

Glucosylation of Low-Density Lipoproteins to an Extent Comparable to That Seen in Diabetes Slows Their Catabolism

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SUMMARY

In previous studies we have shown that extensive glucosylation of low-density lipoproteins (LDL) (40% of lysines modified) completely blocks receptor-mediated degradation in animals and in man. Other studies indicated that in some diabetics up to 5% of lysine residues of LDL were glucosylated. The present study was done to determine if the extent of glucosylation of LDL which can occur in diabetics could alter LDL catabolism. We measured degradation by cultured normal human fibroblasts and turnover in guinea pigs of various LDLs with 2–17% of lysine residues glucosylated. Modification of as few as 2–5% of lysines decreased LDL catabolism by 5–25%, and the degree of inhibition of catabolism was linearly related to the extent of LDL glucosylation. These results indicate that the extent of LDL glucosylation that can occur in diabetes may slow LDL catabolism and hence increase plasma LDL levels. DIABETES 33:130–134, February 1984.

Recognition of low-density lipoproteins (LDL) by the LDL receptor can be inhibited by various chemical modifications of lysine¹ or arginine residues² of apoprotein B, the major protein of LDL. We have previously shown that modification of approximately 40% of lysine residues of LDL by reductive glucosylation completely blocks receptor-mediated LDL catabolism in cultured cells, in animals, and in man.^{3–5} Glucosylation of LDL also occurs *in vivo* in diabetic subjects; however, the extent of modification is considerably less than in the heavily derivatized LDL used in the studies cited above. Preliminary data indicate that less than 2% of lysine residues of LDL are glucosylated in euglycemic individuals, and from 2% to 5% in diabetics attending an outpatient clinic.

The present studies were done to determine if degrees of glucosylation comparable to that seen in some diabetic subjects could also alter LDL catabolism.

METHODS

Isolation and modification of lipoproteins. Human LDL ($d = 1.019 - 1.063$ g/ml) was prepared from pooled plasma obtained from young, healthy, euglycemic donors by sequential ultracentrifugation.⁶ As previously noted, the clearance of both native and modified human LDL in guinea pigs was the same as that of the corresponding homologous LDL, and on this basis human native and glucosylated LDL (GLC-LDL) were used for the turnover studies.³ Radioiodination was carried out by a modification of the iodine monochloride method⁷ using carrier-free Na ¹²⁵I or Na ¹³¹I (Amersham Corp., Arlington Heights, Illinois). After gel filtration chromatography on Sephadex G-25 and dialysis against phosphate-buffered saline with 0.01% ethylenediamine tetraacetic acid (EDTA), pH 7.4 (PBS), >98.5% of radioactivity was precipitable with 10% trichloroacetic acid (TCA). Less than 6% of radioactivity was extractable into organic solvents. The range of specific activities was 102–230 cpm/ng. The same pooled LDL preparation was used to prepare GLC-LDL and control LDL in individual experiments.

In previous studies^{3–5} glucosylation of LDL has usually been done in the presence of a reducing agent such as NaCNBH₃ to increase the degree of glucosylation. However, glucosylation using NaCNBH₃ produces glucitolysine as the glucose adduct; this product differs chemically from fructosyllysine, the form of glucosylation that appears to predominate *in vivo*.^{8,9} To determine if the chemical form of the glucose adduct (as well as the extent of glucosylation) could influence LDL catabolism, we prepared GLC-LDL both in the absence and presence of NaCNBH₃. To prepare GLC-LDL without NaCNBH₃, LDL (2 mg/ml) was incubated with glucose (Mallinckrodt Inc., Paris, Kentucky) in PBS at 37°C for 5–9 days. To vary the extent of glucosylation, glucose concentrations were varied from 10 to 200 mM. For GLC-LDL prepared in the presence of 200 mM NaCNBH₃ (Aldrich

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Chemical Co. Inc., Milwaukee, Wisconsin), glucose concentration was kept at 80 mM and incubation times were varied from 3 to 24 h. For each GLC-LDL, a control LDL preparation was incubated in parallel without glucose. All reagents were sterilized by passage through a 0.45- μ m filter before incubation. After glucosylation, the number of modified lysine residues was measured, and then LDL was radiiodinated and used in experiments. In some experiments, the LDL was iodinated first and then glucosylated. For a given degree of glucosylation, degradation in cultured cells and in vivo clearance of GLC-LDL prepared by either method was the same. On this basis, the turnover results for the two methods of glucosylation were combined.

Measurement of degree of LDL glucosylation was done by three methods: (1) glucitolysine mass measurement on an amino acid analyzer (Beckman 119 CL with Model 126 Data System, Beckman Instruments, Fullerton, California);³ (2) radioimmunoassay using a specific antiserum against glucosylated LDL;¹⁰ and (3) the trinitrobenzenesulfonic acid (TNBS) assay.¹¹ The TNBS assay measures extent of glucosylation indirectly, i.e., as a decrease in reactive amino groups per milligram LDL protein. In our experience, while accurate for more heavily glucosylated preparations it is not reliable at levels of glucosylation of less than 10%. The amino acid analyzer measures glucitolysine directly, but partial hydrolysis of glucitolysine may occur during sample preparation with 6 N HCl. In our hands, however, hydrolysis of glucitolysine appears to be minimal, as exposure of synthetic glucitolysine to the same hydrolysis conditions does not produce any free lysine, and the values derived on more extensively modified samples using the TNBS assay agree well with values from amino acid analysis. To facilitate determination of extent of glucosylation of LDL we established a solid-phase competitive radioimmunoassay (RIA) using a previously described guinea pig antiserum specific for GLC-LDL.¹⁰ The standard curve was calibrated with reference GLC-LDL preparations containing 3–28% of lysines modified as determined by replicate amino acid analyses. Some samples were measured by both RIA and amino acid analysis, and results were in good agreement.

Cell culture studies. Normal human skin fibroblasts obtained from a preputial biopsy were maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum as previously described.^{3,4} Cells taken from the seventh to fourteenth passage were seeded onto 35-mm tissue culture plates and used at 70–80% confluence (80–160 μ g cell protein per dish). To stimulate expression of LDL receptors, the medium was changed to Dulbecco's modified Eagle's medium containing 2.5 mg/ml lipoprotein-deficient human serum 24 h before use in experiments. Cells were harvested after 12–20 h incubation with radiolabeled LDL, and the content of TCA-soluble noniodide radioactivity in the medium was used to calculate the amount of LDL degraded.¹² After removal of media, cells were washed with PBS, dissolved in 0.1 M NaOH, and protein determined by the method of Lowry.¹³

Turnover studies. Male Hartley guinea pigs (450–600 g; Charles River Breeding Laboratories, Inc., Wilmington, Maine) were fed Wayne guinea pig chow (ICN Nutritional Biochemicals, Cleveland, Ohio) ad libitum and KI was added to the drinking water during turnover studies. After gluco-

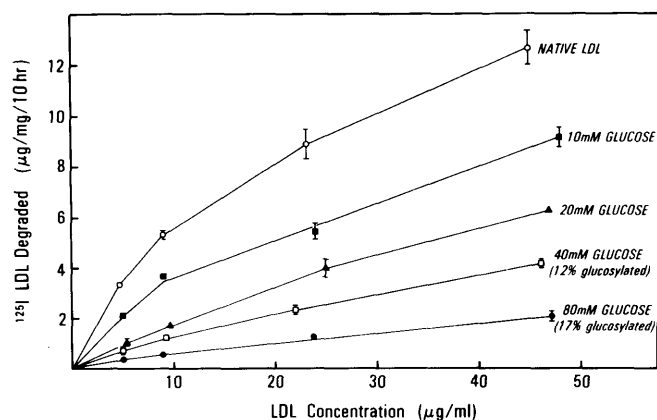


FIGURE 1. Effect of varying degrees of glucosylation on LDL degradation in normal human fibroblasts. Solutions of 125 I-LDL (2 mg/ml in PBS) were preincubated with 10 mM (■), 20 mM (▲), 40 mM (□), or 80 mM (●) glucose (without NaCNBH₃) for 8 days at 37°C. Fibroblasts were grown in DME with 10% fetal bovine serum until 24 h before use, when the medium was changed to DME with 2.5 mg/ml lipoprotein-deficient serum. The indicated concentrations of 125 I GLC-LDL or 125 I native LDL were then added to the cells, and the content of TCA-soluble noniodide radioactivity was determined after a 10-h incubation. In this experiment, 40 mM and 80 mM glucose produced 12% and 17% glucosylation, respectively, when estimated using the TNBS assay.

sylation or control incubation without glucose, LDL was radiiodinated with Na 125 I and native LDL was labeled with Na 131 I. In one set of experiments the isotope assignments were reversed to rule out an isotopic effect as the cause of differences in metabolic behavior. In each animal 125 I-glucosylated (or control) and 131 I native LDL were injected simultaneously into an exposed external jugular vein. Serial blood samples, each 150–200 μ l in volume, were then obtained over a 30-h period by cardiac puncture using a 25-gauge needle, with the animals lightly anesthetized with ether. The samples were anticoagulated with solid EDTA, separated by centrifugation at 2500 \times g for 30 min, and aliquots of plasma counted in a double-channel gamma spectrometer. Two exponential equations were fitted to each plasma decay curve, and fractional catabolic rates (FCR) were then computed as the reciprocal of the area under the normalized radioactivity-time curve.¹⁴

RESULTS

Glucosylation of LDL. When glucosylation of LDL was carried out with 80 mM glucose and 200 mM NaCNBH₃, the degree of derivatization of lysine residues that resulted varied predictably with incubation time. Under these conditions 2–3% of lysines are modified after 3 h, and about 40% after 120 h. In contrast, when glucosylation was done without the reducing agent, great variability was encountered in the extent of modification for different LDL preparations under apparently identical incubation conditions. In most experiments, the maximum extent of glucosylation attained was about 6%, even when concentrations of glucose as high as 200 mM were used. However, in four separate experiments substantially greater derivatization occurred, with up to 17% of lysines modified after incubation with 80 mM glucose for 7–9 days. The reasons for this variability in the extent of glucosylation obtained in the absence of the reducing agent are unknown.

Cell culture studies. The inhibitory effect of glucosylation on receptor-mediated LDL degradation in cultured fibroblasts was demonstrated by direct uptake experiments and by the ability of GLC-LDL to compete for ¹²⁵I-LDL degradation by cultured normal human fibroblasts. Figure 1 shows the effect on high-affinity degradation of ¹²⁵I-LDL produced by preincubation of the LDL with various concentrations of glucose (in the absence of reducing agent) for 7 days. Preincubating the LDL with as little as 10 mM glucose decreased LDL degradation compared with the control degradation, while 80 mM glucose, which resulted in glucosylation of 17% of lysine residues in this experiment, almost completely inhibited high-affinity degradation.

Similar results were obtained in three sets of experiments; however, in four others degradation was decreased only 20–30% by incubation with 80 mM glucose, and no further decrease was seen even with 200 mM glucose. This variability was explained by differences in the degree of glucosylation obtained with different LDL preparations when incubated without NaCNBH₃, as noted above. However, the effect of a given degree of glucosylation on LDL degradation was remarkably consistent, regardless of the incubation conditions or presence of the reducing agent. Figure 2 illustrates the results of an experiment comparing the ability of various unlabeled preparations of GLC-LDL (incubated with NaCNBH₃) to compete for ¹²⁵I-LDL degradation in fibroblasts. In this experiment modification of only 3.2% of lysine residues decreased the effectiveness of the GLC-LDL as a competitor, while GLC-LDL with 10.3% of lysines glucosylated competed very little.

Turnover studies. To determine if small degrees of glucosylation of LDL would also interfere with LDL clearance in vivo, we measured the FCR of GLC-LDL preparations in which 2–12% of lysine residues were glucosylated. In each

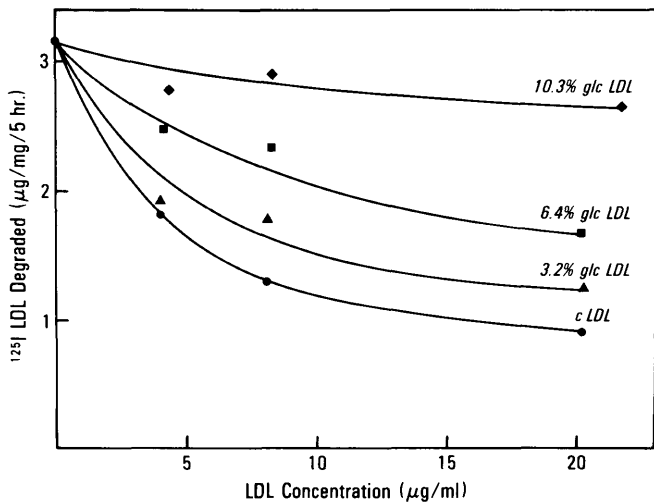


FIGURE 2. Effectiveness of variously glucosylated LDLs as competitors for ¹²⁵I LDL degradation in cultured normal human fibroblasts. LDL was glucosylated by preincubating a 2-mg/ml solution with 80 mM glucose and 200 mM NaCNBH₃ at 37°C. Preincubation times of 3, 8, and 24 h resulted in 3.2%, 6.4%, and 10.3% glucosylation as determined by solid-phase RIA. Fibroblasts were used after 24-h exposure to lipoprotein-free medium as described in the legend to Figure 1. Native ¹²⁵I LDL (2.5 mg/ml) and the indicated quantity of unlabeled control LDL or GLC-LDL was then added, and after 5 h the medium was assayed for TCA-soluble noniodide radioactivity.

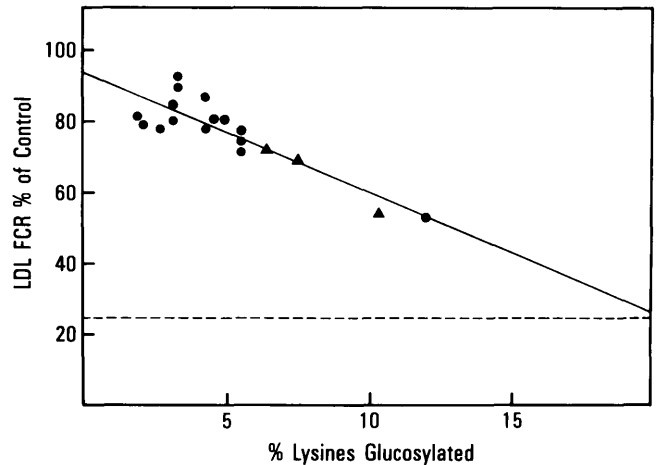


FIGURE 3. Relationship between LDL FCR and degree of glucosylation of LDL in guinea pigs. Each point represents the GLC-LDL FCR as a percentage of the simultaneously measured native LDL FCR in one animal. Human LDL was glucosylated in the presence (▲) or absence (●) of NaCNBH₃, and the degree of glucosylation was measured as indicated in METHODS. The hatched line indicates the receptor-independent component of LDL catabolism (25% of total LDL catabolism) determined in a previous study.⁴

animal a simultaneous measurement of the FCR of native LDL (in which <1% of lysines were glucosylated) was carried out. Figure 3 shows the results of the turnover studies, with the FCR of GLC-LDL in each animal being expressed as a percentage of the simultaneously determined native LDL-FCR. For each set of preparations used, the FCRs of native LDL and control LDL (incubated in parallel without glucose) were the same (data not shown), indicating that the observed differences between native and glucosylated LDL were due to glucosylation and not to some other effect of the incubation. When 2–5% of lysines were glucosylated, LDL catabolism was inhibited 5–25%. It is important to note that all of the GLC-LDL preparations with less than 5% glucosylation were prepared by incubation of LDL and glucose without NaCNBH₃. When the data for all GLC-LDL preparations are combined, the FCR of GLC-LDL was inversely related to the extent of LDL glucosylation ($r = 0.88$, $P < 0.01$), and a consistent decrease compared with native LDL was noted even in LDL in which only 2% of lysines were glucosylated. In previous studies we found that in guinea pigs receptor-independent catabolism accounts for 25% of total LDL catabolism.⁴ This is indicated by the hatched line in Figure 3. The calculated regression line relating FCR and extent of glucosylation crosses this hatched line at 22% glucosylation. This predicts that derivatization of 22% of lysines by glucose is required to completely abolish receptor-mediated LDL degradation in vivo in the guinea pig, and agrees well with our findings in cultured human fibroblasts, as well as with the recent report of Sasaki and Cottam¹⁵ in which it was estimated that 23% of lysines had to be glucosylated to abolish receptor-mediated clearance in rabbits.

DISCUSSION

In preliminary studies based on measurement of glucitolysine in LDL by amino acid analysis, we found that in some diabetics attending an outpatient clinic up to 5% of lysine residues of isolated LDL were glucosylated, while in eugly-

cemic controls less than 2% were glucosylated. Schleicher et al.¹⁶ have also shown greater glucosylation of LDL in diabetics than in normals using an HPLC method. Using monoclonal antibodies to quantify immunoreactive glucitolysine content of LDL, we found that diabetic LDL contained two- to 10-fold more immunoreactive glucitolysine than normal LDL.⁹ Thus, there is considerable evidence that at least in some diabetics LDL is glucosylated to a much greater extent than occurs in LDL isolated from normals.

In the present study we have shown that when as few as 2–5% of lysine residues are glucosylated, there is a 5–25% decrease in the *in vivo* clearance of that LDL. Furthermore, the apparently linear inverse relationship between extent of glucosylation and inhibition of LDL clearance suggests that any degree of glucosylation will impair LDL clearance.

The results of the present studies in cultured fibroblasts and in guinea pigs cannot be extrapolated directly to LDL turnover in human diabetic subjects. However, the finding in some diabetics that LDL is glucosylated to an extent that clearly alters receptor-mediated degradation in human fibroblasts and in animals gives credence to the suggestion that glucosylation might alter LDL catabolism in human diabetics. It is of interest that Kissebah recently reported that turnover of autologous ¹²⁵I-LDL taken from uncontrolled diabetics (fasting plasma glucose 150–300 mg/dl) was decreased by 26% compared with euglycemic controls.¹⁷ Lopes-Virella et al. have also shown that the rate of uptake and degradation by fibroblasts of LDL isolated from markedly uncontrolled diabetics (mean fasting plasma glucose 372 mg/dl) was decreased by 20% compared with LDL isolated from the same patient after good control was achieved.¹⁸

One might ask: what would be the importance of a 20% decrease in LDL FCR in diabetics? If LDL production is constant (and one might even anticipate increased production in this situation) then a corresponding 20% rise in plasma LDL level would result. The data from Framingham¹⁹ and Oslo²⁰ indicate that a sustained change in plasma LDL level of this magnitude could increase the risk of cardiovascular mortality by as much as 45%.

A number of studies now show that improved control of hyperglycemia can lead to an 8–27% lowering of LDL cholesterol levels when each diabetic's pretreatment LDL value is used as control.^{21–24} Obviously numerous variables may have changed when "improved control" was achieved in these studies. For example, VLDL synthesis is increased in uncontrolled diabetics,²⁵ and increased insulinization may itself lead to increased LDL-receptor activity.²⁶ It remains to be determined what proportion of the change in LDL metabolism associated with uncontrolled diabetes is attributable to glucosylation, and what proportion to other effects of the hyperglycemic state. Further studies are in progress to more precisely define the relationship between plasma glucose concentration and extent of LDL glucosylation, and to define the mechanisms and tissue sites of catabolism of GLC-LDL.

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