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NONENZYMATIC GALACTOSYLATION OF HUMAN LDL DECREASES ITS METABOLISM BY HUMAN SKIN FIBROBLASTS

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SUMMARY Incubation of human LDL with galactose in vitro resulted in a glycosylated-LDL containing radiolabel covalently attached to apo B. The rate of radiolabel incorporation was proportional to the time of incubation and concentration of carbohydrate. The rate of incorporation of galactose into apo B was higher than with glucose or mannose. The nonenzymatic glycosylation of LDL decreased its uptake and metabolism by the high affinity, receptor dependent process for LDL in normal human skin fibroblasts.

INTRODUCTION It is well known that the aldehyde of glucose nonenzymatically reacts with amino groups to form a Schiff base. Furthermore, the aldimine linkage of the Schiff base can undergo rearrangement to a more stable ketoamine derivative. Similar non-enzymatic glycosylations do occur in vivo and may contribute to secondary complications in individuals with diabetes mellitus. For instance the level of hemoglobin A_{1c}, a glycosylated hemoglobin, is elevated in diabetes and correlates with the blood glucose concentration (1). Nonenzymatic glycosylation of human LDL in vitro results in a decreased binding, uptake and degradation of the LDL by the high affinity process in normal human fibroblasts (2-5), and decreased clearance in vivo (4,6). Glycosylated-LDL was reported to be elevated in diabetes (4,7). The nonenzymatic glycosylation of lens crystallin has been implicated in the etiology of both diabetic and galactosemic cataracts and cataractous lenses in sugar-induced diabetic or galactosemic animals contain excessive amounts of sugar alcohol, sorbitol or galactitol (8). Galactose also has an aldehydic group and would be

ABBREVIATIONS LDL, low density lipoprotein; LPDS, lipoprotein depleted serum.

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expected to form Schiff bases with amino groups. Recently, glycosylation of albumin in vitro by galactose was reported (9). In this study the nonenzymatic interaction of galactose with LDL decreased its metabolism in vitro by human skin fibroblasts.

MATERIALS AND METHODS Human LDL (d=1.019 to 1.063 g/ml) and human LPDS (d>1.215 g/ml) were prepared by differential ultracentrifugation of plasma from healthy subjects. The concentration of LDL was expressed by its protein content (10). ^{125}I-lipoproteins were prepared via the iodine monochloride procedure (11). Prior to each experiment, the ^{125}I-LDL was diluted with unlabeled LDL and filtered through a 0.45 micron millipore filter.

Derivatization of LDL: Reaction mixtures contained human LDL (11 to 16 mg/ml); 4.4 to 174 mM monosaccharides (D-glucose, D-galactose or D-mannose) and trace amounts of [^{14}C]glucose, [^{3}H]galactose, or [^{14}C] mannose to obtain between 1.6 X 10^5 to 7.7 X 10^7 cpm/μmole, 30 mM $NaCNBH_3$, 50 mM phosphate buffer, pH 7.4, 0.14 M NaCl. Reaction mixtures were filtered through 0.45 micron filters and incubated for 80 hrs. at 37°C. To measure incorporation of radiolabel, 0.05 ml samples were removed and trichloroacetic acid was added to 10% final concentration. The precipitated protein was collected on Whatman GF/C glass filter discs, washed extensively with 10% trichloroacetic acid and dried before radioactivity was determined by scintillation counting in 10 ml of Aquasol II. Derivatives were also prepared by incubation of human LDL (5 mg/ml) with 174 mM glucose-6-P, galactose-6-P or mannose-6-P. These reaction mixtures also contained 30 mM $NaCNBH_3$, 0.14 M NaCl, 50 mM phosphate buffer, pH 7.4 and were incubated 40 hr at 37°C under sterile condition. At the end of the incubation period the LDL derivatives were extensively dialyzed against 0.15 M NaCl containing 0.3 mM EDTA, pH7.4, and sterilized by filtration prior to cell culture studies.

Normal human skin fibroblasts were derived from skin biopsies from a normal subject. Cells were grown in monolayer and used before twenty passages. Cells were maintained at 37°C in a humidified incubator in a 5% CO_2 atmosphere in 75 cm^2 stock flasks containing 10 ml of growth medium: Eagle's minimum essential medium supplemented with 100 IU/ml of penicillin and 100 micrograms/ml of streptomycin, 20 mM Tricine, pH 7.4, 24 mM sodium bicarbonate, 1% nonessential amino acids and 10% fetal bovine serum. On day zero, 1 X 10^5 cells were seeded into each 60 mm petri dish. The growth medium was replaced on day 3. On day 5, the medium was replaced with 2 ml of medium containing 10% (v/v) LPDS and experiments were performed on day 7. The binding, internalization and degradation of ^{125}I-labeled LDL by human skin fibroblasts were carried out as described by Goldstein et al (12).

RESULTS Incubation of glucose, galactose or mannose with LDL result in incorporation of radiolabel into trichloroacetic acid precipitable protein that increases with time (Fig 1). However, the monosaccharides apparently do not react with LDL at the same rate. The stoichiometry of carbohydrate incorporated was calculated from the specific radioactivity of the monosaccharides and assuming 250,000 daltons for apoB. When 4.4 mM monosaccharide was incubated with LDL 1.06, 1.49 and 1.11 moles of glucose, galactose or mannose were incorporated and when the

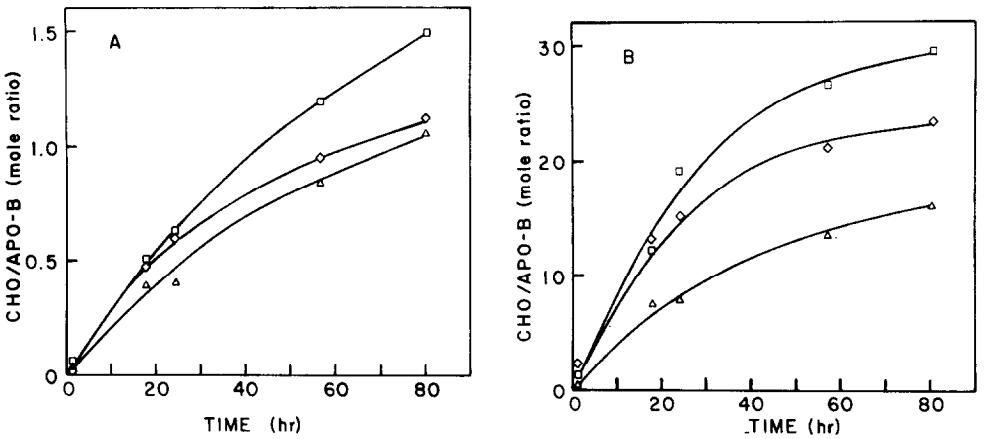


Fig. 1. Nonenzymatic incorporation of radiolabel into LDL by [^{14}C] glucose (Δ), [^{3}H]galactose (□) or [^{14}C]mannose (◇). The reaction mixtures were incubated at 37°C for the times indicated and contained human LDL (11 mg/ml), 30 mM sodium cyanoborohydride, 0.14 M sodium chloride, 50 mM phosphate buffer (pH 7.4) and 4.4mM (A) or 174 mM (B) monosaccharide.

monosaccharide concentration was 174 mM, 16.1, 29.8 and 23.6 moles of the respective carbohydrates were incorporated per mole of apoB. In these latter values, 8 to 15 percent of the total lysyl residues of apoB were modified assuming ∿200 lysyl per apoB calculated from 20 lysyl residues per 250 amino acid residues of apoB (13). However, the values for carbohydrate incorporated should not be considered absolute because preparations of radiolabeled glucose have been reported to contain impurities (14,15).

The results of the time course of binding, internalization and degradation of the iodinated lipoprotein derivatives by normal human fibroblasts are shown in Fig. 2. With ^{125}I-LDL a rapid binding of ^{125}I-LDL was observed which was internalized and acid-soluble degradation products of ^{125}I-LDL appeared in the culture medium at a linear rate after a short lag period. On the other hand ^{125}I-glucose-LDL, ^{125}I-galactose-LDL, ^{125}I-mannose-LDL derivatives containing carbohydrate/apoB mole ratios of 16.1, 29.8 and 23.6, respectively were much less extensively bound,internalized and degraded. In fact almost no measurable degradation was observed.

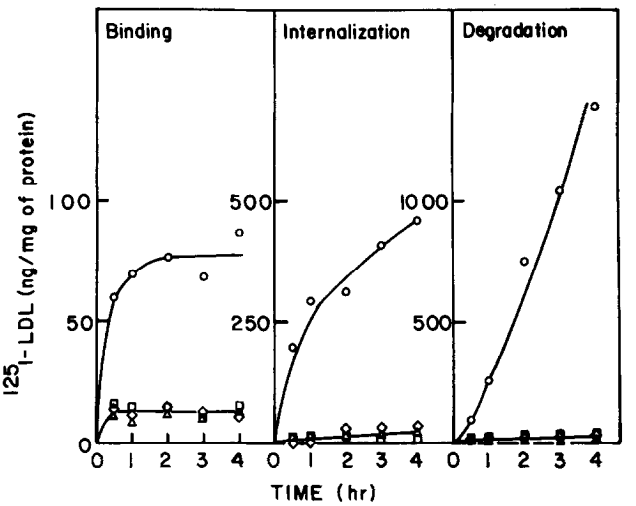
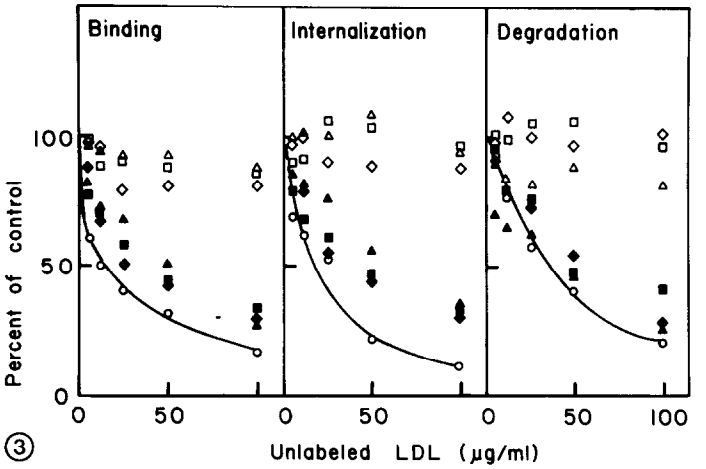


Fig. 2. The time course of binding, internalization and degradation of ^{125}I-glucose-LDL (△), ^{125}I-galactose-LDL (□), ^{125}I-mannose-LDL (◇) and ^{125}I-LDL (○) in normal human skin fibroblasts. At time zero the medium was replaced with fresh LPDS containing 10μg/ml of ^{125}I-glucose-LDL (125cpm/ng), ^{125}I-galactose-LDL (159 cpm/ng), ^{125}I-mannose-LDL (53 cpm/ng) or ^{125}I-LDL (318 cpm/ng). The glycosylated-LDL's contained carbohydrate/apoB mole ratios of 16.1, 29.8 and 23.6 for glucose, galactose and mannose, respectively.

The ability of LDL, glucose-LDL, galactose-LDL, and mannose-LDL to compete with ^{125}I-LDL for binding, internalization and degradation by human skin fibroblasts is shown in Fig. 3. LDL effectively competes with ^{125}I-LDL for binding, internalization and degradation. The modified LDL derivatives containing low mole ratios (1 to 1.5) of carbohydrate per apo B (solid symbols) were slightly less effective than LDL in competing with ^{125}I-LDL. However, no competition was observed between the more extensively modified LDL containing 16 to 30 carbohydrate residues per apo B (open symbols) and the ^{125}I-LDL. Similarly, derivatives of LDL prepared by incubation of LDL with 174 mM glucose-6-phosphate, galactose-6-phosphate or mannose-6-phosphate for 40 hours resulted in modified LDL derivatives which did not compete for ^{125}I-LDL degradation by fibroblasts (Fig 4).

DISCUSSION In this paper, nonenzymatic modification of LDL in vitro by galactose and mannose as well as glucose has been demonstrated. Furthermore, incubation of glucose-6-phosphate, galactose-6-phosphate or



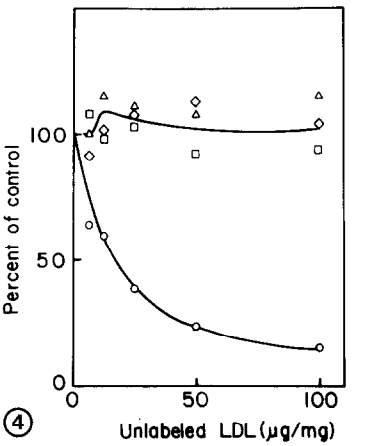


Fig. 3. Competitive displacement of ^{125}I-LDL binding, internalization and degradation by monosaccharide-LDL in normal human skin fibroblasts. Reaction mixtures incubations were carried out for 4 hr at 37°C with 10 μg/ml of ^{125}I-LDL (302 cpm/ng) and the indicated amount of competitors: LDL, (○); glucose-LDL, (▲); galactose-LDL, (□); mannose-LDL (◇). The closed symbols represent carbohydrate/apoB mole ratios of 1.06, 1.49 and 1.11 and the open symbols represent carbohydrate/apoB mole ratios of 16.1, 29.8 and 23.6 for glucose, galactose and mannose derivatives, respectively.

Fig.4. Competition for ^{125}I-LDL degradation by monosaccharide phosphate-LDL derivatives by normal human skin fibroblasts. Incubation was for 4 hr at 37°C in reaction mixtures contained ^{125}I-LDL, 10 μg/ml (23 cpm/ng), in the presence of indicated amount of LDL, (○); glucose-6-P-LDL, (▲); galactose-6-P-LDL, (□); mannose-6-P-LDL, (◇).

mannose-6-phosphate with LDL also resulted in a modification of LDL that did not compete with LDL for the high affinity receptor process on human skin fibroblasts. The incorporation of radiolabel was dependent on the concentration of the monosaccharide and the time of incubation. Galactose and mannose derivatives were formed more rapidly than the glucose derivative under similar incubation conditions. A similar phenomenon has been observed upon incubation of hemoglobin and albumin with monosaccharides (9,16). Reasons for this are not readily evident, perhaps galactose has a higher concentration of the aldehydic structure required for non-enzymatic glycosylation of proteins.

The lysine and arginine residue of apo B are important recognization sites for binding of LDL to high affinity surface receptors (13,17). Carbamylation of $\geq$15% of the total lysine residues of LDL has been

reported to prevent binding, internalization and degradation by high affinity LDL receptors on human skin fibroblasts. In data reported here modification of 8 to 15 percent of the lysyl residues of apoB by glycosylation resulted in a significant decrease in the interaction between the glycosylated-LDL and human skin fibroblasts.

The pathophysiological importance of these monosaccharide LDL derivatives is not completely obvious. However, many complications have been reported in diabetes which may result from in vivo glycosylation. Thus, it is reasonable to speculate that glycosylated-LDL in galactosemic individuals may also occur and result in altered metabolism.

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