

# Nonenzymatic Glycosylation of Low Density Lipoproteins In Vitro Effects on Cell-interactive Properties

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## SUMMARY

**Atherosclerosis occurs at an accelerated rate in patients with diabetes mellitus. Since some proteins undergo nonenzymatic glycosylation in diabetic patients and because certain chemical modifications of low density lipoproteins produced alterations in their interactions with certain cultured cells, a fact that may be relevant to atherogenesis, we investigated the effect of in vitro glycosylation on cell-related properties of low density lipoproteins.**

**Glycosylation was carried out by incubating LDL (1–10 mg LDL-protein/ml) with glucose (0–100 mM) in 0.5 M phosphate buffer, pH 8.0, at 37°C. The amount of glucose incorporated into LDL after 1–2 wk of incubation was estimated to be in the range of 1–10 mol/mol LDL-protein. Amino acid analysis of glycosylated LDL showed that glucose was covalently bound to lysine residues.**

**In studies with cultured human fibroblasts, glycosylated LDL was internalized and degraded significantly less than control LDL, in proportion to the estimated degree of glycosylation (12% of control for the most extensively glycosylated LDL). Glycosylation of LDL also impaired significantly its ability to stimulate cholesteryl ester synthesis by cultured fibroblasts. Glycosylated LDL did not stimulate cholesteryl ester synthesis in rat peritoneal macrophages. If glycosylation of LDL occurs in diabetic patients, some pathophysiological consequences related to the increased incidence of atherosclerosis in these patients may result. DIABETES 30:875–878, October 1981.**

**M**odification of lysine residues of low density lipoprotein (LDL) by certain chemical reactions (e.g., acetylation) leads to reduced ability of LDL to interact with cultured fibroblasts and to a newly acquired ability to bind, become internalized and stimulate cholesteryl ester synthesis in mouse peritoneal macrophages.<sup>1–3</sup> In human monocyte-macrophages, acetyl-LDL, and malondialdehyde-modified LDL can stimulate cholesteryl ester synthesis,<sup>3,4</sup> but native LDL cannot. These

interactions are important because accumulation of cholesteryl esters in macrophages and smooth muscle cells in one of the characteristic features of atherosclerosis,<sup>5</sup> a process that is known to be more prevalent in diabetic patients.<sup>6</sup> Therefore, a search for qualitative change in LDL in diabetic patients that can enhance cholesteryl ester formation in certain cell types is of particular interest. Nonenzymatic glycosylation could represent such a chemical modification, since it is well established that several proteins undergo nonenzymatic glycosylation in diabetic patients.<sup>7,8</sup> We examined, therefore, whether the apoprotein of LDL may be a target of nonenzymatic glycosylation.

In this report, we describe the preparation of glycosylated human LDL and provide structural evidence as to the amino acid residue(s) where glycosylation takes place. We also report changes that occur in LDL following its glycosylation: it has reduced ability to get internalized and degraded and to stimulate cholesteryl ester synthesis in cultured fibroblasts.

## MATERIALS AND METHODS

**Materials.** NaB<sup>3</sup>H<sub>4</sub> (116.6 mCi/mmol) and D-[<sup>14</sup>C] glucose (2.2 mCi/mmol) were purchased from New England Nuclear Co. (Boston, Massachusetts), <sup>3</sup>H-lysine (90 Ci/mmol) from Amersham (Arlington Heights, Illinois). All counts were performed using aquasol-2 as scintillation fluid (New England Nuclear) in a Packard 3255 liquid scintillation counter.

**Lipoprotein isolation.** Plasma was obtained from normolipidemic subjects (in EDTA, 1 mg/ml), and LDL (d = 1.019–1.063) was isolated using sequential ultracentrifugation in a Beckman L2-65B ultracentrifuge. LDL was dialyzed (against EDTA-saline, pH 8.0), filtered through a Millipore filter (0.22 μm) and kept at 4°C until the time of incubation. **Incubations.** LDL (1–10 mg protein/ml) was incubated with glucose (0–100 mM) and/or with <sup>14</sup>C-glucose for various

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lengths of time in 0.5 M phosphate buffer, at 37°C. At the end of incubation, the mixture was dialyzed. To increase the yield of glycosylated LDL, NaBH<sub>3</sub>CN (1 mg/ml) was also included in some experiments.

**Analyses of reaction products.** To measure the incorporation of <sup>14</sup>C-glucose into LDL, an aliquot of the incubation mixture was eluted on a G-50 Sephadex column (30 × 1.5 cm) with 0.5 M phosphate buffer, pH 8.0. Fractions were collected and an aliquot of each fraction was counted. Counts present in the void volume were considered to belong to <sup>14</sup>C-glucose-bound LDL.

Amino acid analysis was performed on control LDL (incubated with buffer alone) and on LDL incubated with <sup>14</sup>C-glucose. LDL or <sup>14</sup>C-glycosylated LDL was reduced with 200-fold molar excess of NaBH<sub>4</sub> (22°C, pH = 8.5, 3 h) and dialyzed against EDTA-saline. Samples were delipidated [chloroform:methanol 2:1], hydrolyzed, rotoevaporated to dryness, taken up in 0.2 N sodium citrate buffer (pH = 2.2), and injected in a D-500 amino acid analyzer (from Dionex Corp., Sunny Vale, California). We collect 1-min fractions for subsequent counting in a scintillation counter. Elution patterns for the standard 20 amino acids were established using the ninhydrin reaction.

In an attempt to identify the observed peaks (of radioactivity), glucositol-<sup>3</sup>H-lysine was synthesized and eluted on the amino acid analyzer following hydrolysis as above. The synthesis of glucositol-<sup>3</sup>H-lysine was carried out using a modified method from Hase et al.<sup>9</sup>

**Cell studies.** Cell reactivities (internalization and degradation) of glycosylated LDL were assessed in competitive binding assays carried out at 37°C over 5 with normal human fibroblasts grown in Eagle's Minimal Essential Medium, buffered at pH 7.4. The media contained 20 μg/ml <sup>125</sup>I-LDL (about 85 cpm/ng) and increasing doses of glycosylated LDL or control LDL.<sup>10</sup> Using the method of McFarlane,<sup>11</sup> <sup>125</sup>I-LDL was prepared. At the end of incubation, cells were washed and dissolved in 0.1 M NaOH, and aliquots were taken for counts and determination of cell protein.<sup>12</sup> The media removed from the cells were analyzed for trichloroacetic acid soluble (noniodide) degradation products according to the method of Goldstein and Brown.<sup>13</sup> The incorporation of <sup>3</sup>H-oleic-acid into cellular cholesteryl esters by normal cultured fibroblasts and rat peritoneal macrophages was measured after their incubation with the different lipoproteins in the presence of <sup>3</sup>H-oleate/albumin (0.1 mM, 20 gpm/pmol) for 18 h. At the end of 18 h, cells were washed and centrifuged. The cell pellet was extracted using chloroform/methanol (2:1) and cholesteryl-[<sup>3</sup>H] oleate was isolated by thin layer chromatography.<sup>14</sup> All experiments were carried out in triplicate. Rat peritoneal macrophages were prepared as described.<sup>2</sup> Acetyl-LDL was prepared according to Weisgraber et al.<sup>1</sup>

## RESULTS

**Incorporation of <sup>14</sup>C-glucose into LDL.** The incorporation of <sup>14</sup>C-glucose into LDL was proportional to the concentration of glucose in the incubation medium and the length of incubation and was in the estimated range of 1–2 mol glucose/mol LDL-protein without NaBH<sub>3</sub>CN. Half-maximal glucose incorporation into LDL occurred after 4–7 days. At 2 wk of incubation, the incorporation of glucose at 37°C was

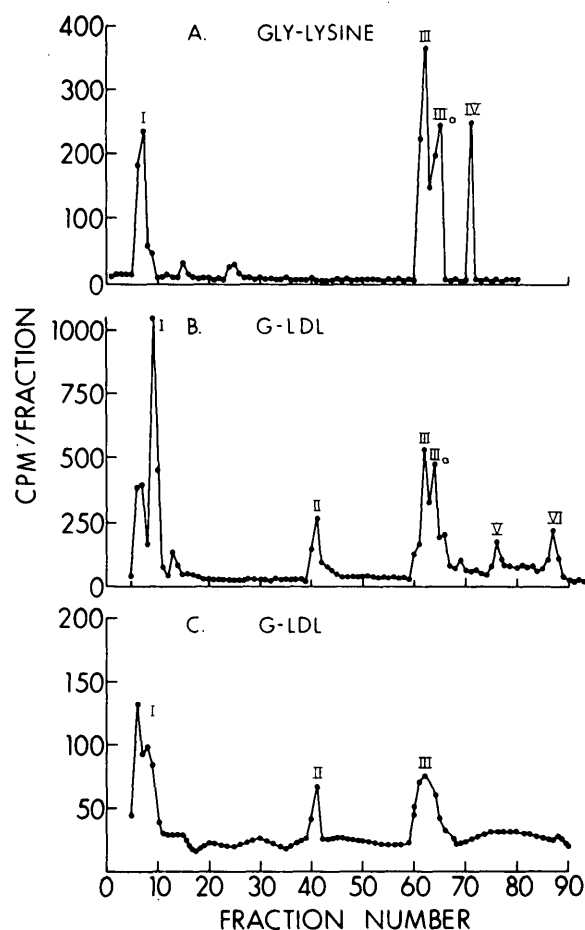
twice as extensive as at 22°C, and eight times as extensive as at 4°C.

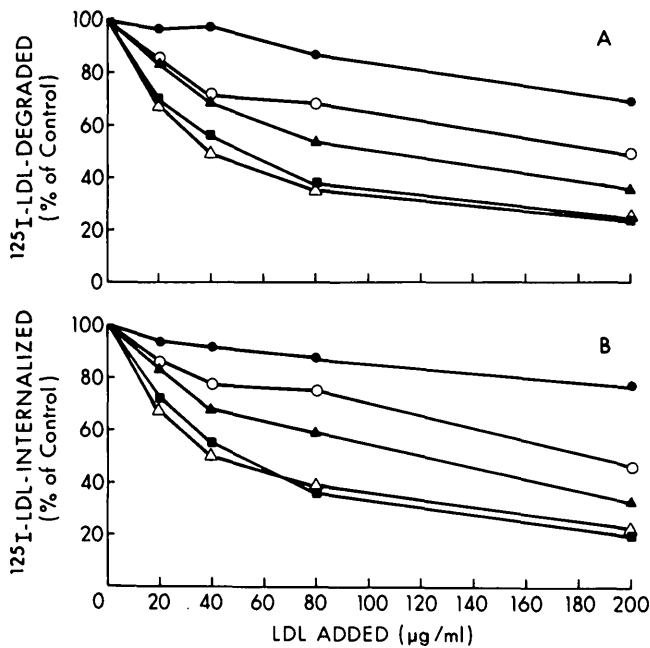
To document that glucose indeed was covalently bound to LDL, we performed amino acid analysis of glycosylated LDL prepared by incubating LDL with <sup>14</sup>C-glucose (20 mM) for 1 wk at 37°C. Analysis was performed on <sup>14</sup>C-glycosylated LDL taken directly from the incubation mixture (following dialysis) and on <sup>14</sup>C-glycosylated LDL taken from pooled void volume fractions following gel filtration on a G-50 sephadex column. The profiles of these LDL preparations, as well as of synthetic glucosyl-<sup>3</sup>H-lysine, are shown in Figure 1. The major peak of radioactivity present in glycosylated LDL (Peak III), coeluted with glucosyl-<sup>3</sup>H-lysine at approximately 62 min. The other minor peaks that we observed have not been identified.

**Cell studies.** Glycosylated LDL has significantly reduced ability (12% of control LDL for the most extensively glycosylated LDL) to compete for the binding (Figure 2A) and degradation (Figure 2B) of <sup>125</sup>I-LDL to cultured human fibroblasts, when compared with control LDL. The more glycosylated the LDL was, the less did it compete for the binding of <sup>125</sup>I-LDL.

Glycosylated LDL also demonstrated reduced ability (18% of activity of control LDL at 40 μg/ml for the most ex-

**FIGURE 1. Amino acid analysis of glycosylated LDL.** LDL (4 mg/ml) was incubated with <sup>14</sup>C-glucose ( $2.6 \times 10^7$  CPM) for 1 wk in 0.5 M phosphate buffer. Top panel (A) shows the profile for synthetic glucosyl-<sup>3</sup>H-lysine. B is the profile of <sup>14</sup>C-glycosylated LDL which was dialyzed following the end of incubation. In C is depicted profile for the pooled void volume of <sup>14</sup>C-glycosylated LDL which was filtered on a G-50 sephadex column, following the end of incubation.



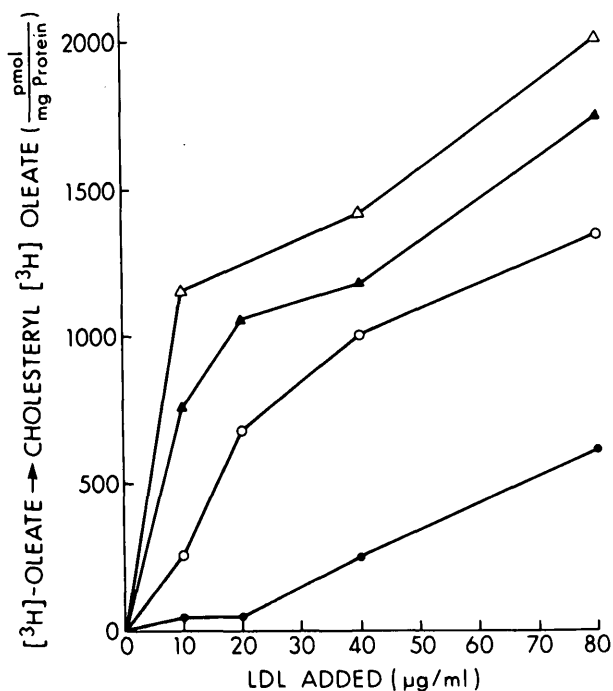


**FIGURE 2.** Competition of glycosylated LDL for the binding (A) and degradation (B) of  $^{125}\text{I}$ -LDL by normal human fibroblasts. ( $\Delta$ — $\Delta$ ) Control LDL (LDL incubated with buffer alone for 2 wk); LDL incubated with: ( $\blacksquare$ — $\blacksquare$ ) 54 mM, glucose, 1 wk; ( $\triangle$ — $\triangle$ ) 54 mM, glucose, 2 wk. ( $\circ$ — $\circ$ ) 54 mM glucose + 1 mg/ml  $\text{NaBH}_3\text{CN}$ , 1 wk; ( $\bullet$ — $\bullet$ ) 54 mM glucose + 1 mg/ml  $\text{NaBH}_3\text{CN}$ , 2 wk. The estimated amount of glucose incorporated into LDL was in the range of 0.5 mol/mol LDL for the least glycosylated preparation, and 10 mol glucose/mol LDL for the most glycosylated preparation.

tensively glycosylated preparation) to stimulate cholesteryl ester formation in normal fibroblasts (Figure 3). Again, this property correlated with the estimated degree of glycosylation.

In rat peritoneal macrophages, both glycosylated LDL

**FIGURE 3** Stimulation of cholesteryl ester synthesis in human fibroblasts by LDL. Symbols same as in Figure 2.



and control LDL did not stimulate cholesteryl ester formation (not shown). In contrast, acetyl-LDL used as a positive control markedly stimulated cholesteryl ester synthesis (at 50  $\mu\text{g}/\text{ml}$  acetyl-LDL,  $25 \pm 5.2$  nmol cholesteryl ester/mg protein were obtained, mean  $\pm$  SD of 3 experiments).

## DISCUSSION

We have shown that LDL can be glycosylated *in vitro* in a slow nonenzymatic reaction, similar to other previously described glucose-protein interactions.<sup>7,8</sup> The amount of glucose that can be incorporated into LDL (in the presence of  $\text{NaBH}_3\text{CN}$ ) is on the order of 5–10 mol of glucose per mol of LDL-protein, representing glycosylation of 2.5–4% of the lysine residues present in LDL protein (there are 192 lysine residues for 1 mol of LDL assuming mol. wt. of 250,000 daltons).<sup>1</sup> However, the amount of  $^3\text{H}$  or  $^{14}\text{C}$ -glucose incorporated into proteins, including LDL, cannot be estimated with accuracy.<sup>15</sup>

We provide evidence that glucose is covalently bound to lysine residues of LDL. This is based on amino acid analyses of  $^{14}\text{C}$ -glycosylated LDL, demonstrating radioactive peaks in the same fractions as synthetic glucositol- $^3\text{H}$ -lysine. In experiments (not reported here) in which glycosylated LDL was reduced with  $\text{NaB}^3\text{H}_4$ , amino acid analysis showed a very similar pattern, that is, most of  $^3\text{H}$ -radioactivity coeluted with synthetic glucositol- $^3\text{H}$ -lysine.

Glycosylated LDL had reduced ability to compete for the binding or degradation of  $^{125}\text{I}$ -LDL to cultured fibroblasts and to stimulate cholesteryl ester synthesis as effectively as does control LDL. The decrease in cell reactivity of glycosylated LDL is proportional to the degree of glycosylation. Of the other chemical modifications of LDL studied, acetyl LDL, acetoacetyl LDL, malondialdehyde-treated LDL, carbamylated LDL, and reductive methylated LDL lose their ability to bind and get degraded by human fibroblasts.<sup>1</sup> Some of these modified LDL (acetyl-LDL, acetoacetyl-LDL) acquire a new ability (not present in native human LDL) to bind, become degraded, and stimulate cholesteryl-ester synthesis in mouse peritoneal macrophages.<sup>2,3</sup> Unlike these modified LDL glycosylated human LDL cannot interact with rodent macrophages. However, because human monocyte-derived macrophages differ from mouse peritoneal macrophages in certain respects, including their interactions with native human LDL,<sup>4,17</sup> glycosylated LDL could conceivably stimulate the accumulation of cholesteryl esters in the human cells and not in the rodent cells. Should this be the case, an increase in cholesteryl esters contents in human monocyte-macrophages (foam cells) within the atherosclerotic lesion may result. Furthermore, slower *in vivo* catabolism of glycosylated LDL (slower catabolism has been demonstrated for methylated LDL<sup>16</sup>), may lead to increased availability of LDL (and cholesterol) for deposition in arterial tissues. In sum, *in vivo* glycosylation of LDL may contribute to the accelerated rate of atherosclerosis present in patients with diabetes mellitus.

In studies currently underway, we are examining LDL isolated from diabetic patients, for the presence of nonenzymatic glycosylation.

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