Glycated Phosphatidylethanolamine Promotes Macrophage Uptake of Low Density Lipoprotein and Accumulation of Cholesteryl Esters and Triacylglycerols

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Non-enzymatic glycation of low density lipoprotein (LDL) has been suggested to be responsible for the increase in susceptibility to atherogenesis of diabetic individuals. Although the association of lipid glycation with this process has been investigated, the effect of specific lipid glycation products on LDL metabolism has not been addressed. This study reports that glucosylated phosphatidylethanolamine (Glc-PtdEtn), the major LDL lipid glycation product, promotes LDL uptake and cholesteryl ester (CE) and triacylglycerol (TG) accumulation by THP-1 macrophages. Incubation of THP-1 macrophages at a concentration of 100 μg/ml protein LDL specifically enriched (10 nmol/mg LDL protein) with synthetically prepared Glc-PtdEtn resulted in a significant increase in CE and TG accumulation when compared with LDL enriched in non-glycosylated PtdEtn. After a 24-h incubation with LDL containing Glc-PtdEtn, the macrophages contained 2-fold higher CE (10.11 ± 1.54 μg/mg cell protein) and TG (285.32 ± 4.38 μg/mg cell protein) compared with LDL specifically enriched in non-glucosylated PtdEtn (CE, 3.97 ± 0.95, p < 0.01 and TG, 185.57 ± 3.58 μg/mg cell protein, p < 0.01). The corresponding values obtained with LDL containing glycated protein and lipid were similar to those of LDL control LDL. Competition studies revealed that acetylated carbocyanine perchlorate (DiI), a 1.6-fold increase was seen in Glc-PtdEtn LDL exposure to copper oxidation. By utilizing the fluorescent probe, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), a 1.6-fold increase was seen in Glc-PtdEtn + LDL uptake when compared with control LDL. Competition studies revealed that acetylated LDL is not a good competitor for DiI Glc-PtdEtn LDL (5-6% inhibition), whereas glycated LDL gave an 80% inhibition, and LDL + Glc-PtdEtn gave 93% inhibition of uptake by macrophages. These results indicate that glucosylation of PtdEtn in LDL accounts for the entire effect of LDL glycation on macrophage uptake and CE and TG accumulation and, therefore, the increased atherogenic potential of LDL in hyperglycemia.

LDL glycation has been proposed to play central role in the atherosclerosis of diabetic hyperglycemia (1). The effect of protein glycation in LDL modification and its oxidation has been extensively investigated. Several groups of investigators have shown that glycated LDL is capable of inducing foam cell formation in a variety of cell culture systems (2–5). Furthermore, LDL from diabetic patients has been shown to increase accumulation of CE in macrophages, and the extent of accumulation has been correlated with the extent of LDL glycation (6, 7). In all these studies it was presumed that the glycated apoB is responsible for the entire altered activity.

By using antibodies to advanced glycation end products (AGE), Bucala et al. (8) have, however, shown that the lipid component of glycated LDL contains most of the AGE present and that the relative amount of this AGE antigen was proportional to the susceptibility of LDL to peroxidation. Since lipid glycation products were not isolated or identified, the significance of lipid glycation in LDL uptake by macrophages could not be investigated or a differentiation attempted between lipid and protein glycation in this process.

We have previously demonstrated that the amino phospholipids of plasma and red blood cells (RBC) from diabetic subjects show a 10-fold increase in glycated PtdEtn over control subjects (9, 10) and that incorporation of Glc-PtdEtn into LDL (11) facilitates peroxidation of LDL lipids. The present study demonstrates that the presence of Glc-PtdEtn in LDL results in an increased uptake of LDL and a dramatic increase in neutral lipid accumulation in THP-1 macrophages.

EXPERIMENTAL PROCEDURES

Cell Culture—THP-1 cells were obtained from the American Type Tissue Culture Collection (TIB 202) and were propagated in RPMI 1640, 10% fetal calf serum/penicillin (100 units/ml)/streptomycin (100 μg/ml) at 37 °C, 5% CO2. Cells were plated at a density of 1 × 106 cells/ml in 10% fetal calf serum medium containing 10−2 M phorbol myristate acetate (PMA) for 72 h. The cells were then washed extensively with serum-free RPMI medium and incubated with or without lipoproteins as indicated for each experiment. In all experiments, cell viability exceeded 90% as determined by trypan blue exclusion.

Synthesis and Isolation of Glc-PtdEtn—Glc-PtdEtn was prepared and purified as described previously (9). Briefly, PtdEtn (2 mg) dissolved in 1 ml of methanol was transferred to a 15-ml test tube, and the solvent evaporated under nitrogen. Four ml of 0.1 M phosphate buffer containing 0–400 mM glucose and 0.1 mM EDTA were added and...
sonicated at low power for 5 min at room temperature, and the mixture was incubated under nitrogen at 37 °C for various periods. Lipids were extracted into chloroform/methanol (2:1, v/v) as described by Folch et al. (12), and the solvents evaporated under nitrogen. Samples were redissolved in chloroform/methanol (2:1, v/v) and kept at −20 °C until HPLC analysis (2 mg) was purified by preparative TLC (20 × 20-cm glass plates) coated with Silica Gel H (250-μm thick layer). The chromato-plates were developed using chloroform/methanol, 30% ammonia (65:35:7, by volume) as described (9). Phospholipids were identified by co-chromatography with appropriate standards, visualizing any lipid bands under ultraviolet light after spraying the plate with 0.05% 2,7-dichlorofluorescein in methanol. Both glucose-labeled and glucosylated lipids were recovered by scraping the gel from appropriate areas of the plate and extracting it twice with the developing solvent.

**Lipoprotein Isolation and Oxidation**—LDL (1.019–1.069 g/ml) was obtained by density gradient ultracentrifugation (13) from plasma of fasted normolipidemic individuals. LDL (2 mg of protein/ml) was subsequently dialyzed against 0.1 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and kept at 4 °C, and used within 1 week. Lipoprotein concentration was determined by the method of Lowry et al. (14) and expressed as mg/ml. Oxidation of LDL (5 mg of protein/5 ml) was performed by dialysis against 5 μM CuSO₄·5H₂O in 0.1 mM phosphate buffer (pH 7.4), for 12 h at 37 °C in the dark. LDL (2 mg of protein/ml) was also incubated with 1 ml of lysis buffer was added (1 g/liter SDS, 0.1 M NaOH). Cells were incubated at room temperature under gentle shaking for 1 h. This allowed both direct fluorescence and protein measurement. The fluorescence of each well was measured in duplicate with a Shimadzu spectrophotometer (RF5000U). The excitation and emission wavelengths were set at 520 and 575 nm, respectively. The detection range for the fluorescence was linear from 0.05 to 20 μg/ml LDL protein. Protein determinations were made in duplicate using the method of Lowry et al. (14) with bovine serum albumin dissolved in lysis buffer as standard. Fluorescence microscopy was performed as described previously (21).

**Determination of Cellular CE and TG Accumulation**—The THP-1 cells were exposed for 24 h to control and modified LDL preparations. After incubation the cells were washed once with ice-cold PBS containing 0.4% bovine serum albumin and twice with PBS alone. Cells were scraped from the culture flask into PBS and sonicated. The cellular lipids were extracted with chloroform/methanol (2:1 v/v) and analyzed by gas-liquid chromatography (22). For this purpose the lipid extract was digested with phospholipase C (Clostridium welchii), and the digestion mixture was extracted with chloroform/methanol (2:1 v/v) containing 100 μg of tridecanoyleglycerol as internal standard. The lipid extracts were then reacted for 30 min at 20 °C with SYLON BPT plus 1 part dry pyridine. This procedure converts the free fatty acids into trimethyl silyl esters and the free sterols, diacylglycerols, and ceramides into trimethyl silyl ethers, leaving the cholesteryl esters and triacylglycerols unmodified. The free cholesterol, cholesterol esters, and triacylglycerols were quantitated using a non-polar capillary column as described previously (23).

**Statistical Analysis**—Cellular uptake and neutral lipid accumulation assays were done in triplicate, and statistical significance was performed with analysis of variance.

## RESULTS

**LDL Phospholipid Analysis**—Fig. 1 shows the results of the LC/ES/MS analysis of phospholipids from control LDL (Fig. 1A) and from LDL preparations containing Glc-PtdEtn (Fig. 1B). The Glc-PtdEtn is well resolved from PtdEtn and from other phospholipids such as diacylglycerols unmodified. The free cholesterol, cholesterol esters, and triacylglycerols were quantitated using a non-polar capillary column as described previously (23). Glucosylated PtdEtn Promotes Macrophage LDL Uptake

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Glucosylated PtdEtn (30 nmol of Glc-PtdEtn/mg of LDL protein) with THP-1 cells leads to a specific increase in CE and TC accumulation when compared with LDL containing non-glycated PtdEtn (10.11 ± 1.54 versus 3.97 ± 0.35 μg/mg cell protein, p < 0.01). The supplementation of LDL with non-glucosylated PtdEtn did not have any significant effect on CE accumulation. In order to differentiate between the contributions of LDL lipid and protein glycation to CE accumulation, these results were compared with those obtained with LDL that had been glycated in both protein and lipid species by incubation with glucose (50 mM) for 7 days, 37 °C. The glycated LDL preparation caused a CE accumulation (11.95 ± 1.38 μg/mg cell protein) that was comparable to that obtained when macrophages were incubated with Glc-PtdEtn LDL (10.11 ± 1.54 μg/mg cell protein).

Fig. 3 shows that incubation of THP-1 cells with Glc-PtdEtn LDL also causes a significant accumulation of TG (285.32 ±
4.38 μg/mg cell protein) which again was indistinguishable from the accumulation obtained by incubation with LDL glycated in lipid and protein (280.78 ± 3.98 μg/mg cell protein). LDL supplemented with non-glucosylated PtdEtn did not stimulate TG accumulation (185.57 ± 3.42 μg/mg cell protein). Furthermore, the increase in TG content of the macrophages seen at 50 μg/ml LDL was only slightly lower than that seen at 100 μg/ml LDL indicating a leveling off in the response. Interestingly, the TG levels in THP-1 cells represented the major neutral lipid pool contributing up to 85% of the cell neutral lipid content.

Stimulation of CE and TG Accumulation by OsLDL Glc-PtdEtn—Previous work done in our laboratory had shown that Glc-PtdEtn is more susceptible to oxidation and also facilitates the oxidation of other LDL phospholipids as well as the CE present in the lipid core of the molecule (11). In the present study we investigated the effects of Glc-PtdEtn on LDL oxidation and on foam cell formation. We oxidized Glc-PtdEtn-enriched LDL by copper and measured the CE and TG deposition in THP-1 cells. Fig. 4A shows the effect of incubation of oxidized PtdEtn LDL and oxidized Glc-PtdEtn-enriched LDL upon the accumulation of free cholesterol, CE, and TC by THP-1 cells in comparison to LDL. The greatest increase is seen in the CE. The oxidized PtdEtn LDL caused a 4-fold increase in the CE content of the cells (18.36 ± 3.25 μg/mg cell protein) compared with control LDL (3.97 ± 1.95 μg/mg cell protein). There was a significant further increase after incubation with oxidized Glc-PtdEtn LDL (28.36 ± 3.25 μg/mg cell protein). Fig. 4B shows the effect of incubation of oxidized PtdEtn-enriched LDL and oxidized Glc-PtdEtn-enriched LDL upon the accumulation of TG by THP-1 cells in comparison to LDL enriched with PtdEtn. The incubation with LDL oxidized after enrichment with Glc-PtdEtn (350.91 ± 26.32 μg/mg cell protein) gave significantly greater accumulation than the incubation with oxidized LDL enriched with PtdEtn (310 ± 21.84 μg/mg cell protein), both values being significantly greater than those obtained for non-oxidized LDL or LDL enriched in PtdEtn. This increase in
neutral lipid deposition correlated with the increase in PtdCho oxidation products due to the presence of Glc-PtdEtn during oxidation. The phospholipids from oxidized Glc-PtdEtn LDL were analyzed by LC/ES/MS. The total positive ion current (Fig. 5A) shows the separation of Glc-PtdEtn LDL phospholipids after oxidation with copper ions. The major phospholipid oxidation products were identified as the PtdCho hydroperoxides (PtdCho OOH), PtdCho core aldehydes (PtdCho Ald), and PtdCho isoprostanes (PtdCho IsoP) in the positive mode of ionization. Fig. 5B shows representative mass chromatograms. In the absence of reference standards the structures proposed for the isoprostanes and core aldehydes containing hydroxyl groups must remain tentative. The pro-oxidative effect of Glc-PtdEtn resulted in a 4-fold increase in the production of total hydroperoxides, core aldehydes, and isoprostanes, when compared with LDL enriched in PtdEtn (Fig. 6).

Cellular Uptake of Dil Labeled Glc-PtdEtn LDL—In order to quantitate the rate of uptake of LDL, we utilized Dil-labeled lipoproteins. After a 4-h incubation with cells, the fluorescence intensity was measured as an indicator of total cell-associated lipoprotein. Cells incubated with Dil LDL enriched with Glc-PtdEtn (10–200 μg/ml) showed a much more rapid increase in cell-associated lipoprotein when compared with control LDL enriched with PtdEtn (Fig. 7). The uptake of Dil LDL enriched in Glc-PtdEtn occurred at the same rate as that for glycated LDL showing that the presence of Glc-PtdEtn in LDL can mimic the properties of glycated LDL. The uptake of all LDL preparations tended to level off in the range of 100–200 μg/ml LDL.

Fig. 8 shows the time course (4, 6, 10, and 20 h) of macrophage accumulation of fluoroescently labeled lipoprotein. At all time points the average accumulation was consistently higher for the LDL preparation containing Glc-PtdEtn when compared with control LDL. The accumulation of Glc-PtdEtn LDL was parallel to that of glycated LDL at all time points. To explore the specificity Glc-PtdEtn LDL interaction with macrophages, we investigated the ability of AcLDL, PtdEtn LDL, glycated LDL, and Glc-PtdEtn at increasing concentrations to compete with Dil-labeled Glc-PtdEtn (50 μg/ml) for uptake by macrophages (Fig. 9). After the 4-h incubation the
AcLDL and control LDL were only able to inhibit 5–6% of the cell association. Glycated LDL led to 80% inhibition of Glc-PtdEtn LDL uptake, whereas the unlabeled Glc-PtdEtn resulted in 93% inhibition of uptake after correction for nonspecific association. Fluorescence microscopy analysis of LDL uptake by THP-1 cells (Fig. 10) showed increased fluorescence in cells incubated with DiI-labeled lipoproteins. At each time point cells were lysed, and fluorescence was measured.

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Increase in Negative Charge of PtdEtn Due to Glycation—Fig. 11 shows that the glycation of PtdEtn increases the negativity of the molecule. Injection of equimolar amounts of PtdEtn and Glc-PtdEtn, chromatographed on normal phase silica column, resulted in a 1.5-fold higher response for Glc-PtdEtn when compared with PtdEtn. The calculated molar response of Glc-PtdEtn was comparable to that of other anionic phospholipids, such as PtdIns and PtdSer (Table I).

**DISCUSSION**

This study demonstrates that incorporation of Glc-PtdEtn into LDL at a level commonly achieved in hyperglycemia can fully account for the increased uptake of glycated LDL by macrophages. Furthermore, the resulting increased accumulation of CE and TG by macrophages exceeds the amounts of neutral lipids anticipated to be transferred to the cells on the basis of uptake of the lipoprotein. This increase was mainly due to accumulation of TG which contributed over 85% of the cell total neutral lipid (TC + TG). The glycated subfraction of LDL, which is elevated in diabetic subjects, has been reported to promote CE deposition and to increase the rate of CE synthesis in macrophages (25), but TG levels had not been measured. Although there was a significant increase in CE deposition in
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**FIG. 11. Increase in negative charge of PtdEtn after glycation.**

LC/ES/MS analysis of synthetic PtdEtn and Glc-PtdEtn total negative ion profile for equimolar amounts of glucosylated and non-glucosylated PtdEtn (A), single ion plots for 16:0–18:2 Gro-PEtn (m/z 714) and 16:0–18:2 Gro Glc PEtn (m/z 876) (B), and ion spectra averaged over both glucosylated and non-glucosylated PtdEtn (C).

**TABLE 1**

| Phospholipid     | Ionization ratio |
|------------------|------------------|
| PtdEtn           | 1                |
| Glc-PtdEtn       | 1.53 ± 0.32      |
| PtdSer           | 1.62 ± 0.41      |
| PtdIns           | 1.57 ± 0.38      |
| CL               | 2.12 ± 0.37      |

The inability of AcLDL to compete with Glc-PtdEtn LDL suggests the participation of receptors other than the LDL or scavenger receptor. Due to the increased negative charge of Glc-PtdEtn compared with PtdEtn and structural similarities to acidic glycerophospholipids, it is possible that receptors for Glc-PtdEtn uptake could be found in the family of receptors that identify anionic phospholipids such as CD 36 (28). It has been shown that CD 36 present in photoreceptor outer segment cells binds PtdSer- and PtdIns-rich liposomes resulting in their uptake (29). LC/ES/MS shows that glucosylation of PtdEtn increases the negative charge of the amino phospholipid, and with structural and physicochemical similarities to acidic glycerophospholipids, Glc-PtdEtn could increase uptake of LDL by CD 36.

In fact, macrophages have already been shown to specifically phagocytose oxidized RBC in which the outer leaflet of the phospholipid bilayer exposes anionic phospholipids, specifically PtdSer. Sambrano et al. (30) have demonstrated that peroxidation of RBC results in disruption of the asymmetry of the plasma membrane phospholipid bilayer resulting in PtdSer exposure and recognition of oxidized RBC by macrophages and their subsequent phagocytosis. Inhibition of uptake of oxidized RBC by OxLDL showed that the scavenger receptors on macrophages were responsible for a major part of the oxidized RBC recognition (31).

Recently another receptor, SRB1, has been shown to have the capability of binding anionic phospholipids. The inhibition of the selective uptake of high density lipoprotein CE in liver parenchymal cells by modified LDL, in particular OxLDL and ionic phospholipids, has suggested that in liver the SRB1 is responsible for the efficient uptake of high density lipoprotein CE (32). The concept that negatively charged molecules can form complexes with LDL is not restricted to negatively charged phospholipids. Basu et al. (15) has demonstrated that complexes containing LDL and large molecular weight dextran sulfates are avidly metabolized by macrophages via a receptor, which appears to be distinct from the AcLDL receptor. Besides the effects of PtdSer on macrophage uptake, association of other negatively charged phospholipids, like cardiolipin, with LDL increases its uptake and the deposition of cholesterol esters by macrophages (33). It has also been claimed that an alteration in the composition of LDL phospholipids caused by phospholipase D (34) and phospholipase A₂ (35) can influence the metabolism of LDL by macrophages, but it is not clear whether the effect is related to a relative increase in acidic phospholipids or lysophospholipids, which would be anticipated to result from the action of these enzymes.

Another possible receptor for Glc-PtdEtn is the newly characterized AGE receptor (36). This receptor has been demonstrated to have specificity toward protein glycation products (37). Many of the binding assays performed with this receptor have only focused on the protein glycation products and the interaction of Glc-PtdEtn with AGE receptor-advanced glycation end products has not been investigated.

The present study demonstrates that peroxidation increases the neutral lipid deposition due to LDL beyond the extent achieved by glucosylation of PtdEtn alone. We have previously shown that glycation of LDL PtdEtn promotes the oxidation of both surface and core components of LDL (11). The increase in susceptibility of LDL in the presence of Glc-PtdEtn to oxidation was characterized by an increase in products of phospholipid oxidation such as PtdCho core aldehydes, which have been

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**our THP-1 cells, the major neutral lipid accumulated was TG.** In this connection it may be noted that recent studies have shown that THP-1 cells contain a large and metabolically active TG pool but a relatively inactive pool of CE (26). A large and active TG pool has also been seen in other human macrophage cultures, but the regulation of TG metabolism in these cells is not known.

The general effects of glycation on LDL interaction with macrophages have been extensively investigated (4, 5). Non-enzymatic glycation of LDL has been shown to increase the fluidity of the phospholipid monolayer, to result in an altered lipid composition (27), and, possibly, in an increased susceptibility to oxidation. These alterations in biological and physico-chemical properties have been attributed solely to ApoB glycation without consideration of LDL phospholipid glycation.

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specifically shown to induce increased monocyte-endothelial interactions in vitro (38).

This is in agreement with the observation that both apolipoprotein and lipid in glycated LDL are more susceptible to oxidation in the presence of Fe$^{3+}$ than non-glycated LDL (39). It was suggested that Fe$^{3+}$ could be coordinated with the endiol group in Amadori compounds and could be converted to ferryl iron with a high redox potential. It was postulated, on the basis of the known chemistry of Schiff base and Amadori products, that during the nucleophilic addition of glucose to protein amino groups, as in the early glycation, the glycation products of proteins deposited in the arterial wall could themselves generate free radicals capable of oxidizing lipids (40). This possibility is supported by the demonstration that both Schiff base and Amadori glycation products generate free radicals in a ratio of 1:1.5 and that these radicals cause increased peroxidation of membrane lipids (40). In similar studies Kobayashi et al. (41) demonstrated that oxidation of glycated LDL results in increased binding and degradation by cultured bovine aortic endothelial cells compared with normal or oxidized LDL.

The present study demonstrates that glucosylation of the PtdEtn component of LDL exerts a dramatic effect on LDL metabolism which mimics that of total LDL glycation. This is the first direct evidence that glycation of LDL lipids results in increased LDL uptake and in CE and TG accumulation in macrophages. In conclusion, this study suggests that the presence of Glc-PtdEtn in LDL plays an important role in the alteration of the biological activity of LDL and reconciles diverse observations regarding the binding of glycated and peroxidized LDL to macrophages and other cells and may account for the increased atherogenesis in diabetes.

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