate of glycation was approximately constant during the incubation time) and that the rate of glycation was first order with respect to LDL and glycatine agent, the rate constants kL, Glycatine agent for glycation of LDL by glucose and methylglyoxal are kL, Glucose = 11.2 (mol/l)^-1\cdot day^-1 and kL, MG = 28,800 (mol/l)^-1\cdot day^-1, respectively, at pH 7.4 and 37°C. This suggests that methylglyoxal is ~2,600-fold more reactive with LDL than is glucose. The predicted in situ rates of glycation of LDL rL, Glycatine agent by glucose and methylglyoxal in plasma, assuming concentrations of LDL, glucose, and methylglyoxal of 1.3 μmol/l, 5 mmol/l, and 100 nmol/l, respectively (8,15), are rL, Glucose = 73 mmol/l\cdot day^-1 and rL, MG = 4 nmol/l\cdot day^-1, suggesting that the rate of LDL glycation by glucose is ~18-fold faster than by methylglyoxal in plasma. The apparent switch of relative reactivity of glucose and methylglyoxal with LDL in situ is due to the markedly lower concentration of methylglyoxal than glucose in plasma: 100 nmol/l vs. 5 mmol/l.

The ratio of FL to methylglyoxal-derived adducts in apoB100 of healthy control was ~56. ApoB100 is probably also glycated prior to assimilation into LDL.

The protein damage marker of highest quantitative content in apoB100 of LDL in healthy human subjects was the early glycation adduct FL, equivalent to 0.42% lysine residues. These levels are threefold lower than reported in earlier studies using the tritiated borohydride reduction technique (1.3% [16]) but similar to the 2–3 nmol FL residues per milligram apoB100 estimates using the fluorescamine technique (17). FL, MG-H1, MetSO, and 3-NT are major adducts of early glycation, advanced glycation, oxidation, and nitration of apoB100, LDL, and also total plasma protein. The rates of damage of LDL and plasma protein can be predicted, assuming these rates are equal to the rate of clearance of adducts in the steady state and taking into account half-lives of LDL and serum albumin—the major plasma protein—are ~3 and 19 days, respectively (18,19). The outcome of these predictions is shown in Table 5. Estimates of kL, Glucose [69 (mol/l)^-1\cdot day^-1] and kL, MG [55,452 (mol/l)^-1\cdot day^-1] from these deductions were not markedly dissimilar from estimates from in vitro glycation studies (see above). Overestimation of rates from in vivo data may be attributed to glycation of apoB100 prior to assimilation in LDL particles. Overall apoB100 of LDL is far more reactive to damage by these modifications than is albumin, even when the eightfold greater molecular mass of apoB100 relative to albumin is taken into account. ApoB100 is highly susceptible to damage and may be a particularly good sensor of it. From the predicted in situ rates of modification, the rate of early and advanced glycation of LDL is only 17 and 4% of that of albumin, whereas the in situ rates of oxidation and nitration are 10 and 76% greater than those of albumin. As LDL has a short plasma half-life, however, the steady levels of protein glycation, oxidation, and nitration adducts in apoB100 represent only a minor part of the total plasma adduct concentration (Table 5). The adduct content in apoB100 of diabetic patients may be increased by effects of both increased rate of modification, caused by increased plasma concentrations of modifying agents, and decreased rate of elimination.

In type 2 diabetic patients, the FL and CML residue

### Table 4

<table>
<thead>
<tr>
<th>Glycemic control</th>
<th>FPG</th>
<th>HbA1c</th>
<th>FL</th>
<th>AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.46**</td>
<td></td>
<td>0.58***</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxidative damage</th>
<th>MetSO</th>
<th>DT</th>
<th>3-NT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.38*</td>
<td>0.60***</td>
<td>0.62***</td>
</tr>
</tbody>
</table>

Data are correlation coefficients (Spearman) with significance: *P < 0.05, **P < 0.01, and ***P < 0.001. Correlation was of glycemic control indicators and protein damage markers of apoB100 in type 2 diabetic patients with and without metformin therapy.
content of apoB100 is not significantly different from that of apoB100 from healthy subjects. Increased plasma glucose concentration in type 2 diabetic patients did not produce a significant increase in FL residue content of apoB100. This may indicate that formation of FL residues in apoB100 by glucose is less favored when the extent of glycation exceeds 2 molar equivalents, limiting further increase of FL residue content in diabetes. Dicarbonyl-derived AGE content of apoB100 from type 2 diabetic patients was, however, increased markedly. This suggests that dicarbonyl glycation is the main cause of increased AGE content of apoB100 of LDL in type 2 diabetic patients.

Arginine-derived AGE residue contents of apoB100 in these patients increased more than threefold. Major quantitative oxidative damage—MetSO residue content—of apoB100 in type 2 diabetic patients was twofold higher than in apoB100 of healthy control subjects. This is commensurate with increased plasma peroxide concentration in type 2 diabetes (21). There is no repair of MetSO by MetSO reductase in plasma, hence plasma MetSO likely reflects increased plasma reactive oxygen species in diabetes and decreased plasma reactive oxygen species production for patients treated with metformin. The 64-fold increase of dityrosine residue content

FIG. 2. Advanced glycation end product and methionine sulfoxide residue contents of apolipoprotein B100 of LDL of type 2 diabetic patients with and without metformin therapy. (A) CEL, (B) G-H1, (C) MG-H1, (D) 3DG-H, (E) CMA, and (F) MetSO. Data are median (lower – upper quartile) except for MetSO, which is mean ± SD. Significance: *P < 0.05, **P < 0.01, and ***P < 0.001, with respect to normal healthy control subjects; and oP < 0.05 and ooP < 0.01, with respect to type 2 diabetic patients not receiving metformin therapy.
of apoB100 in diabetic patients, however, far exceeds this. Dityrosine residues are formed by both spontaneous and enzymatic processes. Enzymatic formation is catalyzed by dual oxidase-1 (22)—a member of the NADPH oxidase family of enzymes implicated in signaling in vascular disease in diabetes (23). Activation of NADPH oxidase/dual oxidase isozymes in diabetes may markedly enhance the formation of dityrosine residues in apoB100 (24). Dityrosine content of apoB100 of diabetic patients (~0.06 mmol/mol Tyr) was intermediate between that of normal control subjects (~0.001 mmol/mol Tyr) and of apoB100 isolated from atherosclerotic plaques (~0.25 mmol/mol Tyr) (25).

For type 2 diabetic patients receiving metformin therapy, there were lower contents of dicarbonyl-derived AGE and MetSO residues than in patients not receiving metformin therapy. CML residue content of apoB100 of LDL was linked to patient sex; imperfect matching of sex may have masked change in CML residue content in patients receiving metformin therapy. Metformin decreased the concentration of methylglyoxal in type 2 diabetic patients (8). It is also expected to react with glyoxal and 3-DG similarly, and thereby decrease plasma levels of these dicarbonyls and prevent related formation of AGE residues. Metformin reacts with methylglyoxal in vivo, forming a triazepinone adduct that has been detected in plasma and urine (26). This decreases methylglyoxal by a scavenging action, although the relatively slow kinetics of this reaction prompted consideration of other mechanisms (27). Improvement of glycemic control by metformin decreases dicarbonyl formation and thereby decreased AGE formation of apoB100 indirectly, as suggested by the correlation of FL residue content of apoB100 with contents of CEL, MG-H, and 3DG-H residues. Both mechanisms are likely involved.

A remarkable finding was the decrease in MetSO residue content of apoB100 in patients receiving metformin therapy. In correlation analysis, there were strong correlations of MetSO with G-H1, MG-H1, and CMA residue contents of apoB100. These correlations were not found in similar analysis of total plasma protein (28), which suggests these relationships are specific to LDL. This likely relates to the most important physiological impact of our findings: our recent research suggests that formation of MG-H1 residues in apoB100 increases binding of LDL to proteoglycan, which may increase the half-life of LDL in the extracellular compartment and thereby susceptibility to oxidation (29). Decreased fractional clearance of apoB100 has been linked to oxidative damage of apoB100 and atherogenicity (12). Increased binding to proteoglycan in the subendothelium is thought to be integral to this process (30). Metformin may decrease dicarbonyl glycation of apoB100 and in so doing prevent decreased plasma clearance and increased oxidation and atherogenicity of LDL in type 2 diabetes. In future studies, it will be of interest to test this hypothesis in prospective placebo-controlled studies.

In this study, 3-NT residue content of apoB100 of LDL was lower in diabetic patients not receiving metformin therapy than healthy control subjects and normalized in patients with metformin therapy. The quantitative amount of 3-NT residues (0.03–0.1 mol%) is unlikely to be damaging, but it may be a marker of nitric oxide bioavailability. Metformin therapy has recently been shown to be linked to activation of endothelial nitric oxide synthase (31). The changes in 3-NT residues of apoB100 of LDL here may reflect bioavailability of nitric oxide in diabetic patients, thereby suggesting that diabetic patients receiving metformin therapy may achieve normal vascular nitric oxide bioavailability. This provides a further mechanism how metformin may be protective to the vasculature in diabetes.

ACKNOWLEDGMENTS

N.R. thanks the British Heart Foundation for an Intermediate Research Fellowship. The authors thank the British Heart Foundation for supporting their research.

No potential conflicts of interest relevant to this article were reported.

Parts of this study were presented in abstract form at the 45th Annual Meeting of the European Association for the Study of Diabetes, Vienna, Austria, 27 September–10 October 2009.

REFERENCES

2. Taskinen MR. Diabetic dyslipidaemia: from basic research to clinical practice. Diabetologia 2004;47:733–749
4. Thorlalcy PJ. Quantitative screening of protein glycation, oxidation, and nitration adducts by LC-MS/MS: protein damage in diabetes, uremia,


27. Xue M, Thormar PE, Rahbani N. LDL glycated by methylglyoxal to physiological extent in vitro has increased binding of proteoglycan without impairment of LDL receptor binding. Diabetologia 2008;51:8299

