Glucosylated Glycerophosphoethanolamines are the Major LDL Glycation Products and Increase LDL Susceptibility to Oxidation

Evidence of Their Presence in Atherosclerotic Lesions

Amir Ravandi, Arnis Kuksis, Nisar A. Shaikh

Abstract—Glycation of both protein and lipid components is believed to be involved in LDL oxidation. However, the relative importance of lipid and protein glycation in the oxidation process has not been established, and products of lipid glycation have not been isolated. Using glucosylated phosphatidylethanolamine (Glc PtdEtn) prepared synthetically, we have identified glycated diacyl and alkenylacyl species among the ethanolamine phospholipids in LDL. Accumulation of these glycation products in LDL incubated with glucose showed a time- and glucose concentration–dependent increase. LDL specifically enriched with Glc PtdEtn (25 nmol/mg protein) showed increased susceptibility to lipid oxidation when dialyzed against a 5-μmol/L Cu²⁺ solution. The presence of this glucosylated lipid resulted in a 5-fold increase in production of phospholipid-bound hydroperoxides and 4-fold increase in phospholipid-bound aldehydes. Inclusion of glucosylated phosphatidylethanolamine in the surface lipid monolayer of the LDL resulted in rapid loss of polyunsaturated cholesteryl esters from the interior of the particle during oxidation. Glycated ethanolamine phospholipids were also isolated and identified from atherosclerotic plaques collected from both diabetic and nondiabetic subjects. The present findings provide direct evidence for the previously proposed causative effect of lipid glycation on LDL oxidation. (Arterioscler Thromb Vasc Biol. 2000;20:467-477.)

Key Words: low-density lipoproteins ▪ peroxidation, lipid ▪ glycation, phospholipid ▪ diabetes ▪ atheroma
solution of either NaCNBH₃ or NaBH₄ to a final concentration of nitrogen. Some samples were reduced by addition of a methanolic as described by Folch et al. Lipids were extracted into chloroform/methanol (2:1, vol/vol) sonicated at low power for 5 minutes at room temperature. The 400 mmol/L glucose and 0.1 mmol/L EDTA were added and nitrogen. Four mL of 0.1 mol/L phosphate buffer containing 0 to tert-butyl hydroperoxide as the oxidant. At different times, aliquots were withdrawn. Lipids were extracted with chloroform-methanol (2:1 vol/vol) and washed repeatedly with buffer to remove residual tert-butyl hydroperoxide. Samples were analyzed by liquid chromatography with online electrospray mass spectrometry (LC/ESI/MS) immediately after being extracted.

**Lipoprotein Isolation**

LDL (1.019 to 1.069 g/mL) was obtained by density gradient ultracentrifugation from plasma of fasted normolipidemic individuals. LDL (2 mg protein per milliliter) was subsequently dialyzed against 0.1 mol/L phosphate buffer (pH 7.4) that contained 0.1 mmol/L EDTA for 24 hours (3 buffer changes). LDL samples were sterilized by being passed through a 0.22-μm filter (Millipore), stored at 4°C, and used within 1 week. Lipoprotein concentration was determined by the method of Lowry et al. and expressed in milligrams per milliliter. Oxidation of LDL (5 mg protein per 5 mL) was performed by dialysis against 5 μmol/L CuSO₄ .5 H₂O in 0.1 mol/L phosphate buffer, pH 7.4, for 24 hours at 37°C in the dark. Lipids were extracted into chloroform/methanol (2:1 vol/vol) as described above.

**Enrichment of LDL With PtdEtn**

Glc and non-Glc PtdEtn was incorporated into LDL in a manner similar to that described by Engelmann et al. for enriching human plasma lipoproteins with phospholipids. Glc PtdEtn (1 mg) in chloroform-methanol (2:1 vol/vol) was transferred to a 15-mL test tube. The solvent was evaporated under nitrogen, and the lipids were dispersed by centrifuging in 1.5 mL buffer that contained 50 mmol/L Tris/HCl, 1 mmol/L DTT, and 0.03 mmol/L EDTA (pH 7.4). Solutions were sonicated in a bath sonicator for 5 minutes at 1-minute intervals while being kept on ice under a stream of nitrogen. The liposome mixture was centrifuged at 3500g, and the supernatant was collected and passed through a 0.45-μm filter. The liposomal mixture (1 mL) was added to fresh plasma (4 mL) that contained 3 mmol/L NaNO₂, under gentle mixing. The mixture was incubated under nitrogen at 37°C for 24 hours. Lipoproteins were isolated as described above.

**LDL Glucosylation**

LDL (2 mg protein per milliliter) in PBS that contained 1 mmol/L EDTA, 0.1 mg/mL chloramphenicol, and 3 mmol/L NaN₃ was incubated with 5 to 400 mmol/L glucose at 37°C for 1 week under nitrogen. Other incubation mixtures contained 3 μCi/mL D-glucose-1-¹⁴C. At the end of incubation, LDL samples were extracted with chloroform/methanol (2:1 vol/vol). LDL samples that contained radiolabeled glucose were reduced with NaBH₄ for 1 hour at 4°C and dialyzed against 0.1 mol/L phosphate buffer that contained 0.1 mmol/L EDTA for 24 hours to remove free radioactive glucose. After dialysis was complete, lipids were extracted and radioactivity measured in both protein and lipid fractions.

**Extraction of Atherosclerotic Plaques**

Atherosclerotic plaques obtained from patients during carotid endarterectomy and from postmortem carotid arteries (24 hours after death) were available from previous studies. Plaques from intermediate to advanced lesions were immediately placed into PBS, pH 7.4, that contained 0.1% EDTA. Plaque material (100 mg) was separated from media and adventitia and minced (0.5 to 1.0 mm² pieces), and total lipid extracts were prepared with chloroform/methanol (2:1 vol/vol). Tissue lipid extracts were stored in chloroform at −20°C under nitrogen.
by guest on October 30, 2017 http://atvb.ahajournals.org/ Downloaded from

mL/min. Effluent was split by 1/50, which resulted in 20 in 14 minutes, then at 100% solvent B for 10 minutes at 1

chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by volume) to 100% solvent B (chloroform/metha-

vent A (chloroform/methanol/30% ammonium hydroxide, 4.6 mm ID) eluted with a linear gradient of 100% sol-

Hydrogen was the carrier gas at 3 PSI.21

gas chromatograph equipped with a flame ionization detector.

Figure 2. LC/ESI/MS analysis of total lipid extract of LDL incu-

bated with 50 mmol/L glucose for 7 days. A, Total negative ion current profile of LDL phospholipids. B, Single-ion plots for

major Glc PtdEtn and PIsEtn species. C, Averaged spectra over the glycated PE peak (15.05 to 15.75 minutes). LDL total lipid extract was dissolved in chloroform/methanol (2:1 vol/vol) and 20 µL of the sample containing 10 µg lipid was analyzed. 

LC/ESI/MS conditions: normal-phase S-µ Spherisorb column (250 mm×4.6 mm ID) eluted with a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by volume) to 100% solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by volume) in 14 minutes, then at 100% solvent B for 10 minutes at 1 mL/min. Effluent was split by 1/50, which resulted in 20 µL/mL being admitted into the mass spectrometer and scanning in the negative ion mode from 400 to 1100 AMU.

Analysis of Fatty Acid Methyl Esters

LDL lipid classes were isolated by preparative TLC on silica gel H with heptane/isopropyl ether/acetic acid (60:40:4 by volume) as solvent.20 In this system, phospholipids were retained at origin, whereas free fatty acids, triacylglycerols, and cholesteryl esters were resolved in order of decreasing polarity. Appropriate areas of the plate were cleared of silica gel, and fatty acid methyl esters (FAME) were prepared by treating the gel with 6% H2SO4 in methanol for 2 hours at 80°C. Heptadecanoic acid was included as an internal standard at ~10% of total fatty acid concentration. After reaction, the FAME were extracted twice with hexane. The solvent was blown down under nitrogen and the samples redissolved in hexane. Fatty acids were analyzed on a polar capillary column (SP 2380, 15 m×0.32 mm ID, Supelco) installed in a Hewlett Packard model 5880 gas chromatograph equipped with a flame ionization detector. Hydrogen was the carrier gas at 3 PSI.21

TABLE 1. Molecular Species of Glucosylated and Nonglycosylated DiradylGroPEtn in LDL

<table>
<thead>
<tr>
<th>Nonglycated, mol%</th>
<th>Glycated, mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo</td>
</tr>
<tr>
<td>Alkenylacyl</td>
<td></td>
</tr>
<tr>
<td>16.0–20:4</td>
<td>4.25±1.39</td>
</tr>
<tr>
<td>18.0–18.3</td>
<td>2.09±0.97</td>
</tr>
<tr>
<td>16.0–20:3</td>
<td></td>
</tr>
<tr>
<td>18.0–20:5</td>
<td>10.75±2.14</td>
</tr>
<tr>
<td>16.0–22:5</td>
<td>19.3±4.53</td>
</tr>
<tr>
<td>18.0–20:3</td>
<td>3.63±0.23</td>
</tr>
<tr>
<td>18.0–22:6</td>
<td>3.45±1.87</td>
</tr>
<tr>
<td>Total</td>
<td>48.98±3.37</td>
</tr>
<tr>
<td>Diacyl</td>
<td></td>
</tr>
<tr>
<td>16.0–18:2</td>
<td>3.84±1.24</td>
</tr>
<tr>
<td>18.0–20:4</td>
<td>3.45±1.32</td>
</tr>
<tr>
<td>18.0–18:3</td>
<td>2.29±1.68</td>
</tr>
<tr>
<td>18.0–18:2</td>
<td>8.11±3.17</td>
</tr>
<tr>
<td>18.0–18:1</td>
<td>1.99±0.79</td>
</tr>
<tr>
<td>16.0–22:6</td>
<td>5.51±2.43</td>
</tr>
<tr>
<td>18.0–20:3</td>
<td>6.1±1.81</td>
</tr>
<tr>
<td>16.0–22:5</td>
<td>12.36±3.92</td>
</tr>
<tr>
<td>18.0–20:3</td>
<td>1.89±0.65</td>
</tr>
<tr>
<td>18.0–22:6</td>
<td>1.41±1.64</td>
</tr>
<tr>
<td>18.0–22:5</td>
<td>2.88±0.85</td>
</tr>
<tr>
<td>Total</td>
<td>51.02±4.34</td>
</tr>
</tbody>
</table>

LDL was incubated with 20 mmol/L glucose for 7 days (pH 7.4) at 37°C (n=4). Lipids were extracted, and different phospholipid classes were separated by normal-phase silica-column HPLC.Resolved peaks were analyzed by online electrospray ms.

Analysis of Phospholipid Classes

Normal-phase HPLC of phospholipids was performed on a 3-µm Spherisorb column (100×4.6 mm ID) or a longer 5-µm Spherisorb column (250×4.6 mm ID; Alltech Associates). Columns were installed into a Hewlett-Packard model 1090 liquid chromatograph and eluted with a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by volume) to 100% solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by volume) for 14 minutes, then in 100% solvent B for 10 minutes.10 Flow was set at 1 mL/min. Peaks were monitored by online ESI/MS.

Analysis of Molecular Species of Phospholipids

Normal-phase HPLC with LC/ESI/MS was performed by splitting HPLC flow by 1/50, which resulted in admission of 20 µL/mL to a Hewlett-Packard model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface (HP 5997A).22 Tuning and calibration of the system was achieved in the mass range of 400 to 1500 atomic mass units (AMU) by using of the standard phospholipid mix dissolved in HPLC solvent A and flow-injected at 50 µL/min into the mass spectrometer. Capillary
voltage was set at 4 kV, endplate voltage at 3.5 kV, and cylinder voltage at 5 kV in the positive mode of ionization. In the negative mode, voltages were 3.5, 3, and 3.5 kV, respectively. Both positive and negative ion spectra were taken in the mass range 100 to 1100 AMU. Capillary exit (Cap Ex) voltage was set at 120 V in the positive and 160 V in the negative ion mode. For fragmentation studies, Cap Ex voltage was raised to 300 V. Nitrogen gas was used as both nebulizing gas (40 PSI) and drying gas (60 PSI, 270°C).

Phospholipids, including glucosylated diradylglycerol phosphoethanolamine, were quantified on the basis of standard curves established for each phospholipid class and for the oxidized phospholipids (core aldehydes, hydroperoxy, and isoprostanes). Oxidized phospholipids that were used as standards were prepared as previously described. The detection limit for Glc PtdEtn with normal scanning range (400 to 1100 AMU) was 20 to 30 pmol, and the response was linear to 100 nmol. Equimolar ion intensities of different species of each phospholipid class varied by <5% in each of the ion modes. LC/ESI/MS response to different phospholipid classes varied greatly and required regular use of standards.

Figure 3. Fragmentation pattern of 16:0 to 18:2 GroPtn generated by increasing the capillary exit voltage from 160 to 300 V. A, Fragmentation pattern in the negative mode of ionization; B, fragmentation in the positive mode of ionization. LC/ESI/MS is as described in Figure 2.

Figure 4. Glucosylation of LDL ethanolamine phospholipids at different incubation times with increases in glucose concentrations. Various time and concentration points represent the sum of PtdEtn and PtdGroEtn in 4 separate incubations. Concentrations were determined with LC/ESI/MS; conditions were as described in Figure 2. *diradylGroPtn represents both the glucosylated and the nonglucosylated diradylGroPtn.

Figure 5. Glycation of LDL lipid (●) and protein (○) as measured by radiolabeled glucose incorporation. LDL was incubated with 20 mmol/L glucose containing 3 mCi/mL D-glucose-1-[14C]. Incubation mixture was reduced with NaBH₄ and dialyzed extensively. Radioactivity was measured by scintillation counting for both lipid and protein fractions and is given as moles of glucose per LDL molecule. Molecular weight of ApoB was assumed to be 500 kDa.

Figure 6. Effect of Glc PtdEtn on phospholipid oxidation by tert-butyyl hydroperoxide. A, Liposomes containing egg yolk Glc PtdEtn and egg yolk PtdEtn; B, Liposomes containing PtdCho in presence and absence of Glc PtdEtn. Peroxidation was measured by monitoring generated monohydroperoxide ions by LC/ESI/MS. LC/ESI/MS is as described in Figure 2.
Measurement of Radioactivity

Radioactivity in glucosylated diradyl GroPEtn and proteins was determined by scintillation counting after lipid extraction and TLC. The silica gel that contained the Glc Etn phospholipids was scraped into scintillation vials with CytoScint (ICN Pharmaceuticals). The radioactivity in the protein precipitate was determined after the precipitate was dissolved in CytoScint.

Results

Isolation of Glc PtdEtn From Glucosylated LDL

Glc PtdEtn may occur in LDL as the Schiff base adduct or its Amadori rearrangement product (Figure 1). The glucose adducts of PtdEtn were originally recognized as part of the front of the PtdCho peak detected by conventional normal-phase LC/ESI/MS as already described for total plasma and red blood cell extracts of diabetic subjects.11 By use of longer HPLC columns, a complete resolution was obtained for the PtdCho, PtdIns, and Glc PtdEtn components overlapping on the shorter column. Furthermore, with the use of the negative ion mode, the major PtdCho peak was seen only as minor PtdCho+Cl adduct, which provided a clear view (Figure 2A) of the Glc diradylGroPEtn peak after incubation of LDL with 50 mmol/L glucose. Figure 2B shows single-ion plots of the major species of both Glc PtdEtn and PlsEtn, which elute with the same retention time as standard Glc PtdEtn. Figure 2C shows the full-mass spectrum averaged over the elution time of the Glc diradyl GroPEtn peak. The spectrum includes many molecular species, which are quantified in Table 1. Table 1 shows that the glucosylation of the ethanolamine phospholipids of LDL was largely indiscriminate, given that both PtdEtn and alkenylacyl GroPEtn components were glucosylated. However, on average, the diacyl species represent a somewhat higher proportion of total glucosylated ethanolamine phospholipids. The species present in untreated LDL show a decrease in PlsEtn glycation compared with LDL incubated with glucose in vitro. The 2 major species in both in vivo and in vitro glycated LDL were 18:0-18:2 and 18:0-20:4 GroPEtn. The difference in glycosylation in untreated LDL shows that the diacyl species represent the majority of the glycated ethanolamine phospholipids.

Although we investigated the possible presence of other aminophospholipid glycation products, such as glycated PtdSer or carboxymethylPtdEtn, in both untreated LDL and LDL incubated with glucose, we did not detect ions in the chromatogram pertaining to these products.

Figure 3 shows the fragment ions obtained for the palmitoyl-linoleoylGroPEtn glucosylate in the negative and positive ionization mode as obtained for the HPLC peak eluting at 15.8

Figure 7. Copper-catalyzed oxidation of LDL phospholipids in presence of PtdEtn or Glc PtdEtn. A, Total positive ion current profile of nonoxidized LDL; B, Total positive ion current profile of oxidized LDL supplemented with PtdEtn (12 hours, 37°C); C, Total positive ion current profile of oxidized LDL supplemented with Glc PtdEtn (12 hours, 37°C). Peak identification is as given in figure. LC/ESI/MS conditions are as described in Figure 2.

Figure 8. Identification of LDL PtdCho hydroperoxy and isoprostane ions generated as a result of copper oxidation (A) spectra averaged over the PtdCho hydroperoxide peak. B, Single-ion plots of the major PtdCho hydroperoxides present in oxidized LDL containing Glc PtdEtn. LC/ESI/MS conditions are as described in Methods.
minutes. In the negative ion mode (Figure 3A), fragmentation of the molecular ion (m/z 876) gave palmitic (m/z 255) and linoleic (m/z 279) acids. The ion at m/z 712 is due to loss of glucose moiety, whereas the ion at m/z 756 results from cleavage of the attached glucose between carbons 2 and 3. In the positive ion mode (Figure 3B), the ion at m/z 303 represents Glc PEtn and the ion at m/z 576 the diacyl Gro fragment. The ion at m/z 180 was due to cleavage of the adduct with a retention of nitrogen with the glucose moiety. The same fragmentation pattern was observed for Glc PtdEtn present in LDL, with more complex fragmentation patterns due to the presence of multiple species. In both modes of ionization, the fragments produced were consistent with either Schiff base or Amadori rearrangement product of Glc PtdEtn, but the relatively higher stability of the complex suggested the Amadori product, as concluded previously by Lederer et al.²⁴

Rate of Glucosylation of LDL

The concentration dependence of LDL lipid glucosylation was investigated by determining the time course of Glc PtdEtn and Glc PtdEtn accumulation in LDL by LC/ESI/MS. Figure 4 shows the concentration dependence of LDL diacylGroPEtn glucosylation. Even at 5 mmol/L glucose (24-hour incubation), 4-nmol/mg LDL protein Glc GroPEtn was present. This represents 8.6% of total LDL diacylGroPEtn and Glc PtdEtn (52 nmol/mg LDL protein) in glucosylated form at physiological glucose concentrations. These levels were close to the amounts found in untreated LDL (3.2 nmol/mg LDL protein), indicative of in vivo levels of PtdEtn glycation. The level of Glc PtdEtn was incubated at 400 mmol/L glucose for 7 days. The concentration dependence of LDL lipid glucosylation was investigated by determining the time course of Glc PtdEtn and Glc PtdEtn accumulation in LDL by LC/ESI/MS. Figure 4 shows the concentration dependence of LDL diacylGroPEtn glucosylation. Even at 5 mmol/L glucose (24-hour incubation), 4-nmol/mg LDL protein Glc GroPEtn was present. This represents 8.6% of total LDL diacylGroPEtn and Glc PtdEtn (52 nmol/mg LDL protein) in glucosylated form at physiological glucose concentrations. These levels were close to the amounts found in untreated LDL (3.2 nmol/mg LDL protein), indicative of in vivo levels of PtdEtn glycation. The level of Glc PtdEtn in LDL incubated at 400 mmol/L glucose for 7 days at 35 nmol/mg LDL protein represents 67% of total LDL diacylGroPEtn being glucosylated. Even at these high glucose concentrations, glycation of LDL diacylGroPEtn was incomplete. With radiolabeled glucose, differences between the rate of lipid and protein glycation in LDL were investigated. Figure 5 shows that the initial rate of incorporation of radioactivity was significantly higher in the lipid than protein fraction. A TLC examination of the lipid phase indicated that ~92% of the radioactivity in the lipid pool was recovered in the band that corresponded to the NaBH₄ reduction product of Glc diacylGroPEtn, and further analysis by LC/ESI/MS gave the correct molecular weights for the reduced Glc diacylGroPEtn products (data not shown). This demonstrates that Glc diacylPEtn is the initial and major LDL glucosylation product.

Relative Rates of Oxidation of PtdEtn and Glc PtdEtn

The effect of glycation on PtdEtn oxidation was determined by comparing the rates of tert-butyl hydroperoxidation of glucosylated and non-Glc PtdEtn incorporated at 10% mass into unilamellar egg yolk PtdCho liposomes (Figure 6). Figure 6A shows the time course of hydroperoxidation of 18:0-18:2 GroPEtn and glucosylated 16:0-18:2 GroPEtn when incorporated into PtdCho liposomes, as determined by LC/ESI/MS. Monohydroperoxides were the major oxidized molecules monitored in both PtdEtn molecules. Glc PtdEtn is noted to be peroxidized 2 to 3 times more readily than non-Glc PtdEtn. Furthermore, Glc PtdEtn displayed prooxidant activity toward non-Glc PtdEtn and PtdCho.

---

Figure 9. Major PtdCho hydroperoxides in oxidized LDL. LDL was supplemented with PtdEtn (15 nmol/mg LDL protein) (E) or supplemented with Glc PtdEtn (20 nmol/mg LDL protein) (F). LDL was oxidized by dialysis against 5 µmol/L copper sulfate solution (37°C). At the end of the oxidation, LDL lipids were extracted and analyzed by LC/ESI/MS in the positive mode of ionization. LC/ESI/MS conditions are as described in Figure 2.
6B shows that 16:0 to 20:4 and 16:0 to 18:2 GroPCho liposomes in presence of Glc PtdEtn were peroxidized at a 2 to 3 times higher rate than the same PtdCho liposomes supplemented with non-Glc PtdEtn.

**Pro-oxidant Activity of Glc PtdEtn in LDL**

To investigate further the apparent pro-oxidant effect of Glc Etn phospholipids, we measured the peroxidation of LDL by copper ions in the presence and absence of added glucosylated 16:0-18:2 GroPCho (10 to 30 nmol/mg LDL protein). This amount corresponds to levels obtained for LDL incubation with 50 mmol/L for 48 hours. Control LDL contained 10 to 20 nmol/mg LDL protein of added nonglucosylated 16:0-18:2 GroPCho. Extent of lipid peroxidation was determined by measuring the hydroperoxides and core aldehydes of the most abundant PtdCho species of the 2 LDL preparations. Figure 7 compares the phospholipid profiles of untreated LDL (Figure 7A) and copper-oxidized LDL plus PtdEtn (12 hours, 37°C) (Figure 7B) and copper-oxidized LDL + Glc PtdEtn (12 hours, 37°C) (Figure 7C) as obtained by positive-ion LC/ESI/MS. Figure 7A shows the resolution of the PtdCho, SM, and lysoPtdCho, which are the major phospholipid components of native LDL. The PtdCho, SM, and lysoPtdCho peaks are split because of a resolution of the short and long carbon-chain species. After copper-ion oxidation (Figure 7B), a dramatic change occurs in the composition of the LDL phospholipids, largely as a result of conversion of the unsaturated PtdCho into the hydroperoxides, core aldehydes, and isoprostanes, which are eluted later than the saturated PtdCho species. In addition, a relative increase has occurred in the proportion of lysoPtdCho, possibly as a result of hydrolysis of the peroxidized PtdCho by the phospholipases present in LDL. After supplementation of the LDL with Glc PtdEtn (Figure 7C), a 4- to 5-fold increase occurs in the lipid peroxidation. The absolute amount of LDL SM remained unchanged in both PtdEtn-supplemented (27.9% total phospholipid) and Glc PtdEtn–supplemented LDL preparations (26.7%), although this is not immediately obvious from either Figure 7B or 7C.

Figure 8A shows the full-mass spectrum averaged over the range of elution times of the hydroperoxy PtdCho (17.070 to 18.603 minutes), whereas Figure 8B shows the single-ion plots corresponding to the monohydroperoxy, dihydroperoxy and isoprostane derivatives of the major PtdCho species. The arachidonate-containing species (16:0-20:4 GroPCho) with 2 hydroperoxy groups (2xOOH) are resolved into 3 subfractions presumably due to the presence of positional and cis, trans isomers of the hydroperoxides.

Figure 9 shows the time course of PtdCho hydroperoxide formation during dialysis against copper ions of LDL supplemented with Glc PtdEtn or PtdEtn. The major PtdCho hydroperoxides, 34:2 1xOOH (m/z 790), 36:2 1xOOH (m/z 818), 34:2 2xOOH (m/z 822), and 36:4 2xOOH (m/z 846), are all increased significantly in Glc PtdEtn supplemented LDL, with 34:2 1xOOH the most abundant. LC/ESI/MS analysis of the NaBH4 reduced peroxidation products indicated that the hydroperoxy PtdCho had been converted into the corresponding hydroxy PtdCho. Addition of Glc PtdEtn to LDL resulted in about 5-fold increase in the formation of phospholipid-bound hydroperoxides calculated from averaged differences from all time points.

Figure 10A shows the full-mass spectrum averaged over the elution range (18.76 to 19.19 minutes), of the C₃ core aldehydes of PtdCho, which are eluted ahead of the C₃ core aldehydes. Figure 10B shows the full spectrum averaged over the entire range (20.78 to 21.39 minutes) of the C₅ core aldehydes which trail into the SM species (m/z 675, palmitoyl sphingosinephosphocholine). Figure 10C shows the single-ion plots for all 4 major molecular species of the PtdCho core aldehydes. The 9-oxononanates and 5-oxo-valerates of the palmitoyl and stearoyl GroPCho represented the major components expected on the basis of the fatty acid composition of the molecular species of LDL PtdCho.

Figure 11 shows the time course of PtdCho core aldehyde formation during copper oxidation of LDL supplemented with Glc PtdEtn or PtdEtn. Major core aldehydes are the 16:0-9:0 Ald and the 18:0-9:0 Ald species, as expected from presence of linoleic acid as the major unsaturated fatty acid (61%) in the sn-2 position of LDL PtdCho. NaBH₄ treatment resulted in a reduction of the aldehydes to the corresponding alcohols, as reflected in the increase of 2 AMU in the molecular ion of each PtdCho core aldehyde (data not
shown). On average, the increase in core aldehyde formation as a result of Glc PtdEtn supplementation was ~4-fold.

Addition of Glc PtdEtn to LDL also promoted oxidation of cholesteryl esters in the interior of the particle. Figure 12 shows a more rapid loss of the 2 major species of polyunsaturated cholesteryl esters during dialysis against copper ions of LDL supplemented with Glc PtdEtn when compared with LDL supplemented with similar amounts of PtdEtn. Destruction of the arachidonoyl ester occurred at a much faster rate than that of the linoleoyl ester of cholesterol. Furthermore, the cholesteryl esters appeared to undergo peroxidation much more rapidly than the glycerophospholipids (compare Figures 10 and 12), which suggests that access to the interior of the particle was facilitated by addition of Glc PtdEtn.

Isolation of Glc PtdEtn and PlsEtn From Atherosclerotic Tissue

Figure 13 shows the total negative ion current profile (Figure 13A), the single-ion chromatograms for major species (Figure 13B), and the mass spectrum averaged over the elution time (15.01 to 15.89 minutes) of the glycated ethanolamine phospholipid peak (Figure 13C), as obtained by normal-phase LC/ESI/MS for a total lipid extract of an atherosclerotic plaque from a diabetic man. To increase the sensitivity of detection, scanning was limited to a mass range of 850 to 1000 AMU, which eliminated any overlap with the PtdEtn, PtdCho+Cl, and SM+Cl ions also present in the total negative LC/ESI/MS profile. As a result, only the dimers of the molecular species of lysoPtdCho remained visible. Table 2 compares the composition of the molecular species of glycated and nonglycated ethanolamine phospholipids isolated from the atheroma. Considerable discrepancy exists between the glycated and nonglycated sets of species, which excludes direct in situ interconversion and suggests possible deposition of these lipids from the circulation. Composition of the molecular species of the ethanolamine phospholipids of the atheroma also differs significantly from that of human LDL (Table 1), with the relative proportion of the polyunsaturated PtdEtn and specially PlsEtn species being reduced. The most abundant glycated PlsEtn species in the plaque was 38:4 (m/z 928). The possibility of selective glycation or oxidation is indicated by the absence of glycated PtdSer species, although PtdSer species were detected in the atheroma. In samples analyzed from nondiabetic individuals, we found 2.4-nmol glycated diradylGroPEtn per milligram total lipid, which represented 1.3% of the total atheroma phospholipid. The atherosclerotic tissue from diabetics contained 11.5-nmol glycated diradylGroPEtn per milligram total lipid, which represented 7.3% of total atheroma phospholipid, whereas an analysis of normal aortic tissue did not show any PtdEtn glycation. These results are comparable to levels of glycated PtdEtn previously measured by our group in plasma of diabetic individuals (4.8% glycated GroPEtn per milligram total phospholipid).11

Discussion

LDL oxidation is claimed to play a central role in atherogenesis, which is exacerbated in diabetic hyperglycemia.1 Hyperglycemia is known to promote LDL glycation6,17 and lipid peroxidation,6,25 but the relative importance of protein and aminophospholipid glycation in these processes has not been established. Using previously prepared standards of Glc PtdEtn,10 we have now identified the lipid glycation product of LDL as a mixture of glycated diacyl and alkenylacylGro-
We had previously shown that the glucose adduct of PtdEtn possesses a molecular weight that corresponds to either the Schiff base or its Amadori rearrangement product. In the present study, we have examined the glucosyl PtdEtn isolated from Glc LDL by pseudo-MS/MS with electrospray and have obtained fragment ions, which are consistent with both Schiff base and its Amadori rearrangement product. On the basis of the relatively high stability of the adduct, we must conclude that the structure is more likely to be that of the Amadori product rather than of the simple Schiff base.

This conclusion is consistent with the recent report of Lederer et al, who have unequivocally proven that Amadori products are formed from D-glucose and PtdEtn in an in vitro model system. Furthermore, Requena et al. have recently demonstrated the formation of carboxymethylEtn after hydrolysis of the aminophospholipid adduct. Bucala et al had shown in vitro that glycation of PtdEtn results in the formation of immunochemically detectable AGE. Furthermore, Pamplona et al. had obtained evidence for glycated aminophospholipids in rat liver and for their increase in animals with streptozotocin-induced diabetes. With Amadori-product formation from glucose and PtdEtn now clearly established, we undertook further investigations on the effect of Glc PtdEtn on LDL lipid oxidation.

Of special interest to LDL lipid oxidation was the presence of the PlsEtn and its fate during glycation. Previous work had attributed both antioxidant and pro-oxidant properties to PlsEtn in various biological systems, although the effect of PlsEtn on LDL oxidation had not been directly studied. Of the total diradylGroPEtn of plasma, 71.8% was due to alkenylacyl, 19.9% to diacyl, and 8.3% to alkylacyl species. The bulk of these phospholipids were assumed to originate in plasma LDL. The LDL preparation used in the present studies contained 49.4% PlsEtn, whereas glucosylated LDL contained 35% PlsEtn of total diradylGroPEtn. Thus, only 70% of the LDL PlsEtn was glucosylated under the present working conditions. Furthermore, the glucosylated species of both PtdEtn and PlsEtn tended to be more saturated than the respective nonglucosylated species. Analysis of diabetic plasma and red blood cells for glycated diradylGroPEtn in our earlier study had indicated a near absence of glycated PlsEtn species, whereas aminophospholipids isolated from red blood cells and plasma were glucosylated in vitro in proportion to their masses. Other studies have shown that hyperglycemic individuals have a significantly lower PtdEtn in LDL compared with normoglycemic controls, but it has not been established whether this is
TABLE 2. Molecular Species of Glycated and Nonglycated DiradylGroPEtn Present in Atherosclerotic Tissue

<table>
<thead>
<tr>
<th>Alkenylacyl</th>
<th>Nonglycated, mol%</th>
<th>Glycated, mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0–18:2</td>
<td>0.53±0.26</td>
<td>1.03±0.52</td>
</tr>
<tr>
<td>16:0–20:4</td>
<td>10.54±4.18</td>
<td>6.46±2.14</td>
</tr>
<tr>
<td>18:0–18:3</td>
<td>2.18±0.83</td>
<td>2.43±0.61</td>
</tr>
<tr>
<td>16:0–20:3</td>
<td>2.31±1.14</td>
<td>0.38±0.14</td>
</tr>
<tr>
<td>18:2–20:4</td>
<td>16.27±3.39</td>
<td>8.31±3.77</td>
</tr>
<tr>
<td>16:0–22:5</td>
<td>28.09±4.57</td>
<td>16.27±3.94</td>
</tr>
<tr>
<td>18:0–20:3</td>
<td>3.63±0.81</td>
<td>1.32±1.56</td>
</tr>
<tr>
<td>18:0–22:6</td>
<td>64.89±4.79</td>
<td>38.33±3.21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diacyl</th>
<th>Nonglycated, mol%</th>
<th>Glycated, mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0–18:2</td>
<td>1.24±0.25</td>
<td>9.35±2.89</td>
</tr>
<tr>
<td>16:0–18:1</td>
<td>1.58±0.47</td>
<td>7.68±3.13</td>
</tr>
<tr>
<td>18:0–18:2</td>
<td>2.31±1.25</td>
<td>4.54±1.22</td>
</tr>
<tr>
<td>16:0–22:6</td>
<td>8.22±0.54</td>
<td>10.68±2.35</td>
</tr>
<tr>
<td>18:2–20:4</td>
<td>0.64±0.21</td>
<td>3.76±1.64</td>
</tr>
<tr>
<td>16:0–22:5</td>
<td>18.34±2.57</td>
<td>19.73±3.52</td>
</tr>
<tr>
<td>18:1–20:3</td>
<td>1.63±0.33</td>
<td>4.42±1.14</td>
</tr>
<tr>
<td>18:0–22:6</td>
<td>1.15±0.48</td>
<td>1.51±0.68</td>
</tr>
<tr>
<td>18:0–20:4</td>
<td>35.11±4.23</td>
<td>61.67±4.89</td>
</tr>
</tbody>
</table>

Lipid extracts of atherosclerotic tissue (n=6) analysed by LC/ESI/MS are as described in Methods.

due to decreased PlsEtn. The loss of alkenylacyl species from glycated ethanolamine phospholipid could be due to their greater susceptibility to peroxidation, given that it has been demonstrated that the vinyl ether bond of alkenylacyl-GroPEtn is highly sensitive to peroxidation and can act as an antioxidant in protection of other phospholipids.32

Our study shows a more rapid and more extensive glycosylation of PtdEtn compared with apoprotein B in LDL. The distribution of radioactive glucose suggests that PtdEtn is more susceptible to glucosylation than apoB possibly due to more favorable environment for trapping the initial Schiff base adduct in the lipid phase. Our measurements of radioactive glucose distribution are consistent with the results of Bucala et al, who showed markedly increased levels of AGE in lipid compared with the aqueous (apoprotein) phase in diabetic and end-stage renal disease patients.

The present study shows that Glc PtdEtn is more easily peroxidized than PtdEtn, and that Glc PtdEtn, included in a liposomal mixture, promotes the oxidation of PtdEtn and PtdCho. Oxidation of phospholipids proceeds by way of hydroperoxides to the core aldehydes, all of which were isolated and identified as products of LDL oxidation. Likewise, addition of Glc PtdEtn to LDL caused more extensive oxidation of PtdEtn and PtdCho than addition of an equal amount of PtdEtn. This oxidation took place in the absence of protein glycation and free glucose in the incubation medium. The increased peroxidation of glycosylated ethanolamine phospholipids can probably be attributed to an increased exposure of these molecules to the oxidizing agent when incorporated into a liposome along with other phospholipids. The increased bulk of the polar-head group in the glycosylated aminophospholipid might permit a more ready access of the oxidizing agent to the unsaturated fatty chains of all glycerophospholipids in the liposomes and in the lipid monolayer of LDL. Furthermore, addition of Glc PtdEtn to LDL in absence of protein glycation resulted in increased oxidation of cholesteryl esters, which are located in the interior of the LDL particle. Clearly, Glc PtdEtn or its oxidation in the LDL lipid monolayer facilitates the access of free radicals to the cholesteryl esters in the interior of the LDL particle, which leads to a rapid destruction of the polyunsaturated cholesteryl esters. Previously it has been reported that LDL molecules from diabetic individuals show increased phospholipid layer fluidity, which could explain the increased susceptibility to oxidation.33

In the past glucose, has been claimed to possess pro-oxidant activity in both free and bound form, but more recently, increasing evidence has been obtained favoring the bound glucose pathway of oxidation of biological macromolecules. On the basis of albumin as a model protein, Hunt et al have concluded that oxidative alterations in experimental diabetes mellitus are due to protein-glucose adduct oxidation. The promotion of LDL lipid peroxidation by Glc PtdEtn demonstrated in the present study would also be consistent with pro-oxidant action of bound glucose. However, direct measurements of the oxidation potential of aminophospholipid-bound glucose in the absence of protein glycosylation suggest that the role of lipid glycation may have been overlooked. Hunt et al did not examine the effect of PtdEtn glycation in their study. In any event, the interplay between glycation, glycoxidation, and lipid peroxidation may be more complex than revealed by model studies, and the separate contributions of each step of the overall process to the final result have yet to be determined. Thus, complications may arise from the interaction of the oxidation products (hydroperoxides and core aldehydes) with free amino groups of both proteins and aminophospholipids as shown elsewhere. Greenspan et al have demonstrated that LDL associated with negatively charged phospholipids causes a dramatic increase in uptake and deposition of cholesteryl esters in J774 macrophages. We have recently shown that the glycation of PtdEtn not only results in increased negative charge but also promotes cholesteryl ester and triglyceride accumulation in macrophages. Extensive data have been reported on the involvement of protein AGE in lipid peroxidation in atheroma development. The identification of glycated PtdEtn and PlsEtn in atheroma suggests that glycated phospholipids may also be involved in plaque formation. The presence of PtdCho core aldehydes is of special interest because these compounds are found in oxidized LDL and in atherosclerotic tissue. PtdCho core aldehydes have been specifically shown to induce increased monocyte-endothelial interactions in vitro and PtdCho with a short chain aldehyde in the sn-2 position can mimic platelet-activating factor. In conclusion, the present study demonstrates that Glc PtdEtn is an early product of LDL glucosylation and that it is more readily peroxidized than PtdEtn. Furthermore, Glc PtdEtn pro-
motes the peroxidation of LDL phospholipids and cholesterol esters when included at a level of 10% of total diradylGroPEtn of LDL. These studies lend experimental support to previous speculation about the role of aminophospholipid glycation in LDL oxidation. This is the first report of isolation and identification of glycosylated diradylGroPEtn from LDL and human atherosclerotic tissue. The present study provides evidence that GlcPdEttn may promote oxidation of LDL phospholipids and cholesterol esters in hyperglycemia.

Acknowledgments

This work was supported by the Heart and Stroke Foundation of Ontario, Toronto, Canada (A.K.) and Spectral Diagnostics Inc, Toronto, Canada (N.A.S.).

References

1
5. Shaikh NA. Assessment of various techniques for the quantitative extraction of neutral and charged phospholipids with low-density lipoprotein (LDL) increases its uptake and the deposition of cholesterol-esters by macrophages. Biochim Biophys Acta. 1995;1257:257–264.
29. Hofner G, Lichtenberg D, Kostner GM, Hermeter A. Oxidation of fluoroc-
32
34
36
Glucosylated Glycerophosphoethanolamines are the Major LDL Glycation Products and Increase LDL Susceptibility to Oxidation: Evidence of Their Presence in Atherosclerotic Lesions

Amir Ravandi, Arnis Kuksis and Nisar A. Shaikh

doi: 10.1161/01.ATV.20.2.467
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/2/467

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at: http://atvb.ahajournals.org//subscriptions/